

Miriam Melis *Editor*

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*To my family, for their love and support and
for brightening up the day when it was needed*

Endocannabinoids and Lipid Mediators in Brain Functions

The science of cannabinoids is 50 years old. These past years provided a remarkable and constant number of breakthroughs, showing that the signalling mediated by endocannabinoids and lipid mediators impacts almost every function of the body. Indeed, this represents a special field of research, which allows tackling the complexity of biological functions and provides potential therapeutic frameworks for a plethora of diseases.

Endocannabinoids and lipid mediators, acting on multiple receptors in both CNS and periphery, are involved in the control of neuronal firing, synaptic plasticity, local circuit activity and behaviour. In fact, these lipid molecules, being intimately connected with diverse metabolic and signalling pathways, differently affect various functions of neurons and glial cells through activation not only of surface receptors, such as cannabinoid and vanilloid receptors, but also of nuclear receptors such as peroxisome proliferator-activated receptors. Although the endocannabinoid system plays a fundamental role in making short- and long-term modifications to neural circuits and related behaviours, many facets of this system are still unclear.

The number of exciting discoveries brought up to the scientific community almost on a daily basis highlights the importance of an updated volume on this topic, particularly given that potential therapeutic benefits of cannabis and cannabinoids are currently under heavy analysis in many countries worldwide. Hence, the main objective of this book is to explore not only some of the many functions of endocannabinoids (and lipid mediators) in physiological control of networks at a cellular and molecular level but also to extend this knowledge for the potential use of cannabinoids and/or drugs regulating endocannabinoid levels *in vivo* as therapeutic target(s) in neurological and neuropsychiatric disorders. In this book, new findings and ideas about the endocannabinoid system and its roles as neuronal circuit modulator related to human brain pathologies characterized by alterations in neuroplasticity will be highlighted. Endocannabinoid roles in key systems controlling appetite, pain, learning and memory, as well as stress responses, will be presented. In addition, pathological processes associated with changes in endocannabinoid signalling will be discussed in the context of anxiety, autism,

depression and addiction. Such an integrated science has important therapeutic implications because it explores the interactions among the environment, the brain and the body. Finally, given that nowadays *Cannabis* use is increasingly prevalent among adolescents and that this (mis)use interferes with endogenous endocannabinoid system, which is critically involved in both pre- and post-natal neurodevelopment, the consequences on normal development, thus predisposing to motivational, affective and psychotic disorders, will be discussed.

Given the tremendous amount of information available and the pace of research progress in this field, it is impossible for the current volume to be fully comprehensive. Nonetheless, this book will provide an excellent background to researchers looking for extending their areas of interest and to newcomers in the field.

As the book was being prepared for publication, Dr. Loren (Larry) Parsons, who contributed to this book, passed away in his sleep at only 52 years old. He brought light both to the field of endocannabinoid system, in particular with regard to the mechanisms involved in drug abuse, and to everyone who interacted with him at a personal or professional level. He will be deeply missed.

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The Endocannabinoid System in Leptin-Driven Changes of Orexinergic Signaling Under Physiological and Pathological Conditions

Luigia Cristino, Roberta Imperatore, Letizia Palomba,
and Vincenzo Di Marzo

Abstract In this chapter, the neuroanatomical overlap in the distribution of orexin and endocannabinoid systems, as well as the functional interaction between leptin-driven synaptic rewiring of orexinergic neurons and the orexin receptor-mediated activation of 2-arachidonoylglycerol synthesis, will be presented in the context of their role in the regulation of appetite, reward, sleep/wake, and analgesia. This chapter attempts to piece together what is known about this important cross talk and points out its potential therapeutic implications.

Abbreviations

2-AG	2-arachidonoylglycerol
AA	arachidonic acid
AC	adenylyl cyclase
AEA	anandamide
AgRP	agouti-related peptide
ARC	arcuate nucleus
BBB	blood-brain barrier
cAMP	cyclic adenosine monophosphate
CART	cocaine- and amphetamine-regulated transcript
CB1	cannabinoid receptor 1
CNS	central nervous system
CSF	cerebrospinal fluid
CTB	cholera toxin B subunit
DA	dopaminergic
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DMH	dorsomedial hypothalamic nucleus
DR	dorsal raphe nucleus

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DSE	depolarization-induced suppression of excitation
DSI	depolarization-induced suppression of inhibition
ECs	endocannabinoids
ERK	extracellular signal-regulated kinases
FAAH	fatty acid amide hydrolase
FRET	fluorescence resonance energy transfer
GPCRs	G-protein-coupled receptors
Hcrt	hypocretin
HFD	high-fat diet
IP3	inositol trisphosphate
LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
LH	lateral hypothalamus
LHA	lateral hypothalamic area
LPA	lysophosphatidic acid
MAGL	monoacylglycerol lipase
MAPKKK	mitogen-activated protein kinase kinase kinase
MCH	melanin-concentrating hormone
MSH	melanocyte-stimulating hormone
NAcc	nucleus accumbens
NMDA	<i>N</i> -methyl-D-aspartate receptor
NPY	neuropeptide Y
NSCCs	nonselective cation channels
OX-1R	orexin-1 receptor
OX-2R	orexin-2 receptor
OX-A	orexin-A
OX-B	orexin-B
PA	phosphatidic acid
PAG	periaqueductal gray
PAP	phosphatidic acid phosphohydrolase
PC	phosphatidylcholine
PFCx	prefrontal cortex
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA1	phospholipase A1
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
POMC	pro-opiomelanocortin
PPO	prepro-orexin
PPT	pedunculopontine nucleus
PVN	paraventricular nucleus

REMS	rapid eye movement sleep
RHT	retinohypothalamic tract
ROCCs	receptor-operated calcium channels
SOCCs	store-operated calcium channels
SON	supraoptic nucleus
THC	tetrahydrocannabinol
TMN	tuberomammillary nucleus
TRP	transient receptor potential channels
TRPV	vanilloid transient receptor potential channels
VMH	ventromedial hypothalamus
VTA	ventral tegmental area

1 Endocannabinoids and Cannabinoid Receptor Type 1: An Overview

The endocannabinoids (ECs) are arachidonic acid-containing messengers generated by esterase and phosphoesterase action, produced on demand at the site of need. The most important ECs are *N*-arachidonyl ethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al. 1992; Sugiura et al. 1995). In the CNS, ECs are often synthesized by postsynaptic neurons in response to a depolarization-induced increase in intracellular Ca^{2+} levels before being rapidly released to act in an autocrine or paracrine manner (Di Marzo 2009). Through the paracrine mode of action, ECs bind CB1 at presynaptic sides and inhibit both inhibitory and excitatory neurotransmission, thereby inducing a retrograde suppression of inhibition (DSI) or suppression of excitation (DSE), respectively, of the cellular target (Kano et al. 2009). After EC synthesis and release, EC signaling is terminated by neuronal reuptake and intracellular hydrolysis of AEA and 2-AG by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. A plethora of studies report that the ECs modulate other neurotransmitters like endogenous opioids (Robledo et al. 2008; Parolaro et al. 2010), dopamine and adenosine (Carriba et al. 2007; Ferré et al. 2009; Fernández-Ruiz et al. 2010). Interestingly, strongly emerging evidence points to a cross talk between ECs and orexins in the overall regulation of appetite, nociception, and sleep/wake cycle, thus opening new horizons to potential therapeutic applications of currently existing drugs targeting dysfunctions of these functions. The cannabinoid receptors of type 1 (CB1) are the major cannabinoid receptors expressed in the brain, where they are found both in neurons and astrocytes. CB1 belongs to the superfamily of G-protein-coupled receptors (GPCRs), which inhibit adenylyl cyclase (AC) activity with consequent decrease of cAMP and reduction, among others, of inhibitory c-Raf phosphorylation by PKA activity (Melck et al. 1999; Davis et al. 2003). Through Gi, CB1 regulates ion channels via activation of K^{+} channels and inhibition of N-,

P-/Q-, and L-type voltage-gated Ca^{2+} channels (Deadwyler et al. 1995; Hampson et al. 1995). However, CB1 activation is also able to regulate AC via Gs, as well as Ca^{2+} fluxes and phospholipases with subsequent modulation of mitogen-activated protein kinases (p42/p44, p38, and c-Jun N-terminal kinase) regulating nuclear transcription factors (Howlett et al. 2002).

2 Orexin/Hypocretin: Discovery and Characterization

The discovery of orexin/hypocretin neuropeptides and their receptors dates back to 1998 and is attributed to two independent groups. By using subtraction hybridization, de Lecea with colleagues identified the mRNA sequence encoding prepro-hypocretin, the putative precursor of two putative peptide transmitters produced in the mammalian hypothalamus. Simultaneously, while searching for endogenous peptide ligands for multiple orphan G-protein-coupled receptor HFGAN72, Sakurai and Yanagisawa identified the same neuropeptides (Sakurai et al. 1998). De Lecea et al., named these peptides “hypocretins” by combining the suffix “hypo” of hypothalamus, the main source of these peptides in the brain, and “cretin” of “secretin,” the gut hormone with high amino acid homology with hypocretins (de Lecea et al. 1998). Sakurai et al., referred to these peptides as orexins from the Greek word “orexis” meaning appetite, in view of their stimulatory action on food intake following central administration (Sakurai et al. 1998). It soon became clear that the peptides isolated by these two groups were identical and both sets of names are still in use. However, the Nomenclature Committee of the International Union of Basic and Clinical Pharmacology (IUPHAR) recommends the use of “orexin” for peptides and receptors and “hypocretin” for the genes encoding them (Gotter et al. 2012). Thus, the peptides are known as prepro-orexin (PPO), orexin-A and orexin-B, and the receptors as OX_1 and OX_2 receptors (OX-1R and OX-2R , respectively), while the rodent genes encoding PPO and the receptors are *Hcrt*, *Hcrtr1*, and *Hcrtr2*, respectively. Mammalian (human, pig, dog, rat, mouse) PPO is composed of 130–131 aa (de Lecea et al. 1998; Sakurai et al. 1998). OX-A is a 33-amino acid peptide of 3562 Da with two intrachain disulfide bonds (Sakurai et al. 1998), which make the peptide particularly prone to misfolding following exposure to free radicals like nitric oxide (Nobunaga et al. 2014). OX-B is a 28-amino acid of 2937 Da with 46% sequence homology with OX-A . The amino acid sequences for OX-A and OX-B are highly conserved in mammals: OX-A is identical in pigs, dogs, rats, mice, and humans, whereas in OX-B there are few changes in one or two amino acids among these species (Kukkonen 2013). Since OX-A and OX-B are very ancient neuromodulators, a strong phylogenetic conservation of their amino acid sequences occurs among nonmammalian vertebrates (Sakurai 2007; Tsujino and Sakurai 2009). Both OX-A and OX-B are cleaved from the precursor PPO (de Lecea et al. 1998; Sakurai et al. 1998), whose expression is restricted to a small number of neuronal cell bodies in the LHA (Peyron et al. 1998), which send widely distributed projections to the rest of the brain (Peyron et al. 1998; Date et al. 1999; Nambu et al. 1999). Indeed, each brain region contains at

least one of the two orexin receptors, except for the cerebellum (Trivedi et al. 1998). Orexinergic neurons send widespread projections to other neurons of the brain, including dopaminergic neurons of the reward areas (VTA, NAcc), noradrenergic neurons controlling arousal at LC, serotonergic neurons of the DR, cholinergic neurons of the striatum, and multiple populations of neuropeptide-expressing neurons in the cerebral cortex, mesopontine tegmental nuclei, periaqueductal gray, tuberomammillary nucleus, septal nucleus, and paraventricular thalamus (Peyron et al. 1998; Horvath et al. 1999). Orexinergic neurons also project to regions regulating energy balance in the brain (ARC, VMH, DMH, PVN, LHA) (Lee et al. 2013) and periphery (pancreas, gastrointestinal system, liver, brown and white adipose tissue) (Wu et al. 2004; Grabauskas and Moises 2003; Oldfield et al. 2002; Adler et al. 2012). Notably, the orexins colocalize with many neuropeptides, such as prolactin (Risold et al. 1999), pentraxin (Reti et al. 2002), dynorphin (Chou et al. 2001), and galanin (Håkansson et al. 1999). Many orexin neurons are also glutamatergic as shown by their expression of the vesicular glutamate transporters (Rosin et al. 2003; Torrealba et al. 2003). Of the two peptides, OX-A is the most lipophilic, has been detected in the CSF and plasma, and is able to cross the BBB in both directions (Kastin and Akerstrom 1999).

3 Orexin Receptors: Common Signaling with CB1

Orexins act via OX-1R and OX-2R, which are GPCRs belonging to the rhodopsin family (Sakurai et al. 1998). OX-2R couples to the G_q , G_s , or $G_{i/o}$ subclasses of G proteins and has equal affinity for OX-A and OX-B (reviewed in Kukkonen and Leonard 2014). OX-1R preferentially couples to G_q (Sakurai et al. 1998) and is activated ten times more potently by OX-A than by OX-B. In many regions of the brain, OX-1R and/or OX-2R are distributed complementary to OX-positive projections, especially in the prefrontal and infralimbic cortex, CA of hippocampus, amygdala, BST, paraventricular thalamic nucleus, anterior hypothalamus, DR, VTA, LC, LDT/PPT nucleus (Trivedi et al. 1998; Lu et al. 2000; Marcus et al. 2001), ARC, TMN, DMH, PVN, LH, and medial septal nucleus (Lu et al. 2000; Marcus et al. 2001). OX-1R and OX-2R are also present in peripheral tissues (kidney, adrenal, thyroid, testis, ovaries, lung, pituitary, and jejunum) (Jöhren et al. 2001). Ca^{2+} signaling is among the first functional response triggered by OX-A-mediated stimulation of OX-1R (Sakurai et al. 1998). Indeed, orexin receptors regulate receptor-operated calcium channels (ROCCs), which include nonselective cation channels (NSCCs, such as transient receptor potential (TRP) channels) and Na^+/Ca^{2+} exchanger channels (Louhivuori et al. 2010). In neurons and other excitable cells, unlike CB1, OX-1R also activates N-type voltage-gated Ca^{2+} channels (Uramura et al. 2001). OX-A-mediated elevation of intracellular Ca^{2+} concentration is dependent from many different pathways, including ROCCs, SOCCs, and IP3-mediated Ca^{2+} release (Fig. 1). Unlike CB1, which inhibits AC activity, OX-1R regulates AC activity in both a positive and negative manner (Tang et al. 2008). Both CB1 and OX-1R activate ERK1/2 (Milasta et al. 2005; Ammoun

et al. 2006a) and PI3K (Ammoun et al. 2006a; Skrzypski et al. 2011). Upon activation by OX-1R, PKC activates NSCCs (Xia et al. 2009), PLD (Jäntti et al. 2012), and L- and N-type Ca^{2+} channels and inhibits the inwardly rectifying K^{+} channels (Nakajima and Nakajima 2010). Notably, OX-1R modulates the CB1-mediated synaptic plasticity (Borgland et al. 2006; Selbach et al. 2010; Yang et al. 2013) by being able to activate PKC which, in turn, inhibits CB1 activity (Garcia et al. 1998; Uramura et al. 2001). Furthermore, OX-1R activates PLC, PLA2, and PLD (Lund et al. 2000; Johansson et al. 2007, 2008; Turunen et al. 2010, 2012; Jäntti et al. 2012). PLC activation leads to the production of DAG, which can be directly deacylated by DAGL generating a monoacylglycerol (possible 2-AG) or phosphorylated to phosphatidic acid (PA). Both DAG and PA are important lipid mediators with many targets (Fig. 1). PLA2 hydrolyzes glycerophospholipids at the *sn*-2 position by releasing a free fatty acid, mainly arachidonic acid (AA), a precursor of prostaglandins and leukotrienes. AA inhibits the activity of K^{+} and Na^{+} channels and of L- and N-type Ca^{2+} channels, while it can activate TRP channels such as the vanilloid TRPs, TRPV1 and TRPV4 (Kukkonen 2011) (Fig. 1) (reviewed in Meves 2008). Orexin receptors activate PLD, which produces PA and free choline starting from phosphatidylcholine (PC) as a substrate. PA activates many kinases such as phosphatidylinositol-4-phosphate 5-kinase, which generates PIP2, thereby stimulating mitogen-activated protein kinase kinase kinase (MAPKKK) Raf1 and protein kinase C ζ . Furthermore, PA can be converted to DAG by phosphatidic acid phosphohydrolase (PAP) or in lyso-PA (LPA) by PLA1 or PLA2 (Fig. 1). Orexin

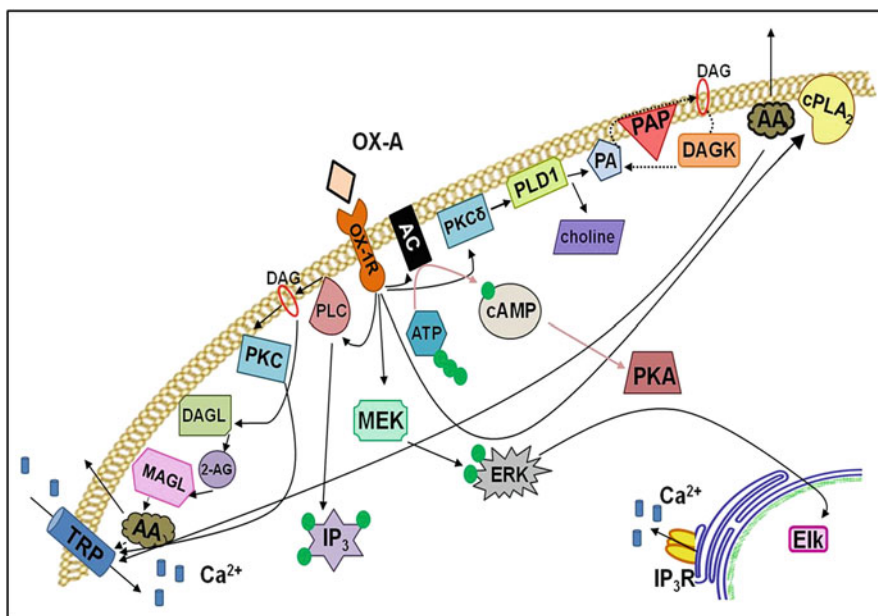


Fig. 1 Simplified overview of orexin receptor signaling

receptor-induced production of DAG and PA may affect CB1 activity through the formation of 2-AG, but the physiological relevance of this remains to be shown.

4 Anatomical Overlap Between Cannabinoid and Orexin Receptor Distribution in the Brain

To the best of our knowledge, no extensive study has been yet performed to describe the anatomical map of cannabinoid and orexin co-distribution in the brain. Evidence from separate studies concerning CB1 or OX-1R or OX-2R suggests an overlap of expression in critical areas involved in the overall regulation of appetite and metabolism, reward, nociception, and sleep/wake cycle. Thus, OX-1R and OX-2R, as well as CB1, are expressed with different densities in all the hypothalamic nuclei, including ARC, DMH, LHA, PVN, SON, and VMH. Prefrontal and infralimbic cortex, CA of hippocampus, amygdala, BST, paraventricular thalamic nucleus, DR, VTA, LC, LDT/PPT nucleus TMN, and medial septal nucleus also express both cannabinoid and orexin receptors (Fig. 2).

5 Molecular Interactions Between Cannabinoid and Orexin Receptors

In 2003, Hilairiet et al. provided for the first time, in CHO cells stably co-transfected with CB1 and OX-1R, evidence for a functional cross-talk between these receptors. Indeed, co-expression of both receptors enhanced the ability of OX-A to stimulate

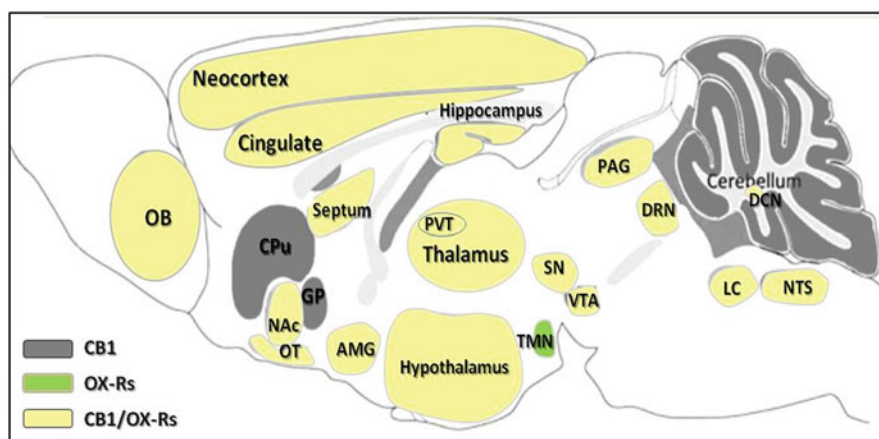


Fig. 2 Anatomical map showing different densities of cannabinoid and orexin receptor co-distribution in the brain

the ERK1/2 pathway ~100-fold more as compared to what is observed when only one of the receptors is expressed in cells and in a manner prevented by SR141716A (a CB1 antagonist/inverse agonist, also known as rimonabant), as well as by pertussis toxin. The authors explained this effect with a direct functional involvement of CB1 in the formation of heteromeric complexes with OX-1R also on the basis of electron microscopy study showing these receptors closely enough to form hetero-oligomers in CHO cells (Hilairt et al. 2003). This possibility was further investigated by Ellis et al. (2006) by using HEK293 cells co-expressing both receptors and displaying spontaneous OX-1R and CB1 internalization following cell exposure to OX-A. In this study, rimonabant reduced the potency of OX-A to activate the MAP kinases ERK1/2 in cells expressing both receptors. Likewise, the OX-1R antagonist SB674042 reduced the potency of the CB1 agonist WIN55,212-2 to phosphorylate ERK1/2. More importantly, orchestrated OX-1R and CB1 trafficking occurred in HEK293 cells following inducible expression of these receptors since (i) treatment with rimonabant resulted in the re-localization of CB1 to the cell surface and (ii) treatment with SB674042 (a selective OX-1R antagonist) induced redistribution of both receptors to the cell surface. Further evidence concerning physical interactions between OX-1R and CB1 was demonstrated by labeling covalently the extracellular domains of CB1 and OX-1R with SNAP-tag® and CLIP-tag™, respectively (Ward et al. 2011). By this approach CB1/OX-1R heteromerization was clearly identified by single-cell fluorescence resonance energy transfer (FRET) assay, which established that CB1 and OX-1R were close enough to produce true heteromers (dimers or oligomers). Besides heteromerization, a further mechanism has been proposed to explain CB1-dependent potentiation of OX-A signaling to ERK1/2. This is based on the enhancement of 2-AG synthesis by OX-A-mediated activation of the PLC-DAGL pathway via OX-1R (Turunen et al. 2012). Since both OX-1R and CB1 activate ERK1/2 (Bouaboula et al. 1995; Ammoun et al. 2006b), the heterologous or constitutive expression of OX-1R and CB1 in cells very strongly potentiates OX-A signaling to ERK1/2 (Turunen et al. 2012; Jäntti et al. 2013; Kukkonen and Leonard 2014). Therefore, the orexinergic potentiation of ERK1/2 phosphorylation in OX-1R-/CB1-CHO-transfected cells could be due to CB1 receptor activation by 2-AG and hence to the amplification of OX-1R intracellular signaling by endocannabinoid activation of CB1, instead of receptor di-/oligomerization. Emerging studies in rodents provide pharmacological evidence that OX-A enhances 2-AG levels in different regions of the brain including LH (Cristino et al. 2013a, b), PAG (Cristino et al. 2016), and ARC (Morello et al. 2016), possibly by Gq(OX-1R)-PLC-DAGL α -2-AG pathway which is crucial in the modulation of autocrine and paracrine functions and regulation of synaptic transmission (Fig. 3).

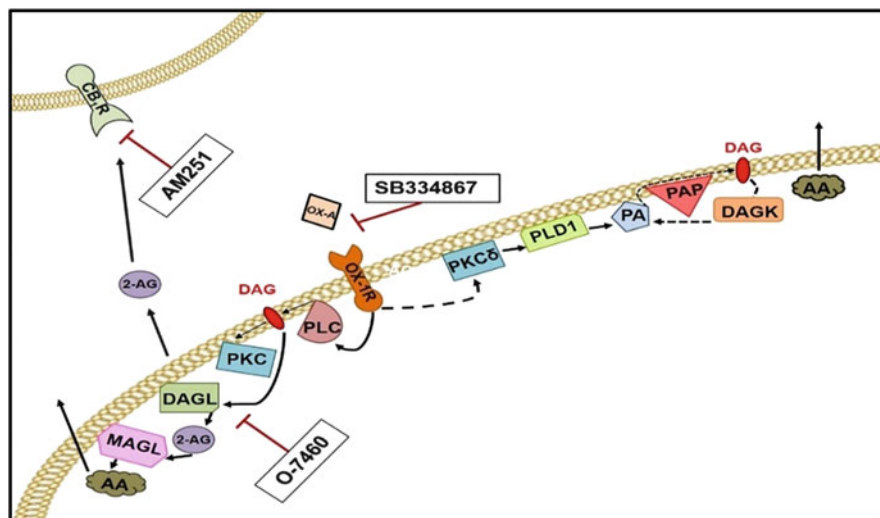


Fig. 3 OX-A signaling cascade regulates 2-AG synthesis and affects CB1 signaling in autocrine and paracrine way

6 Functional Interaction Between Cannabinoid and Orexin Receptors Under Leptin Signal Deficiency: Emerging Studies

Because of their local fast and fine regulation of production, endocannabinoid levels change according to rapid changes in metabolic requirements of the body. Thanks to the pivotal study of Di Marzo and colleagues (Di Marzo et al. 2001), an inverse relationship between leptin and the endocannabinoids (2-AG or AEA) has been established in the hypothalamus of leptin signaling-deficient obese (*ob/ob* and *db/db*) mice. A previous study found that the CB1 antagonist/inverse agonist rimonabant was able to reduce the food consumption in fasted mice (Colombo et al. 1998). Subsequently, both AEA and 2-AG levels were found to increase or decrease in fasted and sated mice, respectively (Kirkham et al. 2002), whereas CB1-deficient mice were shown to exhibit a leaner phenotype and resistance to diet-induced obesity, also because of lower food intake (Cota et al. 2003). Leptin is a protein of 167 amino acids mainly produced by adipocytes (Fox 2006) and released into the bloodstream. Leptin crosses the BBB by a saturable system (Banks et al. 1996) and signals to the brain to stop eating by inhibiting NPY/AgRP neurons and activating POMC/CART neurons in the arcuate nucleus of the hypothalamus (Schwartz et al. 2000; Sahu 2003) (Fig. 4). Notably, the amount of the circulating leptin is proportional to adiposity and body weight, both in mice and humans (Considine et al. 1996), and leptin administration in rodents causes a profound decrease in food intake and weight loss (Friedman and Halaas 1998). Rodents with genetic defective leptin signaling are obese, as in the case of *db/db* mice (knockout

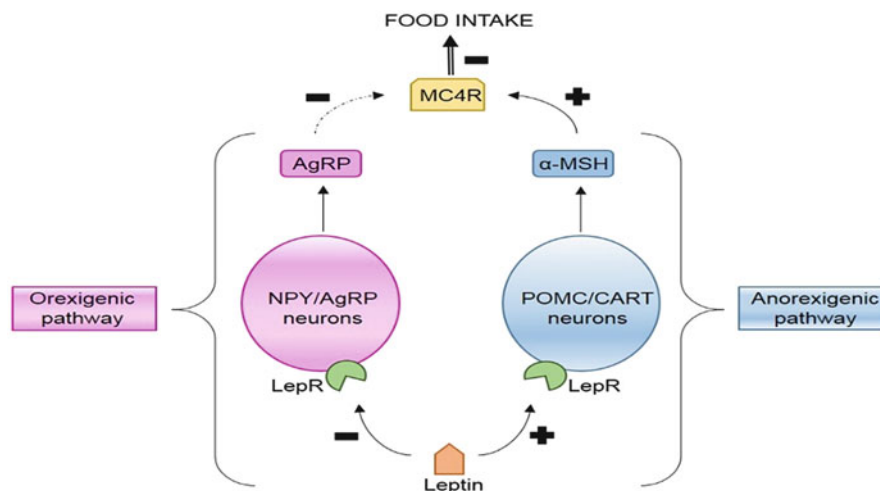


Fig. 4 Schematic representation of leptin signaling at hypothalamic neurons of ARC

for the *Ob-Rb* gene coding the leptin receptors) or *ob/ob* mice (knockout for the *Ob* gene coding for leptin) (Hill et al. 2010).

6.1 Appetite and Energy Homeostasis

Unlike anatomical studies, growing data are accumulating to unravel the functional cross talk between orexinergic and cannabinoid systems in the regulation of appetite and energy balance. In this regard, the first study concerning the regulation of appetite was by Crespo et al. (2008). By using i.c.v. injection of orexin in pre-fed rats, they reported a dose-dependent increase in the short-term feeding behaviors. The authors also tested the effect of the CB1 inverse agonist SR141716 (rimonabant) when given alone and observed a decrease in feeding behaviors. Finally, the combined effect of orexin and SR141716 was tested. Intraperitoneal injection of SR141716, 10 minutes prior to i.c.v. orexin injection, blocked the orexin-induced feeding already at doses that did not reduce feeding when used alone (Crespo et al. 2008). Similar to a CB1 agonist, also OX-A or OX-B administration stimulates food intake in mice, whereas the OX-1R antagonist, SB334867, reduces feeding (Sakurai et al. 1998; Haynes et al. 2000; Shiraishi et al. 2000). As with endocannabinoid levels, an inverse relationship occurs between circulating leptin and orexin levels. Indeed, fasting results in the upregulation of PPO mRNA (Sakurai et al. 1998), also in obese mice (Yamanaka et al. 2003). However, the regulation of food intake by endocannabinoids at the hypothalamic level results more complex than what was initially believed, because of the occurrence of bimodal orexinergic vs. anorexigenic effects of CB1 activation, depending on its

expression at glutamatergic or GABAergic inputs. In this scenario, the study of Huang et al. (2007) is clearly understandable, as the authors suggest that fasting-related reduction of leptin levels controls arousal by increasing of orexinergic neuron activity. By using patch-clamp recordings, Huang et al. demonstrated that WIN 55,212-2, a cannabinoid agonist, was able to depolarize MCH neurons, whereas it reduced the spontaneous firing of OX neurons in a manner prevented by AM251 and tetrodotoxin. Using conditions inducing DSI or DSE applied to MCH or OX neurons, the authors revealed that both MCH and OX neurons release endocannabinoids and are innervated by CB1-expressing inhibitory and excitatory inputs, respectively. Notably, the regulation of food intake controlled by endocannabinoids appears further complicated by the observation that hypothalamic circuits are affected by synaptic rewiring regulated by several circulating hormones (leptin, ghrelin, glucocorticoids, etc.). Cristino et al. (2013a, b) investigated the obesity-associated changes in the hypothalamic circuits of orexinergic neurons in the LH. This study employed leptin signaling-deficient mice such as *ob/ob* mice and mice with leptin insensitivity in the ARC caused by high-fat diet (HFD)-induced obesity. It was found that, in these obese mice, in comparison with respective lean control mice, (i) CB1-expressing presynaptic inputs to orexin-A neurons change from predominantly excitatory to inhibitory inputs; (ii) OX-A neurons are able to synthesize 2-AG more than in lean control mice because of elevation of DAGL α expression; and (iii) DSI occurs at OX-A neurons with consequent disinhibition of OX-A neurons and elevation of OX-A trafficking and release to many LH target areas like ARC, PVN, PAG, NAcc, and VTA. The authors also preliminarily investigated the mechanism of the synaptic remodeling at OX-A neurons and found that lack of leptin signaling in the ARC of obese mice, where from most of the fibers innervating orexinergic neurons originate, was the most likely cause. Leptin treatment reversed the synaptic remodeling only in *ob/ob* and not in HFD mice, indicating that this phenomenon is a consequence of leptin deficiency or leptin resistance in the ARC. Interestingly, possibly because *ob/ob* mice lacked endogenous leptin when weaned, but they received it from their heterozygous mothers during lactation, the remodeling of their synapses only occurred after weaning and was reversed by exogenous leptin injection (Cristino et al. 2013a, b) (Fig. 5). Recent findings from Morello et al. (2016) showed a CB1 and OX-1R interaction also in the regulation of POMC neurons, the master subset of the ARC anorexigenic hypothalamic neurons which act to reduce appetite and body weight (Schwartz et al. 2000; Cone 2005). Indeed, ablation of POMC neurons, or loss of α -MSH production, leads to obesity (Yaswen et al. 1999; Balthasar et al. 2004). Manyfold anatomical evidence, including our previous study (Cristino et al. 2013a, b), reveals that POMC neurons send projections to OX-A and regulate OX-A expression (López et al. 2007). OX-A neurons, in turn, send inputs back to POMC neurons (Chemelli et al. 1999) underlying a neuronal circuit perfectly organized to ensure food seeking accompanied by alertness. Notably, CB1 agonism at POMC neurons acutely enhances feeding in a dose-dependent manner (Gómez et al. 2002; Koch et al. 2015) without affecting α -MSH release at low doses (Koch et al. 2015). Furthermore, α -MSH, at doses effective at reducing food intake, does

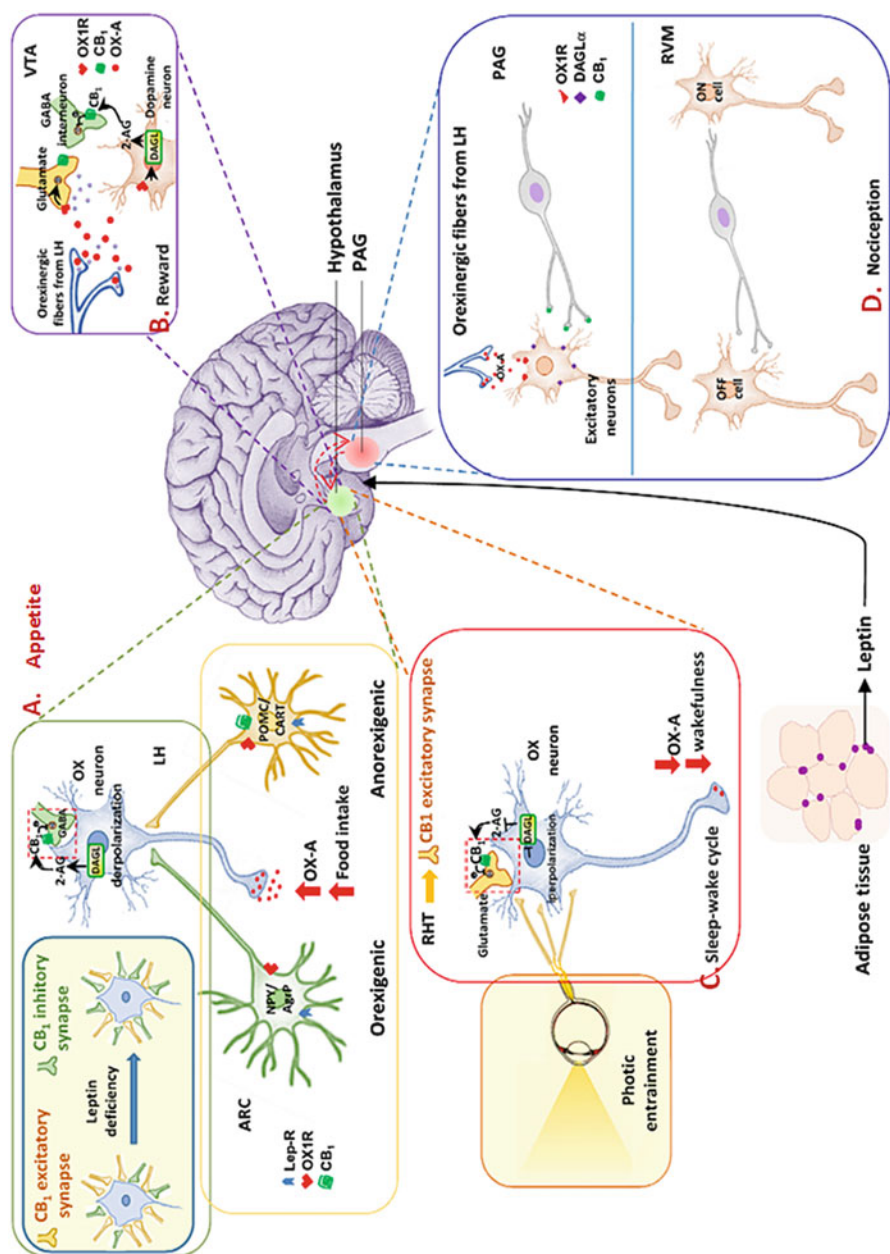


Fig. 5 (continued)

not alter hypothalamic endocannabinoid levels (Matias et al. 2008). In this scenario Morello et al. (2016) found that prolonged OX-1R activation by OX-A promotes appetite by blunting POMC production and α -MSH release as a consequence of OX-A-induced potentiation of 2-AG synthesis and CB1 activation at POMC neurons of mice under satiety state. This pathway relies its mechanism on the potentiation of ERK1/2-mediated Ser-727 phosphorylation/inhibition of STAT3 which affects negatively the expression of the *Pomc* gene (Fig. 6). To further support the occurrence of this autocrine mechanism, the authors also verified its existence in cultured POMC primary neurons. Several preclinical and clinical observations showing an association between obesity and ECS dysregulation in both central and peripheral tissues (Matias and Di Marzo 2007; Bermudez-Silva et al. 2010), as well as the occurrence of a positive correlation between the plasma endocannabinoid levels and markers of obesity or metabolic disorders, have been reported (Engeli et al. 2005; Cota 2007; Di Marzo et al. 2009; Abdunour et al. 2014). The finding that an endogenously produced peptide such as OX-A can modulate the function of CB1 might shed light on the hyperphagic action of endocannabinoids and on the functional consequences of the inverse relationship between leptin and endocannabinoid levels occurring during obesity also in humans (Monteleone et al. 2005; Nicholson et al. 2015). Indeed, during obesity, the aberrant activation of OX-A-mediated endocannabinoid biosynthesis triggered by deficits in leptin signaling at POMC neurons causes a vicious circle with inhibition of POMC synthesis, hyperphagia, further body weight gain, hypertension, and dysmetabolism, such as hepatosteatosis (Morello et al. 2016; Imperatore et al. 2017). The discovery of this leptin/orexin/endocannabinoid/ α -MSH signaling loop and its impact on hyperphagia, obesity, and fatty liver now call for investigations on potential synergies between OX-1R antagonists, CB₁R antagonists, and MC4R agonists for the reduction of body weight. This, in turn, might pave the way



Fig. 5 (continued) Scheme of the leptin-mediated interaction between cannabinoid and orexinergic systems in the control of food intake, sleep/wake cycle, nociception, and reward. Overview of the proposed anatomical pathways, synaptic receptor distribution, and mechanism of endocannabinoid-/orexin-modulated functions. **A.** Appetite. A leptin-driven synaptic rewiring occurs at OX-A neurons. In obese mice, the CB1-positive inputs to OX-A neurons shift from predominantly excitatory to inhibitory drive with consequent disinhibition of OX-A release to LH target areas and increase in food intake. **B.** Reward and seeking behavior. The OX-A-mediated activation of the GqCPR(OX-1R)-PLC-DAGL α -2AG pathway at VTA leads to DSI at CB1-positive inputs to DA neurons and consequent disinhibition of DA release and decrease on reward and seeking behavior. **C.** Wakefulness. OX-A neurons are entrained by a blue light-mediated activation of the retinohypothalamic pathway (RHT). This phenomenon induces a 2-AG synthesis at the OX-A target neurons and consequent DSE-mediated inhibition of OX-A neurons. These effects reduce both OX-A release and wakefulness. **D.** Analgesia. Incoming nociceptive signals are under the control of descending excitatory projections from vPAG neurons to RVM. These projections undergo disinhibition by glutamate release and 2-AG-mediated DSI, respectively, at OX-A- and CB1-positive inputs to vPAG output neurons to ON and OFF cells in the RVM. Hypothalamic leptin signal deficiency potentiates OX-A release to PAG and facilitates disinhibition of descending antinociceptive pathway and analgesia

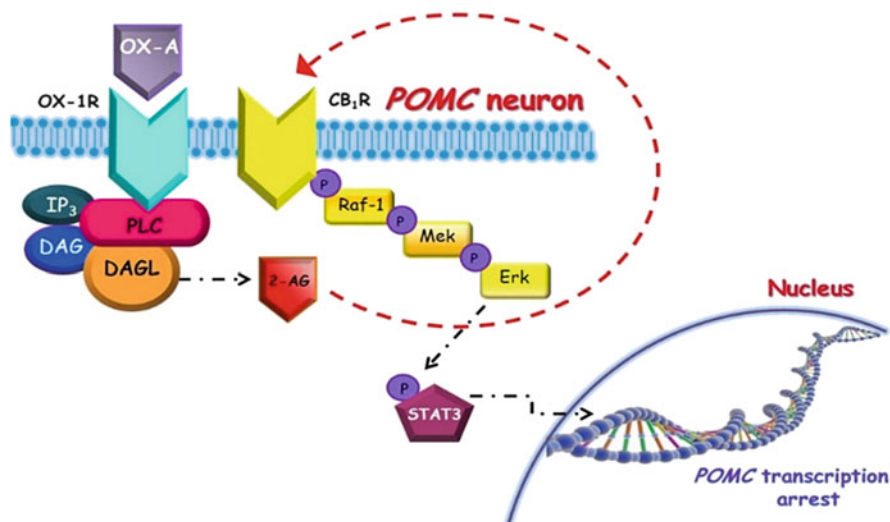


Fig. 6 Scheme of the molecular pathway underlying OX-A-mediated CB₁ activation at POMC neurons. OX-A-mediated CB₁R activation via 2-AG production contributes to increase the OX-A potency to phosphorylate ERK1/2 (pERK1/2^{Thr202/Tyr204}) in POMC neurons concurrently with enhancement of STAT3 phosphorylation (pSTAT3^{Ser727}). These effects result in the lowering of POMC production and POMC-derived α -MSH levels

to new safer and more efficacious polypharmacological or multi-target treatments against metabolic disorders (Kälin et al. 2015).

6.2 Reward, Arousal, and Seeking Behavior

Orexin and endocannabinoids have been shown to be involved in reward processes, drug-seeking behavior, and addiction in animal models and human (España 2012; Oleson et al. 2012; Baimel et al. 2015; Hernandez and Cheer 2015). Orexinergic neurons project to the VTA and NAcc, the master regions of the brain critical for motivation and reward behavior (Wise and Rompre 1989; España 2012) whose activation is associated with preferences for cues linked with drug and food rewards (Harris et al. 2005). In particular, in the VTA, orexin directly activates dopaminergic neurons through OX-1R (Nakamura et al. 2000) or affects glutamate release onto dopaminergic neurons, inducing excitatory synaptic plasticity (Borgland et al. 2010; Baimel and Borgland 2012) and causing DA release in VTA target regions, such as the NAcc (Vittoz and Berridge 2006; Narita et al. 2006). More recently, orexin was also linked to cannabinoid-induced reward. Chiou and collaborators have proposed another cellular mechanism through which orexin can increase VTA dopaminergic activity by triggering endocannabinoid synthesis (Chiou et al. 2013). Endocannabinoid production and release have been found to occur at VTA-DA

neurons (Alger 2002; Melis et al. 2004). Electrophysiological studies demonstrated that activation of OX-1R in VTA-DA neurons initiates a Gq/11-coupled PLC-DAGL pathway leading to the biosynthesis of 2-AG, which, retrogradely, activates CB1 at both inhibitory and excitatory inputs to DA neurons, thus regulating burst firing (French 1997; Wu and French 2000) and neurotransmitter release (Chen et al. 1990; Cheer et al. 2007; Oleson et al. 2012) of VTA-DA neurons. In resting conditions, around 50% of DA neurons are innervated by inhibitory GABAergic inputs (Grace and Bunney 1984). The retrograde activation of CB1 on GABA inputs results in increase of VTA-DA firing and DA release to target areas (Overton and Clark 1997; Zweifel et al. 2009; Máttyás et al. 2008; Chiou et al. 2013). However, although in normal conditions the final endocannabinoid-mediated modulation of DA neuron activity depends on the functional balance between inhibitory and excitatory inputs to these neurons, acute restraint stress activates orexinergic neurons leading to downstream release of OX-A to the VTA and consequent activation of OX-1R of DA neurons. This event triggers the synthesis of 2-AG, which, through a retrograde inhibition of GABA release, induces disinhibition of VTA-DA neurons and initiation of seeking behavior. The opposite scenario occurs in the DR, where the OX-B-mediated inhibition of glutamate release is due to OX-2R-induced enhancement of 2-AG release and activation of CB1 at excitatory inputs and depression of glutamate-mediated synaptic currents of DR serotonergic neurons, as demonstrated by patch-clamp study in male rats (Haj-Dahmane and Shen 2005) (Fig. 5). This effect was mimicked by WIN55,212-2 and abolished by AM-251, confirming the involvement of CB1 receptors. The inhibition of glutamate release was also counteracted by inhibition of G-protein signaling in postsynaptic neurons by GDP β S, a non-hydrolyzable analog of GDP. These results were the first to suggest an orexin/endocannabinoid interaction in the regulation of DR serotonergic neurons by orexin-induced postsynaptic release of ECs. Since serotonergic neurons are activated during wakefulness, the inhibitory action of OX-B, an arousal-increasing neuropeptide, on these neurons therefore seems paradoxical. The authors, however, speculated that this negative feedback induced by endocannabinoid release is necessary for preventing excessive neuronal excitation, therefore ensuring a stable firing (Haj-Dahmane and Shen 2005). Accordingly, the orexin and endocannabinoid interaction is important in the physiological regulation of arousal. In agreement with the role of endocannabinoids and orexins in the regulation of anxiety-like responses, the overlapping distribution of both their receptors has been described in the VTA, NAcc, PFCx, septal nuclei, and amygdaloid nuclei (Maldonado et al. 2006; Aston-Jones et al. 2010; Plaza-Zabala et al. 2012; Flores et al. 2015), besides the DR and LC. Therefore, it can be speculated that orexins exert orexigenic functions by controlling both appetite and reward circuits through stimulation of endocannabinoid release. This is of special relevance to the observation that orexins, via mesolimbic circuits, promote ingestion of highly salient substances (e.g., high-fat diet, drugs of abuse), at least in part, via direct projections onto VTA-DA neurons (Petrovich et al. 2012; Cason and Aston-Jones 2013), which promote NMDA receptor-mediated excitatory postsynaptic potentials at DA neurons and DA release into the NA and PFCx (Borgland

et al. 2006; Vittoz and Berridge 2006; España et al. 2010). However, release of orexin and glutamate is required for long-term potentiation of DA signaling that underlies cue-induced reinstatement (seeking) of rewards. Notably, given that both OX-A and OX-B are co-released with dynorphin and glutamate, it has been proposed that both the orexins potentiate the glutamate-mediated long-term modifications that underlie natural reward and addiction to drugs (Mahler et al. 2013). By contrast, lowering the orexins vs. dynorphin ratio suppresses reward responses (Muschamp et al. 2014) by inhibiting DA release to the NAcc and drug, food, or sucrose seeking (Abizaid et al. 2006; España et al. 2010; Sharf et al. 2010; Smith and Aston-Jones 2012; Srinivasan et al. 2012).

6.3 Sleep/Wakefulness

It has been observed that orexinergic neurons are active during wakefulness (Modirrousta et al. 2005), while MCH neurons are active in REMS (Verret et al. 2003). Notably, OX knockout (KO) mice exhibit a narcoleptic-like phenotype, with sudden transitions from wakefulness into REMS (Chemelli et al. 1999). Furthermore, it is known that eCBs have been implicated in sleep regulation because of their strong hypnogenic properties (Pérez-Morales et al. 2012). Indeed, THC, the primary psychoactive agent in marijuana and hashish, tends to generate a distorted sense of time (Tinklenberg et al. 1976). The CB1 agonist CP55940 attenuates light-induced clock-phase advance in hamsters (Sanford et al. 2008), and endocannabinoids, both 2-AG and AEA, show a circadian variation in the brain (Valenti et al. 2004). Interaction between the orexinergic and endocannabinoid systems in the sleep regulation has been described by Pérez-Morales et al. (2013) starting from evidence indicating that WIN55-212-2 depolarizes MCH neurons and hyperpolarizes OX-A neurons in *in vitro* LH slices (Huang et al. 2007). By injection of 2-AG in the LH of rats, Pérez-Morales and colleagues found that 2-AG increases REMS through a CB1 activation and increases c-Fos expression in MCH neurons, without affecting c-Fos expression in OX-A neurons. Furthermore, Cristino et al. (2013a, b) studied the orexinergic and endocannabinoid interaction in wakefulness. They found the endocannabinoid biosynthesis in the LH of mice induced by a light-modulated excitation of the retinohypothalamic tract (RHT). By electron and confocal microscopy, the authors found CB1 expression at RHT projections to OX-A neurons and identified the retinal ganglion cells/OX-A light-mediated circuit activated by blue light pulse by inducing *c-Fos* expression in the target OX-A neurons (Fig. 5). This activation was paralleled by 2-AG synthesis in a manner prevented by antagonism of mGluR5 receptors which were, in turn, found at OX-A neurons. Collectively, these data suggest that orexinergic and endocannabinoid interaction, in specialized neuronal pathways, regulates different aspects of sleep/wake cycle including the REMS onset and the blue light entrainment of circadian functions.

6.4 Nociception

One common function for both orexin and endocannabinoid systems is the modulation of pain perception at spinal and supraspinal levels. The antinociceptive descending PAG-RVM pathway is crucial in the regulation of pain. PAG contains glutamatergic neurons receiving inhibitory GABAergic inputs from the local interneurons. PAG-mediated control of pain occurs concomitantly with the modulation of pain-responding neurons of the RVM: the ON cells, which are activated, and the OFF cells, which are inhibited, by nociceptive stimuli. In the ventrolateral PAG (vlPAG), activation of excitatory output neurons projecting monosynaptically to OFF cells in the rostral ventromedial medulla (RVM) causes antinociceptive responses via OFF cell stimulation and ON cell inhibition in the RVM, which send inhibitory projections to the dorsal horn of the spinal cord (Behbehani and Zemlan 1990). By morphological approach based on the injection of the retrograde tracing CTB-Alexa488 in the RVM of mice, Cristino and colleagues revealed the occurrence of monosynaptic projections from vlPAG neurons to the OFF cells in the RVM. More importantly, by high-resolution electron microscopy, the authors found that these OX-A neurons targeted by RHT express DAGL α at the cytoplasmic side of the membrane, near to OX-1R at postsynaptic side of excitatory OX-A-positive inputs, and are innervated by CB1-positive inhibitory inputs coming from local GABAergic interneurons (Cristino et al. 2016) (Fig. 5). This study provided the anatomical contribution to understand the OX-1R-mediated PLC-DAGL-2-AG disinhibition of the PAG-RVM descending antinociceptive pathway found by Ho et al. (2011). Indeed, Ho and colleagues were the first to demonstrate that 2-AG can be generated by OX-A-mediated activation of OX-1R in the rat PAG by using O-7460 (a DAGL α inhibitor) during patch-clamp recording of vlPAG slices. They found that, unlike OX-B, OX-A depressed IPSCs in PAG slices in a manner dependent on OX-1R, CB1, PLC, and DAGL activity (Ho et al. 2011) and was mimicked by a MAGL inhibitor. These data suggest that activation of OX-1R at postsynaptic site, as well as activation of mGluR5 and M1/M3 mAChRs, initiates the OX-1R-mediated PLC-DAGL-2-AG disinhibition mechanism in the PAG, thereby contributing to analgesia induced by intra-vlPAG injection of OX-A during the rat hot plate test (Chiou et al. 2013). More recently, Lee et al. found that 30-min restraint stress in mice was able to induce analgesia during hot plate test in a manner prevented by intra-vlPAG injection or intraperitoneal injection of SB334867 or AM251, but not TCS-OX2-29 or naloxone. They found that, during stress, orexins lead to endocannabinoid generation in the vlPAG by engaging the OX-1R-mediated PLC-DAGL α -2-AG pathway, thereby inducing the establishment of stress-induced analgesia (Lee et al. 2016). Accordingly, Cristino et al. found that reduced-pain sensitivity and enhanced OFF-decreased ON cell activity occur in *ob/ob* mice wherein the leptin deficiency strongly enhances OX-A release to vlPAG, among other LH targets. In *ob/ob* mice, these alterations result in (i) increased OX-1R-mediated PLC-DAGL-2-AG disinhibition of vlPAG output neurons by DSI of the neighboring CB1-expressing GABAergic inputs; (ii) subsequent increase of OFF

and decrease of ON cell activity in the RVM, as assessed by patch clamp and in vivo electrophysiology; and (iii) analgesia, in both healthy and neuropathic mice. Notably, in HFD obese mice, analgesia was only unmasked following leptin receptor antagonism because of the leptin insensitivity of these mice. All these effects were mimicked by i.c.v. OX-A injection in lean mice and were markedly reduced by treatment with AM251 or SB in *ob/ob* mice at a dose inactive in wt mice (Cristino et al. 2013a, b). According to the orexin/endocannabinoid synergistic effect in the control of analgesia, Kargar et al. found that intra-LC microinjection of OX-A exerted antinociceptive function in the rat formalin test (a model of inflammatory pain), in a manner prevented by intra-LC microinjection of either SB334867 or AM251 (Kargar et al. 2015).

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How CB1 Receptor Activity and Distribution Contribute to Make the Male and Female Brain Different Toward Cannabinoid-Induced Effects

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Abstract Sex-dependent differences have been consistently reported in the prevalence and frequency of use of *Cannabis*, with patterns of use, subjective effects, and progression to dependence being different in male and female smokers. As confirmed by animal studies, cannabinoids exert sex-dependent effects in many physiological and behavioral aspects, including food intake and energy balance (stronger in males) and emotional regulation (stronger in females). Following chronic THC exposure during adolescence, cannabinoid receptors undergo desensitization, which is greater in adolescent female animals than in male animals. Preclinical research is greatly contributing to elucidate the neurobiological bases for sex differences in cannabinoid effects, among which different cannabinoid pharmacodynamic and pharmacokinetic and gonadal hormones play crucial roles. The sexual dimorphism of the brain in cannabinoid subtype 1 receptor (CB1R) distribution and function massively contributes to the variety of differences reported in cannabinoid-induced effects between the two sexes. The distribution and activity of neuronal CB1Rs in the male and female brains and how they can be differently affected by stress and drugs of abuse have been investigated in many brain areas. In this chapter, we will first provide an overview on the brain sexual dimorphism and the sex-dependent effects of cannabinoids. Then we will review both clinical and laboratory-based research evidence revealing important sex-related differences in CB1 receptor level and function in different brain areas. Finally, the influence of gonadal hormones in determining such differences will be discussed.

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1 Brain Sexual Dimorphisms and the Endocannabinoid System

Sex hormones begin to exert their influence on the brain and behavior during the development of the fetus and continue during adulthood (Gore et al. 2014). Various brain regions, like those implicated in sexual and reproductive behaviors (e.g., hypothalamus), display evident differences between males and females. Important differences between men and women have been described in brain circuits associated to stress and emotions as well as to memory and cognition (Cahill 2005). For example, men show larger volumes in the parietal cortex (which is involved in space perception), amygdala (which regulates sexual and social behavior), and hypothalamus (which controls sex drive and food intake), while women show larger volumes in the frontal cortex (responsible for problem-solving and decision-making) and the medial paralimbic cortex (responsible for emotion regulation) (Goldstein et al. 2001). Compelling human neuroimaging *in vivo* studies have revealed sex-specific differences in brain functions, neural structure, and neurochemistry, thus highlighting the need of considering sex as a modulating factor in brain-related data interpretation (Sacher et al. 2013). Sex-dependent differences also exist at the cellular level, with men and women differing in both cell type and receptor density.

The endogenous cannabinoid system emerges relatively early during brain development. Similar to other brain neurotransmitter systems, the endocannabinoid system is sexually dimorphic, with male and female brains displaying different levels of endocannabinoids (Bradshaw et al. 2006), different endocannabinoid-mediated glial cell genesis in the developing brain (Krebs-Kraft et al. 2010), and different cannabinoid subtype 1 receptor (CB1R) density and affinity (Rodríguez de Fonseca et al. 1994). Parolaro and Viveros' groups, for example, showed that sex affects the endocannabinoid system in many brain areas and that CB1R density and functionality vary in the two sexes in a region-dependent manner (Zamberletti et al. 2012; Llorente-Berzal et al. 2013). Preclinical studies have consistently demonstrated that the density of CB1Rs fluctuates during the estrous cycle and that the affinity of agonist binding is also affected by sex hormones (Rodríguez de Fonseca et al. 1994; Riebe et al. 2010). Moreover, treatment with delta9-tetrahydrocannabinol (delta9-THC) induced greater desensitization of brain CB1Rs in female than in male rats, an effect more evident in adolescent than in adult animals (Burston et al. 2010). These and other more recent findings support the need of considering sex and menstrual phases when analyzing CB1R level and activity in humans (Normandin et al. 2015). The endocannabinoid system is also particularly sensitive to early life stress in a sex- and brain region-dependent manner, with changes evident at gene expression level already in the adolescent brain (Marco et al. 2014).

Human studies revealed that the number of CB1Rs increased with aging in females but not in males (Van Laere et al. 2008). In particular, women show an age-dependent increase of CB1Rs in the basal ganglia, lateral temporal cortex, and

hippocampus, while men display an enhanced binding in the limbic system and cortico–striato–thalamic–cortical circuit. Intriguingly, adolescent cannabinoid exposure induces long-term and sex-dependent effects on adult hippocampal neurogenesis and response to stress (Lee et al. 2014). Recently, Woolley's group showed that the compound URB597, a fatty acid amide hydrolase inhibitor which blocks the breakdown of the endogenous cannabinoid anandamide, strongly suppressed inhibitory synapses in female but not male brains, indicating tonic endocannabinoid release in females that is absent in males (Tabatadze et al. 2015). Noteworthy, such a difference is not related to circulating reproductive hormones.

2 Sex-Dependent Effects of Cannabinoids

While there are no racial or ethnic differences for *Cannabis* use worldwide, the prevalence of marijuana smoking, frequency of use, and vulnerability to develop dependence are different between sexes (UNODOC 2015), in both humans and animals (McGregor and Arnold 2007; Fattore et al. 2009; Cooper and Haney 2014). Marijuana is the first illicit drug to be experienced by adolescents, with boys being in general more likely than girls to become “heavy users” of *Cannabis* (Kohn et al. 2004). In all European countries, more male students than females use *Cannabis*, while females are more likely to use sedative drugs (EMCDDA 2006). In Canada, among high school students smoking marijuana, more males than females report poor family relationships and problems at school (Butters 2005). In South America, the gender ratio of *Cannabis* use is lower for the adolescent than the adult population, with a higher proportion of female users in the most developed countries (UNODOC 2015). Gender differences have also been found in self-reported health-related quality of life among marijuana users (Lev-Ran et al. 2012).

Cannabinoids have been shown to influence physiology and behavior in a sex-dependent manner (Craft et al. 2013; Wagner 2016). The effects of cannabinoids on food intake and energy balance, for example, are more manifest in males, while their effects on anxiety-like states and depression are more evident in females (Diaz et al. 2009; Fattore and Fratta 2010). Female marijuana smokers show lower circulating levels of delta9-THC (Jones et al. 2008) and weaker cardiovascular and subjective effects than male smokers (Leatherdale et al. 2007). Notably, male non-marijuana smokers are more sensitive than women to the subjective effects of delta9-THC (Haney 2007). Yet, female marijuana smokers report more frequent dizziness and higher susceptibility to cannabinoid-induced hemodynamic changes and visuospatial memory impairment than male smokers (Mathew et al. 2003). Moreover, women smoking marijuana show less evident withdrawal symptoms than men (Crowley et al. 1998), are more likely to smoke marijuana mostly when under pressure or anxious (Patton et al. 2002), avoid using other drugs (i.e., use *Cannabis* only), and have a lower prevalence of panic attacks and personality disorders (Hasin et al. 2008). The beneficial effects of *Cannabis* use on sexual

functioning are consistently reported by women, while men report both facilitatory and inhibiting effects following *Cannabis* use (Gorzalka et al. 2010). In rodents, catalepsy, antinociception, and changes in locomotor activity typically induced by cannabinoids are more evident in females than in males (Tseng and Craft 2004), while cannabinoid-induced effects on exploratory behavior and anxiety are manifest in females only (Biscaia et al. 2003). Differences related to gender (i.e., sociocultural distinctions between men and women) and sex (i.e., anatomical and physiological distinctions between males and females) have also been reported in impulsive actions and compulsive behavior (Fattore and Melis 2016a) as well as in other dysregulated behaviors (Fattore and Melis 2016b).

3 CB1R Density and Function in the Male and Female Brain

Within the brain, neuronal CB1Rs are not distributed homogeneously, being present at higher concentrations in areas regulating cognition and short-term memory (cerebral cortex and hippocampus) and motor functions (basal ganglia and cerebellum) (Herkenham et al. 1990; Tsou et al. 1998; McPartland et al. 2007). CB1Rs are also present in astrocytes and microglia (Stella 2010; Bosier et al. 2013). Similarly, the cannabinoid subtype 2 receptors (CB2Rs) have been detected in microglia, in dendritic cells, in brain endothelial cells, and in subgroups of neurons in various brain regions (Mackie 2005; Maresz et al. 2005; Van Sickle et al. 2005). However, whether sex affects the density and activity of glial CB1Rs or brain CB2Rs is still not known.

This chapter focuses on the distribution and activity of neuronal CB1Rs in brain areas where sex-dependent differences have been explored both at physiological conditions and in response to different events. In particular, sex-dependent differences in CB1R level and function following stressful events (e.g., maternal deprivation) and exposure to drugs of abuse (e.g., cocaine) are reviewed and discussed for each brain area.

3.1 Prefrontal Cortex

The prefrontal cortex is associated with high-order cognitive and memory functions, encodes relevant information in decision-making and goal-directed behavior, and is involved in the storage of information and in control processes, e.g., monitoring and selection (Dembrow and Johnston 2014). Together with the hippocampus and the amygdala, the medial part of the prefrontal cortex regulates fear conditioning and extinction processes likely exerting a top-down control over subcortical structures to induce appropriate behavioral responses. CB1Rs are widely represented in the prefrontal cortex (Glass et al. 1997), which could explain

why marijuana use is associated to impairments in working memory, mental flexibility, and selective and sustained attention.

Sex differences in the rat prefrontal cortex have been described for both CB1R density and functionality. One study reported females showing lower basal levels of CB1Rs but higher CB1R activity when compared to male counterparts (Llorente-Berzal et al. 2013), while other studies reported discrepant findings (Zamberletti et al. 2012; Xing et al. 2014). Notably, early maternal separation and adolescent exposure to drugs of abuse were found to affect prefrontal CB1R density and functionality in a sex-dependent manner. That is, after early life stress (i.e., maternal separation) followed by adolescent cocaine exposure, reduced expression and functionality of CB1Rs in the prefrontal cortex were observed in male rats but not in females (Llorente-Berzal et al. 2013). Conversely, following adolescent exposure to nicotine, both male and female rats showed increased CB1R activity (Mateos et al. 2010), while after repeated tail-shock stress, CB1R mRNA expression was decreased in females only (Xing et al. 2014). Also a high-fat diet influences the functionality of CB1Rs in the prefrontal cortex, since it increased CB1R function, but not density, in females (Rojo et al. 2013). Yet, whether the same occurs in males, and to which extent, remains to be determined. Cycling female rats have lower density of CB1Rs than male and ovariectomized female rats in the prefrontal cortex, whereas no sex-dependent differences are present in the functionality of CB1Rs (Castelli et al. 2014). Notably, while ovariectomy increases CB1R expression, replacement with estradiol reduces the amount of CB1Rs to the levels of cycling females, suggesting that ovarian hormones might negatively affect CB1R density in brain areas involved in cognition and emotional processing.

3.2 *Thalamus*

The thalamus is located in the central portion of the diencephalon and is formed by groups of distinct nuclei, e.g., anterior, posterior, medial, lateral, pulvinar, and reticular nuclei. Extensively connected to virtually all areas of the cerebral cortex, to the brain stem, and to the spinal cord, the thalamus acts as a brain–body *gateway* and represents the most important relay station in the brain (Wolff et al. 2015). By filtering the unnecessary information and allowing conscious mind to register relevant signals only, it is considered to crucially regulate sensory information processing, pain perception, attention, sleep, arousal, learning, cognition, memory, and decision-making (Jones 2007; Baxter 2013; Funahashi 2013; Mitchell and Chakraborty 2013; Mitchell et al. 2014; Mitchell 2015).

This brain area shows a distinctive heterogeneous distribution of CB1Rs, with the highest level located in thalamic nuclei connected with the associational cortical areas (Glass et al. 1997). Curiously, synthetic cannabinoid users show a gray matter density reduction in the right and left thalamus when compared with the healthy control group (Nurmedov et al. 2015). The endocannabinoid system modulates the sleep–wake cycle and the level of arousal, by affecting thalamic rhythms and

oscillations (Dasilva et al. 2014), and pain perception, by decreasing nociceptive neurotransmission at the level of the spinal cord and the thalamus (Bradshaw et al. 2006). Altered levels of endocannabinoids in the brain are associated to altered pain sensitivity: activation of thalamic CB1Rs takes part to the antinociceptive efficacy of cannabinoids (Martin et al. 1996), and chronic pain may lead to a specific upregulation of thalamic CB1Rs (Siegling et al. 2001; Knerlich-Lukoschus et al. 2011). Low to moderate levels of estrogen receptor subtypes α - and β -mRNA were detected, respectively, in the subthalamic nucleus and the ventral lateral nucleus of the thalamus (Osterlund and Hurd 2001). It is therefore possible that, being sexually dimorphic, endocannabinoid levels could account for sex differences in pain perception that women reported during different phases of the menstrual cycle (Iacovides et al. 2015).

Unfortunately, to date only few studies examined sex-dependent differences in the expression and function of CB1Rs in thalamic nuclei, most of them being performed on male subjects only. For example, social isolation (Sciolino et al. 2010), but not chronic stress (Hill et al. 2009), was found to affect the density of CB1Rs in the thalamus of male animals, but it is not known whether females are also affected and, if so, to which extent. Conversely, other studies were performed on females only, thus making not possible a direct comparison between the two sexes. A preclinical study that proposed inadequate playful interactions in adolescent animals as a novel model for studying social peer rejection, for example, demonstrated that in play-deprived female rats, the expression levels of CB1Rs were increased in the thalamus (Schneider et al. 2014). But whether the same occurs in male animals, or whether this phenomenon is sexually dimorphic, remains to be demonstrated. Sex-dependent differences in basal CB1R density and function in the thalamus were provided by Zamberletti et al. (2012), who found higher CB1R level and functionality in female rats than in males, and by Llorente-Berzal et al. (2013) that confirmed the higher level of CB1R functionality in females but reported higher CB1R density in males. Importantly, this latter study showed that thalamus CB1R density is significantly affected by early life stress and adolescent exposure to drugs of abuse in a sex-dependent manner. In fact, authors showed that early maternal deprivation caused a reduction in the expression of thalamic CB1Rs in males but not in females, while adolescent cocaine administration induced an increase in thalamic CB1R functionality in females but not in males (Llorente-Berzal et al. 2013).

3.3 *Hypothalamus*

The hypothalamus is a region comprised of several nuclei that is deputed to the integration of body functions for the maintenance of homeostasis, which is essential for survival and reproduction of the species (Seoane-Collazo et al. 2015). This brain region maintains homeostatic balance in physiology and behavior by integrating massive volumes of information (concerning, among other functions, feeding,

satiety, stress, and motivational states) and by assigning appropriate instructions to downstream effector systems (Haas and Lin 2012). The hypothalamus regulates the release of pituitary hormones and the body heat in response to variations in external temperature, influences caloric intake and weight regulation, determines wakefulness/sleep cycles, and regulates fluid intake and sensation of thirst.

The endocannabinoid system plays an important regulatory role on the hypothalamus and broadly interacts with the hypothalamic–pituitary–adrenal axis (Hill and Tasker 2012). The expression of CB1Rs in the hypothalamus is among the lowest in the brain; yet, activation of these receptors is particularly efficient and strongly affects the cross talk between the hypothalamic nuclei and peripheral organs. Among the hypothalamic nuclei, the ventromedial hypothalamic nucleus (involved in homeostatic and behavioral functions including food intake) and the paraventricular nucleus (controlling stress, metabolism, growth, reproduction, immune and autonomic functions) contain CB1Rs (Castelli et al. 2007; Reguero et al. 2011). The endocannabinoid system is intimately involved in appetitive and reward-related behavior. In the hypothalamus, the synthesis of endocannabinoids increases during brief starvation and decreases following food intake (Bellochio et al. 2006), while expression of CB1Rs increases during fasting (Burdyga et al. 2006).

In the hypothalamus, basal CB1R expression was reported to be higher in female than in male rats (Zamberletti et al. 2012; Xing et al. 2014). Hypothalamic CB1R functionality, but not density, is significantly influenced by neonatal manipulation in a different manner in male and female rats (Llorente-Berzal et al. 2013). Indeed, although females display a higher basal level of CB1R functionality than males (Llorente-Berzal et al. 2013), maternal deprivation significantly diminishes CB1R functionality in males (Llorente-Berzal et al. 2013) and slightly increases CB1R activity in females (Zamberletti et al. 2012). Similar to early life stress, also repeated stress during adolescence (i.e., tail-shock stress) is able to modify CB1R expression in the hypothalamus, by decreasing and increasing CB1R mRNA in stressed females and males, respectively (Xing et al. 2014). Such sex-dependent differences might be explained, at least in part, by the effects of estrogen on CB1R binding. Males and ovariectomized females, in fact, show higher levels of CB1R binding than intact females and estradiol-treated ovariectomized females (Riebe et al. 2010). Findings that ovariectomized females are not different from males in terms of hypothalamic CB1R binding and similar to cycling females when treated with high doses of estradiol suggest that estradiol decreases the expression of CB1Rs in the hypothalamus (Riebe et al. 2010).

3.4 *Hippocampus*

Located within the brain medial temporal lobe (underneath the cortical surface), the hippocampus is crucial for spatial navigation and storage of long-term memory (Hölscher 2003). It possesses an extraordinary capacity for structural

reorganization and controls different types of learning and habit formation processes. In many mammalian species, the total volume of the hippocampus is significantly larger in males than in females (Keeley et al. 2015), a phenomenon that could be due in part to sex-dependent changes in neurogenesis or sex hormone-specific effects.

The hippocampus is among the richest regions of CB1Rs, located presynaptically on the axons of GABAergic interneurons (Mackie 2005). Activation of hippocampal presynaptic CB1Rs has been reported to strongly inhibit GABA release from inhibitory networks in both humans and rats and to reduce the power of hippocampal network oscillations (Hájos et al. 2000). CB1Rs located in the hippocampus contribute to the amnesic-like effects produced in animals by cannabinoid agonists (Akirav 2011) and to impairments in cognition and learning produced in humans by acute or chronic use of marijuana (Paton and Pertwee 1973).

Hippocampal volume was reported to be larger in adult females exposed to delta9-THC during adolescence (Keeley et al. 2015) and to be related to gonadal hormone levels (Galea et al. 1999). Significant sex effects were found in both CB1R density and functionality within the hippocampus, although discrepant findings were also reported. In female rats, for example, Llorente-Berzal et al. (2013) found a slightly lower CB1R expression and a markedly higher CB1R activity than in males, while Zamberletti et al. (2012) reported higher levels of CB1Rs in females and no significant sex differences in CB1R functionality. A third study that used Lister hooded rats instead of Sprague Dawley or Wistar albino strains as in the above studies, found no significant differences between males and females in both CB1R density and function (Castelli et al. 2014). Discrepancies in CB1R density and function in different rat strains have been reported also for other brain areas and are in line with the numerous evidence showing that behavioral (Deiana et al. 2007; Renard et al. 2013; Manduca et al. 2014; Wakeford and Riley 2014), biochemical (Chen et al. 1991; Cadoni et al. 2015), and molecular (Arnold et al. 2010) effects of cannabinoids are strain dependent.

Recently, sex-dependent alterations in hippocampal CB1R expression following adolescent exposure to delta9-THC treatment have been reported in rats (Silva et al. 2015). Specifically, a downregulation of CB1Rs was found in both sexes at 24 h and 2 weeks post chronic treatment with delta9-THC in the CA2 region; however, in females it also persisted in CA1 and CA3 areas. Also Weed et al. (2016) reported sex-dependent long-term alterations in hippocampal CB1R levels after chronic adolescent treatment with delta9-THC. Yet, sex-dependent differences were not found in adolescent rats immediately after the last delta9-THC administration (Weed et al. 2016). Importantly, early life stress induces a significant reduction in CB1R expression in CA1 region of both sexes, but to a greater extent in male than in female rats, and a marked decrease in the CA3 region only in males (Suárez et al. 2009). Conversely, repeated stress during adolescence induces a decreased expression of CB1R mRNA in the hippocampus of female but not male rats (Xing et al. 2014). Notably, the same study found that baseline CB2R mRNA levels were significantly higher in the hippocampus of female animals than in males but were not affected by stress. Another study demonstrated that chronic unpredictable mild

stress induces a downregulation of CB1Rs in the hippocampus of male rats (an effect more evident in the dorsal than in the ventral hippocampus), but an upregulation of CB1Rs in the dorsal hippocampus of females (Reich et al. 2009). The above described studies support the notion that the endocannabinoid system is differentially organized in the male and female brains to respond to chronic stress. Finally, Riebe et al. (2010) found no difference in CB1R density between males and females in the hippocampus, although ovariectomized female rats show higher CB1R density compared to cycling female and estradiol-treated ovariectomized female and male rats. Finding that the difference between ovariectomized and cycling female rats is reversed by estradiol replacement not only confirms that CB1R expression is regulated by estradiol in a brain region-dependent manner but also that estradiol has an inhibitory effect on CB1R density in the hippocampus. Similarly, differences in CB1R density between males and ovariectomized females indicate that testosterone may exert an inhibitory effect on the expression of hippocampal CB1Rs.

3.5 Amygdala

The amygdala is a complex area that embraces several interconnected regions including the basolateral amygdala, the central amygdala, and the medial amygdala (Sah et al. 2003). It is a key site for the assignment of emotional salience to external stimuli and the coordination of affective, autonomic, and behavioral responses to such stimuli, i.e., it acts as an interface between sensory inputs and cortical processing (Herman et al. 2003). Activation of the amygdala is associated to the generation of fear and anxiety responses and to the activation of the hypothalamic–pituitary–adrenal axis (Davis 1997; Shin and Liberzon 2010).

Within the cortico–limbic system, and together with the hippocampus and the prefrontal cortex, the basolateral amygdala shows the most prominent expression of CB1Rs, while lower levels of CB1R mRNA expression are present in the central amygdala (McPartland et al. 2007). The endocannabinoid signaling plays an important role in controlling exploratory behavior, motivational states, conditioned fear, and extinction of aversive memories (Marsicano et al. 2002; Chhatwal and Ressler 2007; Ramikie and Patel 2012). A number of evidence revealed a sexually dimorphic amygdala in *Cannabis* users. Female marijuana users, for example, show a larger right amygdala than female controls, while male users have similar volume as male controls (McQueeney et al. 2011). In the amygdala, women show lower baseline cerebral CB1R availability than men; notably, low CB1R availability is related to a more evident novelty-seeking phenotype (Van Laere et al. 2009). An interesting study by Krebs-Kraft et al. (2010) revealed a sexually dimorphic endocannabinoid system within the medial amygdala of developing rats. Females, for example, have higher level of the endocannabinoid degradation enzymes (fatty acid amid hydrolase and monoacylglycerol lipase) than males, lower amounts of endocannabinoids (anandamide and 2-arachidonoylglycerol), and more *ex novo*

generated glial cells (but not neurons) than males. Curiously, treatment of neonate rats with cannabinoid agonists reduces glial cell genesis in females but not in males (Krebs-Kraft et al. 2010).

Animal data available so far on sex-dependent differences in CB1R density and function in the amygdala of adult male and female rats are somewhat discrepant. Zamberletti et al. (2012) reported higher density and functionality of CB1Rs in female than in male rats. Yet, Llorente-Berzal et al. (2013) confirmed a higher level of CB1R functionality in females but detected no sex differences in CB1R density in the amygdala. Female animals also exhibited higher basal levels of CB1 mRNA expression in the amygdala (Xing et al. 2014) but significantly reduced CB1R level and function following exposure to delta9-THC (Rubino et al. 2008). Moreover, CB1R density in the amygdala has been reported to be either higher (Riebe et al. 2010) or lower (Castelli et al. 2014) in cycling females than in males and in ovariectomized females. Notably, such a difference appeared to be estradiol dependent, since it was reversed by replacement with estrogen regardless of the direction of effect (Riebe et al. 2010; Castelli et al. 2014). Interestingly, early life stress induced a CB1R downregulation in the basolateral amygdala of male but not female rats (Alteba et al. 2016).

3.6 *Periaqueductal Gray*

The periaqueductal gray (PAG) matter is a midbrain structure that surrounds the Sylvius aqueduct, receives afferents from nociceptive neurons in the spinal cord, and sends projections to thalamic nuclei that process nociception. It is an important site in ascending pain transmission and a key component of a descending pain inhibitory system. However, it also interacts with the amygdala to regulate fear and anxiety processing and is implicated in modulation of cardiovascular, respiratory, and motor control (Benarroch 2012).

The endocannabinoid system plays an important role in the regulation of aversive responses in the PAG by exerting a “fine-tuning” regulatory control of defensive responses (Moreira et al. 2009, 2012; Fogaça et al. 2012). PAG is also a crucial supraspinal site of action for the CB1R-mediated analgesic effects of cannabinoids (Hu et al. 2014).

Analysis of the density of CB1Rs in the PAG of male and female rats reported discrepant results. Indeed, CB1R density was found to be higher in female rats by Zamberletti et al. (2012), while Llorente-Berzal et al. (2013) reported no differences between the two sexes. However, both studies failed in detecting significant sex-dependent differences in CB1R functionality in the PAG of male and female rats (Zamberletti et al. 2012; Llorente-Berzal et al. 2013). Moreover, adolescent exposure to delta9-THC induces downregulation of CB1Rs in rats, which was greater in female than male adolescent rats (Rubino et al. 2008). Notably, female and male adolescent rats showed higher and lower desensitization in the PAG, respectively, than same-sex adults, while in adults CB1R desensitization was

greater in males than in females (Burston et al. 2010). Why CB1R desensitization after adolescent exposure to delta9-THC occurs in the PAG to a greater extent in adolescent females and adult males remains to be elucidated.

3.7 *Nucleus Accumbens*

The mesolimbic dopamine system that projects from the ventral tegmental area to the nucleus accumbens is crucial for the regulation of the rewarding effects of drugs of abuse. In particular, the nucleus accumbens is required not only for regulating most reward-related behaviors but also for making associations between salient environmental events and rewarding outcomes (Fattore and Diana 2016).

CB1Rs are densely distributed in the human and rat nucleus accumbens, located on both GABAergic and glutamatergic axon terminals (Hoffman and Lupica 2001; Robbe et al. 2001). Noteworthy, although women were reported to be more sensitive to the subjective effects of marijuana (Cooper and Haney 2014), no differences were found between daily marijuana users and nonusers in the morphology (i.e., volume and shape) of the nucleus accumbens (Weiland et al. 2015). During reward anticipation in a monetary reward task, *Cannabis* users displayed attenuated activity in the nucleus accumbens compared to controls (van Hell et al. 2010), but since only male volunteers were employed, whether or not this effect is similar in the two sexes is not known. A more recent study confirmed that marijuana use is associated with decreased nucleus accumbens neural response to anticipation of nondrug rewards (Martz et al. 2016). Unfortunately, although both male and female young adults participated to this study, sex-dependent differences were not evaluated, thus leaving unaddressed the question of potential sex differences in anticipatory reward processing in the nucleus accumbens.

In rats, CB1R density and function are reduced after exposure to cannabinoids during adolescence (Rubino and Parolaro 2008; Marco et al. 2009). Sex-dependent differences have been reported in the density and activity of CB1Rs in the nucleus accumbens, but, as seen for other brain regions, findings are somewhere discrepant among studies. Zamberletti et al. (2012), for example, reported higher CB1R density in females than males with no differences in functionality, while Llorente-Berzal et al. (2013) found similar density of CB1Rs in the two sexes but significantly higher CB1R functionality in females than in males. Conversely, Castelli et al. (2014) did not find any difference between male and female rats as concerns the density and function of CB1Rs in either the shell or the core subregion of the nucleus accumbens.

3.8 Ventral Tegmental Area

The ventral tegmental area receives important dopaminergic inputs from the amygdala and prefrontal cortex and projects relevant dopaminergic efferences back to the amygdala and prefrontal cortex but also to the nucleus accumbens (Fattore and Diana 2016). It is crucially involved in the control of motivated behavior, reward-related learning, salient sensory, and emotional information processing. The tail of the ventral tegmental area, better known as the rostromedial tegmental nucleus, was recently hypothesized to exert a major inhibitory drive on dopaminergic systems (Bourdy and Barrot 2012).

Within the ventral tegmental area, CB1Rs are more numerous on inhibitory terminals rather than on excitatory synapses and disinhibit dopamine neurons in reward-related contexts (Lupica and Riegel 2005; Oleson et al. 2012). CB1R activation results in the inhibition of GABAergic neurotransmission: depression of the GABAergic inhibitory input of dopaminergic neurons increases their firing rate and dopamine release in the projection region of nucleus accumbens (Szabo et al. 2002). Chronic exposure to cannabinoid activates CB1Rs of this area inducing transient depression in locally activated glutamate synapses onto dopamine neurons through activation of glutamate NMDA receptors (Liu et al. 2010).

Both endocannabinoid signaling and CB1Rs within the ventral tegmental area have been reported to be affected by sex by preclinical research (Melis et al. 2013). Yet, no clinical study has been performed so far to specifically assess the existence of sex-dependent differences in the morphology or activation of this area in marijuana smokers. One study, for example, examined whether marijuana smoking was associated with greater cue reactivity in the ventral tegmental area and reported that, when compared to sporadic *Cannabis* users and controls, frequent users show higher neural responses (Cousijn et al. 2013). However, although male and female marijuana users were involved in this study, sex-dependent differences were not evaluated.

In rats, CB1R density and activity in the ventral tegmental area have been found to be higher in females than in males (Zamberletti et al. 2012), although no differences were reported by Castelli et al. (2014). Importantly, delta9-THC exposure during adolescence significantly decreased CB1R level (Rubino et al. 2008; Zamberletti et al. 2012) and function (Zamberletti et al. 2012) in the ventral tegmental area of adult female rats, while no significant alterations were observed in adult male rats (Rubino et al. 2008; Zamberletti et al. 2012). The same cannabinoid treatment significantly decreased CB1R density in both female and male adolescent rats, while CB1R function was significantly reduced in adolescent females only (Rubino et al. 2008).

3.9 *Cerebellum*

The cerebellum has long been acknowledged for its role in motor coordination, but it is also involved in the temporal control of action sequences and in complex cognitive behavior (Schmahmann and Caplan 2006). Along with the basal ganglia, the hippocampus, and the cortex, the cerebellum is a brain area displaying very high expression of CB1Rs, consistent with the profile of motor effects typically induced by cannabinoid agents. The cerebellar cortex is particularly enriched of CB1Rs, located primarily on axons in the molecular layer (Kawamura et al. 2006). Chronic marijuana users show decreased glucose metabolism in the cerebellum compared with controls at baseline, but increased metabolism after acute intravenous delta9-THC administration (Volkow et al. 1996). In the cerebellum, other studies reported decreased regional cerebral blood flow in abstinent marijuana users (Block et al. 2000), but increased regional cerebral blood flow after either intravenous delta9-THC administration or marijuana smoking (Mathew et al. 1998; O'Leary et al. 2002).

Animal studies showed that CB1R levels within the cerebellum are higher in females than in males (Zamberletti et al. 2012) but that CB1R functionality is not significantly different between the two sexes (Zamberletti et al. 2012; Llorente-Berzal et al. 2013). CB1R density and functionality can be differently affected by early life stress. Maternal deprivation, for example, significantly increases the number of CB1Rs exclusively in females and CB1R activity exclusively in males (Llorente-Berzal et al. 2013). Yet, Zamberletti et al. (2012) reported no significant effect of sex on cerebellar CB1R level and function in maternally deprived adult rats. On the other hand, the effect induced on CB1R density and activity by chronic delta9-THC in adolescent animals was similar in the two sexes (Rubino et al. 2008).

3.10 *Basal Ganglia*

CB1Rs are also present in the caudate putamen, substantia nigra, and globus pallidus of the human brain, localized on presynaptic terminals of GABAergic striatonigral and striatopallidal neurons (Hurley et al. 2003; McPartland et al. 2007). In the caudate putamen, the level of CB1R mRNA was significantly lower in marijuana users than in controls (Villares 2007).

In rats, few sex differences were found in the caudate putamen, substantia nigra, and globus pallidus, but, as in other brain regions, data are mostly controversial. Zamberletti et al. (2012), for example, reported a significantly higher CB1R density in females than in males, while the opposite was found by Llorente-Berzal et al. (2013). The two studies reported discrepant data also for CB1R function, but only in the caudate putamen. Rubino et al. (2008) showed a significant reduction of CB1R density following adolescent chronic treatment with delta9-THC in the caudate putamen and substantia nigra of adolescent female rats only. Moreover, the same

treatment induced a significant reduction in the function of CB1Rs in the caudate putamen, substantia nigra, and globus pallidus of female rats, but only in the substantia nigra in males (Rubino et al. 2008). As concerns the effect of exposure to drugs of abuse and/or early life stress, it was shown that adolescent exposure to cocaine, but not maternal deprivation, significantly affected CB1R function in all these three regions in male rats while only in the globus pallidus in females (Llorente-Berzal et al. 2013). Notably, CB1R density was not affected by cocaine administrations in both males and females, but it was significantly increased in the substantia nigra of maternally deprived animals of both sexes (Llorente-Berzal et al. 2013).

A third study, however, reported no significant differences in CB1R density and function in the caudate putamen of male and female rats (Castelli et al. 2014). Differences in CB1R binding and function of male and female adult rats among different studies might be due to differences in the methodological approaches (homogenate binding vs. autoradiographic analysis vs. *in situ* hybridization) and in the strains of rats used (Castelli et al. 2014).

4 Hormonal Influence on CB1Rs

Sex steroids, e.g., estrogens and progestogens, are critical player during brain development since they shape the central nervous system during development and their activating effects strongly influence brain function and behaviors. To further complicate the complex scenario of brain region-dependent differences in the level and activity of CB1Rs in males and females, gonadal hormones have been found to strongly affect CB1R binding and endocannabinoid levels in both humans and animals (Fattore and Fratta 2010; Fattore 2013; Sanchez et al. 2016). Ovarian hormones, for example, mediate the long-lasting behavioral and pharmacodynamic effects induced by chronic exposure to delta9-THC during adolescence in female rats (Winsauer et al. 2011). Estradiol modulates the endocannabinoid tone and rapidly suppresses inhibitory synaptic transmission in hippocampus in a sex-specific manner, i.e., in females but not males (Huang and Woolley 2012). In turn, cannabinoids impinge upon hypothalamic–pituitary–gonadal axis activity, negatively impacting on sexual behavior and sexual motivation (Ferrari et al. 2000; López 2010; López et al. 2010).

In humans, female marijuana smokers appear to be more sensitive to the behavioral and physiological effects of cannabinoids (Craft 2005), with treatment-seeking women presenting more severe withdrawal symptoms than treatment-seeking men (Herrmann et al. 2015). The existence of a reciprocal influence between marijuana smoking and the menstrual cycle is supported by the findings that women who smoke marijuana are at higher risk for delayed ovulation and anovulatory cycles (Jukic et al. 2007) and for reduced fertility due to ovulatory abnormalities (Mueller et al. 1990).

Table 1 Gender- and sex-dependent differences in the regional distribution of CB1 receptors in basal conditions and their modifications in males and/or females after exposure to early life stress or THC

Density	Basal	Early life stress	THC
Prefrontal cortex	F < M (Castelli et al. 2014; Llorente-Berzal et al. 2013)		↓ F (Zamberletti et al. 2012)
	F > M (Zamberletti et al. 2012; Xing et al. 2014)		
Thalamus	F > M (Zamberletti et al. 2012)	↓ M (Llorente-Berzal et al. 2013)	↓ M (Zamberletti et al. 2012)
	F < M (Llorente-Berzal et al. 2013)		
Hypothalamus	F < M (Riebe et al. 2010)		↓ F (Zamberletti et al. 2012)
	F > M (Zamberletti et al. 2012; Xing et al. 2014)		
Hippocampus	F < M (Llorente-Berzal et al. 2013)	↓ F ↓ M (Suárez et al. 2009)	↓ F ↓ M (Silva et al. 2015)
	F > M (Zamberletti et al. 2012; Xing et al. 2014)		
	F ≈ M (Castelli et al. 2014; Riebe et al. 2010)		
Amygdala	F < M (humans) (Van Laere et al. 2009)		↓ F ↓ M (Rubino et al. 2008) ↓ F (Zamberletti et al. 2012)
	F < M (Castelli et al. 2014)		
	F > M (Zamberletti et al. 2012; Xing et al. 2014; Riebe et al. 2010)		
	F ≈ M (Llorente-Berzal et al. 2013)		
Periaqueductal gray	F > M (Zamberletti et al. 2012)		↓ F ↓ M (adolescent rats, Rubino et al. 2008)
	F ≈ M (Llorente-Berzal et al. 2013)		
Nucleus accumbens	F > M (Zamberletti et al. 2012)		↓ F ↓ M (Zamberletti et al. 2012) ↓ F (adult rats); ↓ M (adolescent rats, Rubino et al. 2008)
	F ≈ M (Llorente-Berzal et al. 2013; Castelli et al. 2014)		
Ventral tegmental area	F > M (Zamberletti et al. 2012)		↓ F (adult rats, Rubino et al. 2008; Zamberletti et al. 2012)
	F ≈ M (Castelli et al. 2014)		

(continued)

Table 1 (continued)

Density	Basal	Early life stress	THC
Cerebellum	F > M (Zamberletti et al. 2012)	↑ F (Llorente-Berzal et al. 2013)	↓ F ↓ M (adolescent rats, Rubino et al. 2008)
Basal ganglia (CPu, SN, GB)	F > M (CPu, SN, GP) (Zamberletti et al. 2012)	↑ M ↑ F (SN) (Llorente-Berzal et al. 2013)	↓ F (CPu, SN, adolescent rats, Rubino et al. 2008)
	F < M (CPu, SN, GP) (Llorente-Berzal et al. 2013)		↓ F (CPu, SN, GP); ↑ M (SN); ↓ M (CPu) (Zamberletti et al. 2012)
	F ≈ M (CPu) (Castelli et al. 2014)		

In rats, CB1R density and affinity have been reported to be influenced in certain brain areas by the levels of estradiol, progesterone, and testosterone (Rodríguez de Fonseca et al. 1994). Ovarian hormones have been described to significantly influence cannabinoid taking (Fattore et al. 2007) and cannabinoid seeking (Fattore et al. 2010) in rats and to regulate CB1R density and function in the rat prefrontal cortex and amygdala (Castelli et al. 2014). Interestingly, adolescent exposure to cannabinoid agonists caused an immediate (transient) reduction in CB1R expression in the hypothalamus and amygdala of adult rats but did not alter CB1R expression in the nucleus accumbens and globus pallidus at two different time points (Chadwick et al. 2011).

5 Conclusions

Sex-dependent differences in brain CB1R expression and functionality have been reported in almost all brain areas (see Tables 1 and 2). CB1R binding was also found to vary in a sex-specific manner after exposure to both stressful events and drugs of abuse, which suggests a differential activation and functionality of the endocannabinoid system in males and females, and might explain the divergent responses to exogenous cannabinoids often described in the two sexes. The different CB1R distribution and function within the male and female brain and the different gonadal hormonal milieu in the two sexes surely contribute to the different subjective effects induced by exogenous cannabinoids in men and women. Factors determining different CB1R expression in males and females are not well understood; yet, sex steroids have been suggested as potential modulators of CB1R expression both in the brain and periphery. In the anterior pituitary gland, for example, CB1R gene expression was found to be influenced by estrogens

Table 2 Gender- and sex-dependent differences in the functionality of CB1 receptors in basal conditions and their modifications in males and/or females after exposure to early life stress or THC

Functionality	Basal	Early life stress	THC
Prefrontal cortex	F > M (Llorente-Berzal et al. 2013)	↓ M ↓ F (Llorente-Berzal et al. 2013)	↓ M ↓ F (Zamberletti et al. 2012)
	F ≤ M (Zamberletti et al. 2012)		
	F ≈ M (Castelli et al. 2014)		
Thalamus	F > M (Llorente-Berzal et al. 2013, Zamberletti et al. 2012)	↑ M (Zamberletti et al. 2012)	↓ F (Zamberletti et al. 2012)
Hypothalamus	F > M (Llorente-Berzal et al. 2013)	↓ M (Llorente-Berzal et al. 2013) ↑ F (Zamberletti et al. 2012)	↓ F (Zamberletti et al. 2012)
Hippocampus	F > M (Llorente-Berzal et al. 2013)	↑ F ↑ M (Zamberletti et al. 2012)	↓ F ↓ M (Zamberletti et al. 2012) ↓ M (adult rats, Rubino et al. 2008)
	F ≈ M (Castelli et al. 2014; Zamberletti et al. 2012)	↑ F ↑ M (Zamberletti et al. 2012)	↓ F ↓ M (Zamberletti et al. 2012) ↓ M (adult rats, Rubino et al. 2008)
Amygdala	F > M (Zamberletti et al. 2012; Llorente-Berzal et al. 2013)	↓ F ↑ M (Zamberletti et al. 2012)	↓ F (adult rats, Rubino et al. 2008) ↓ M (adolescent rats, Rubino et al. 2008) ↓ F ↓ M (Zamberletti et al. 2012)
Periaqueductal gray	F ≈ M (Zamberletti et al. 2012; Llorente-Berzal et al. 2013)	↑ F (Llorente-Berzal et al. 2013)	↓ F ↓ M (Zamberletti et al. 2012)
Nucleus accumbens	F ≈ M (Castelli et al. 2014, Zamberletti et al. 2012)	↓ M (Llorente-Berzal et al. 2013)	↓ F ↓ M (Zamberletti et al. 2012) ↓ F (Rubino et al. 2008)
	F > M (Llorente-Berzal et al. 2013)		
Ventral tegmental area	F > M (Zamberletti et al. 2012)	↑ M (Zamberletti et al. 2012)	↓ F (adolescent rats, Rubino et al. 2008) ↓ F (Zamberletti et al. 2012)
	F ≈ M (Zamberletti et al. 2014)		
Cerebellum	F ≈ M (Llorente-Berzal et al. 2013; Zamberletti et al. 2012)	↑ M (Llorente-Berzal et al. 2013)	↓ F ↓ M (Zamberletti et al. 2012) ↓ F ↓ M (adolescent rats, Rubino et al. 2008)

(continued)

Table 2 (continued)

Functionality	Basal	Early life stress	THC
Basal ganglia (CPu, SN, GB)	F > M (GP) (Llorente-Berzal et al. 2013; Zamberletti et al. 2012)		↓ F (CPu, SN, GP); ↓ M (SN) (adolescent rats, Rubino et al. 2008) ↓ F ↓ M (CPu, SN) (Zamberletti et al. 2012)
	F < M (SN) (Llorente-Berzal et al. 2013; Zamberletti et al. 2012)		
	F < M (CPu) (Zamberletti et al. 2012)		
	F > M (CPu) (Llorente-Berzal et al. 2013)		
	F ≈ M (CPu) (Castelli et al. 2014)		

(González et al. 2000). Similarly, CB1R density is reduced in parotid glands of castrated male rats and is restored following testosterone replacement (Busch et al. 2006), confirming that sex hormones can modulate CB1R expression. It should be also kept in mind that estradiol differentially modulates cannabinoid regulation of amino acid neurotransmission (Nguyen and Wagner 2006), regulates emotional behavior through an endocannabinoid mechanism (Hill et al. 2007), and negatively modulates cannabinoid-induced effects on appetite, core body temperature, and neurotransmission at proopiomelanocortin (POMC) synapses (Kellert et al. 2009). The numerous evidence of region-dependent fluctuations of brain CB1R density and/or function along the ovarian cycle and following gonadectomy and sex steroid replacement (Bonnin et al. 1993; Rodríguez de Fonseca et al. 1994) strongly support the hypothesis of sex hormone-dependent differences in the sensitivity to cannabinoid treatment (Craft and Leidl 2008).

A conspicuous progress has been made during the recent years in the appreciation of (i) the sexual dimorphic nature of the endocannabinoid system, (ii) the influence of the gonadal hormones on the density and activity of brain CB1Rs, (iii) the necessity to include female subjects in clinical and preclinical research, and (iv) the need for gender-tailored prevention and treatment options for marijuana male and female smokers. Yet, current knowledge on sex-dependent differences in the endocannabinoid system and in response to cannabinoids is only in its infancy, and further rigorous research is needed to understand what makes the female and male brain different to these regards.

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Endocannabinoids, Stress, and Negative Affect

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Abstract The stress response is an evolutionarily conserved mechanism to both allow an organism to cope with a threat and to restore homeostasis following exposure to a stressor. With respect to this response, preclinical research demonstrates that the endogenous cannabinoid (ECB) system constrains the hypothalamic-pituitary-adrenal axis and plays a major role in the habituation to stressors. Specifically, anandamide tonically constrains activation of stress responsive circuits in the brain under basal conditions; however, exposure to stress or glucocorticoids initiates a cascade of events whereby corticotropin-releasing hormone (CRH) rapidly reduces anandamide metabolism through CRHR1-mediated activation of fatty acid amide hydrolase (FAAH) in the basolateral amygdala (BLA), which ultimately facilitates activation of the neuroendocrine axis and emotional response to stress. On the other hand, 2-arachidonoylglycerol (2-AG) provides on-demand synaptic modulation and promotes short-term adaptation to stress via glucocorticoid receptor-dependent mobilization in the hypothalamus and extrahypothalamic inhibitory feedback centers such as the medial prefrontal cortex (mPFC) and hippocampus. Additionally, 2-AG is recruited in the BLA to facilitate habituation to chronic homotypic stress, thus facilitating long-term adaptation as well. Accordingly, impairments in ECB signaling within the hypothalamus, BLA, mPFC, and/or hippocampus may confer maladaptive neuroendocrine and behavioral responses to stress, thereby contributing to the emergence of stress-related disorders such as anxiety, depression, and substance abuse. Moreover, sexual dimorphism and genetic differences in the ECB system may contribute to individual differences in stress coping strategies, which can have profound ramifications

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for susceptibility and resilience to stress-related mental disorders. This chapter will review the growing preclinical and clinical evidence demonstrating that the ECB system may be a promising therapeutic target for a host of human affective disorders.

1 Neuroendocrine Response to Stress

The stress response consists of a cascade of events that occur to both prime an organism to deal with a real or perceived threat in the environment and restore bodily homeostasis following resolution of that threat (Cannon 1935; Selye and Fortier 1950). Activation of the stress response initiates a number of behavioral and physiological changes that improve an individual's chance of survival when faced with homeostatic challenges. This occurs through two distinct biological pathways that govern the autonomic and neuroendocrine response to stress. The sympathoadrenal medullary axis coordinates the immediate autonomic response to stress, which is characterized by a release of catecholamines and functions to coordinate a constellation of physiological events, such as increased blood pressure, heart rate, respiratory rate, and pupil dilation, which maximize survival during fight-or-flight-type situations. By contrast, the hypothalamic-pituitary-adrenal (HPA) axis orchestrates the neuroendocrine stress response, which is characterized by the release of glucocorticoids that promote homeostatic recovery. This review will focus more specifically on the neuroendocrine arm of the stress response.

The HPA axis represents the primary branch of the neuroendocrine response to stress (Goel et al. 2014; Handa and Weiser 2014). The HPA response to stress is initiated by activation of corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) neurosecretory cells in the parvocellular region of the hypothalamic paraventricular nucleus (PVN) (Dunn and Berridge 1990). CRH and AVP are released into portal circulation of the median eminence, which results in the mobilization of ACTH from the anterior pituitary into the blood stream (Antoni 1986). Finally, ACTH induces the release of glucocorticoids (corticosterone in rodents, cortisol in humans) from the adrenal glands (Axelrod and Reisine 1984; de Kloet 1984), which subsequently act on glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) to mobilize energy stores, thus increasing arousal and focusing attention on the threat at hand (Chrousos and Gold 1992; Tsigos and Chrousos 2002).

Apart from the hypothalamus, GRs are expressed in high levels in the basolateral amygdala (BLA), medial prefrontal cortex (mPFC), and the hippocampus; three brain regions intimately involved in the activation, and subsequent termination, of the neuroendocrine stress response (McEwen 2012; Goldstein et al. 1996). The amygdala primarily exerts a stress-activating role during the processing of environmental stimuli, while the hippocampus and mPFC largely exert inhibitory roles over the stress response. Projections arising from the hippocampus and mPFC serve to dampen HPA axis activity through the activation of a GABAergic relay in the

bed nucleus of the stria terminalis (BNST), which inhibits neurosecretory cells in the PVN (Radley et al. 2009, 2013; Radley and Sawchenko 2011, 2015). Acting as an interface between the endocrine stress response and the response of the brain to stress, glucocorticoids acting on GRs in the mPFC (Diorio et al. 1993), hippocampus (Jacobson and Sapolsky 1991), anterior pituitary (Keller-Wood and Dallman 1984), and hypothalamus (Hinz and Hirschelmann 2000) serve to deactivate the HPA axis, thereby promoting stress recovery.

Dysfunction of the HPA axis can lead to overproduction of glucocorticoids, which can produce structural and functional changes that may increase susceptibility to psychiatric disorders, such as anxiety and major depressive disorder (de Kloet et al. 2005; Hammen 2005; Miller et al. 2007; Selye and Fortier 1950; Bodnoff et al. 1995; Checkley 1996; Chrousos and Gold 1992). Chronic stressors result in atrophy and compromised function of the mPFC and hippocampus while increasing dendritic complexity, synaptic connectivity, and functionality of the amygdala (McEwen et al. 2015). The result of this impaired top-down inhibitory control is that amygdala-based mechanisms of information processing predominate, resulting in a sustained state of stress and anxiety and persistent drive onto the HPA axis.

Interestingly, female rats, compared to their male counterparts, have higher basal corticosterone and higher restraint stress-induced corticosterone secretion (Aloisi et al. 1998; Goel and Bale 2008, 2010; Mitsushima et al. 2008; Sterrenburg et al. 2012; Viau 2002). Moreover, corticosterone remains elevated for longer in female rodents following stress offset (Heinsbroek et al. 1991; Iwasaki-Sekino et al. 2009; Weinstock et al. 1998). Human research into sex differences in the HPA axis are less conclusive (Collins and Frankenhaeuser 1978; Earle et al. 1999), but women do tend to exhibit higher cortisol secretion than men in response to social stressors (Stroud et al. 2002). This chapter will review the preclinical evidence supporting a role for the endocannabinoid (ECB) system in regulating the neuroendocrine and behavioral response to acute and chronic stress and also briefly describe potential sex differences in ECB signaling that may underlie discrepancies in the prevalence of stress-related disorders in males and females in the clinical population.

2 Introduction to the Endocannabinoid System

The *Cannabis sativa* plant, or marijuana, has long been used for its mood-enhancing properties (Bonn-Miller et al. 2007; Schofield et al. 2006); however, the biological system that is responsible for its physiological and behavioral effects has only recently been characterized (Matsuda et al. 1990). This system, collectively referred to as the ECB system, is primarily composed of the presynaptically expressed cannabinoid 1 receptor (CB1R), one of the most abundant G-protein-coupled receptor in the central nervous system (Devane et al. 1992; Herkenham et al. 1990; Matsuda et al. 1990) as well as the cannabinoid 2 receptor (CB2R), which is thought to be primarily expressed on glial cells in response to an

inflammatory challenge (Munro et al. 1993). Neuroanatomical data has demonstrated that these receptors are widely expressed throughout the forebrain, basal ganglia, and limbic system, which suggests a significant neuromodulatory role for this receptor subtype in humans (Glass et al. 1997) and rodents (Herkenham et al. 1991). The CB2R, on the other hand, is primarily located in the periphery on immune cells (Howlett 2002; Pertwee 2005) and, compared to CB1Rs, is expressed in much lower densities in the central nervous system (Van Sickle et al. 2005). CB1Rs are located on presynaptic axon terminals (Freund et al. 2003; Chevalleyre et al. 2006) of glutamatergic principal neurons as well as on a subpopulation of calbindin-negative and cholecystokinin-positive GABAergic basket cells (Freund et al. 2003; Tsou et al. 1998). Activation of the CB1R suppresses neurotransmitter release through the inhibition of adenylate cyclase, the inhibition of calcium channels, and the activation of potassium channels (Howlett and Fleming 1984; Howlett 1987; Mackie and Hille 1992; Twitchell et al. 1997; Mackie et al. 1995), making them vital in the balance of excitation and inhibition within a given neural circuit. Additionally, CB1R mRNA (Berrendero et al. 1999; Mailleux and Vanderhaeghen 1992; Tsou et al. 1998; Egertová and Elphick 2000; Katona et al. 2001) as well as the CB1R itself (Dalton and Zavitsanou 2010; Glass et al. 1997; Herkenham et al. 1990, 1991) has been localized in areas associated with hypothalamic-pituitary-adrenal (HPA) axis control such as the hypothalamus, BLA, mPFC, and hippocampus in both rodents and humans.

The ECB system is unique in that many endogenous ligands exist for the same receptor. The two primary endogenous ligands that have been characterized to date are N-arachidonylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al. 1992; Sugiura et al. 1995). AEA is primarily synthesized by a specific phospholipase D (NAPE-PLD) (Okamoto et al. 2004), though other redundant biosynthetic pathways exist (Liu et al. 2008). AEA is primarily metabolized by fatty acid amide hydrolase (FAAH) (Cravatt et al. 1996), which is predominantly located in postsynaptic membrane (Tsou et al. 1998). On the other hand, 2-AG is synthesized by two sn-1-selective DAG lipases (DAGL- α and DAGL- β) (Bisogno et al. 2003) and is primarily metabolized by monoacylglycerol lipase (MAGL), which is localized within presynaptic terminals (Dinh et al. 2002). There is also a small but relevant role of alpha-beta hydrolase 6 (ABHD6) in the metabolism of 2-AG (Marrs et al. 2010). Moreover, both AEA and 2-AG are also oxygenated by cyclo-oxygenase-2 (COX-2) to form bioactive prostamides (Hermanson et al. 2014).

ECBs are not stored in vesicles like traditional neurotransmitters but are instead synthesized in postsynaptic cells in an “on-demand” fashion following depolarization of the postsynaptic neuron (Piomelli 2003). They are then transported into the synapse to activate the presynaptically located cannabinoid receptors (Freund et al. 2003). The on-demand nature of ECB release, as well as the expression profile of CB1R on both inhibitory and excitatory terminals (Kano et al. 2009), makes this system ideally positioned to regulate synaptic feedback and is thus a prime target for the treatment of disorders where disruptions in homeostatic function are a hallmark symptom.

Although AEA and 2-AG both share affinity for the same receptor, these two bioactive lipids are functionally distinct. It is unknown why two endogenous ligands exist to compete for the same receptor, but the pharmacokinetic profile of these two ECBs has offered some insight. For instance, AEA has a high affinity for the CB1R but is a low efficacy agonist with respect to inducing intracellular signal transduction. By contrast, 2-AG appears to have a lower affinity for the CB1R but a much higher signaling efficacy than AEA (Hillard 2000). Based on these characteristics, it has been proposed that AEA functions under basal conditions to maintain normal functioning (i.e., a tonic signal), while 2-AG is recruited to modulate activity-induced synaptic plasticity according to changes in the environment (i.e., a phasic signal) (Morena et al. 2016; McLaughlin et al. 2014).

3 Role of the Endocannabinoid System in the Acute Effects of Stress

The anatomical localization and unique functional profile of the ECB system suggests that it could be an important regulator of the acute response to stress. At the level of the hypothalamus, CB1Rs are located on the axon terminals of glutamatergic neurons which activate CRH-expressing neurons in the PVN, making the ECB system ideally positioned to gate excitatory drive of the HPA axis (Di et al. 2003). For instance, *in vitro* application of corticosterone to hypothalamic slices induces the synthesis and release of ECBs, which then act to inhibit glutamatergic release onto CRH neurosecretory cells in the PVN, thereby dampening activation of the HPA axis (Di et al. 2003; Malcher-Lopes et al. 2006). Notably, CB1R knockout mice show not only increased CRH mRNA in the PVN of the hypothalamus (Cota et al. 2003) but also increased circulating ACTH and corticosterone (Haller et al. 2004a, b; Barna et al. 2004; Steiner et al. 2008). This relationship of glucocorticoids and ECBs is also recapitulated in rats subjected to acute restraint stress, such that CB1R antagonism significantly exacerbates both basal and stress-induced corticosterone secretion (Patel et al. 2004) and increases 2-AG in the hypothalamus (Evanson et al. 2010). Moreover, *in vivo* administration of corticosterone also increases AEA and 2-AG content in the hypothalamus (Hill et al. 2010; Evanson et al. 2010), while intra-PVN blockade of CB1Rs prevents glucocorticoid-mediated suppression of the HPA axis (Evanson et al. 2010). Thus, CB1Rs appear to play a pivotal role in negatively regulating excitatory inputs to the hypothalamic PVN, inactivation of which increases excitability of the PVN in a manner similar to what is seen following stress exposure.

ECB signaling in extrahypothalamic regions, such as the amygdala, hippocampus, and mPFC, also serves an important regulatory role in stress-induced HPA axis activation and recovery. With respect to activation of the HPA axis, the amygdala has emerged as a critical region governing stress reactivity and anxiety-like behavior, and a role for the ECB system in constraining amygdala activity has recently

been demonstrated. For instance, acute restraint stress leads to reductions in AEA in the amygdala via enhanced FAAH activity, and AEA content is negatively correlated with serum corticosterone levels (Hill et al. 2009). Additionally, inhibition of FAAH activity in the BLA partially reverses stress-induced increases in serum corticosterone (Hill et al. 2009). These effects of stress are also recapitulated by central administration of CRH, which was found to significantly reduce AEA content in the amygdala through an increase in FAAH activity and subsequently contribute to increased anxiety-like behavior in rats (Gray et al. 2015). Furthermore, the ability of systemic FAAH inhibition to reduce HPA axis activation is mediated by the local activation of CB1R in the BLA (Bedse et al. 2014). The mechanisms by which AEA signaling modulates BLA excitability are likely via the ability of CB1R signaling to gate excitatory inputs to the BLA, such that under ambient conditions, an AEA tone constrains excitatory inputs to the BLA, while following exposure to stress, when AEA levels decline, disinhibition of these excitatory inputs facilitates stress-induced activation of the BLA (Morena et al. 2016; Ahn et al. 2008; Gorzalka et al. 2008). These findings appear to hold true in humans as well given that individuals possessing a gene variant in FAAH, which results in reduced FAAH activity and elevated AEA levels (Sipe et al. 2002; Dincheva et al. 2015), exhibit reduced activation, and accelerated habituation, of the amygdala in response to stressful threat cues (Hariri et al. 2009; Gunduz-Cinar et al. 2013). Quite interestingly, a recent report has similarly demonstrated that variants in CRH receptor signaling seem to moderate the effects of changes in FAAH activity to regulate amygdala activation (Demers et al. 2016), which is consistent with the relationship between CRH and FAAH detailed above. These data collectively suggest that the actions of CRH in the amygdala serve to enhance FAAH-mediated AEA hydrolysis, which ultimately removes the break on the system and allows for an exaggerated neuroendocrine response to stress.

Moreover, AEA and 2-AG appear to serve distinct functions in the amygdala with respect to the regulation of HPA axis activity in response to acute stress (Hill and Tasker 2012). For instance, while acute stress results in relatively rapid suppression in AEA content in the BLA (Hill et al. 2009; Rademacher et al. 2008), 2-AG content is only increased in the BLA after repeated homotypic stress (Patel et al. 2009), suggesting that changes in amygdalar AEA signaling contribute to initiation of the stress response, whereas 2-AG becomes recruited to promote stress habituation. The importance of 2-AG signaling in the amygdala has further been shown using mice deficient for DAGL α , which is the enzyme primarily responsible for the synthesis of 2-AG in the brain. Male and female DAGL α knockout mice exhibit increased anxiety-like behavior as well as a female-specific depression-like phenotype as measured by decreased sucrose preference in the sucrose preference test (Shonesy et al. 2014). These authors further established that DAGL α deletion impaired retrograde eCB signaling within the amygdala, highlighting the importance of ECB-mediated inhibition of this brain region.

ECBs in the hippocampus and mPFC appear to play a major role in stress recovery. In the hippocampus, acute stress results in reduced AEA and increased 2-AG content in the hippocampus (Wang et al. 2012). These authors also

demonstrated that stress-induced suppression of GABAergic transmission in the hippocampus occurs through an ECB mechanism, which is dependent on activation of glucocorticoid receptors. Specifically, these data suggest that glucocorticoids increase 2-AG content, which in turn acts to reduce GABA release and, potentially, increase excitability of the hippocampus (Wang et al. 2012). Thus, glucocorticoids regulate synaptic function in response to stress via the ECB system. In the mPFC, stress results in increased 2-AG content, which is also prevented with a glucocorticoid receptor antagonist (Hill et al. 2011a), thus suggesting that stress-induced changes in ECB signaling are also dependent on glucocorticoid release in the mPFC. Moreover, intra-mPFC CB1R blockade delays termination of the corticosterone response to acute restraint stress, and bath application of corticosterone to mPFC cortical slices suppresses GABA release in a CB1R-dependent manner (Hill et al. 2011a). This suggests that, like in the hippocampus, glucocorticoids recruit 2-AG signaling to suppress GABA release and promote excitability of a circuit. Thus, 2-AG is particularly important for promoting termination of the stress response, presumably by increasing the activation of afferents arising from the mPFC and hippocampus which act in concert to inhibit the HPA axis.

Whereas stress exposure leads to a delayed increase in 2-AG in the mPFC following the termination of stress (Hill et al. 2011a), stress also elicits a rapid reduction in AEA content in the mPFC (McLaughlin et al. 2012). As AEA and 2-AG exhibit a “yin-yang” relationship, where AEA is depressed quickly after stress followed by a delayed increase in 2-AG, it has been proposed that AEA functions under basal conditions to maintain normal functioning, while 2-AG is recruited to shut down the stress response once the environment is deemed no longer threatening (Hill and Tasker 2012). Consistent with this hypothesis, intra-mPFC administration of a FAAH inhibitor can blunt the HPA axis response to stress (McLaughlin et al. 2014).

While the majority of preclinical data has been collected in male animals, significant sex differences exist in how men and women respond to an acute stressor. For example, men and women use different coping strategies to deal with stress (Schmied et al. 2015), and the psychological impact of stress may be greater in women (Schmied et al. 2015; Taylor 2014), which may indicate sex differences in HPA axis reactivity. Interestingly, ECBs and gonadal hormones interact in a bidirectional manner to modulate behavior (Hill et al. 2007a; Carroll and Anker 2010; Gorzalka and Dang 2012), so sexual dimorphisms in the stress response may arise, at least partially, from sex differences in the ECB system. Acute stress increases circulating ECBs in men and women, but the effect depends on hormone stage in women (Dlugos et al. 2012). Specifically, ECBs are highest in women during the luteal phase, when levels of gonadal hormones are also high. In ovariectomized female rats, estradiol reduces anxiety, but this effect is blocked by a CB1R antagonist and recapitulated by the administration of an FAAH inhibitor (Hill et al. 2007a), suggesting that gonadal hormones such as estradiol may alleviate stress-induced negative affective symptoms through an ECB mechanism. Additionally, a series of elegant studies from Catharine Woolley’s group have recently demonstrated that estradiol increases AEA levels in the hippocampus via

mGluR1-dependent stimulation of phospholipase C and IP3R activation, which ultimately serves to dampen GABAergic inhibition of hippocampal principal outputs (Huang and Woolley 2012; Tabatadze et al. 2015). Notably, this estradiol-mediated stimulation of hippocampal AEA synthesis only occurs in females, thus providing a mechanism by which estradiol may modulate hippocampal-dependent stress recovery processes in a sex-specific manner, which could have important implications for the sexually dimorphic response to stress.

4 Role of the ECB System in the Effects of Chronic Stress

As one might expect, exposure to chronic stress also results in alterations to multiple components of the ECB system in both hypothalamic and extrahypothalamic structures. For instance, 7 days of social defeat stress reduces AEA and increases 2-AG content in the hypothalamus of defeated mice (Dubreucq et al. 2012). Chronic stress has also been shown to increase basal and stress-induced corticosterone secretion in parallel with these decreased AEA levels in the hypothalamus (Hill et al. 2010). Furthermore, CB1Rs are downregulated in the hypothalamus following both postweaning social isolation (Sciolino et al. 2010) and repeated restraint stress through the actions of corticosterone (Wamsteeker et al. 2010). Similar to acute stress, these findings indicate that chronic stress causes functional adaptations in the hypothalamic ECB system that serve to reduce the threshold for HPA axis activation and render the system less sensitive to heightened glucocorticoid-mediated negative feedback.

Changes in the ECB system following chronic stress seem to both contribute to alterations in amygdalar function and also function to maintain homeostasis in the face of repeated stress. Following chronic restraint stress, pyramidal neurons of the BLA exhibit an increase in dendritic arborization (Hill et al. 2013a, b), indicative of enhanced BLA output. These changes were mediated by reductions in AEA via elevated FAAH, as this phenotype was reversed by genetic and pharmacological ablation of FAAH (Hill et al. 2013a, b). Repeated restraint stress also causes an increase in basal circulating corticosterone, while decreasing AEA content throughout the corticolimbic circuit, specifically in the PFC and amygdala (Hill et al. 2010; Patel et al. 2005; Rademacher et al. 2008). Inhibition of FAAH does not modulate HPA dynamics during habituation to repeated stress; however, it does prevent basal hypersecretion of corticosterone, suggesting that the decrease in AEA signaling contributes to changes in basal HPA axis function. Exposure to repeated restraint (Patel et al. 2005; Hill et al. 2010), or chronic treatment with corticosterone, results in increased 2-AG content in the amygdala (Hill et al. 2005), implying that the increase in 2-AG levels could also be a byproduct of chronically elevated corticosterone. A putative mechanism underlying this enhancement of 2-AG signaling is the downregulation of MAGL, the 2-AG hydrolase, in the BLA following chronic restraint stress (Sumislowski et al. 2011). This increase in 2-AG content within the amygdala appears to contribute to the adaptive process of stress habituation as local

blockade of CB1R within the BLA can dishabituate the HPA axis to repeated stress (Hill et al. 2010). Together, these studies highlight a key, albeit functionally distinct, role for AEA and 2-AG in regulating the amygdalar response to chronic stress by preventing basal corticosterone hypersecretion and promoting habituation to stress, respectively.

In the hippocampus and mPFC, exposure to chronic stress or corticosterone similarly produces an increase in FAAH activity, a decrease in AEA content, and an increase in 2-AG (Bowles et al. 2012; Patel et al. 2005; Rademacher et al. 2008; Hill et al. 2006, 2007b; Gray et al. 2016). However, the CB1R response to chronic stress in the PFC is unique with respect to the rest of the corticolimbic circuit. Whereas chronic stress unanimously decreases CB1R binding in subcortical areas, CB1R binding in the PFC becomes paradoxically upregulated (Hill et al. 2008a, b; McLaughlin et al. 2013). Moreover, this increase in CB1R binding and functionality has been recapitulated in postmortem tissue samples from depressed (Choi et al. 2012; Hungund et al. 2004) and alcoholic (Vinod et al. 2010) suicide victims. When the initial postmortem studies were published, the prevailing notion was that the increase in CB1R functionality was a detrimental consequence of prolonged exposure to stress that ultimately contributed to the depressive-like phenotype (Hungund et al. 2004). Other research has arrived at the conclusion that despite higher receptor number, receptor functionality may be reduced (see Chapter “Endocannabinoid-Dependent Synaptic Plasticity in the Striatum”). However, an additional explanation could be that this region-specific increase in the number of CB1R binding sites may serve an adaptive purpose that is recruited in an attempt to dampen the negative effects of chronic stress. In support of this notion, we have shown that following chronic unpredictable stress exposure, intra-mPFC CB1R blockade significantly exacerbates behavioral despair in the forced swim test as operationalized by immobility behavior during the session (McLaughlin et al. 2013). These data suggest that negating the chronic stress-induced increase in CB1R binding in the mPFC promotes despair-like coping responses beyond that which would be expected from chronic stress alone. In line with this protective role for CB1Rs in the mPFC, glial cells have been shown to release an increased amount of ECBs and over-express CB1Rs in the PFC under neuropathological conditions, which may constitute an endogenous defense mechanism that prevents additional cell damage (Massi et al. 2008). Moreover, CB1R knockout mice, which exhibit pronounced neuroinflammatory responses in the PFC (Zoppi et al. 2011), also show significant dendritic retraction in the PFC and expansion in the BLA, thus mirroring the effects of chronic stress exposure (Hill et al. 2011b). These data collectively suggest that endocannabinoid signaling protects against the detrimental effects of chronic stress in a region-specific manner, with breakdown of this system likely contributing to the development of stress-related psychiatric disorders.

Little is known regarding how ECB signaling is altered in females under conditions of chronic stress, although the consensus is that the response is unique from that of males. For instance, chronic unpredictable stress has been shown to actually increase CB1R binding in the dorsal hippocampus of intact and ovariectomized female rats (Reich et al. 2009). Similarly, hippocampal CB1R

immunoreactivity is decreased in neonatal male rats, but not female rats that experienced maternal deprivation (Suárez et al. 2009). Thus, sex differences in the ECB response to chronic stress could contribute to the wide gap in prevalence rates for stress-related illnesses among men and women in the clinical population.

5 Stress-Related Negative Affect and Drug Withdrawal

5.1 *Stress-Related Negative Affective Behavior*

Considering that ECB signaling becomes depressed after chronic stress resulting in hyperactivity of the HPA axis, as well as the fact that high levels of stress are a risk factor for many psychiatric disorders such as anxiety, depression, and PTSD (Smoller 2015), it is not surprising that perturbation of the ECB system results in affective symptoms of stress-related disorders (Gray et al. 2015; Gorzalka and Hill 2011; Neumeister et al. 2015). For instance, CB1R knockout mice exhibit increased depressive-like passive coping responses (i.e., immobility) in the forced swim test and tail suspension test (Steiner et al. 2008; Aso et al. 2008), two commonly implemented paradigms used to assess the antidepressant potential of novel pharmacotherapeutic compounds. Similarly, these mice are particularly susceptible to the anhedonic effects of chronic stress (Martin et al. 2002) and exhibit reduced responsiveness to rewarding stimuli such as alcohol (Poncelet et al. 2003) and sucrose (Sanchis-Segura et al. 2004; Cota et al. 2003), in addition to reductions in food intake and weight gain (Haller et al. 2002). CB1R-deficient mice also display an increase in anxiogenic-like traits in tests such as the light-dark box (Martin et al. 2002) and elevated plus maze (Haller et al. 2002, 2004a, b) and increased aggression in the resident-intruder test (Martin et al. 2002). Moreover, these animals exhibit strongly impaired short-term and long-term extinction in fear-conditioning tests (Marsicano et al. 2002) but show no differences during extinction of appetitive motivated tasks (Hölter et al. 2005). Lastly, these mice exhibit impaired hippocampal neurogenesis (Jin et al. 2004) and reduced brain-derived neurotrophic factor (BDNF) release in response to neurotoxic insults (Khaspekov et al. 2004). Therefore, CB1R-deficient mice exhibit a phenotype that is strikingly reminiscent of the symptomatic profile of both depression and anxiety (Hill and Gorzalka 2005), suggesting that disruptions in ECB signaling fundamentally contribute to stress-related affective disorders.

In line with these data, chronic CB1R antagonist administration mimics the effects of chronic unpredictable stress, including increased behavioral despair in the forced swim test, reduced sucrose consumption, decreased serotonin levels in the frontal cortex, reduced hippocampal cell proliferation, survival, and BDNF levels, and increased concentrations of pro-inflammatory cytokines (Beyer et al. 2010). Thus, much like the emotional phenotype displayed by CB1R-deficient mice, chronic CB1R blockade is capable of recapitulating the symptom profile of major

depression in preclinical models (Hill and Gorzalka 2005; Valverde and Torrens 2012). Evidence for the detrimental effects of chronic CB1R blockade has since been demonstrated in human clinical trials for the CB1R antagonist rimonabant as a prospective treatment for obesity. A significant proportion of individuals taking the drug spontaneously developed anxiety, adverse depressive-like symptoms, and suicidal ideations that inevitably led to the suspension of clinical trials in both North America and Europe (Christensen et al. 2007; Hill and Gorzalka 2009). Moreover, these effects were also observed in clinical trials for rimonabant in the treatment of atherosclerosis (the STRADIVARIUS, or Strategy to Reduce Atherosclerosis Development Involving Administration of Rimonabant: The Intravascular Ultrasound Study) (Nissen et al. 2008). In these trials, 43% of individuals taking rimonabant developed adverse mood and anxiety responses, compared to 28% in the placebo condition (Nissen et al. 2008). In fact, the emergence of these symptoms was sufficient for one in 13 individuals to discontinue use of rimonabant, compared to one in 47 individuals who discontinued placebo treatment for these same reasons (Nissen et al. 2008). A likely explanation for the strikingly high incidence of adverse depressive-like symptoms observed in these trials is the non-exclusion of patients with prior psychiatric disorders. This inevitably resulted in a less selected study population that more closely reflects the risks of depression and anxiety with rimonabant treatment in routine clinical practice (Rumsfeld and Nallamothu 2008). Thus, chronic CB1R blockade confers symptoms of stress-related affective disorders in both preclinical models and clinical populations, underscoring the importance of this system for proper physiological, behavioral, and emotional responses to environmental adversity.

5.2 *Drug Withdrawal*

While drug and alcohol dependence is associated with altered motivational states, tolerance, and escalated consumption, withdrawal is associated with a profound negative affective state, the alleviation of which serves as the driving factor in relapse (Boden and Fergusson 2011; Sánchez-Peña et al. 2012). Specifically, the self-medication hypothesis predicts that drug use is perpetuated by these negative affective symptoms present during withdrawal (Markou et al. 1998). Given that chronic substance abuse alters the neuroendocrine stress response and that stress hormones recruit the ECB system to modulate emotional behavior, it is unsurprising that the ECB system also fundamentally contributes to both drug dependence and withdrawal symptomology.

Most evidence demonstrates a role for the ECB system in the adverse effects of drug dependence, and withdrawal comes from studies using alcohol-dependent rodents. Rats exposed to 52 days of forced alcohol consumption show reduced CB1R gene expression in the caudate putamen, hypothalamus, and hippocampus (Ortiz et al. 2004), indicating that the downregulation of CB1Rs may be the result of a compensatory mechanism in response to dynamic changes in ECB mobilization.

Moreover, after two cycles of withdrawal, chronic alcohol exposure leads to reductions in CB1R mRNA expression in the BLA (Serrano et al. 2012), which likely contributes to the anxiety-like phenotype observed during withdrawal. Accordingly, a similar phenomenon has also been observed in human alcoholics abstaining from alcohol who show reduced CB1R occupancy and lower AEA levels throughout the brain (Hirvonen et al. 2013; Ceccarini et al. 2014).

In preclinical models, CB1R antagonists prevent acquisition of alcohol drinking in alcohol-preferring rats (Serra et al. 2001) and decrease drinking, as well as sucrose intake, in outbred mice and rats (Arnone et al. 1997; Freedland et al. 2001). Furthermore, some studies have demonstrated that CB1R antagonists decrease escalated alcohol self-administration in alcohol-dependent rats (Rodriguez de Fonseca et al. 1999). Enhanced AEA in mice with a human FAAH polymorphism exhibiting decreased enzymatic activity has also recently been shown to display enhanced binge alcohol intake and preference (Zhou et al. 2016), suggesting that the ECB system is not only important for the affective symptoms of withdrawal but also for the induction of alcohol dependence.

An interaction between the opioid and ECB systems has recently been expanded on (Scavone et al. 2013; Coutts and Pertwee 1998), with preclinical evidence demonstrating that treatment with Δ -9-tetrahydrocannabinol (THC), AEA, or 2-AG can alleviate the symptoms of morphine withdrawal (Cichewicz and Welch 2003; Vela et al. 1995; Yamaguchi et al. 2001; Ramesh et al. 2011, 2013). Additionally, a CB1R antagonist reduces heroin self-administration in rats and morphine-conditioned place preference in mice (Navarro et al. 2001), indicating that reducing ECB signaling can alleviate the rewarding and motivating properties of opioids. This has led researchers to propose the investigation of cannabinoids in the treatment of opioid dependence (Scavone et al. 2013).

Furthermore, after chronic nicotine exposure, AEA and 2-AG is increased in the brainstem and decreased in the hippocampus and cortex (Gonzalez et al. 2002). This same study showed that AEA is also decreased in the striatum after chronic nicotine exposure, with no changes in 2-AG in this region. Thus, the symptomatology of nicotine withdrawal may be driven by changes in ECB signaling. Conversely, FAAH inhibition enhances nicotine-conditioned place preference in mice (Merritt et al. 2008) while also decreasing nicotine withdrawal-induced anxiety without affecting the physical signs of withdrawal (Cippitelli et al. 2011). Therefore, increasing AEA signaling is a potential therapeutic target for specifically alleviating mood disruptions during nicotine withdrawal.

Finally, there is some evidence to indicate that ECB system changes contribute to cocaine withdrawal. Animals chronically treated with cocaine show a decrease in 2-AG content in the limbic forebrain (Gonzalez et al. 2002). Interestingly, this study also evaluated alcohol and nicotine, and cocaine withdrawal resulted in the least amount of changes in ECBs. Furthermore, CB1R antagonists do not reduce cocaine self-administration but do block reinstatement of cocaine seeking and cue- or stress-induced relapse (Filip et al. 2006; Chauvet et al. 2014; De Vries et al. 2001; McReynolds et al. 2016; Vaughn et al. 2012). There has been some speculation as to why CB1R antagonists do not block the reinforcing effects of cocaine,

as they do for alcohol, nicotine, and opioids. It has been suggested that the difference may result from how cocaine modulates dopamine, by directly increasing dopamine in the nucleus accumbens as opposed to acting through the VTA (Parolaro et al. 2007). For example, CB1R deletion does not block cocaine-induced increases in nucleus accumbens dopamine (Soria et al. 2005), suggesting that ECBs are not directly involved in the rewarding effects of cocaine, but play a role in mechanisms driving relapse. On the whole, however, drug withdrawal depresses ECB signaling likely via the CRH-mediated mechanism described above, which ultimately serves a permissive role in the induction of withdrawal-induced negative affective symptoms.

5.3 Sex Differences in Negative Affect and Drug Withdrawal

Women report mood disorders approximately twice as often as men, and sexual dimorphism of the ECB system may contribute to these differences. Multiple lines of evidence suggest that gonadal hormones, such as estradiol, modulate the ECB system (Gorzalka and Dang 2012). For instance, long-term ovariectomy induces depressive-like behaviors in rats (de Chaves et al. 2009). As mentioned previously, the anxiolytic effect of estradiol is blocked by a CB1R antagonist (Hill et al. 2007a), indicating that estrogens may provide anxiolytic-like effects through an ECB mechanism. Clinical data has also demonstrated that major depressive disorder is correlated with reductions in circulating ECBs in women (Hill et al. 2008a, b). Additionally, circulating 2-AG is also depressed in men and women diagnosed with PTSD (Hill et al. 2013a, b), perhaps suggesting an impaired ability to terminate the stress response. Overall, sex differences seen in psychiatric disorders, especially those related to disruptions in mood, may arise from sexual dimorphisms in the ECB system.

Sex differences also exist in the way men and women develop and express the symptoms of alcohol dependence. Alcohol dependence may develop more rapidly in women, and adolescent girls show greater cognitive deficits after excessive alcohol use than adolescent boys (for review see Sharrett-Field et al. 2013). Women also report significantly more psychiatric symptoms in response to chronic alcohol use (Hernandez-Avila et al. 2004), as well as fertility issues and menstrual cycle disruptions (Wilsnack et al. 1984; Emanuele and Emanuele 2001). Consequently, gonadal hormones, and thus changes in ECB signaling, may contribute to sex differences in alcoholism. For example, female wild-type mice drink significantly more than male wild-type mice, but this sex difference is abolished by CB1R deletion (Hungund et al. 2003). Additionally, estradiol protects against the motoric symptoms of withdrawal, as well as neuronal damage to the cerebellum observed following chronic alcohol exposure (Jung et al. 2002). As estradiol has been shown to stimulate AEA biosynthesis in the brain (Huang and Woolley 2012; Tabatadze et al. 2015) and downregulate FAAH expression, thereby increasing AEA levels

(Maccarrone et al. 2000), estradiol may protect against the affective symptoms of alcohol withdrawal through an ECB mechanism.

6 Implications for the Development of ECB-Based Therapeutics

There is some evidence that exogenous cannabinoid use can alleviate mood disorders. For example, a recent controlled trial examining the effects of cannabis consumption on chronic neuropathic pain revealed significant improvements in measures of anxiety and depression, in addition to reduced pain and better quality of sleep (Ware et al. 2010). Unfortunately, a major obstacle in the implementation of cannabis-derived drugs for mood disorders is that long-term heavy cannabis use, particularly during adolescence, is associated with increased risk of experiencing depressive-like symptoms that persist into adulthood (Bovasso 2001; Patton 2002). However, considerable evidence suggests that enhancement of AEA or 2-AG signaling specifically may attenuate the symptoms of stress-related affective disorders.

Inhibition of FAAH reduces anxiety behavior in mice and rats, which is coupled with an increase in AEA in the frontal cortex, striatum, and hippocampus (Marco et al. 2015; Kathuria et al. 2003; Hill et al. 2007a). FAAH inhibition has also been shown to reverse the anxiety and depressive phenotype expressed in rats exposed to chronic stress (Hill et al. 2013a, b; Bortolato et al. 2007; Rossi et al. 2010; Lomazzo et al. 2015). For example, FAAH knockout mice do not show the same anxiety behavior, decreases in AEA, and structural remodeling of the amygdala seen in wild-type mice exposed to chronic stress (Hill et al. 2013a, b). Systemic FAAH inhibition also prevents the negative affective phenotype induced by social defeat stress in rodents (Rossi et al. 2010), suggesting that FAAH inhibition may alleviate symptoms of affective disorders.

In fact, in humans, anxiety is negatively correlated with circulating AEA (Dlugos et al. 2012), suggesting that increasing AEA signaling could be therapeutic for mood disorders. Additionally, a polymorphism of the FAAH gene (C385A) in humans is correlated with both decreased anxiety and increased resting state functional connectivity between the mPFC and BLA (Dincheva et al. 2015; Hariri et al. 2009; Gunduz-Cinar et al. 2013). These authors recapitulated this polymorphism in the mouse and observed an anxiolytic phenotype as well as increased projections from the mPFC to the BLA. Thus, AEA signaling in the mPFC may be constraining BLA activity, perhaps reducing stress-induced negative affect.

In terms of 2-AG, DAGL α -deficient mice show increased anxiety- and depressive-like behavior, which is reversible with a MAGL inhibitor (Shonesy et al. 2014). However, it is important to note that the negative affective behaviors observed were sex specific, with only female DAGL α -deficient mice showing depressive-like behavior. Conversely, systemic MAGL inhibition reduces the

anxiety- and depressive-like behavior seen in mice following chronic unpredictable stress exposure (Zhang et al. 2015), indicating that increasing 2-AG signaling during stress may also be efficacious for treating anxiety and depression. A reduction in circulating 2-AG levels has been reported in humans who have experienced chronic stress of simulated space travel (Yi et al. 2016), supporting the notion that 2-AG tone may play a role in stress-related pathology. Overall, considering the preclinical and clinical data, impairments in ECB signaling may well contribute to stress-related affective disorders, making the use of enzyme inhibitors an attractive option for pharmacotherapeutic development as they avoid the pitfalls of global agonism seen in THC or synthetic CB1R agonists by specifically augmenting AEA or 2-AG mobilization (Gunduz-Cinar et al. 2013).

7 Summary

The stress response is an evolutionarily conserved mechanism to both allow an organism to cope with a threat and to restore homeostasis following the occurrence of a stressor. In addition, preclinical research demonstrates that the ECB system constrains the HPA axis and plays a major role in the habituation to stressors. AEA serves to tonically activate the ECB system, and the reduction of AEA, via increased FAAH activity, in the BLA serves a permissive role in the initiation of the stress response. 2-AG signaling, on the other hand, is recruited to activate CB1Rs and promote termination of the stress response in the short term and to facilitate habituation to stressors in the long term.

The impairment of the ECB system following stress may contribute to stress-related disorders such as anxiety, depression, and substance abuse. Chronic stress downregulates the ECB system, resulting in reduced inhibitory control to the amygdala and a hyperactive stress response. Sex-dependent and genetic differences in the ECB system speak both to the detriment of having reduced ECB signaling and the conferred resiliency to stressors when ECB signaling is augmented.

The growing body of preclinical and clinical evidence demonstrates that the ECB system represents a promising therapeutic avenue for a host of affective human disorders. Pharmacological inhibition of ECB hydrolases has emerged as an effective strategy for managing behavioral and neuroendocrine responses to acute and chronic stress. Moreover, genetic polymorphism in genes that encode these enzymes has offered considerable insight into the stress-resilient phenotype conferred by increased circulating AEA in humans. This piece of information, in addition to extensive animal research, points to selectively augmenting ECB activity via FAAH inhibition as an attractive therapeutic strategy for treating human stress-related affective illnesses.

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Cell-Autonomous Endocannabinoid Production Shapes Polarized and Dynamic Distribution and Signaling Patterns of Cannabinoid CB₁ Receptors in Neurons

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Abstract Interaction with the highly regulated local lipid environment is emerging as key dynamic component of cellular function through the control of the structure, conformation, and function of cell-membrane-embedded proteins, such as G-protein-coupled receptors (GPCRs). The type-1 cannabinoid receptor CB₁, because of a relatively unstable GPCR structure and specific entry sites for lipids diffusing from the plasma membrane, may be particularly sensitive to such effects. In this chapter, we will discuss the first level of this lipid–protein interaction: the cell-autonomous scale, the foundation on which other important layers, such as paracrine or transsynaptic signaling systems, are built in vivo. Recent studies reveal an intricate balance between the polarized production of endocannabinoid (eCB) lipids and the polarized targeting and signaling of CB₁. The endocannabinoid 2-arachidonoylglycerol (2-AG), which is specifically produced in the somatodendritic plasma membrane, exerts cell-autonomous tonic activation on somatodendritic CB₁ receptors. This activation, in addition to important local signaling effects, also regulates CB₁ responses to other cannabinoids and provides the driving force for important basal endocytosis, which is followed by transcytotic CB₁ delivery to the axonal plasma membrane, where the large majority of CB₁Rs accumulate at steady state. This cell-autonomous tonic CB₁ activation is based on two important properties of the endocannabinoid system: the elevated basal production of eCBs in specific regions of the plasma membrane (i.e., basal activation) and the structural instability of the CB₁ protein (i.e., constitutive activity). Key elements of this unusually dynamic functional model are valuable to better understand activation mechanisms of presynaptic CB₁ receptors and may also explain the high diversity of reported CB₁ ligands, ranging from peptide and lipid allo- and orthosteric regulators to the phytocannabinoid Δ^9 -THC, the psychoactive component of marijuana.

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1 Polarized Distribution of Neuronal Plasma Membrane Components

Most differentiated cells organize their plasma membrane into distinct domains, and this compartmentalization of membrane proteins and lipids is critical for proper cell functioning. According to an emerging view, membrane proteins and lipids are organized in constitutive dynamic molecular assemblies, regulated by a combination of protein–protein and protein–lipid interactions (Saka et al. 2013). The highly regulated lipid environment controls the structure, conformation, and function of embedded proteins (Phillips et al. 2009), and the dynamics of the underlying actin cytoskeleton regulate the mobility of many of the membrane constituents, playing a key role in controlling receptor signaling through the regulation of their interactions with functional protein partners (Mattila et al. 2016). As compared to other cell types, archetypal neurons possess a vast plasma membrane surface, which, similarly to the underlying neuronal cytoplasm, is highly compartmentalized and heterogeneous both in physical structure and chemical composition. The polarized neuronal architecture, crucial to establish the directionality of information flow through neuronal networks, is maintained by a nonuniform distribution of organelles and proteins (Craig and Banker 1994). Accordingly, the protein and lipid composition of the plasma membrane also differs greatly between axons and somatodendritic compartments (Horton and Ehlers 2003). This polarization is established by specific targeting mechanisms as discussed below and is maintained by the axon initial segment (AIS), which acts as a surface diffusion barrier and an intracellular traffic filter for both lipids and proteins (for review see Letierrier and Dargent (2014)).

Importantly, neuronal proteins are constantly renewed, and the polarity of their distribution is maintained due to specific targeting processes following their synthesis in the Golgi apparatus, typically localized in the soma (reviewed in Lasiecka and Winckler (2011)). Most dendritic proteins are targeted to the somatodendritic plasma membrane through direct *selective delivery*. For example, cargoes transporting the dendritic transferrin receptor from the Golgi apparatus to the plasma membrane are excluded from the axon, leading to an exclusively dendritic delivery (Burack et al. 2000). For axonal targeting, three main processes have been described, *selective delivery*, *selective fusion*, and *selective retention/elimination* (Horton and Ehlers 2003). While direct targeting through *selective delivery* is predominant in dendrites, it seems that only a few axonal proteins reach the axon through this mechanism (Lasiecka and Winckler 2011). As microtubule orientation-based carriers cannot traffic selectively to the axon due to the mixed dendritic microtubule orientation, either *selective fusion* or *selective retention/elimination* may be used to achieve specific axonal targeting. The latter process assumes that carriers fuse to the dendritic membrane, but proteins are rapidly removed by endocytosis and delivered to the axon in endosomes through *transcytosis* (Lasiecka and Winckler 2011). Experimentally, the difference between both mechanisms is hard to detect but recent studies tend to demonstrate that

transcytosis is the predominant axonal targeting mechanism, as first shown for the neuronal sodium channel $\text{Na}_v1.2$, where a specific C-terminus sequence is recognized by the clathrin-dependent endocytic pathway in the somatodendritic compartment, leading to transcytosis and axonal targeting (Garrido et al. 2001). Subsequently, similar targeting processes were found for various other axonal proteins, such as vesicle-associated membrane protein 2 (VAMP2), involved in synaptic vesicle fusion with the presynaptic membrane (Sampo et al. 2003); tropomyosin-related kinase A (TrkA) receptors, involved in growth and survival of developing neurons (Ascano et al. 2009); contactin-associated protein 2, a cell adhesion molecule (Bel et al. 2009); and β -site APP-cleaving enzyme 1 (BACE1) of amyloid precursor protein (Buggia-Prévot et al. 2014). Recent results, discussed below, also indicate that several important members of the G-protein-coupled receptor (GPCR) family, a large group of sensory and signaling membrane proteins with a vast array of critical functions in neurons, are addressed to axons through a similar mechanism, but with the notable exception that their targeting depends on their pharmacological activation state (Carrel et al. 2011; Simon et al. 2013).

2 GPCR Activation State Depends on the Plasma Membrane Environment

GPCRs are dynamic sensory molecules, composed of rigid alpha-helix transmembrane domains (TMs) that are connected by supple loops, leading to a highly flexible three-dimensional structure (Deupi and Kobilka 2010; Venkatakrisnan et al. 2013). Evidence from both functional and biophysical studies suggests that GPCRs are not simple bimodal switches but permanently sample multiple conformations (Deupi and Kobilka 2010; Venkatakrisnan et al. 2013). They are mostly held in a basal conformational equilibrium at steady state by intervening loops and non-covalent intramolecular interactions such as the proposed “ionic lock” between the third and sixth TMs (Ballesteros et al. 2001). These interactions, as well as others, may collectively form a central hydrophobic core in most Family A GPCRs (Tehan et al. 2007).

At the basal state, inactive conformations typically display a lower energy level than active conformations (Kobilka and Deupi 2007; Deupi and Kobilka 2010). This free-energy difference between active and inactive states, called *activation energy barrier*, leads to a lower probability for active states to appear spontaneously. Agonist-binding level modifies the energy landscape resulting in increased number of receptors in active conformations, i.e., showing rearrangement of the cytoplasmic side of the receptor, which leads to mobilization of intracellular signaling pathways such as the characteristic activation of cognate heterotrimeric G-proteins (Deupi and Kobilka 2010; Venkatakrisnan et al. 2013). Signaling by stabilized activated states is typically terminated in seconds by phosphorylation of specific intracellular serine and threonine residues, which leads to decoupling of the

receptor from G-proteins (desensitization) and to the recruitment of scaffolding/effector proteins such as β -arrestins, ultimately resulting in GPCR endocytosis in the timescale of minutes, typically through the clathrin-mediated endocytic pathway (Hanyaloglu and Zastrow 2008). After elimination of bound ligands and dephosphorylation in endosomes, GPCRs are either recycled back to the plasma membrane or become degraded in lysosomes, depending on the receptor subtype, its level of activation, and the cell type (Hanyaloglu and Zastrow 2008). While the above-described agonist-induced GPCR endocytosis was initially associated with rapid desensitization of G-protein-mediated cellular responses, accumulating evidence now indicates both G-protein-independent signaling (Reiter et al. 2012) and G-protein-mediated signaling (Irannejad and Zastrow 2014) also occur from endosomal compartments.

Theoretical energy landscapes and experimental results both suggest that the multitude of possible GPCR conformational states leads to an energy continuum (Deupi and Kobilka 2010). Thus, at the basal state, even if active conformations are not favored due to a high activation energy barrier, some receptors may still display active conformations. This ligand-free basal activity is called *constitutive activity*. This concept leads to the characterization of antagonist ligands by their ability to promote inactive states: an *inverse agonist* ligand stabilizes inactive receptor conformations, decreasing the basal biological response, while a *neutral antagonist* binds the receptor without inducing changes in the conformational state. Finally, *allosteric modulators* modify either the active (positive allosteric modulators or PAMs) or the inactive (negative allosteric modulators or NAMs) state of the receptor by binding to other parts of the GPCR than the major ligand-binding (orthosteric) site.

The above model shows that constitutive GPCR activity, which could be perceived as counterintuitive in the now outdated bimodal switch model of GPCR function, is rather a logical result of the inherent characteristics of molecular GPCR mechanisms. Notably, the level of basal activity of a given GPCR depends not only of the level of the activation energy barrier but also of the presence of active conformations at low energy levels, since high conformation dynamics may lead to smaller energetic differences between inactive, intermediate, and active states (Manglik and Kobilka 2014). Depending on the GPCR, one or both of these mechanisms can explain constitutive activity. Importantly, the level of basal activity in ligand-free conditions depends both on factors intrinsic to the receptor protein—mostly the ensemble of inactive-state conserving non-covalent interactions between its transmembrane domains, which determine *constitutive activity*—and on extrinsic local factors of *basal activation*, such as cytosolic signaling and regulatory proteins, plasma membrane lipids, pH, ions, transmembrane voltage gradients (reviewed in Manglik and Kobilka (2014)), and even the curvature of the plasma membrane (Brown 2012). Notably, direct and indirect interactions with membrane lipids are now thought to control various essential aspects of GPCR function (reviewed in Oates and Watts (2011)). For example, it was recently speculated that cholesterol-induced structural changes might induce important changes in GPCR ligand binding (Zocher et al. 2012) and, very recently,

differences in the phospholipid composition of the plasma membrane—a function of the cell type, subcellular domains, or nanoscale lipid domains—were shown to play a key role in GPCR activation through genuine allosteric mechanisms (Dawaliby et al. 2016). In conclusion, the combination of intrinsic *constitutive activity* and extrinsic *basal activation* yields the level of *tonic activation*, which is typically low for many but not all GPCRs (Seifert and Wenzel-Seifert 2002).

The above-described lipid-induced interactions may be particularly relevant for GPCRs with a relatively unstable structure (i.e., high conformational flexibility), such as the type-1 cannabinoid receptor CB₁.

3 The CB₁ Cannabinoid Receptor: A Structurally Instable GPCR

The type-1 cannabinoid receptor (CB₁), a class A (or rhodopsin-like) GPCR, is the major neuronal target of endocannabinoid lipids (eCBs) and of exogenous cannabinoids, such as Δ^9 -tetrahydrocannabinol (THC), the main psychoactive constituent of marijuana (Pertwee et al. 2010). CB₁, like numerous other GPCRs, displays a high level of constitutive activity both when heterogeneously expressed in non-neuronal cells and in neurons where CB₁ receptors are endogenous (Pertwee 2005). However, in contrast to other constitutively active GPCRs, where constitutive activity is characteristically a result of structural instability (Gether et al. 1997; Alewijnse et al. 2000; Zhang 2004; Holst et al. 2004; Engelstoft et al. 2014; Gilliland et al. 2015), for CB₁, the lipid composition of the surrounding plasma membrane contributes significantly to tonic activation, blurring the distinction between classical constitutive activity and tonic activation, as discussed below.

The cannabinoid receptor family shares several highly conserved stretches of residues, such as the aspartic acid-arginine-tyrosine (DRY) amino acid motif as well as several neighboring residues with the GPCR consensus sequence. However, Debra Kendall's group has observed (D'Antona et al. 2006) that all cannabinoid receptors identified to date differ from the consensus GPCR sequence by containing Thr at position 3.46 according to the Ballesteros–Weinstein numbering scheme (Ballesteros and Weinstein 1995), named T210 in the original article (D'Antona et al. 2006), one helical turn above Arg 3.50, which is part of the basal-state stabilizing, relatively conserved ionic lock in other GPCRs (Ballesteros et al. 2001). Strikingly, mutations of this single residue lead to dramatic effects in CB₁ activity: substitution of Thr 3.46 with Ala (T3.46A or T210A), a residue with a higher helical packing moment, results in a receptor that shows diminished constitutive activity, while mutation to isoleucine (T3.46I or T210I) yields a hyperactive receptor (D'Antona et al. 2006). These mutations were proposed to result in changes in the conformational state of CB₁, yielding receptor forms that display significantly different levels of constitutive activity but preserve responsiveness to agonist and inverse agonist ligands (D'Antona et al. 2006). Indeed, we found that

treatment of T210I-CB₁ expressing neurons with the antagonist/inverse agonist AM281 or treatment of T210A-CB₁ expressing neurons with the agonist WIN22,512-2 resulted in a significant redistribution toward the wild-type (WT) phenotype, confirming that T210-CB₁R mutants are true conformational CB₁ isoforms (Simon et al. 2013).

Further studies that used modeling and experimental confirmation with double mutant CB₁R have significantly extended our comprehension of the key conformational states of CB₁R and probably also of other GPCRs (Scott et al. 2012; Ahn et al. 2013). These results, yet to be directly confirmed by crystallography, suggest a simple and elegant mechanistic explanation for the graded levels of constitutive activation of CB₁: inactive mutant receptor forms have two salt bridges between transmembrane domains 2 and 6 (TM2–TM6) and TM3–TM6. These interactions between opposed TMs keep the receptor in a tightly packed form with low conformational flexibility and high thermal stability and presumably preclude efficient activation of intracellular effectors in the absence of an agonist. Fully active receptor forms have no salt bridges between opposing TMs but do between neighboring TMs, leading to a loosely packed instable form that efficiently activates intracellular signaling. Finally, partially active receptor forms, such as the wild-type CB₁, contain only one salt bridge between the opposing TM3 and TM6, accompanied by a salt bridge between neighboring TMs (Scott et al. 2012; Ahn et al. 2013). This model indicates that wild-type CB₁ receptors are partially destabilized GPCRs at the basal state.

Due to this structural instability, the activation energy barrier of wild-type CB₁ is lower than in typical GPCRs, providing a mechanistic explanation for constitutive CB₁ activity and possibly also for the elevated sensitivity of the receptor to ortho- and allosteric ligands. Indeed, to date, *in vitro* evidence suggests that there are at least 15 endogenous lipidic compounds that can target cannabinoid receptors either orthosterically or allosterically (Pertwee 2015; see more details below). Other ligands with reported CB₁-activating effects include sodium, peptides such as pepcan-12, a 12-amino-acid neuropeptide or hemopressin (for a recent review, see Morales et al. (2016)), and pregnenolone (Vallee et al. 2014) which has traditionally been viewed as an inactive precursor of neurosteroids. Some of these compounds also target non-cannabinoid receptors or ion channels, at concentrations at which they seem to interact with cannabinoid receptors (Pertwee 2015). Notably, plasma membrane lipids that are either considered to be the orthosteric ligands of CB₁, such as anandamide (N-arachidonoyl ethanolamide or AEA) and 2-arachidonoylglycerol (2-AG), or endogenous allosteric modulators, such as oleamide (an endogenous fatty acid amide) or the arachidonic acid derivative lipoxin A₄, may also nonspecifically modulate GPCR conformations, as suggested by their allo- or orthosteric modulator role for several other membrane proteins (for recent reviews, see van der Westhuizen et al. (2015)). For example, AEA, first described as the *bona fide* CB₁ ligand, now is recognized as an activator of at least 19 other plasma membrane proteins at micromolar concentrations (Pertwee 2005), and 2-AG was recently shown to potentiate GABA-mediated activation of the GABA_A ligand-gated ion channel, through positive allosteric modulation of its

$\beta 2$ -subunit (Sigel et al. 2011; Baur et al. 2013). Moreover, in pancreatic β -cells, 2-AG blocks K_{ATP} potassium channels through a CB_1 -independent mechanism, given that the CB_1 -specific antagonist/inverse agonist AM251 does not remove this effect (Spivak et al. 2012). Finally, recent reports have failed to confirm CB_1R -related allosteric modulator effects of pregnenolone and lipoxin A4 in a standard model cell line (Khajehali et al. 2015) and, together with hemopressin, also by using physiologically relevant cultured neurons and the endocannabinoid 2-AG ligand (Straiker et al. 2015). These diverging results underline the delicate pharmacology of CB_1 and indicate that standard experimental approaches developed for typical GPCRs, which are more stable at steady state and have well-known specific endogenous agonists, should be used under enhanced scrutiny for the naturally destabilized CB_1 .

4 Ubiquitous Neuronal Membrane Component Endocannabinoids Produce Tonic CB_1 Activation

The lipophilic eCBs were proposed to be synthesized on demand from ubiquitous plasma membrane components through various biosynthetic pathways, either in response to intracellular calcium elevations or combined with activation of several $G_{q/11}$ -protein-coupled receptors (for recent detailed reviews, see Alger and Kim (2011), Pertwee (2015)). The first eCBs identified were AEA and 2-AG, both belonging to the eicosanoid family of polyunsaturated fatty acids. AEA is synthesized mainly by hydrolysis of the corresponding N-acyl-phosphatidylethanolamines (NAPEs), catalyzed by a phospholipase D selective for NAPEs (NAPE-PLD), and is degraded by hydrolysis by fatty acid amine hydrolase (FAAH). The other major endocannabinoid, 2-AG, is released by hydrolysis of the cell membrane diacylglycerols (DAGs) by two isoforms of DAG lipases (DAGL α and DAGL β). The degradation of 2-AG is mainly due to monoacylglycerol lipases (MAGLs). Importantly, DAGL α , the major DAGL in the postnatal brain, is segregated to axonal tracts during embryonic development but was shown to accumulate after birth in the somatodendritic plasma membrane in several brain areas, such as the cerebellum (Bisogno et al. 2003), striatum (Uchigashima et al. 2007), hippocampus (Yoshida et al. 2006; Katona et al. 2006), and amygdala (Yoshida et al. 2011). Similarly, we found somatodendritic segregation of DAGL α in fully polarized mature cultured hippocampal neurons (Ladarre et al. 2015), indicating local production of 2-AG in the plasma membrane of the somatodendritic compartment but not in its axonal counterpart.

Neuronal eCBs are generally considered to be retrograde transsynaptic signals, produced in the postsynaptic cell, released and traveling “backwards” across the synaptic cleft, through still undetermined mechanisms, to activate presynaptic CB_1 , as shown by a large body of indirect experimental evidence, gathered using electrophysiological, anatomical, and genetic tools (reviewed in Freund et al.

(2003), Kano et al. (2009)). Studies aimed at understanding eCB transport—by focusing mostly on AEA—have proposed protein carriers (Chicca et al. 2012), such as fatty acid-binding proteins, heat shock proteins, or albumin (Maccarrone et al. 2009), as potential transporters to move eCBs intracellularly and across the plasma membrane (Chicca et al. 2012; Fowler 2013). However, the actual role of such a transporter, the FAAH-like AEA transporter (FLAT) (Fu et al. 2012), has also been questioned (Leung et al. 2013). Neo-synthesized AEA was also suggested to be released into the extracellular fluid and to reach its targets bound to circulating proteins, such as lipocalins or albumin (Piomelli 2003). Interestingly, a recent report suggests vesicle-based extracellular transport for anandamide by showing its enriched presence in exosomes and ectosomes (i.e., shed microvesicles), produced by microglia (Gabrielli et al. 2015). Such lipidic-vesicle-based transport may well explain efficient distribution of lipid eCBs in aqueous environments and opens up an entire new field in neurotransmitter research aimed at studying the liberation and metabolism of these extracellular vesicle transporters.

Importantly, however, in addition to this established retrograde transsynaptic signaling role, it was also reported that significant amounts of 2-AG are present in resting neurons, that only a fraction of stimulation-induced 2-AG is released from cells, and that basal levels of AEA are sufficient to constitutively induce CB₁ signaling (for review refer to Alger and Kim (2011)). Notably, both AEA and 2-AG are directly derived from membrane phospholipids and synthesized in the lipid bilayer of the plasma membrane, and no effective intramembrane sequestration of the important basal pool of eCBs has yet been identified. Consequently, these endocannabinoids may reach CB₁ through two-dimensional diffusion in—and through direct entry from—the lipid bilayer, a long-held assumption in the field recently confirmed by experimental and computational results (Reggio 2010). Indeed, analogously to the now structurally resolved S1PR1 lipidergic GPCR, 2-AG was shown to access the binding pocket of the type-2 cannabinoid receptor CB₂ through entry between TM6 and TM7 (Hurst et al. 2013). As the CB₁ amino acid sequence is highly similar to that of CB₂, we may assume that their structure, binding sites, and agonist entry mode are analogous. Structure–activity relationship studies investigating the interaction of cannabinoid receptors with eCBs have also indicated in AEA, 2-AG, and congeners high “dynamic plasticity,” indicating that they are able to extend as well as form tightly curved conformations (Reggio 2010). Strikingly, only analogs that are capable of these two extremes in conformation are recognized by CB₁, suggesting that one of these conformations is important for getting to the receptor in the membrane while the other is important for getting into the receptor (Reggio 2010). Due to these unusual ligand characteristics, the basal activation stemming from the steady-state endocannabinoid tone in the plasma membrane may be a major contributor to tonic CB₁ activation (reviewed in Howlett et al. (2011)). Indeed, blocking the synthesis of 2-AG, the major endocannabinoid in the mammalian brain (Stella et al. 1997), abolishes (or strongly decreases) both constitutive CB₁ signaling and CB₁ internalization in heterologous systems (Turu et al. 2007). These results were recently further refined by our laboratory in cultured hippocampal neurons by showing that the antagonist/inverse agonist AM281

produces a marked rapid increase of basal PKA activation in somata and dendrites, but not in axons, and that DAGL inhibition abolishes this effect, suggesting that tonic activation of somatodendritic CB₁ is generated by local somatodendritic production of the endocannabinoid 2-AG (Ladarré et al. 2015). Interestingly, the 2-AG content of the somatodendritic plasma membrane also modulates CB₁ responses to exogenous cannabinoids, as we have observed that the presence of 2-AG is necessary for WIN55-212,2 and THC-mediated activation of somatodendritic CB₁, but, on the contrary, CP55,940-mediated decrease of PKA activity in the somatodendritic compartment is higher in the absence of 2-AG (Ladarré et al. 2015). These results suggest that 2-AG, which is specifically produced in the somatodendritic plasma membrane, has important cell-autonomous local actions on somatodendritic CB₁ receptors, both by achieving significant basal activation and by regulating CB₁ activation by other cannabinoids.

Taken together, several lines of experimental evidence show that eCBs acting on CB₁ may be, in many instances, of cell-autonomous origin. This mode of action is often referred to in the endocannabinoid literature as “autocrine” (from Greek *krīnō*, “to separate” or “to secrete”), a term usually applied to hormones or other chemical messengers that are secreted to the extracellular milieu before binding to receptors on the same cell. However, as seen above, eCBs do not need to leave the neuronal plasma membrane to gain access to the ligand-binding site of CB₁ receptors on the same neuron. We have also shown previously that tonic CB₁ activation does not depend on eCBs in the extracellular milieu (Leterrier et al. 2004). Consequently, “cell-autonomous” appears to be a more adapted term for this important element of eCB action.

In conclusion, the above-detailed results suggest that important CB₁R-activating lipids may modify relatively nonspecifically the conformation of plasma-membrane-embedded proteins. Indeed, the lack of clearly understood storage and release mechanisms for eCBs, the structural instability of CB₁, the high level of cellular eCB content in membranes of non-stimulated neurons, the multiple reports of cell-autonomous CB₁ activation mechanisms, and high level of CB₁ receptors outside of presynaptic specializations (Mátyás et al. 2006; Leterrier et al. 2006) suggest that in addition to the classical *modus operandi* of presynaptic GPCR function (Starke 1981), novel functional paradigms should also be envisioned for CB₁, both in the adult and developing brain. An interesting possibility is that CB₁ may be particularly sensitive to the lipid composition of the plasma membrane, due to its structural instability and the presence of specific entry sites that are accessible for lipids present in the lipid bilayer. As a consequence, CB₁ may be able to continuously translate the plasma membrane concentration of highly regulated lipid eCBs into the activation of specific intracellular signaling pathways (and be the subject of steady-state endocytosis, as discussed below). However, this relative nonspecificity of CB₁ activation mechanisms does not exclude their physiological pertinence: cannabinoid receptors may have been evolutionary optimized to become highly sensitive but relatively nonspecific sensory molecules, able to report changes in the level of a broad range of lipidic and non-lipidic components, several of them being synthesis intermediates of other important molecules such as

arachidonic acid. Importantly, recent insights into GPCR function have started to reveal that the very efficient and rather on–off mode of rhodopsin activation, traditionally considered as the gold standard of GPCR action, may correspond only to one extreme of the GPCR function spectrum, being optimized for the highly efficient (i.e., very low signal-to-noise ratio) response to light (Manglik and Kobilka 2014). On the contrary, the relatively inefficient coupling of the β 2AR receptor may be important for its more complex functional repertoire and its diversity of responses to different ligands (Manglik and Kobilka 2014). In this context, the instable and promiscuous CB₁ would rather be located at the opposite side of the GPCR function spectrum, as compared to rhodopsin. Finally, the structural characteristics of CB₁ may also explain its role as a major drug target: the phytocannabinoid Δ^9 -THC, which was likely not optimized by evolution in plants to act on a human brain receptor, is able to efficiently activate CB₁, because of its high sensitivity that results from structural instability.

5 Subcellular Distribution of Neuronal CB₁ Receptors

CB₁ is one of the most abundant GPCRs in the brain. Its distribution has been studied with various techniques such as autoradiography, *in situ* hybridization, and immunohistochemistry, all reporting high receptor densities in the cerebral cortex, hippocampus, amygdala, basal ganglia, and cerebellum, in accordance with behavioral effects of cannabis (Freund et al. 2003). CB₁ receptors are highly expressed in GABAergic neurons and display a lower but significant expression in glutamatergic neurons (Marsicano and Lutz 1999; Kawamura et al. 2006; Katona et al. 2006). Interestingly, while most CB₁ receptors are localized to the plasma membrane in axons—typically outside of synaptic terminals as discussed below—of mature neurons both *in vitro* (Coutts et al. 2001; Leterrier et al. 2006; McDonald et al. 2007) and *in vivo* (Katona et al. 1999, 2001; Pickel et al. 2004; Nyiri et al. 2005; Bodor 2005; Kawamura et al. 2006) (Mátyás et al. 2006; Thibault et al. 2013), high levels of intracellular CB₁ localization were reported in non-polarized cells, both when endogenously expressed (McIntosh et al. 1998; Hsieh et al. 1999; Graham et al. 2006; Ellis et al. 2006; Tappe-Theodor et al. 2007) or transfected (Wu et al. 2008; Rozenfeld and Devi 2008; Grimsey et al. 2010). Similar prominent intracellular CB₁ localization was reported in the somatodendritic domain of mature neurons (Fig. 1) (Coutts et al. 2001; Katona et al. 2001; Pickel et al. 2004; Bodor 2005; Leterrier et al. 2006; McDonald et al. 2007; Scavone et al. 2010; Thibault et al. 2013) as well as in the somatodendritic and axonal domain of embryonic neurons (Vitalis et al. 2008).

The exact origin and role of this intracellular CB₁ population is a hotly debated subject in the cannabinoid field. Since eCBs are lipid ligands that are thought to easily cross the plasma membrane, several teams have proposed “stationary” intracellular localization of functional CB₁ receptors on different intracellular organelles. First, a stationary and functional intracellular localization guided by

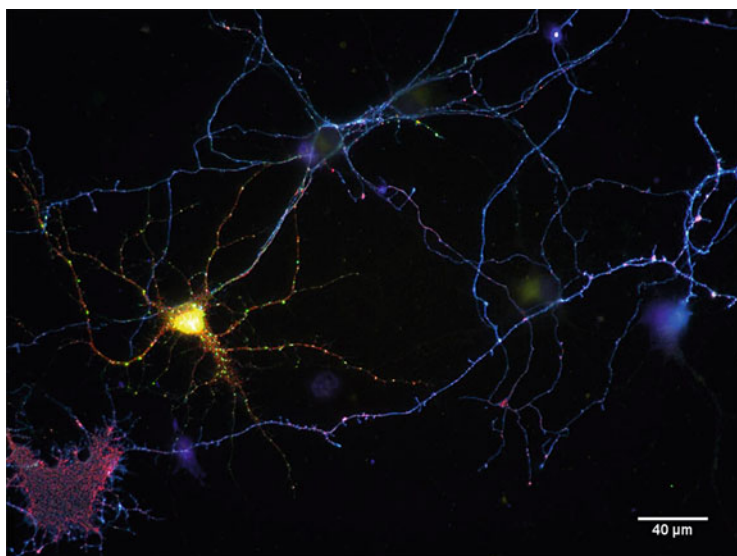


Fig. 1 CB₁ receptors are constantly endocytosed in the somatodendritic region but not on the axonal plasma membrane. Living neurons co-expressing *Flag-CB₁-eGFP* (green) and a *clathrin light chain-DsRed* (red) fusion proteins at day in vitro 8 were incubated for 1 h with an anti-Flag M1 antibody to bind to the extracellular Flag epitope (i.e., antibody feeding), revealed after fixation and permeabilization in blue to label CB₁ that transited at the neuronal surface in the last 1 h. This figure shows that in the somatodendritic compartment (at the left side of the image), CB₁ receptors are predominantly internalized and often co-localize with clathrin, indicating their internalization through a clathrin-dependent mechanism. On the contrary, in axons (network of thin neurites at the right side of the image), CB₁ accumulates on the plasma membrane. In the lower left corner, a glial cell expresses *clathrin light chain-DsRed* but not *Flag-CB₁-eGFP*. For experimental details and a higher magnification example of a similar preparation, showing endocytic vesicles in the soma and proximal dendrites co-labeled for *Flag-CB₁-eGFP*, *clathrin-DsRed*, and M1 antibody, please refer to Leterrier et al. (2006). Endogenous CB₁Rs show similar activation-dependent constitutive endocytosis as shown by live labeling with a N-terminus-targeted antibody (Simon et al. 2013)

the adaptor-protein AP-3 to late endosomal/lysosomal compartments was proposed (Rozenfeld and Devi 2008). Lysosomal CB₁ was subsequently suggested to release calcium from the endoplasmic reticulum and lysosomal calcium stores following anandamide stimulation (Brailoiu et al. 2011). Next, functional CB₁ receptors, found by immunohistochemistry to be expressed on the mitochondrial outer membrane, were shown to inhibit mitochondrial respiration (Benard et al. 2012) reducing it by 20% at saturating ligand concentrations (Hebert-Chatelain et al. 2014). However, others have also reported that both agonist and inverse agonist cannabinoid ligands may inhibit mitochondrial respiration through off-target (i.e., CB₁-independent) mechanisms (Fišar et al. 2014; Schirris et al. 2015; Singh et al. 2015) and that anti-CB₁ antibodies show off-target labeling of another important mitochondrial protein, which is upregulated by neuronal anoxia (Morozov et al. 2013, 2015). In addition, important aspects of general GPCR function, such as targeting,

signaling, desensitization by phosphorylation, and resensitization/downregulation through endocytic pathways, remain poorly understood for mitochondrial CB₁ receptors, warranting further studies to assess their physiological relevance.

Contrarily to this “static” view of intracellular CB₁ localization, several studies, including reports from our laboratory, have indicated a different and dynamic source for intracellular CB₁ receptors: constitutive endocytosis from the plasma membrane. First, live imaging of GFP-tagged CB₁ expressing HEK293 cells shows highly dynamic endosomal labeling with traffic from and to the plasma membrane (see Fig. 3 and Supplementary Movie 1 in Leterrier et al. 2004). Second, the endosomal CB₁ population is readily labeled by incubation of non-permeabilized living cells with antibodies directed toward the extracellular N-terminus, both for transfected (Fig. 1 and Leterrier et al. 2004, 2006; Koch et al. 2006; Ellis et al. 2006; Wu et al. 2008; Grimsey et al. 2010; Ward et al. 2011; Simon et al. 2013) and endogenous CB₁ (Leterrier et al. (2006) and Supplementary Figure S2 in Simon et al. (2013)). Third, pharmacological or genetic inhibition of the endocytic machinery upregulates the proportion of plasma-membrane-located CB₁ receptors and diminishes the intracellular CB₁ population (Leterrier et al. 2004, 2006).

What is the driving force of this constitutive CB₁ internalization, which was reported both in a wide range of immortalized cell lines and in neurons? Results obtained by either pharmacological (Leterrier et al. 2004, 2006; Ellis et al. 2006; Ward et al. 2011; Simon et al. 2013) or genetic (D’Antona et al. 2006; Simon et al. 2013) tools show that constitutive CB₁ endocytosis is a consequence of tonic CB₁ activation at steady state and indicate that the presence of both structural CB₁ determinants (D’Antona et al. 2006; Simon et al. 2013) and basal activation through steady-state cell-autonomous production of endocannabinoids such as 2-AG (Turu et al. 2007) are necessary. These results are in line with the view that the important intracellular pool of CB₁ receptors, a distribution pattern characteristic also for several other constitutively active GPCRs (Morisset et al. 2000; Marion et al. 2004; Morris 2004; Jacquier et al. 2006; Mohammad et al. 2007; Holliday et al. 2007; Chanrion et al. 2008), is a dynamic phenomenon and is related to tonic CB₁ activity. Finally, similarly to other GPCRs, exposure to agonists induces additional internalization of the plasma-membrane-located CB₁ population in non-polarized cell lines (Hsieh et al. 1999; Leterrier et al. 2004; Tappe-Theodor et al. 2007; Wu et al. 2008; Grimsey et al. 2010).

Poststimulation endocytosis is important both for resensitization and downregulation of CB₁ receptors, either through recycling to the plasma membrane or through lysosomal destruction mediated by association of post-endocytic sorting proteins such as GASPI, respectively (Whistler et al. 2002; Martini et al. 2007, 2010). The choice between recycling and degradation may depend both on the concentration of the agonist ligand and on the treatment duration (Hsieh et al. 1999; Whistler et al. 2002; Leterrier et al. 2004; Martini et al. 2007, 2010; Tappe-Theodor et al. 2007; Wu et al. 2008; Grimsey et al. 2010). However, GPCR recycling is difficult to measure directly, putatively contributing to a debate about this specific point of the CB₁ life cycle (Rozenfeld and Devi 2008; Grimsey et al. 2010).

What is the *in vivo* relevance of the above results, which, by using highly resolved *in vitro* experimental approaches, indicate elevated activation-dependent plasticity of subcellular CB₁ distribution phenotypes? The first indication of activation-dependent plasticity in brain CB₁ distribution came from reports showing that chronic cannabis use leads to downregulation of CB₁ in humans and that these effects subside within days to several weeks of sustained abstinence (for review see Curran et al. (2016)). Similarly, chronic cannabinoid treatment in rodents leads to a reduction in CB₁ function throughout the brain (Breivogel and Childers (1999) and reviewed in Sim-Selley (2003), González et al. (2005)) that persists for days to weeks before functional recovery that varies between brain regions (Sim-Selley et al. 2006). These results suggested the occurrence of agonist-induced CB₁ internalization *in vivo*, and we have indeed reported that acute systemic cannabinoid treatment of rats induces marked CB₁ internalization into somatodendritic endosomes (Thibault et al. 2013). On the contrary, antagonist/inverse agonist treatment decreased the basal level of endosomal CB₁ receptors in the somatodendritic compartment, strikingly demonstrating that at steady state CB₁ is already significantly internalized through basal activation (Thibault et al. 2013). Notably, at 16h after a single cannabinoid treatment, the number of perikarya displaying CB₁-containing endosomes returned near to control levels in the neocortex without change in brain CB₁ protein levels, suggesting that CB₁ are efficiently recycled after moderate activation (Thibault et al. 2013). However, more sustained activation led to enhanced endosomal labeling and a parallel decrease in brain CB₁ protein levels (Thibault et al. 2013), suggesting redirection of CB₁ to degradation pathways, leading to the characteristic downregulation reported to parallel the development of tolerance (Sim-Selley et al. 2006).

Interestingly, near-maximal increase of the somatodendritic endosomal labeling after a single agonist treatment was not accompanied by a significant decrease in axonal labeling, which required repeated daily agonist treatments even in the most responsive regions, such as the neocortex and hippocampus (Thibault et al. 2013). These results were recently confirmed by showing slow activation-dependent elimination of CB₁ from synaptic boutons following chronic CB₁ activation in rats (Dudok et al. 2015). The relatively slow axonal translocation kinetics likely results from restrained endocytosis of axonal CB₁ receptors. Indeed, maximal agonist-induced CB₁ internalization in axons of cultured neurons is obtained only after 16 hours of continuous agonist treatment (Coutts et al. 2001; Leterrier et al. 2006), while immortalized cell lines display maximal levels of CB₁ endocytosis already at 15–30 minutes following activation (Rinaldi-Carmona et al. 1998; Hsieh et al. 1999; Leterrier et al. 2004), indicating the relatively low efficacy of the axonal endocytotic machinery. Notably, endocytic capacity at the neuronal surface is also highly polarized and compartmentalized, plasma membrane endocytosis at the axonal cell membrane is restricted to nerve terminals and varicosities, whereas dendrites and immature axons are capable of internalization along their whole length, as shown in cultured hippocampal neurons (Sinclair et al. 1988; Parton et al. 1992; Parton and Dotti 1993). Thus, CB₁ receptors, located mainly at preterminal axons and axon shafts (Katona et al. 1999, 2001; Pickel et al. 2004;

Nyiri et al. 2005; Bodor 2005; Kawamura et al. 2006; Mátyás et al. 2006; Thibault et al. 2013), may need to be transported for considerable distances following activation in order to reach synapses and varicosities and access the endocytic machinery. In certain pathways of the basal ganglia that express functional CB₁ receptors on thin, unmyelinated fibers devoid of synaptic specializations, such as the pallido-nigral and striato-pallidal projections (Mátyás et al. 2006), this distance may reach several hundreds of micrometers. Indeed, we have found a striking lack of CB₁ redistribution in pallido-nigral axons after prolonged agonist treatment (Thibault et al. 2013), indicating their pronounced inability to internalize. Interestingly, basal ganglia display also very moderate downregulation of CB₁ following chronic exposure to Δ^9 -THC or synthetic agonists, as compared to other brain regions (Sim-Selley 2003; González et al. 2005). Since GPCR endocytosis is the first step of receptor downregulation, a simple mechanistic model may explain the marked region-specific differences in downregulation of CB₁ receptors by suggesting that endocytosis and downregulation of axonal CB₁ receptors are inversely proportional to their distance from potential internalization sites, such as terminals and axonal varicosities (Thibault et al. 2013). Finally, the reduced internalization capacity of the basal ganglia may also explain the reported lack of difference in desensitization in this region after treatment with agonists having high and low capacity to induce endocytosis (Wu et al. 2008): in the neocortex and hippocampus, efficient internalization, dephosphorylation, and recycling allow partial CB₁ resensitization in animals treated with high, but not low, endocytotic agonists (Wu et al. 2008), while the basal ganglia mostly lack this internalization-dependent resensitizing mechanism (Sim-Selley and Martin 2002).

In conclusion, CB₁ displays polarized distribution in neurons: in the somatodendritic compartment, CB₁ is mainly internalized due to a combined effect of structural instability and basal activation stemming from the constitutive endocannabinoid tone in the plasma membrane, while in the axon CB₁ accumulates on the plasma membrane due to a lack of endocannabinoids and the lower endocytic capacity of this neuronal compartment.

6 Polarized CB₁ Targeting in Neurons

Since polarized distribution of GPCRs to one of the main neuronal sub-domains—soma, dendrites, or axons—has critical functional consequences, considerable effort has been deployed in the last decades toward both classifying GPCRs as pre- or postsynaptic and identifying protein motifs ensuring targeting and anchoring of receptors either to presynaptic (axonal) or postsynaptic (somatodendritic) domains. However, this enterprise has only yielded the identification of a limited number of targeting motifs (reviewed in Lasiecka and Winckler (2011), Winckler (2012)), so specific targeting and anchoring signals have not yet been identified for the vast majority of neuronal GPCRs. In addition, detailed ultrastructural analysis revealed that most GPCRs are located outside of pre- and postsynaptic

specializations (for references see Simon et al. (2013)). Finally, by demonstrating an often surprising and yet unexplained diversity of distribution phenotypes, GPCRs may display different polarized distribution patterns depending on the neuronal subtype or on the neurochemical environment (for references see Simon et al. (2013)). Such differences in polarized distribution of neuronal GPCRs may have important but presently mostly unexplored consequences on intraneuronal mobilization of signaling pathways.

Recent studies have started to reveal an intricate dynamic relationship between neuronal polarization and GPCR activation, reporting previously unexpected polarized and dynamic patterns of CB₁ targeting and signaling. The first approach to study CB₁ trafficking in neurons was the comparison of steady-state (i.e., non-stimulated) endocytosis of endogenous and transfected CB₁, by using “antibody feeding” of live neurons, which showed significantly higher CB₁ endocytosis from the somatodendritic surface as compared to the axonal plasma membrane (Leterrier et al. 2006; Simon et al. 2013), yielding two consistently offset sigmoid curves of CB₁ endocytosis over a large scale of CB₁ activation levels (Simon et al. 2013). This finding is notable since, as described above, in neurons surface-localized CB₁ receptors are mostly found in axons, so one could expect higher levels of axonal CB₁ endocytosis assuming similar endocytic efficiency from the somatodendritic and axonal plasma membranes. Therefore, this result directly confirms the above-proposed difference in CB₁R internalization capacity between dendrites and axons. We also established that, similarly to the above-reported findings in non-polarized cells, the driving force of this constitutive somatodendritic endocytosis is tonic CB₁ activation, as shown by using either pharmacological (Leterrier et al. 2006; Simon et al. 2013) or genetic (Simon et al. 2013) tools. This tonic activation depends both on structural CB₁ determinants (Simon et al. 2013) and steady-state cell-autonomous production of the eCB 2-AG (Turu et al. 2007). The substantial difference between the somatodendritic and axonal endocytosis rates, present at a broad range of CB₁ activation levels (Simon et al. 2013), suggests that polarized CB₁ distribution may be a result of selective elimination of CB₁ from the somatodendritic plasma membrane, as previously described for several axonal proteins which are also first delivered to the dendritic plasma membrane but rapidly removed by endocytosis and translocated to the axon through transcytosis (Lisiecka and Winckler 2011). This hypothesis was confirmed by using microfluidic devices that allow separate incubation of the somatodendritic and axonal plasma membrane, allowing us to demonstrate that CB₁ receptors transiently present on the somatodendritic plasma membrane are constitutively endocytosed and transported to the axonal surface through transcytosis (Simon et al. 2013). Direct axonal export of CB₁ is also present but does not lead to efficient GPCR delivery to distal axonal portions. Conversely, the transcytotic route originating from somatodendritic recycling endosomes delivers CB₁ in vesicles specialized for long-range axonal delivery directly to distal portions of axons (Simon et al. 2013). Interestingly, once delivered to the axonal plasma membrane, CB₁ receptors appear to diffuse relatively freely, as shown both by diffusion analysis using single-quantum dot imaging (Mikasova et al. 2008) and by our

FRAP analysis (Simon et al. 2013), suggesting the lack of anchoring to scaffolding proteins. Interestingly, a recent study showed, by using STORM super-resolution microscopy, a uniform distribution of CB₁ in presynaptic terminals of GABAergic neurons in the mouse hippocampus (Dudok et al. 2015) without specific enrichment in nanoscale domains in synaptic terminals. This is in line with our model of passive accumulation of CB₁ on the axonal plasma membrane, which suggests that CB₁ receptors are “retained” in the axonal plasma membrane simply through a reduced basal endocytosis rate. Based on the ensemble of the above results, we suggest that the major driving force of polarized CB₁ targeting to axons is a polarized difference in steady-state endocytosis, which is higher in the somatodendritic region (Leterrier et al. 2006; Simon et al. 2013). This model was confirmed by an independent report (McDonald et al. 2007), and we have tested its general validity for other axonal GPCRs by showing that the 5-HT_{1B} serotonin receptor is addressed to axons by similar mechanisms (Carrel et al. 2011).

The pharmacological activation state of CB₁ as a driving force of this transcytotic targeting was debated initially (McDonald et al. 2007), possibly due to the important natural variability of polarized distribution patterns of CB₁ in cultured hippocampal neurons and to a yet unexplained mixed phenotype of CB₁ expression following inverse agonist treatment (Simon et al. 2013). We have overcome this variability in our reports (Leterrier et al. 2006; Simon et al. 2013) by using a Brefeldin A (BFA) release protocol, a standard experimental approach in protein targeting studies, which enhances experimental sensitivity by first accumulating newly synthesized proteins in the Golgi apparatus before synchronized release through washout of BFA (Cid-Arregui et al. 1995; Wisco et al. 2003; Fache et al. 2004). By using this tool, antagonist/inverse agonist AM281 treatment led to the accumulation of newly released CB₁ on the somatodendritic plasma membrane, showing that the pharmacological conformational state of CB₁ is a major regulator of steady-state somatodendritic endocytosis and transcytotic axonal targeting (Leterrier et al. 2006; Simon et al. 2013), a finding also confirmed by our subsequent studies, which used additional pharmacological and genetic tools, the T210I-CB₁ and T210A-CB₁ mutants (Simon et al. 2013). Notably, we have shown that tonic activation-dependent constitutive endocytosis is also crucial for axonal targeting of at least one other GPCR, the 5-HT_{1B} serotonin receptor, since inverse agonist treatment, which prevents tonic activation, leads to atypical accumulation of newly synthesized 5-HT_{1B}s on the somatodendritic plasma membrane (Carrel et al. 2011). Strikingly, kinetic modeling confirmed by our experimental results indicated that gradual changes of basal activation lead to gradual shifts of polarized CB₁ localization and that changes in basal activation can completely switch distribution phenotypes from somatodendritic (i.e., postsynaptic) to axonal (i.e., presynaptic) and vice versa, for several GPCRs (Simon et al. 2013). Finally, as discussed above, *in vivo* inhibition of basal CB₁ activation by inverse agonist treatment decreases the number of cell bodies containing CB₁-immunoreactive endosomes, confirming that a substantial amount of CB₁ receptors is permanently activated and internalized in the brain (Thibault et al. 2013).

Taken together, our results (summarized in Fig. 2A) demonstrate an unexpectedly high activation-dependent plasticity of polarized CB₁ distribution. We have

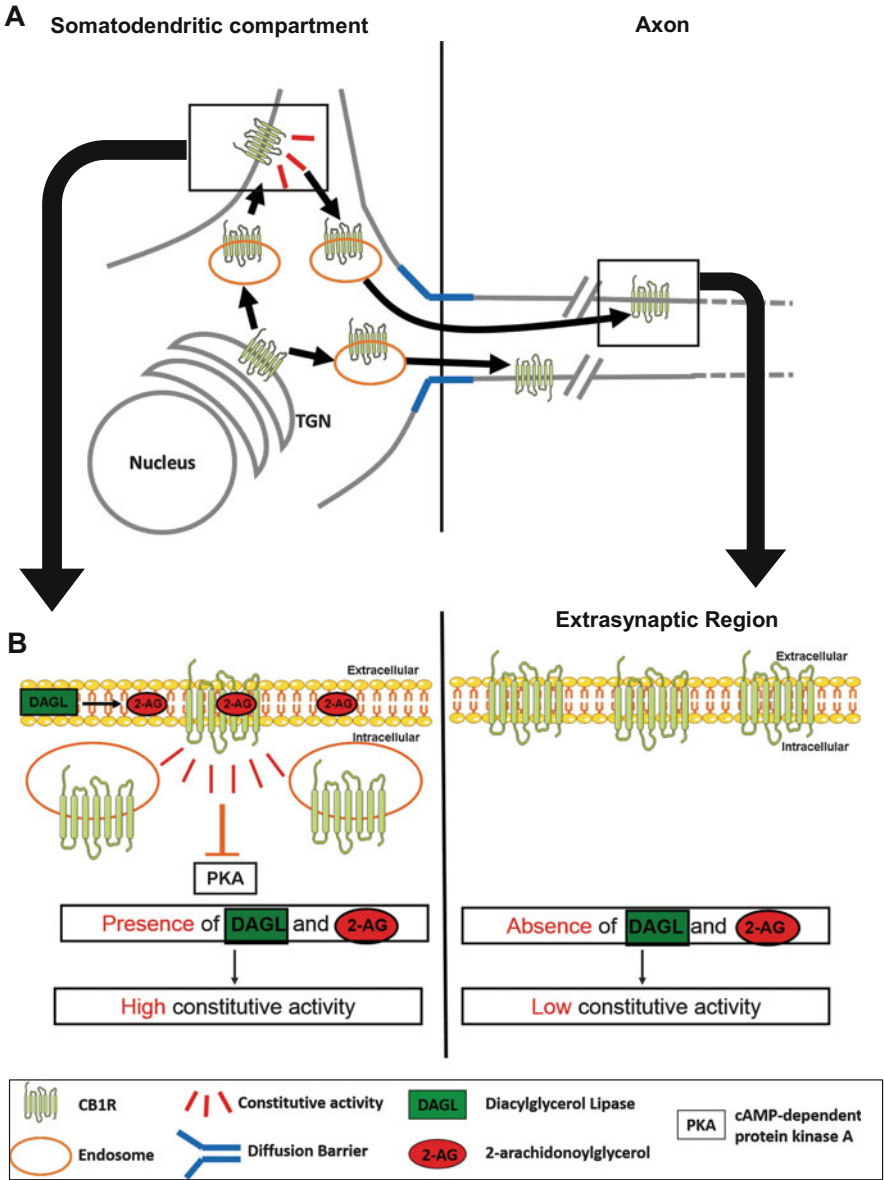


Fig. 2 Polarized patterns of CB₁ targeting and signaling in neurons. **(a)** Schematic representation of CB₁ targeting in neurons. After their synthesis, CB₁ is targeted both to the axonal plasma membrane and to the somatodendritic plasma membrane. Somatodendritic CB₁ receptors are rapidly internalized by constitutive endocytosis and transferred by transcytosis to the axonal plasma membrane where they accumulate. Importantly, transcytosis enables CB₁R transport to distal axonal regions, as compared to direct targeting to proximal axonal regions. **(b)** Schematic representation of basal CB₁ activation in the somatodendritic compartment and extrasynaptic axonal regions, where the majority of axonal CB₁ locate. In the somatodendritic compartment, CB₁ receptors constitutively inhibit cAMP/PKA activity and are constitutively endocytosed because of basal activation, both due to elevated local levels of the endocannabinoid 2-AG,

characterized several key points of neuronal GPCR targeting by first showing how prolonged pharmacological treatments modify neuronal GPCR distribution (Simon et al. 2013). We have also shown that the change of basal activation levels may lead to a striking reversion of polarized GPCR distribution and that the natural constitutive activation of CB₁ is near to the optimal moderate level to ensure polarized distribution to the axonal plasma membrane (Simon et al. 2013).

7 Polarized CB₁R Signaling in Neurons

The above studies show that trafficking of CB₁ differs between the somatodendritic compartment, where it is mostly internalized by constitutive activation, and axons, where CB₁ receptors are predominantly localized on the axonal plasma membrane. We have recently asked whether these polarized differences in traffic and localization are accompanied by polarized differences in signaling. By using cultured hippocampal neurons of rat embryos, we measured the PKA activity downstream of endogenous CB₁ receptors with FRET (Förster resonance energy transfer) probes in individual axons, dendrites, and neuronal somata (Ladarre et al. 2015). In both somata and dendrites, CB₁ activation with WIN55-212,2 led to a decrease of PKA activity while CB₁ blockade with antagonist/inverse agonist AM281 produced a rapid and significant increase of PKA activity. Thus, somatodendritic CB₁ receptors, in transient passage at the plasma membrane (Leterrier et al. 2006; Simon et al. 2013), are available to exogenous cannabinoids, potentially explaining important local effects such as endocannabinoid-mediated somatodendritic slow self-inhibition (SSI) (Bacci et al. 2004; Marinelli et al. 2008) and the tonic 2-AG-mediated enhancement of the h-current through the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in superficial CA1 pyramidal cells (Maroso et al. 2016). In addition, somatodendritic CB₁ also constitutively inhibit PKA activity (Ladarre et al. 2015), which is consistent with their constitutive activation responsible for their endocytosis in this compartment, as described above. Importantly, inhibiting DAGL α before AM281 application removes the effect of the antagonist/inverse agonist, suggesting that the tonic activation of somatodendritic CB₁ receptors requires local cell-autonomous production of 2-AG (Ladarre et al. 2015). Interestingly, as already mentioned above, the 2-AG content of the somatodendritic plasma membrane also modulates CB₁ responses to exogenous cannabinoids, being necessary for WIN55-212,2- and THC-mediated CB₁R activation but dispensable (and competitively antagonist) for CP55,940

Fig. 2 (continued) synthesized by DAGL α . On the contrary, in axons, CB₁ receptors accumulate on plasma membrane of axonal shafts (i.e., preterminal axons) and are not tonically activated due to a lack of DAGL α

action on CB₁ (Ladarré et al. 2015). Therefore, 2-AG, which is specifically produced in the somatodendritic plasma membrane (Bisogno et al. 2003), has important cell-autonomous local actions on somatodendritic CB₁ receptors, by regulating, in addition to the significant basal activation of downstream signaling pathways, their activation by other cannabinoids and by providing the driving force for significant basal endocytosis followed by transcytotic delivery to the axonal plasma membrane.

Interestingly, in axons the rapid decrease of PKA activity following CB₁ activation is significantly stronger than in dendrites (Ladarré et al. 2015). This result is consistent with the higher level of CB₁ receptors on axonal plasma membrane as compared to somatodendritic plasma membrane (Coutts et al. 2001; Pickel et al. 2004; Leterrier et al. 2006). Moreover, inverse agonist application does not lead to an increase of PKA activity, confirming the absence of cell-autonomous tonic activation for axonal CB₁ receptors (Ladarré et al. 2015). Notably, DAGL α is absent in mature axons (Bisogno et al. 2003). Thus, absence of 2-AG in this compartment may explain the lack of cell-autonomous tonic CB₁ activation (Fig. 2B), further proving that tonic CB₁ activation, reported in a wide range of cell lines and in the somatodendritic domain, requires cell-autonomous production of eCBs in addition to the structural instability of CB₁.

8 A Special Case: CB₁ at the Presynaptic Terminal

CB₁ was characterized originally as a major presynaptic GPCR implicated in retrograde synaptic signaling (recent review in Katona and Freund (2012)), and this discovery is registered among the most important advances of the last decades in neuroscience (Südhof and Malenka 2008). Intriguingly, this key physiological role is apparently assured by only a small percentage of CB1Rs, since subcellular localization studies have consistently found a significantly higher density of CB1Rs in axon shafts (also called preterminal axons) than in terminals, indicating a predominantly extrasynaptic cell membrane localization of axonal CB1Rs both in vitro (Leterrier et al. 2006) and in vivo (Bodor et al. 2005; Nyiri et al. 2005; Mátyás et al. 2006). The physiological role of the large extrasynaptic CB₁ population, often located at great distances from synapses (Thibault et al. 2013), is still enigmatic. Nevertheless, endocannabinoids induce critical changes in synaptic strength both in a tonic and in a phasic, activity-dependent manner (Katona and Freund 2012; Soltesz et al. 2015). While early studies focused more on phasic synaptic modulation, in which eCBs transiently depress neurotransmission after being produced “on demand” in response to specific postsynaptic signals, such as an increase in Ca²⁺ concentration or activation of G_{q/11}-coupled GPCRs, tonic eCB effect on the probability of GABA release is increasingly recognized as a major feature of brain eCB function (Katona and Freund 2012; Soltesz et al. 2015).

What is the probable relevance of the results, reviewed above in the chapter, to the presynaptic terminal, a small and highly specialized subcellular compartment?

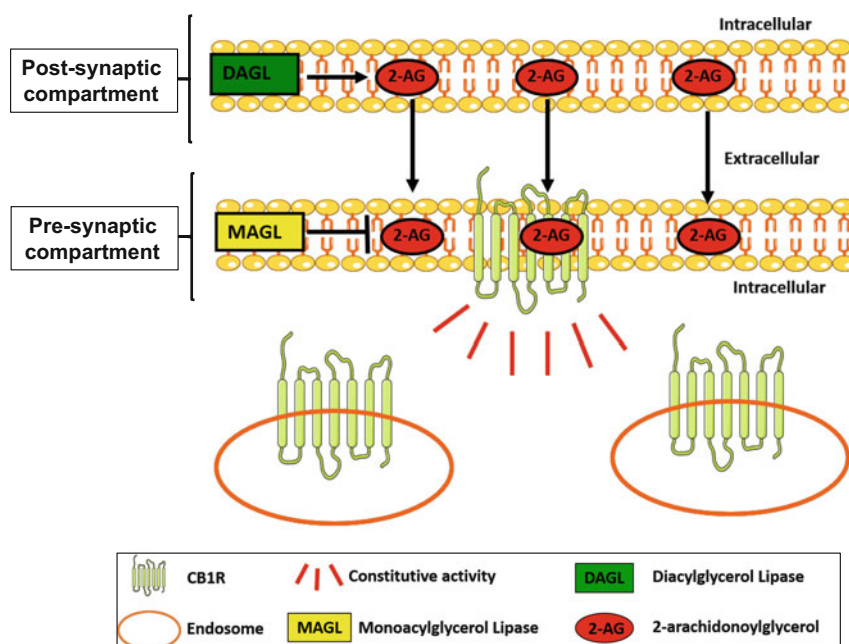


Fig. 3 Schematic representation of CB₁ receptor activation in synapses. CB₁ in the presynaptic compartment is tonically activated by paracrine 2-AG secretion and still not well-understood extracellular 2-AG transport from the postsynaptic membrane. In the presynaptic compartment, 2-AG levels are tightly controlled by MAGL-mediated degradation, and activated CB₁ is likely to endocytose into endosomes

These results (summarized in Fig. 2) were typically obtained in isolated neurons and are useful to understand the cell-autonomous components of tonic CB₁ activation, localization, and signaling. They indicate that tonic activation of CB₁ receptors in axons, where presynaptic terminals are located, is not of cell-autonomous origin. Accordingly, a key role for constitutive release of endocannabinoids from the postsynaptic neuron was demonstrated by showing that postsynaptic BAPTA chelation of intracellular Ca²⁺ signals abolished tonic eCB signaling (Hentges et al. 2005; Neu et al. 2007). Indeed, in the brain, tonic paracrine liberation of eCBs from neighboring neurons or glial cells adds a major additional layer of regulation for tonic CB₁ activation (Losonczy et al. 2004; Neu et al. 2007; Szabó et al. 2014; Lenkey et al. 2015). As a representative example, constitutive presynaptic CB₁ activity in the hippocampus, having a pivotal function in the tonic control of GABA release, is generated by continuous postsynaptic production of 2-arachidonoylglycerol (2-AG) and is strictly regulated by presynaptic monoacylglycerol lipase activity (Fig. 3) (Lee et al. 2015).

Unfortunately, the difficulty in reconstructing physiologically relevant synapses *in vitro* and the small size of the presynaptic terminal—close to the resolution limit of optical microscopy—has hindered us in the past from finding direct answers to

several important questions. First, while it has been shown that the presence of eCBs is needed for tonic activation, we do not know whether the structural instability of CB₁ has also a key role in the regulation of presynaptic plasticity. Recently, the contribution of structural determinants (i.e., constitutive activity) and endocannabinoid tone (i.e., basal activation) in tonic CB₁ activation was started to be evaluated by measuring *ex vivo* effects of a recently developed neutral antagonist NESS0327 on the release of GABA from perisomatic GABAergic axon terminals in the mouse hippocampus (Lee et al. 2015). Interestingly, NESS0327 inhibited CB₁ activation by both exogenous and endogenous cannabinoids, but a residual tonic CB₁ activation was still measured. However, it is not clear if this remaining constitutive activation is due to a genuine intrinsic constitutive activity or to the activation of CB₁ receptors bound to 2-AG before NESS0327 application. This question could be investigated in the future by genetically introducing stabilizing mutations to create, for example, mice expressing T3.46A (or T210A) mutant CB₁ receptors. Second, we do not know whether presynaptic CB₁ receptors show the activation-dependent subcellular targeting patterns described in the somatodendritic region, which would lead to a relatively low level of surface-located CB₁ in synapses presenting high levels of tonic CB₁ activation. However, several lines of indirect evidence suggest that this may be the case. CB₁ containing endosomal structures were reported in presynaptic terminals not only in agonist-treated (Thibault et al. 2013) but also in untreated animals (Bodor 2005). Chronic cannabinoid treatment leads to a measurable downregulation of CB₁ from terminals and axons, suggesting that CB₁ activation leads to local endocytosis at the presynaptic terminal (Thibault et al. 2013; Dudok et al. 2015). Finally, a recent study reported a striking lack of correlation between CB₁ levels at the presynaptic plasma membrane and the level of tonic endocannabinoid-mediated modulation of GABA release at single synapses, a result that may probably be explained by tonic activation-induced endocytic elimination of CB₁ receptors from the presynaptic plasma membrane (Lenkey et al. 2015). Undoubtedly, further technical development of highly resolved dynamic experimental approaches is needed to achieve a better understanding of the dynamic behavior of CB₁ receptors in the presynaptic terminal.

9 Conclusion

Taken together, the results presented in this chapter suggest that basal activation of the structurally instable CB₁ through cell-autonomous somatodendritic 2-AG production, which is polarized in neurons, is essential in obtaining tonic activation of somatodendritic CB₁. This tonic activation, besides other important local effects, drives the correct, i.e., axonally polarized, CB₁ distribution. Thus, the polarized production of a neuronal membrane lipid, 2-AG, leads to dynamic and polarized distribution and signaling patterns of CB₁, a major neurotransmitter receptor. Key elements of this functional model are also valuable to better understand activation

of presynaptic CB₁ receptors. The ensemble of the reviewed data strongly differentiates CB₁ from other, more classical GPCRs, since the combined result of ubiquitous eCB lipids and the instable CB₁ structure leads to an unusually dynamic model of receptor function, providing a novel conceptual framework for the comprehension of the brain endocannabinoid system.

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Endocannabinoid-Dependent Synaptic Plasticity in the Striatum

Brady K. Atwood and David M. Lovinger

Abstract The striatum plays a critical role in mediating the goal-directed and habitual behaviors involved in the development of drug abuse and addiction. Cannabinoid receptor-dependent synaptic plasticity is a prominent regulator of striatal circuit function. It is increasingly clear that striatal endocannabinoid signaling impacts the development of drug abuse. The expression of endocannabinoid-mediated plasticity in the striatum is affected by disparate types of drugs of abuse, such as opiates and alcohol. Understanding the mechanism of endocannabinoid-mediated plasticity and its effects on striatal circuit function may elucidate how endocannabinoids shape addiction-related behavior and perhaps offer new therapeutic avenues for treating drug abuse.

1 Introduction

The striatum is a set of large subcortical nuclei with key roles in the cortico-basal ganglia circuitry that control goal-directed and habitual actions (Gerfen and Bolam 2010; Yin and Knowlton 2006). As the entryway for information flow from the cortex to the basal ganglia, the striatum processes information relevant to all sensory, motor, and emotional functions and communicates with downstream basal ganglia nuclei. The striatum also receives the strongest dopaminergic innervation of any part of the brain, and these inputs arise from midbrain neurons (Gerfen and Bolam 2010). Ultimately, the basal ganglia feedback to the cortex controls production and structure of nonreflexive actions.

The striatum can be roughly separated into at least three large subregions that are part of distinct cortico-basal ganglia circuits (although more extensive divisions have been suggested) (Joel and Weiner 2000; Yin and Knowlton 2006). The

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dorsomedial striatum (DMS, in rodent, caudate nucleus in primates) is part of an “associative” circuit in which cortical regions such as the prefrontal cortex and other associative cortices innervate the DMS. This circuit has a number of functions, including integration of spatial information and control of behaviors that are driven by outcomes (i.e., goal-directed behaviors) (Yin and Knowlton 2006). The “sensorimotor” circuit includes projections from primary sensory and motor cortices to the dorsolateral striatum (DLS in rodents, putamen nucleus in primates). This circuit controls the production of well-learned behaviors, especially those that are driven by stimuli and context and have become habitual (Yin and Knowlton 2006; Gerdeman et al. 2003; Everitt and Robbins 2016). The limbic circuit includes connections from both allocortical and neocortical regions to the ventral striatum (also known as nucleus accumbens, NAc). In the mammalian brain, this cortico-basal ganglia circuit is involved in affective control of actions, and also has prominent roles in learning and memory driven by conditioned cues, including learning related to the actions of drugs of abuse (Yin and Knowlton 2006). The different striatal subregions can be further subdivided into smaller areas including the striosome/matrix subcompartments of the dorsal striatum, and the core/shell subcompartments of the NAc. These subcompartments are distinguished by differences in afferent inputs and efferent outputs (Eblen and Graybiel 1995; Kincaid and Wilson 1996; Langer and Graybiel 1995).

As described later in this chapter, the striatum has a rich mixture of afferent inputs and microcircuits that ultimately determine output to downstream BG regions. A variety of neurotransmitters, neuromodulators, and neuropeptides act within these circuits, including glutamate, GABA, dopamine, acetylcholine, serotonin, dynorphin, enkephalin, somatostatin, and substance P, among others (Emson et al. 2010). The role of endocannabinoids and CB1 receptors within these circuits is to modulate the actions of the other neurotransmitters, predominantly through presynaptic inhibition of neurotransmitter release (Lovinger et al. 2010). These modulatory roles are the subject of this review.

As extensively reviewed in this volume, endocannabinoids (eCBs) are modified fatty acids produced by all cells in the body, as they are metabolites of arachidonoyl-containing membrane lipids (Pertwee 2015). The endocannabinoids are synthesized and released from cells where they have juxtacrine and paracrine actions on other cells and cellular elements. A few subtypes of cell surface receptors, described later in this review, mediate eCB actions (Pertwee 2015). Within the nervous system, the cannabinoid 1 (CB1) receptor is the predominant mediator of eCB effects. This is a G protein-coupled receptor (GPCR) that generally modulates cells in a way that inhibits neuronal activity and neurotransmitter release. The CB1 receptor is one of the most strongly expressed GPCRs in the central nervous system, with widespread expression across the brain (Hu and Mackie 2015; Allen Brain Atlas, <http://mouse.brain-map.org>). The eCB signaling CB receptor system has a huge variety of actions within the body and brain, including influences on cognitive functions, action control, emotional regulation, cardiovascular function, and regulation of the immune system (Pertwee 2015). Given the ubiquity of this signaling system in humans, understanding eCB-CB functions has the potential to provide new avenues for therapeutic intervention in a variety of disorders.

The name of the eCBs is derived from the fact that the CB receptors are the major target for delta9-tetrahydrocannabinol (THC), the major psychoactive “phytocannabinoid” compound in *Cannabis sativa* (and hence in marijuana, hashish, and other cannabis drugs) (Pertwee 2015). Cannabis derivatives are among the most widely used and abused drugs in the world, and thus it is important to determine how THC acts through its receptors to produce intoxication, cognitive impairment, habit formation, and abuse liability. This review will focus on phytocannabinoid, synthetic cannabinoid, eCB and CB receptor actions in the striatum, and the ways in which these actions contribute to behavior and alterations in behavior induced by drugs of abuse.

1.1 The Endocannabinoid System in the Striatum

1.1.1 Striatal Neurons

Over 90% of neurons within the striatum are the GABAergic medium spiny neurons (MSNs) and these are the only projection neurons (Tepper et al. 2007). Figure 1 depicts these MSNs and their synaptic inputs. MSNs of the direct pathway express D1 dopamine receptors and primarily project to the substantia nigra pars reticulata, whereas MSNs of the indirect pathway express D2 dopamine receptors and project to the external globus pallidus (Gerfen 1992). MSN activity is driven by cortical and intralaminar thalamic glutamatergic inputs. These inputs mostly synapse onto dendritic spine heads of the MSNs that are found on the outer two thirds of the dendrites, though some intralaminar thalamic inputs synapse onto dendritic shafts (Gerfen and Bolam 2010; Tepper and Bolam 2004). The striatum and nucleus accumbens are also home to a sparse population of GABAergic interneurons (Tepper et al. 2007, 2010) along with one class of large, aspiny, tonically active cholinergic interneurons (CINs) (Bennett and Wilson 1999; Kawaguchi 1992; Zhou et al. 2002). The population of GABAergic interneurons can be subdivided in a number of different ways, one of the simplest is based on the expression of specific proteins and their distinct firing patterns. Fast-spiking interneurons (FSIs) express the calcium-binding protein parvalbumin (Kawaguchi 1993; Kubota et al. 1993; Tepper and Bolam 2004; Tepper et al. 2010). FSIs are electrotonically coupled via gap junctions (Koos and Tepper 1999) and their activity is driven mostly by cortical inputs (Kita 1993), but FSIs also receive input from dopaminergic and cholinergic neurons and other striatal GABAergic neurons. FSIs make strong synaptic connections with MSNs of both pathways and act as mediators of feed-forward inhibition of MSN activity (Tepper et al. 2010). Low-threshold-spiking interneurons (LTSNs) express neuropeptide Y, somatostatin, and nitric oxide synthase (NOS). They also receive input from cortical, dopaminergic, and cholinergic neurons. These LTSNs also form synapses with MSNs, although not as abundantly as with FSIs (Kubota et al. 1993; Partridge et al. 2009; Tepper and Bolam 2004; Tepper et al. 2010). A third class of GABAergic interneurons are the calretinin-positive interneurons (Tepper et al. 2010). Although little is known of their role in striatal function,

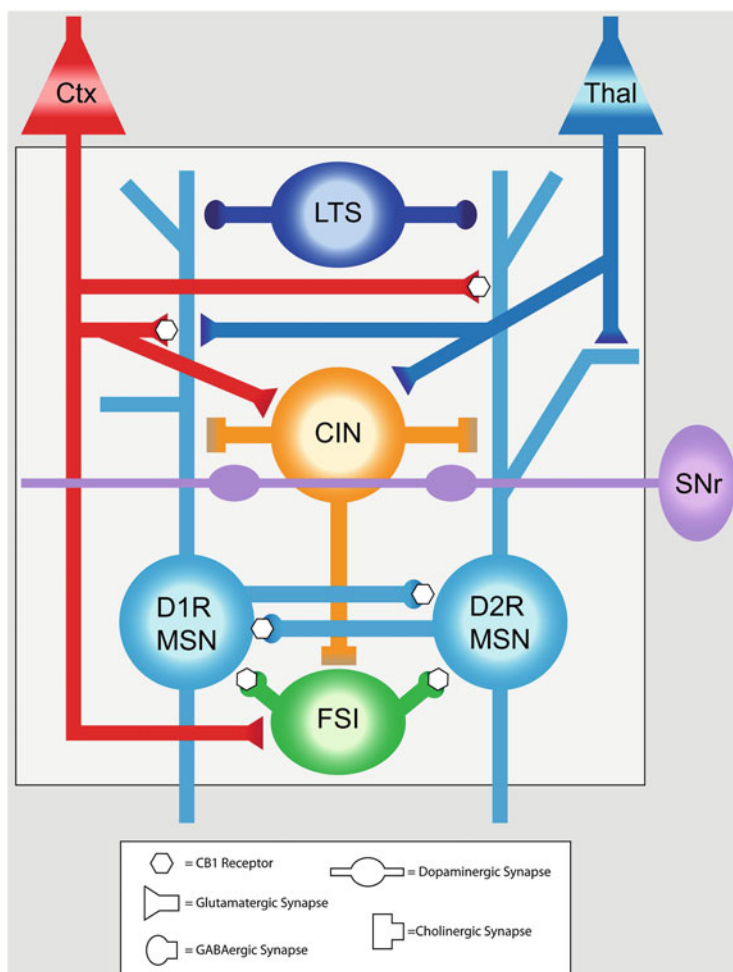


Fig. 1 CB1 cannabinoid receptors at striatal synapses. The striatum is home to a variety of synapse types. Excitatory synapses in the dorsal striatum arise from the neocortex and thalamus. The nucleus accumbens has additional prominent excitatory inputs that have their sources in the allocortex (including the hippocampus and amygdala). The GABAergic medium spiny neurons (MSNs) are the projection neurons of the striatum. MSNs of the direct pathway primarily express D1 dopamine receptors, whereas MSNs of the indirect pathway predominantly express D2 dopamine receptors. Fast-spiking interneurons (FSI) and low-threshold spiking interneurons (LTS) are GABAergic interneurons that modulate striatal output (in addition to other GABAergic interneuron types not pictured). Tonicly active cholinergic interneurons (CIN) and dopaminergic input from the midbrain (substantia nigra pars compacta in the dorsal striatum and ventral tegmental area in the nucleus accumbens) further modulate striatal function. CB1 receptors are found at many types of glutamatergic and GABAergic synapses and are predominantly presynaptically expressed

they do not express D1 or D2 dopamine receptors (Petryszyn et al. 2014). The striatum can also be subdivided into intercalated striosome and matrix regions differentiated on their inputs and outputs and enrichment of specific proteins in one region over the other (Bolam et al. 1988; Davis and Puhl 2011; Desban et al. 1993; Eblen and Graybiel 1995; Kincaid and Wilson 1996; Langer and Graybiel 1989). Striosome MSNs receive more prominent input from associative and limbic brain regions and project to the substantia nigra pars compacta (Canales 2005; Gerfen 1992; Joel and Weiner 2000). Matrix MSNs receive input from sensorimotor regions and project predominantly to external globus pallidus (Berretta et al. 1997; Donoghue and Herkenham 1986; Ebrahimi et al. 1992; Gerfen 1992; Malach and Graybiel 1986).

1.2 Endocannabinoid System Expression in the Striatum

The striatum is home to some of the most abundant CNS expression of the CB1R cannabinoid receptor (CB1R) (Herkenham et al. 1991a, b; Tsou et al. 1998), best known as the receptor responsible for mediating the psychoactive properties of Δ^9 -THC (Matsuda et al. 1990; Monory et al. 2007). Anatomical techniques such as in situ hybridization, receptor autoradiography, and immunohistochemistry as well as functional studies have identified CB1R expression throughout the striatum. Figure 1 outlines the synapses at which CB1R has been detected thus far in the striatum. CB1R mRNA is highly expressed in MSNs (Julian et al. 2003; Matsuda et al. 1993). CB1R is found in both direct and indirect MSNs (Hohmann and Herkenham 2000; Oude Ophuis et al. 2014). Some of the densest expression of CB1R in the CNS is in the terminal fields of MSNs in the substantia nigra and globus pallidus (Herkenham et al. 1991a, b) and this expression is not due to its expression in intrinsic neurons in these brain regions, but is in the MSNs originating from the dorsal striatum (Egertova and Elphick 2000; Julian et al. 2003; Matsuda et al. 1993; Tsou et al. 1998). The highest levels of CB1R expression within the striatum are found in the dorsolateral striatum with a decreasing gradient of expression ventromedially (Egertova and Elphick 2000; Marsicano and Lutz 1999; Oude Ophuis et al. 2014; Tsou et al. 1998; Van Waes et al. 2012). CB1R receptors are found in both striosome and matrix subdivisions of the striatum where they may be more abundantly expressed within matrix MSNs (Fusco et al. 2004; Rodriguez et al. 2001).

CB1Rs are also found on cortical inputs to the dorsal striatum (Gerdeman and Lovinger 2001; Huang et al. 2001; Rodriguez et al. 2001; Wu et al. 2015) and nucleus accumbens (Robbe et al. 2001). CB1R mRNA is not found in the intralaminar nuclei of the thalamus, suggesting an absence of CB1R expression in thalamic inputs to the dorsal striatum, and functional data support this conclusion (Wu et al. 2015). Anatomical and functional studies demonstrate that FSIs abundantly express CB1R (Fusco et al. 2004; Hohmann and Herkenham 2000; Mathur et al. 2013; Uchigashima et al. 2007; Winters et al. 2012). There are conflicting reports regarding the degree to which LTSNs and CINs express CB1R (Azad et al.

2001; Fusco et al. 2004; Hohmann and Herkenham 2000). Calretinin interneurons are not reported to express CB1R (Fusco et al. 2004). One recent study found that FSIs were the only neurons within the nucleus accumbens to express CB1R (Winters et al. 2012). Dopaminergic inputs to the dorsal striatum from the substantia nigra pars compacta do not express CB1R (Herkenham et al. 1991a, b; Mailleux and Vanderhaeghen 1992b; Matsuda et al. 1993; Romero et al. 1997), although there is the possibility that they do transiently during development as CB1R expresses in cultured mesencephalic TH-positive neurons (Hernandez et al. 2000). VTA dopaminergic inputs to nucleus accumbens also do not express CB1R (Mailleux and Vanderhaeghen 1992b; Matsuda et al. 1993; Romero et al. 1997). CB1R is localized predominantly on presynaptic elements in the striatum and is most abundant at inhibitory synapses, such as MSN and FSI terminals, although it is found on glutamatergic terminals as well (Narushima et al. 2006a, b; Rodriguez et al. 2001; Uchigashima et al. 2007). CB1R is also found on glutamatergic terminals in the nucleus accumbens (Matyas et al. 2007; Pickel et al. 2004). In addition to its prevalence on presynaptic elements, CB1R may be found postsynaptically to some degree as well (Kofalvi et al. 2005; Pickel et al. 2006).

One study found that CB1R mRNA levels in the striatum peak at postnatal day 25 and then decrease into adulthood (Van Waes et al. 2012). The highest CB1R mRNA levels in the striatum were found in areas receiving more sensorimotor input, whereas levels were lower in associative and limbic areas. Interestingly, the striatal areas with most CB1R mRNA expression received inputs from cortical regions with lower CB1R expression and vice versa. The authors of this study suggest that CB1Rs in sensorimotor areas are mostly involved in modulating MSN collaterals, whereas in limbic and associative regions, they are more involved in regulating glutamatergic input and interneuron neurotransmission. However, it is important to know if protein expression patterns align with this mRNA expression pattern. In addition to CB1R, other cannabinoid receptors include the type 2 cannabinoid receptor CB2R as well as the ionotropic TRPV1 receptor. CB2R expression in neurons in the striatum is controversial (Atwood and Mackie 2010), and reports of its presence may be due to expression in microglia (Concannon et al. 2015; Palazuelos et al. 2009; Sagredo et al. 2009) or blood vessels (Dowie et al. 2014). TRPV1 is expressed in both the dorsal striatum and nucleus accumbens (Grueter et al. 2010; Kauer and Gibson 2009; Koles et al. 2013; Roberts et al. 2004; Starowicz et al. 2008). TRPV1 expression on inputs to the striatum may be restricted to juvenile stages of development, suggesting that studies of TRPV1 signaling in older animals may be of postsynaptic TRPV1 (Koles et al. 2013).

The endocannabinoid system consists of not just cannabinoid receptors but also the enzymes that are responsible for the production and degradation of endocannabinoids, the endogenous ligands for the cannabinoid receptors and these enzymes are also present in the striatum. Endocannabinoids are produced on demand by biosynthetic enzymes that cleave plasma membrane lipid precursors to form them (Alger 2002; Wilson and Nicoll 2002). There are two primary endocannabinoids. Anandamide (AEA) is an endogenous ligand for both CB1R as well as TRPV1 with little efficacy at CB2R (Devane et al. 1992; Howlett et al.

2002; Zygmunt et al. 1999). 2-arachidonylglycerol (2-AG) is the second major endocannabinoid and it appears to be the primary mediator of endocannabinoid retrograde signaling in the CNS (Gao et al. 2010; Gonsiorek et al. 2000; Howlett et al. 2002; Mechoulam et al. 1995; Stella et al. 1997; Sugiura et al. 1995; Tanimura et al. 2010). 2-AG acts at CB1R and CB2R (Gonsiorek et al. 2000; Howlett et al. 2002). N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) is a calcium-sensitive enzyme that can produce the endocannabinoid AEA and is thought to be primarily responsible for its production (Ligresti et al. 2005; Okamoto et al. 2004, 2007; Piomelli et al. 2000). Other biosynthetic pathways do exist to produce AEA, such as through α/β -hydrolase4, or phospholipase A2 or phospholipase C (PLC) pathways (Ligresti et al. 2005; Liu et al. 2006; Simon and Cravatt 2006, 2008, 2010). There are little data as to the subcellular expression of NAPE-PLD in neurons of the striatum, but some evidence indicates that it has at least some level of expression (Castelli et al. 2007; Ferrer et al. 2007; Marco et al. 2014). In other brain regions, NAPE-PLD is predominantly a presynaptically localized enzyme (Nyilas et al. 2008; Pickel et al. 2012). AEA may be released by stimulating striatal neurons in culture and in vivo (Di Marzo et al. 1994; Giuffrida et al. 1999). 2-AG on the other hand is formed by sn-1-specific diacylglycerol lipases (DGL- α and DGL- β) (Bisogno et al. 2003). DGL α is primarily responsible for producing the 2-AG that is involved in modulation of synaptic transmission, whereas DGL β may have little role (Gao et al. 2010; Tanimura et al. 2010). Striatal DGL- α expression is almost exclusively expressed postsynaptically and is found most abundantly expressed on dendrites and especially dendritic spines in the striatum, consistent with findings in other brain regions (Hashimoto-dani et al. 2013; Katona 2009; Matyas et al. 2007; Shonesy et al. 2013, 2015; Uchigashima et al. 2007). Some functional evidence exists suggesting that endocannabinoid release following production occurs via an endocannabinoid membrane transporter (AMT) in the striatum (Adermark and Lovinger 2007b; Ronesi et al. 2004; Seif et al. 2011). Termination of endocannabinoid signaling is accomplished via uptake by the AMT (Beltramo et al. 1997; Fegley et al. 2004a, b; Gerdeman et al. 2002; Hillard et al. 1997; Robbe et al. 2002b) and subsequent hydrolysis by intracellular enzymes. Fatty-acid amide hydrolase (FAAH) degrades AEA (Cravatt et al. 1996; Ueda et al. 1995), whereas monoacylglycerol lipase (MGL) degrades 2-AG (Dinh et al. 2002a, 2002b). Numerous studies demonstrate the anatomical and functional expression of these enzymes in the striatum (Castelli et al. 2007; Gubellini et al. 2002; Maccarrone et al. 2001, 2003; Malone et al. 2008; Marco et al. 2014; Matyas et al. 2007; Micalé et al. 2009; Seif et al. 2011; Soria-Gomez et al. 2007). In other brain regions it has been established that FAAH is predominantly a postsynaptic enzyme, whereas MGL is presynaptically localized (Gulyas et al. 2004). Although there are little data to suggest their subcellular distribution in the striatum, it is likely that these enzymes are expressed in a similar manner.

2 Cannabinoid Receptor-Mediated Regulation of Synaptic Transmission

2.1 *Effects of Cannabinoids on GABAergic and Glutamatergic Synaptic Transmission*

CB1R is a G protein-coupled receptor (GPCR) that primarily couples to the Gi/o class of G proteins, and as such its activation leads to an inhibition of adenylyl cyclase and subsequent decrease in cellular cAMP levels (Howlett et al. 2002). CB1R activation also inhibits voltage-gated calcium channels and activates G protein-coupled inwardly rectifying potassium channels (Caulfield and Brown 1992; Mackie et al. 1993, 1995; Mackie and Hille 1992; Shen and Thayer 1998; Twitchell et al. 1997). Cumulatively, these effects on intracellular signaling result in reduced cellular excitability and, due to its proximity to synaptic terminals (Nyiri et al. 2005), a reduction in the probability of neurotransmitter release (Shen et al. 1996). This ability to inhibit neurotransmission allows both exogenous cannabinoid agonists (such as Δ^9 -THC) and endogenous cannabinoids to have a profound impact on neuronal communication. Since CB1R is found on both glutamatergic and GABAergic terminals in the striatum and other brain regions, its activation can suppress both inhibitory and excitatory synaptic transmission.

Despite earlier hypotheses that CB1R receptor activation in the striatum would increase GABAergic transmission in the striatum via reduced GABA uptake (Glass et al. 1997), the first evidence that cannabinoids could influence striatal fast synaptic transmission demonstrated that synthetic cannabinoid agonists, such as WIN55,212-2, actually reduce the magnitude of inhibitory postsynaptic currents (IPSCs) recorded in MSNs (Szabo et al. 1998). The inhibitory effect of WIN55,212-2 was prevented by the CB1R receptor antagonist SR141617A but WIN55,212-2 failed to inhibit calcium currents in MSNs, suggesting that presynaptic CB1R receptors mediated the inhibition of GABAergic transmission. Subsequently others demonstrated that CB1R agonists had similar effects on inhibitory transmission in nucleus accumbens MSNs (Hoffman et al. 2003; Manzoni and Bockaert 2001). Here it was shown that CB1R receptor activation reduces evoked IPSC amplitudes and changes the frequency, but not the amplitude of miniature IPSCs, also suggesting presynaptic CB1R receptors as the mediators of the cannabinoid ligands' effects. The first studies of the effects of cannabinoids on glutamatergic transmission demonstrated that exogenously applied AEA and synthetic cannabinoids also decrease excitatory postsynaptic current (EPSC) magnitudes in MSNs of the dorsal striatum via CB1R receptor activation (Gerdeman and Lovinger 2001; Gerdeman et al. 2002; Huang et al. 2001). CB1R receptor activation increases paired-pulse ratios and coefficients of variation and reduces the frequency, but not amplitude, of spontaneous EPSCs and evoked asynchronous EPSCs all indicative of a presynaptic locus for inhibition of neurotransmission (Gerdeman and Lovinger 2001; Huang et al. 2001). Once again, it was also demonstrated that CB1R receptor

activation via synthetic cannabinoids has similar inhibitory effects on excitatory transmission in nucleus accumbens MSNs (Robbe et al. 2001, 2002b).

Following these studies demonstrating that exogenous application of cannabinoid ligands could inhibit synaptic transmission in both the dorsal striatum and in nucleus accumbens, two studies published very close together demonstrated that endocannabinoids produced via specific stimulation paradigms could also inhibit neurotransmission as well. In the dorsal striatum a pairing of high-frequency stimulations (four 1 s 100 Hz stimulations) with postsynaptic depolarizations induces presynaptically expressed synaptic depression (Choi and Lovinger 1997a, b) that was later demonstrated to be prevented by a CB1R receptor antagonist and absent in CB1R null mutant mice (Gerdeman et al. 2002). In the nucleus accumbens, moderate-frequency stimulation (10 min, 13 Hz) also induces presynaptically expressed synaptic depression that is also blocked by a CB1R receptor antagonist and is absent in CB1R null mice. Furthermore the authors of this study demonstrated that a CB1R agonist occluded the inhibitory effects of the stimulation (Robbe et al. 2002b). In addition both studies demonstrated that the AMT blocker AM404 could induce or enhance synaptic depression, further supporting the evidence for endocannabinoid-mediated inhibition of neurotransmission (Gerdeman et al. 2002; Robbe et al. 2002b). Furthermore it was realized that the synaptic depression elicited by high-frequency stimulation in field recordings that was discovered in earlier studies was actually cannabinoid receptor-mediated (Calabresi et al. 1993, 1994; Lovinger et al. 1993; Walsh 1993).

2.2 Types of Synaptic Depression Mediated by Cannabinoid Receptors

There are different forms of synaptic depression that occur. Short-term depression (STD) in response to specific postsynaptic activation patterns, stimulation paradigms, or receptor ligands lasts for seconds to minutes and then returns to pre-stimulation or pre-ligand neurotransmission levels. On the other hand, long-term depression (LTD) lasts from minutes to hours or possibly longer. LTD can be further subdivided into two different forms of LTD. Static LTD is resistant to reversal by a receptor antagonist and is indicative of long-lasting changes in synaptic signaling elements independent of ongoing receptor activation (Atwood et al. 2014b). Labile LTD is long-lasting synaptic depression that is readily reversed by a receptor antagonist and thus is dependent on either persistent receptor activation by a ligand or is mediated by sustained receptor activation independent of the continuous presence of a receptor ligand (Atwood et al. 2014b). The differences between STD and labile LTD are nuanced, but it is clear that for certain receptors such as the Group II mGluRs, at certain synapses, LTD mediated by these receptors can be reversed only so long as the antagonist is present in the preparation (Lodge et al. 2013). Removal of the antagonist restores synaptic depression back to its

pre-antagonist application levels in what has been referred to as “reversibly reversible LTD” (Lodge et al. 2013), but can be classified as an example of labile LTD.

It is clear that cannabinoid receptors can induce both STD and LTD (both reversible and irreversible) in multiple brain areas. The studies identifying endocannabinoid-mediated synaptic depression described above determined that endocannabinoids induce LTD of excitatory transmission (eCB-LTD). CB1R receptors also can induce LTD of inhibitory transmission (eCB-iLTD) as first evidenced by Adermark and coworkers (Adermark and Lovinger 2007b, 2009; Adermark et al. 2009) in the dorsal striatum; however, the same has not been demonstrated in the nucleus accumbens. Dorsal striatal eCB-iLTD occurs at both FSI-MSN and MSN-MSN synapses, although different mechanisms induce LTD at each type of synapse (Mathur et al. 2013). Nonetheless, it is now well established that CB1R receptor activation can induce LTD in the striatum. On the other hand, other studies demonstrated that CB1R receptor activation can also induce STD. Cannabinoid receptor-mediated depolarization-induced suppression of inhibition (DSI) and excitation (DSE) were originally described in the hippocampus and cerebellum (Kreitzer and Regehr 2001; Wilson and Nicoll 2001). Cannabinoid receptor-mediated DSE may occur at glutamatergic inputs onto MSNs (Narushima et al. 2006a; Uchigashima et al. 2007), although others have failed to observe this (Gerdeman et al. 2002; Kreitzer and Malenka 2005; Yin and Lovinger 2006). DSI weakly occurs at MSN-MSN synapses in the dorsal striatum (Freiman et al. 2006). DSI at FSI-MSN synapses has been identified by some, but not others in the dorsal striatum (Freiman et al. 2006; Narushima et al. 2006b, 2007; Uchigashima et al. 2007). CB1R-mediated DSI does occur at FSI-MSN synapses in nucleus accumbens (Winters et al. 2012). DSE and DSI utilize differing mechanisms (discussed below). It is clear though that the CB1R receptor is not restricted to mediating one form of synaptic depression. Even at the same synapses, different forms of synaptic depression can exist. Both LTD and STD (DSE) occur at excitatory inputs onto dorsal striatal MSNs (Gerdeman et al. 2002; Narushima et al. 2006a, Yin et al. 2006), and LTD is only reversible (labile LTD) if the antagonist is applied shortly after the LTD-inducing stimulation after which it becomes static LTD (Ronesi et al. 2004). It is also likely that CB1R receptor activation alone is insufficient to induce LTD at these synapses, but requires other signaling components (Ronesi et al. 2004). Excitatory synapses appear to be less sensitive to cannabinoids than inhibitory synapses. Low-frequency stimulation (LFS) induces LTD of only inhibitory transmission onto dorsal striatal MSNs, and inhibitory synapses respond more to lower concentrations of CB1R agonist (Adermark and Lovinger 2009). CB1R-mediated LTD of excitatory inputs onto dorsal striatal and nucleus accumbens MSNs is sometimes irreversible and sometimes reversible by a CB1R receptor antagonist depending on the experimental conditions (Adermark and Lovinger 2007b, 2009; Adermark et al. 2009; Huang et al. 2001; Kreitzer and Malenka 2005, 2007; Robbe et al. 2001; Yin et al. 2006, 2008). CB1R-mediated synaptic depression at inhibitory synapses is also irreversible in some experimental conditions and reversible in others (Adermark and Lovinger 2007b, 2009; Adermark et al. 2009; Freiman et al. 2006).

Whether or not LTD is reversible (labile LTD) or not (static LTD) may depend upon a number of factors. CB1R activation alone is usually insufficient to induce eCB-LTD (Huang et al. 2001; Ronesi et al. 2004; Singla et al. 2007; Yin and Lovinger 2006). One determination of whether CB1R activation induces LTD or not may be the level of presynaptic activity. For example, in stimulating two pathway inputs to a single MSN, the pathway that received 5 min 1 Hz stimulation during application of WIN55,212-2 displayed LTD, whereas the pathway with no stimulation during agonist application did not display LTD. Similar results were found when one pathway received constant 0.05 Hz stim rather than 1 Hz for 5 min. HFS-induced eCB-LTD occurred in a pathway that received 0.05 Hz stimulation, but did not occur in a pathway that received no stimulation for 10 min after the induction protocol (Singla et al. 2007). Indeed postsynaptic loading with AEA induces LTD at excitatory synapses onto dorsal striatal MSNs that also requires presynaptic activity, as does eCB-LTD induced by pharmacological activation of postsynaptic L-type VGCCs (Adermark and Lovinger 2007a, b, 2009; Adermark et al. 2009). On the other hand, AEA loading-induced eCB-iLTD onto dorsal striatal MSNs does not require presynaptic activity, but it is enhanced by it (Adermark and Lovinger 2007b). eCB-iLTD induced by an L-type VGCC activator also occurs independently of presynaptic activity (Adermark et al. 2009). Cannabinoid modulation of net striatal output is the product of the balance of inhibitory and excitatory cannabinoid receptor-mediated synaptic plasticity. For instance, low- to moderate-frequency stimulation induces an LTP-like increase in neuronal output due to endocannabinoid-LTD of inhibitory synapses, whereas HFS induces net LTD by inhibiting excitatory synapses (Adermark 2011; Adermark and Lovinger 2009). The type of depression CB1R mediates is likely a result of differing synaptic environments. The composition and activation state of the presynaptic intracellular signaling pathways that couple CB1R to its mediators of neurotransmitter release inhibition are likely candidates for determining the type of synaptic depression that occurs. This likely explains why LTD is inducible at inhibitory synapses without needing presynaptic activity, whereas the opposite is true for excitatory synapses (Adermark and Lovinger 2007a; Adermark et al. 2009; Singla et al. 2007). Additionally a certain length of CB1R activation may be necessary for the receptor to engage the signaling pathways that are responsible for LTD expression rather than the typical STD. The composition of the machinery responsible for producing endocannabinoids (the type and levels of production) may differ from synapse to synapse and therefore may play some role in determining whether LTD or STD is induced. This could also explain why different synapses require different stimulation patterns to induce CB1R-mediated plasticity. This may also determine to some degree the role of endocannabinoid signaling in filtering striatal transmission. Some data suggest that endocannabinoids only inhibit neurotransmitter release from less active terminals in the dorsal striatum and nucleus accumbens core (Wang et al. 2012; Wong et al. 2015), thus acting as a high-pass filtering mechanism, though in dopamine-depleted mice, this filtering capacity is lost (Wong et al. 2015).

Multiple types of stimulation paradigms can induce endocannabinoid-mediated LTD. As indicated above 100 Hz, HFS paired with postsynaptic depolarization induces LTD of excitatory synapses in the dorsal striatum (Atwood et al. 2014a, b; Choi and Lovinger 1997a, b; Gerdeman et al. 2002; Kreitzer and Malenka 2007; Ronesi et al. 2004; Wang et al. 2006; Yin et al. 2006), and 10 min of 13 Hz MFS induces LTD of excitatory synapses in the nucleus accumbens (Mato et al. 2005, 2008; Robbe et al. 2002b). A multitude of studies have demonstrated that in field recordings HFS is sufficient to induce LTD that is cannabinoid-mediated, similar to the HFS pairing with postsynaptic depolarization utilized in whole-cell recordings (e.g., see Gerdeman et al. 2002). In addition 60–80 s of 1 Hz LFS induces eCB-iLTD in MSNs in the dorsal striatum (Adermark and Lovinger 2009; Mathur et al. 2013). HFS can also induce iLTD when MSNs are held in a slightly depolarized “up-state” (Mathur et al. 2013). 20 Hz stimulation paired with postsynaptic depolarization induces LTD in dorsal striatal indirect pathway MSNs (Lerner et al. 2010; Lerner and Kreitzer 2012). 20 min of LFS 4 Hz stimulation in nucleus accumbens induces CB1R-dependent LTD as well (Burattini et al. 2014). In addition, 5 min of MFS 10 Hz stimulation induces CB1-mediated LTD in the nucleus accumbens (Hoffman et al. 2003; Mato et al. 2005) and in the dorsal striatum where it is presynaptically expressed static LTD and is not occlusive with HFS-mediated eCB-LTD (Ronesi and Lovinger 2005). Another study that used this same LFS paradigm in the nucleus accumbens core found LTD only in indirect pathway MSNs and found that a CB1R receptor antagonist only partially blocked this LTD (Grueter et al. 2010). Instead a complete block was achieved when the CB1R antagonist was paired with a TRPV1 antagonist. TRPV1 is one of the other targets of AEA (Smart and Jerman 2000; Zygmunt et al. 1999). LFS-LTD was reduced in the TRPV1 null mutant mouse (Grueter et al. 2010). Unlike the CB1R receptor-mediated LTD component, the TRPV1 component was postsynaptically expressed. This is consistent with a recent study that found that presynaptic TRPV1 receptors stimulate glutamate release, but this effect is absent after postnatal day 27 (Koles et al. 2013). The presynaptic TRPV1 receptors do not cross talk with presynaptic CB1R signaling as well.

Spike-timing protocols have also been utilized to induce cannabinoid receptor-mediated plasticity in the striatum. An electrical and an optogenetic negative-timing spike-timing-dependent plasticity protocol (postsynaptic depolarization prior to presynaptic stimulation) can induce presynaptic LTD of glutamatergic inputs onto MSNs that requires CB1R receptor activation (Shen et al. 2008; Wu et al. 2015). The conditions that determine whether or not LTD is induced using these protocols are discussed below. The direction of the spike timing can change depending on whether GABAergic transmission is blocked. In the absence of GABA_A receptor antagonists a positive-timing paradigm (presynaptic stimulation precedes postsynaptic depolarization) leads to CB1R-mediated LTD (Fino et al. 2005, 2010). A recent study found that altering the number of stimulation pairings using STDP could produce both LTD at pairings greater than 100, but at low pairing numbers (5–10 pairings), the stimulation actually induced long-term potentiation (LTP) (Cui et al. 2015). The LTP required CB1R and TRPV1 activation as well as

postsynaptic endocannabinoid production. eCB-LTD and eCB-LTP could be sequentially induced in the same MSN.

2.3 Local Effects of Cannabinoids on Striatal Dopamine Release

It is well established that CB1Rs in the midbrain can alter synaptic transmission onto dopaminergic neurons that project to the striatum, thus modulating dopamine release in the striatum (French et al. 1997; Gardner 2005; Lupica et al. 2004; Parsons and Hurd 2015). In addition to their effects on glutamate and GABAergic transmission, there is some limited evidence that cannabinoids can also modulate striatal dopaminergic transmission locally within the striatum and not just indirectly via midbrain CB1Rs. CB1R agonists can inhibit electrically evoked dopamine release in striatal slices, and their effects were blocked by CB1R antagonists (Cadogan et al. 1997). CB1R activation had no effect on dopamine uptake or acetylcholine release. Similar findings were obtained when dopamine release was induced via NMDAR activation (Kathmann et al. 1999). However, Szabo and colleagues (Szabo et al. 1999) found conflicting results, showing that CB1R agonists and a CB1R antagonist had no effect on electrically evoked dopamine release in the dorsal striatum and nucleus accumbens. These findings were later reconciled to some degree by findings that a CB1R agonist indeed does not inhibit dopamine release in response to single electrical stimuli, but dopamine release elicited by trains of stimulation (30 pulses 10 Hz) was inhibited by a CB1R agonist (Sidlo et al. 2008). This effect was blocked by a CB1R antagonist but persists in the presence of a dopamine transporter inhibitor and occurs equally in guinea pig, mouse, and rat. These data are all curious given the absence of CB1Rs on striatal dopaminergic inputs (Herkenham et al. 1991a, b; Mailleux and Vanderhaeghen 1992b; Matsuda et al. 1993; Romero et al. 1997). Therefore the effects of cannabinoids are likely indirect. In support of this, additional experiments suggested a role of GABA_AR signaling as well as hydrogen peroxide and KATP channels (Sidlo et al. 2008). The authors of this study hypothesized that CB1R activation reduces GABA release, which decreases GABAAR activity to increase H₂O₂ production. This would cause more opening of presynaptic KATP channels on dopaminergic terminals, which reduces dopamine release. Additional work will be needed to conclusively determine if this is indeed the mechanism. One additional minor note is that TRPV1 activation enhances dopamine release in the striatum, although its ability to do so decreases during development. By postnatal day 27, it has no impact (Koles et al. 2013).

3 Mechanisms of Cannabinoid Receptor-Mediated Synaptic Plasticity

3.1 *Postsynaptic Production of Endocannabinoids*

Endocannabinoid signaling as indicated above involves postsynaptic production of endocannabinoids that transmit in a retrograde fashion. A number of different mechanisms exist in the striatum that initiate the formation of endocannabinoids by their biosynthetic enzymes (Fig. 2). It is probable that different types of synapses utilize distinct pathways leading to the production and release of endocannabinoids. Ultimately endocannabinoids are formed by enzymatic cleavage of membrane phospholipids by specific phospholipases (such as NAPE-PLD), although the identity of these enzymes and the routes by which they are activated vary. It is well established, however, that increases in postsynaptic calcium levels and activation of specific GPCRs are primarily involved in initiating the endocannabinoid production process, although other molecular players may contribute.

Increases in postsynaptic calcium levels are important for the expression of some forms of eCB-induced plasticity, such as some forms of eCB-LTD. Postsynaptic calcium chelation prevents eCB-LTD in the dorsal striatum and in the nucleus accumbens (Choi and Lovinger 1997a, b; Robbe et al. 2002b; Yin et al. 2008; Yin and Lovinger 2006). In the striatum the predominant source of postsynaptic calcium are the L-type VGCCs that are localized postsynaptically at synapses onto MSNs (Olson et al. 2005) and have no impact on presynaptic function (Lovinger et al. 1994; Kupferschmidt et al. 2015). L-type VGCC blockade prevents eCB-LTD (Calabresi et al. 1992b, 1993; Choi and Lovinger 1997a, b; Kreitzer and Malenka 2005; Wang et al. 2006). The most convincing data for a role of L-type VGCCs in eCB-LTD though is that this LTD does not occur in a mutant mouse that is null for the low-threshold, Ca_v1.3 form of the L-type channel VGCC (Wang et al. 2006). Furthermore, direct pharmacological activation of L-type VGCCs can induce eCB-LTD as long as it is paired with presynaptic activity (Adermark and Lovinger 2007a). A recent study in the nucleus accumbens demonstrated that 5HT₂R activation induces LTD that requires L-type VGCCs. 20 min 4 Hz stimulation induces 5-HT release, which activates postsynaptic 5HT₂Rs to increase L-type VGCC activity to induce 2-AG release (Burattini et al. 2014). eCB-mediated long-lasting disinhibition (DLL) of striatal output is also prevented by an L-type channel blocker, suggesting that L-type VGCCs are important for eCB-iLTD as well (Adermark 2011). Indeed these channels are critical for the “up-state” form of eCB-iLTD, but interestingly not the “down-state” form of eCB-iLTD that occurs when MSNs are held at more hyperpolarized potentials (Mathur et al. 2013). Blocking L-type VGCCs converted the “up-state” eCB-iLTD to become like the “down-state” eCB-iLTD. Additional experiments (discussed below) determined that the “up-state” L-type VGCC-dependent eCB-iLTD is mediated by 2-AG, whereas the “down-state” eCB-iLTD is mediated by AEA and is calcium independent. Another source of postsynaptic calcium are the intracellular stores (Yin and

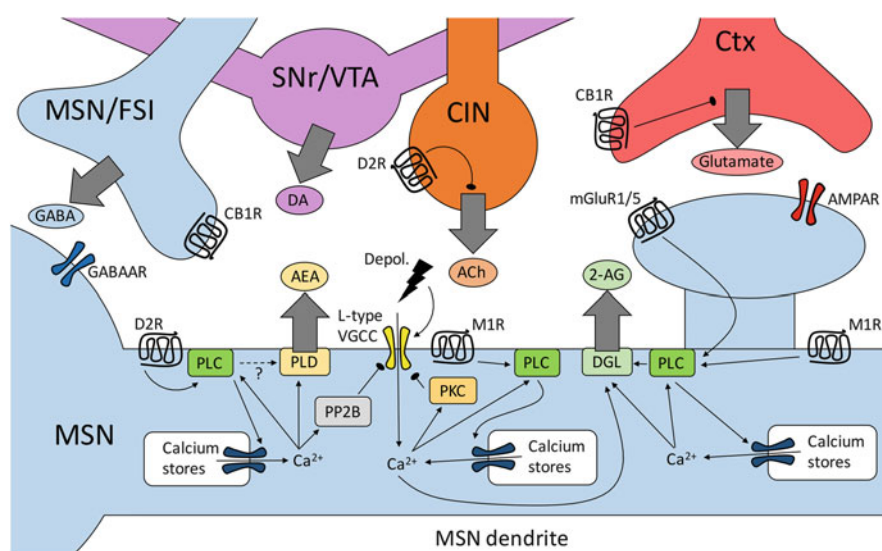


Fig. 2 Endocannabinoid production and signaling in the striatum. Endocannabinoids are produced from postsynaptic neurons through a number of parallel and intersecting pathways. 2-arachidonoyl glycerol (2-AG) can be produced via activation of Gq-coupled G protein-coupled receptors, such as Group I metabotropic glutamate receptors (mGluR1/mGluR5) or M1 muscarinic receptors (M1Rs). Activation of phospholipase C (PLC) and diacylglycerol lipase (DGL) along with increased intracellular calcium, via IP₃ receptor-regulated intracellular stores or through L-type voltage-gated calcium channels (VGCC), can result in 2-AG production. Activation of postsynaptic D2 dopamine receptors may also result in anandamide (AEA) production via PLC and phospholipase D (PLD) activation coupled with increased intracellular calcium levels. M1R may exert an inhibitory influence on endocannabinoid production via indirect inhibition of VGCCs. D2Rs may relieve this inhibition through activation of calcineurin (PP2B) or by inhibiting acetylcholine release from CINs. Presynaptic CB1 cannabinoid receptors are targeted by the postsynaptically released endocannabinoids to inhibit GABA and glutamate release

Lovinger 2006). HFS-induced eCB-LTD in indirect pathway MSNs requires some calcium from L-type VGCCs and RyR stores (Lerner and Kreitzer 2012). On the other hand, STDP LTD requires postsynaptic Ca²⁺ arising from L-type VGCCs and IP3R-regulated stores, but not RyR stores (Fino et al. 2010). Blockade of Ca²⁺ stores with thapsigargin and a RyR antagonist, but not an IP3R antagonist, prevents MFS (13 Hz 10 min) eCB-LTD in nucleus accumbens (Robbe et al. 2002b).

Like the “down-state” eCB-iLTD described above, other forms of eCB-mediated plasticity in the striatum appear to operate independently of L-type VGCCs. L-type VGCC blockade does not prevent MFS (13 Hz 10 min) eCB-LTD in nucleus accumbens (Mato et al. 2008), although as indicated above, this form of LTD does still require postsynaptic calcium from RyR stores (Robbe et al. 2002b). Other forms of LTD are even completely independent of postsynaptic calcium. LFS (20 Hz)-LTD in indirect MSNs does not require postsynaptic Ca²⁺ (Lerner and Kreitzer 2012). The MFS-induced (5 min 10 Hz stimulation) eCB-LTD in the dorsal striatum is not prevented by L-type VGCC antagonists or intracellular calcium chelation (Ronesi and Lovinger 2005). AEA loading LTD of inhibitory

transmission does not require postsynaptic calcium signaling, but this is likely due to bypassing the need for calcium-stimulated AEA production (Adermark and Lovinger 2007b, 2009).

In addition to postsynaptic calcium signaling being requisite for many forms of endocannabinoid-mediated plasticity, activation of specific types of GPCRs are also needed for endocannabinoid production to occur. One of these is the Group I class of mGluRs (mGluR1 and mGluR5) that couple to the Gq class of G proteins. In the dorsal striatum, mGluR5 is postsynaptically expressed (Narushima et al. 2007; Uchigashima et al. 2007) and is localized near postsynaptic DGL- α (Uchigashima et al. 2007), suggesting a predominant role of this receptor in initiating 2-AG production. mGluR5 is mostly perisynaptic and closely localized to the PSD (Uchigashima et al. 2007). Activation of Group I mGluRs is sufficient to induce eCB-LTD in the dorsal striatum (Chepkova et al. 2009) and in the nucleus accumbens (Robbe et al. 2002b). Antagonists of group I mGluRs can block LTD in the dorsal striatum (Adermark 2011; Calabresi et al. 1999a, b; Robbe et al. 2002b; Sung et al. 2001; Yin et al. 2008). A Group I mGluR agonist has little effect on transmission in neurons held at -70 mV, but produces eCB-LTD in neurons held at -50 mV, suggesting that there might be state dependence to mGluR-mediated eCB-LTD (Kreitzer and Malenka 2005). Indeed, blocking L-type VGCCs prevents mGluR1/5-induced eCB-LTD in the dorsal striatum (Kreitzer and Malenka 2005). This suggests that, for at least this form of eCB-LTD, a convergence of G protein and calcium signaling is necessary. In addition, via their Gq coupling, mGluRs can stimulate release of calcium from internal stores, linking mGluR activation with calcium signaling in eCB production. mGluR1/5 activation in nucleus accumbens occludes LFS-induced eCB-LTD (Grueter et al. 2010). eCB-LTD can often be blocked by antagonists of Group I mGluRs. mGluR1/5 antagonists block eCB-LTD of excitatory transmission induced by MFS (13 Hz 10 min) in nucleus accumbens (Robbe et al. 2002b). HFS-induced eCB-LTD at excitatory inputs to indirect pathway MSNs in the dorsal striatum is mGluR1/5-dependent (Kreitzer and Malenka 2007; Lerner and Kreitzer 2012; Sung et al. 2001) as is MFS/LFS (20 Hz)-induced eCB-LTD onto the same neurons (Lerner and Kreitzer 2012). Neurotensin-induced eCB-LTD in the dorsal striatum is blocked by a Group I mGluR antagonist (Yin et al. 2008). On the other hand, eCB-LTD induced by L-type VGCC activators occurs independently of mGluRs, suggesting that with sufficient postsynaptic calcium levels, the need for mGluR to stimulate endocannabinoid release is bypassed (Adermark and Lovinger 2007a). mGluR1/5 activation could induce eCB-LTD by stimulating calcium release from intracellular stores or also by more directly activating endocannabinoid-producing enzymes. eCB-LTD induced by mGluR I activation occurs at corticostriatal but not thalamostriatal inputs (Wu et al. 2015). eCB-iLTD in DLS also requires Group I mGluRs (Adermark and Lovinger 2009), and the long-lasting disinhibition (DLL) of striatal output that results from this eCB-iLTD also requires mGluR1/5 signaling (Adermark 2011). One recent study found that mGluR I agonist-induced eCB-LTD occurs in the nucleus accumbens in adolescent, but not adult mice, and there is no LTD in either age in DLS, suggesting that there may be an age dependence to

eCB-LTD (Zhang et al. 2015). However, the dorsal striatal data could explain an age dependence of DLL (Adermark and Lovinger 2009; Clarke and Adermark 2010; Zhang et al. 2015). An age dependence of eCB-LTD has been observed by others as well, but contrasts to some degree with others' findings (Ade and Lovinger 2007; Choi and Lovinger 1997a, b). These age-dependent differences may be explained by reduced CB1R and mGluR1 expression in nucleus accumbens and DLS in adults (Ade and Lovinger 2007; Mailleux and Vanderhaeghen 1992a; Van Waes et al. 2012; Zhang et al. 2015) and increased AEA tone in DLS of adults (Zhang et al. 2015). Group I mGluR activation induces CB1R-mediated depression of FSI-MSN and MSN-MSN synapses (Freiman et al. 2006). Interestingly, Group I mGluR activation is reported to preferentially induce 2-AG production (Jung et al. 2005), fitting with the data that suggest that the 2-AG-mediated "up-state" eCB-iLTD may be induced via mGluR1/5 activation (Mathur et al. 2013). eCB STD also involves Group I mGluRs. DSE in MSNs requires mGluR1/5 activation (Narushima et al. 2006a; Uchigashima et al. 2007), and DSI that occurs at FSI inputs to MSNs is enhanced by mGluR1/5 activation as is DSI at MSN-MSN synapses (Freiman et al. 2006; Narushima et al. 2006b).

mGluR1 and mGluR5 are the two members of the Group I class of mGluRs. Which one is responsible for mediating endocannabinoid production in the striatum and nucleus accumbens? STDP induces presynaptic cannabinoid LTD that requires mGluR5 receptor activation (Fino et al. 2010; Shen et al. 2008). mGluR5 is involved in Group I mGluR-driven eCB-LTD in the dorsal striatum of adolescent and adult mice, whereas mGluR1 is only involved in older mice (Chepkova et al. 2009). This may explain other data from older animals that HFS-induced eCB-LTD requires mGluR1, but not mGluR5 (Gubellini et al. 2001). Disruption of mGluR5 regulation by knocking out the PSD protein SAPAP allows for eCB-LTD in the dorsal striatum to be more readily inducible (Chen et al. 2011b). In the nucleus accumbens, an mGluR5-selective antagonist blocks eCB-LTD induced by multiple types of stimulation protocols (Grueter et al. 2010; Robbe et al. 2002b). Disruption of mGluR5 surface expression (via an in vivo cocaine exposure) in nucleus accumbens disrupts eCB-LTD (Fourgeaud et al. 2004). mGluR5 is likely the mGluR involved in DSE in the striatum (Uchigashima et al. 2007). There are also some forms of eCB-LTD that appear to be independent of mGluR activation. Clearly, loading a postsynaptic cell with AEA bypasses the need to form endocannabinoids and as such AEA loading-induced LTD does not require mGluR signaling (Adermark and Lovinger 2007b, 2009). The MFS-induced (5 min 10 Hz stimulation) LTD in the dorsal striatum that is CB1-dependent but not occlusive with eCB HFS LTD is not dependent on mGluRs (Ronesi and Lovinger 2005).

Cholinergic signaling also influences eCB-mediated synaptic plasticity. The Gq-coupled mAChRs (e.g., M1R), for instance, can play similar roles in the induction of eCB production as mGluRs (Kano et al. 2009; Martin et al. 2015) albeit the evidence is less abundant for such in the striatum and nucleus accumbens. In the dorsal striatum, M1Rs are postsynaptically expressed (Narushima et al. 2007; Uchigashima et al. 2007) and are localized near postsynaptic DGL- α , although not

as close to the PSD as mGluR5 (Uchigashima et al. 2007). STDP-induced eCB-LTD is blocked by an M1R antagonist (Fino et al. 2010). eCB-LTD in the dorsal striatum is blocked by an M1R antagonist, but does not occlude a CB1R agonist's inhibitory effect, suggesting that M1R activation is upstream of CB1R activation (Tozzi et al. 2011). An mAChR agonist enhances eCB-LTD in the striatum (Calabresi et al. 1992b). eCB-mediated long-lasting disinhibition (DLL) of striatal output is not prevented by an mAChR antagonist, but is occluded by an mAChR agonist (Adermark 2011). DSE in MSNs requires mGluR1/5 activation but not mAChR activation (Narushima et al. 2006a), but a combination of mGluR5 and mAChR activation enhances DSE (Uchigashima et al. 2007). On the other hand, DSI that occurs at FSI inputs to MSNs (Narushima et al. 2006b) is dependent on postsynaptic M1R signaling (Narushima et al. 2007) and is enhanced by mGluR1/5 activation (Uchigashima et al. 2007). Activation of M1R or inhibiting AChE enhances DSI, whereas an M1R antagonist blocks DSI, and DSI is absent in M1 null mutant mice. Reducing the firing of CINs decreases DSI as well. On the other hand, M1R activation might actually impair CB1-induced LTD. M1R activation reduces L-type VGCC opening in MSNs via PLC-mediated induction of calcium release from IP3R-sensitive intracellular calcium stores and subsequent activation of PKC (Howe and Surmeier 1995; Perez-Burgos et al. 2008). Thus activation of CINs could prevent LTD induction by activating M1Rs and thus decrease Ca^{2+} influx from L-type VGCCs (Wang et al. 2006).

D2 dopamine receptors (D2Rs) are another critical component for eCB-mediated synaptic plasticity, at least in the dorsal striatum. In this region D2R activation induces synaptic depression of excitatory and inhibitory synapses (Bamford et al. 2004a, b; Calabresi et al. 1992a, b; Centonze et al. 2004b; Cepeda et al. 1993; Flores-Hernandez et al. 1997; Hsu et al. 1995; Tang et al. 2001). Some of these studies identified D2R-mediated synaptic depression as presynaptically expressed, and this may indeed be the case to some degree. Presynaptic D2R activation can reduce MFS but not LFS-induced synaptic depression of corticostriatal transmission, which may allow them to act as a low-pass filter, although D2R activation alone in these experiments was insufficient to induce LTD (Bamford et al. 2004b). However, D2Rs are only sparsely expressed at presynaptic sites in DMS and absent in DLS (Wang and Pickel 2002), but are abundantly expressed postsynaptically in the striatum (Ambrose et al. 2004; Fuxe et al. 2007; Wang and Pickel 2002). This suggests then that D2R activation induces retrograde messenger-induced presynaptic depression, which suggests endocannabinoids may be involved. Indeed in vivo D2 activation stimulates AEA release but not 2-AG release in the dorsal striatum, whereas a D1R dopamine receptor agonist has no effect (Centonze et al. 2004a; Giuffrida et al. 1999). This is via stimulation of NAPE-PLD and inhibition of FAAH (Centonze et al. 2004a). Indeed, D2R activation in MSNs can result in increases in postsynaptic calcium levels that arise from a PLC-IP3R-mediated calcium release from intracellular stores (Hernandez-Lopez et al. 2000). Increased intracellular calcium could stimulate NAPE-PLD and other calcium-sensitive enzymes that play a role in AEA production (Okamoto et al. 2004, 2007). The age-dependent shift from LTP to LTD

in response to HFS in the dorsal striatum is paralleled by an increase in AEA levels. AEA induces LTD in young rats demonstrating that CB1R receptors are present and functional at these synapses by the early stages of postnatal development. Modulating 2-AG levels using THL has no effect on the HFS-induced expression of eCB-LTD, supporting the evidence that AEA mediates HFS-induced LTD (Ade and Lovinger 2007). Cocaine has the same effect as the D2R agonist on AEA levels. In addition cocaine-mediated inhibition of GABAergic transmission in the striatum is via D2R and endocannabinoids (Centonze et al. 2004a). D2R-mediated STD is occluded by a CB1R agonist, blocked by a CB1R antagonist, and absent in a CB1R null mutant mouse (Yin and Lovinger 2006). Interestingly this effect is also blocked by an mGluR1/5 antagonist suggesting a convergence of dopaminergic and glutamatergic signaling. Indeed D2R-mediated synaptic depression is enhanced when paired with stimulation of glutamatergic striatal inputs (Bamford et al. 2004b; Yin and Lovinger 2006). In addition, D2R agonist-induced inhibition of corticostriatal neurotransmitter release is blocked by CB1 antagonists in control and dopamine-depleted mouse dorsal striatum and mGluR5 antagonists in dopamine-depleted mice (Wong et al. 2015). The role of mGluR5 in control mice was not addressed in this study. In the nucleus accumbens core, D2R-mediated inhibition of neurotransmitter release from cortical inputs onto MSNs is also dependent on endocannabinoid signaling as well as mGluR1 and mGluR5 (Wang et al. 2012). These studies also indicate that CB1R activation is downstream of D2R and mGluR activation. In the dorsal striatum, D2Rs are often as requisite as mGluRs in determining whether eCB-LTD takes place. HFS-induced eCB-LTD is blocked by D2R antagonists or occluded by D2R activation (Kreitzer and Malenka 2005, 2007; Lerner and Kreitzer 2012; Yin and Lovinger 2006). This LTD is also lost in dopamine-depleted states (Calabresi et al. 1993). In D2R null mice, HFS fails to induce LTD (Tang et al. 2001) or may induce NMDAR-dependent LTP instead of eCB-LTD (Calabresi et al. 1997). It has also been shown that eCB-LTD at excitatory inputs to indirect pathway MSNs is D2R-dependent (Kreitzer and Malenka 2007). Neurotensin-induced CB1-mediated inhibition of corticostriatal transmission is blocked by a D2 antagonist (Yin et al. 2008). eCB-mediated long-lasting disinhibition (DLL) of striatal output is prevented by a D2R antagonist in addition to the previously mentioned block by an mGluR antagonist (Adermark 2011). The MFS-induced (5 min 10 Hz stimulation) eCB-LTD in the dorsal striatum is blocked by a D2R antagonist (Ronesi and Lovinger 2005). D2R antagonists block LFS (20 Hz) LTD (Lerner and Kreitzer 2012). As is the case with mGluR1/5 in L-type VGCC activator-induced LTD, D2Rs are also not required for this form of eCB-LTD, suggesting that increasing intracellular calcium also bypasses the cellular consequences of activating D2Rs (Adermark and Lovinger 2007a). Negative-timing STDP induces presynaptic cannabinoid LTD that requires both D2 and mGluR5 receptor activation in indirect MSNs. The same protocol fails to induce LTD in direct MSNs unless D1 dopamine receptors are blocked after which eCB-LTD is induced (Shen et al. 2008). The case for D1R having an inhibitory role in eCB-LTD is curious considering early reports that D1-like receptors block eCB-LTD (Calabresi et al. 1992a, b). Later work determined, however, that a D1R

antagonist inhibited LTP, whereas an antagonist for the D1R-like D5R prevented LTD induction (Centonze et al. 2003a, b). The D5Rs involved were possibly localized in NOS+ interneurons (discussed in further detail below).

Also of interest is whether D2R-mediated eCB-LTD is pathway dependent. eCB-LTD is induced in the dorsal striatum in both direct and indirect pathway MSNs and is inhibited in both by a D2R antagonist (Tozzi et al. 2011; Wang et al. 2006). This is comparable to what has been found by others (Bagetta et al. 2011; Picconi et al. 2011; Shen et al. 2008; Wu et al. 2015), but contrasts with others who only find LTD in indirect pathway MSNs (Kreitzer and Malenka 2007; Nazzaro et al. 2012). This may be due to the transgenic mice used (Bagetta et al. 2011; Kramer et al. 2011; Kreitzer and Malenka 2007) or the form of stimulation (Kreitzer and Malenka 2007, 2008). It bears mentioning here that up-state eCB-iLTD occurs equally in both pathways, whereas down-state eCB-iLTD only occurs in direct pathway MSNs (Mathur et al. 2013). Although there is no clear role for dopamine receptors in these forms of iLTD, the pathway dependence may implicate some differential involvement of dopamine receptors. STDP-induced eCB-tLTP in the dorsal striatum is also dopamine dependent and occurs in both direct and indirect pathways (Cui et al. 2015). On the other hand, in nucleus accumbens, dopamine receptor antagonists do not appear to block eCB-LTD (Robbe et al. 2002b).

Postsynaptic depolarization is not needed for D2R-mediated CB1R-dependent STD in the dorsal striatum (Yin and Lovinger 2006). For eCB-LTD, depolarization is not sufficient to induce LTD (Calabresi et al. 1999a; Kreitzer and Malenka 2005); however, the state-dependent mGluR-induced LTD that requires modest depolarization in conjunction with 25 Hz stimulation requires D2Rs and is enhanced by a D2R agonist (Kreitzer and Malenka 2005). This LTD requires L-type VGCCs, indicating that the depolarization is permissive for L-type VGCC opening. Despite not requiring depolarization though, the D2R-induced STD still requires calcium release from postsynaptic stores (Yin and Lovinger 2006). D2R antagonists block both HFS and LFS (20 Hz) LTD by altering cAMP/PKA signaling, and this requires RGS4 functionality (Lerner and Kreitzer 2012). D2-mediated eCB-LTD may involve AC5 in the striatum. AC5 is expressed in MSNs and AC5 null mutant mice have disrupted LTD, suggesting that postsynaptic cAMP signaling is important for D2-mediated (but not mGluR-mediated) eCB-LTD (Kheirbek et al. 2009). An interesting study in adult rat nucleus accumbens core demonstrates that a combination of D1R and D2R agonists increase neuron firing, but neither alone does (Seif et al. 2011). This effect is blocked by CB1R and PLC antagonists. The agonist effects are independent of GABAergic, glutamatergic, and cholinergic transmission. MGL, but not FAAH, inhibition allows subthreshold concentration of the agonists to increase neuron firing. mGluR5 inhibition prevents agonist effects, but not when the postsynaptic cell is filled with 2-AG and an AMT blocker prevents the effect of 2-AG. These agonist effects are mediated by reduction of A-type K⁺ currents. The authors of this study suggest that postsynaptic CB1Rs mediate the dopamine receptor effects (Kofalvi et al. 2005; Pickel et al. 2006; Seif et al. 2011).

Additional studies implicate other signaling systems in D2R-mediated CB1-dependent synaptic depression. One of these is the striatal adenosine system. A2A adenosine receptors (A2ARs) are mostly postsynaptic receptors and co-localize with D2Rs, but not D1Rs (Fuxe et al. 2007; Quiroz et al. 2009). A2AR activation is necessary for induction of STDP-induced striatal LTP in striatal indirect pathway MSNs (Shen et al. 2008). On the other hand, antagonism of A2ARs coupled with a D2R agonist is permissive for presynaptically expressed synaptic depression (Tozzi et al. 2007, 2011). In addition inhibition of A2AR activation enhances D2R-mediated synaptic depression (Ferre et al. 1997; Kim and Palmiter 2008; Stromberg et al. 2000; Tozzi et al. 2007, 2011, 2012). A2AR inhibition coupled with D2R activation induces LTD that is at least in part dependent on CB1R (Tozzi et al. 2011, 2012). This LTD is presynaptically expressed, requires postsynaptic calcium, and occurs in both direct and indirect pathways (Tozzi et al. 2011). Some glutamatergic terminals that contact direct pathway MSNs contain A2ARs, and activation of these receptors increases glutamatergic transmission and counters the effects of CB1R activation (Martire et al. 2011; Quiroz et al. 2009). Thus A2ARs counter the effects of D2R activation in indirect pathway MSNs via postsynaptic signaling mechanisms and antagonize the effects of CB1R activation presynaptically in direct pathway MSNs. An additional site of action of D2Rs and A2ARs may be on CINs in the striatum. There is some evidence that D2R and A2ARs are co-expressed in CINs (Brown et al. 1990; James and Richardson 1993; Song et al. 2000; Tozzi et al. 2011), but other studies suggest that CINs might not express A2ARs (Svenningsson et al. 1997). A2AR activation stimulates ACh release in the striatum (Brown et al. 1990). Activation of D2Rs and inhibition of A2ARs reduces firing of CINs (Tozzi et al. 2011, 2012). Reduced firing of CINs decreases ACh release. Additionally CINs also express the inhibitory A1 adenosine receptor which reduces ACh release, offering another avenue for adenosine to reduce ACh release (Brown et al. 1990; Song et al. 2000). One hypothesis that connects D2R activation with cholinergic transmission is that D2R-mediated inhibition of acetylcholine release from CINs relieves M1R-mediated tonic inhibition of L-type VGCCs on MSNs as discussed above, thus allowing LTD to occur (Wang et al. 2006). Additional data demonstrate that an M1R antagonist reduces EPSC amplitude, as does application of AChE. The effect of the M1R antagonist requires presynaptic activity and a postsynaptic potential more depolarized than -90 mV. The M1R and AChE effects are preserved in the presence of a D2R antagonist. In the presence of a D2R antagonist, HFS paired with depolarization usually does not induce eCB-LTD, but does when a M1R antagonist or AChE is applied prior to the induction protocol (Wang et al. 2006). Interestingly, postsynaptic D2Rs exert an inhibitory effect on L-type VGCCs via calcineurin/PP-2B, suggesting that these receptors actually could provide some antagonistic effects against the relief of M1R signaling (Hernandez-Lopez et al. 2000; Perez-Burgos et al. 2008). Much work remains to understand how D2Rs, Group I mGluRs, and cholinergic signaling interact to differentially produce specific endocannabinoids and subsequent forms synaptic plasticity.

Another element to D2R-, cholinergic-, and mGluR-dependent CB1R-mediated plasticity in the striatum is nitric oxide (NO) signaling (Centonze et al. 2003a, b). NO arises from the NOS-positive interneurons. In addition to membrane depolarization, mGluR activation, and dopamine, NO appears to be required for HFS-induced eCB-LTD (Calabresi et al. 1999a). This LTD is mutually occlusive with LTD induced by cGMP-dependent phosphodiesterase inhibitor (Calabresi et al. 1999b) and is prevented by NO signaling blockade. Activating intracellular PKG induces LTD as do NO donors. mGluR1/5 activation-induced eCB-LTD can be blocked but not reversed by nNOS inhibitors, and this LTD is absent in the eNOS null mutant mouse. The NO donor SNAP mimics the effect of mGluR-driven LTD. The NO effect is likely downstream of CB1R activation as a NOS inhibitor can block the effect of a CB1R agonist (Sergeeva et al. 2007). NO is critical for the expression of mGluR1/5-driven eCB-LTD (Chepkova et al. 2009). Other data implicate the D1-like D5R inducing NO release from interneurons (Centonze et al. 2003a, b). While other findings indicate that D2R-dependent LTD in the striatum is reduced in eNOS KO mice, NOS inhibitor enhances LTD expression, although this LTD is NMDAR-dependent, suggesting it may be a different entity than CB1R-mediated LTD (Doreulee et al. 2003).

Some combination of receptor activation (mGluR1/5, mAChR, D2R) and calcium signaling ultimately leads to the activation of enzymes that cleave membrane lipids to produce anandamide (AEA) and 2-arachidonylglycerol (2-AG). These GPCRs may signal through a number of pathways to induce eCB production. One such pathway is through activation of phospholipases such as PLC, most likely PLCbeta (Hernandez-Lopez et al. 2000; Perez-Burgos et al. 2008; Uchigashima et al. 2007). STDP LTD is blocked by a PLC inhibitor (Fino et al. 2010). Postsynaptic PLC is critical for MFS/LFS (20Hz)/Depolarization eCB-LTD on indirect MSNs, but interestingly does not appear to be required for HFS LTD onto the same neurons (Lerner and Kreitzer 2012). D2R activation-induced eCB signaling in the dorsal striatum is dependent on PLC (Yin and Lovinger 2006). Neurotensin-induced CB1-mediated plasticity is blocked by a PLC inhibitor (Yin et al. 2008). L-type VGCC activator-induced eCB-LTD occurs independently of PLC (Adermark and Lovinger 2007a). PLD may be another phospholipase necessary for the expression of HFS-induced eCB-LTD (Lerner and Kreitzer 2012), but it is not clear that this is the NAPE-PLD implicated in AEA production.

HFS in young rats (<P14) induces long-term potentiation (LTP), but induces LTD in older rats. HFS induces LTP in older rats in the presence of a CB1R receptor antagonist (Ade and Lovinger 2007). This shift from LTP to LTD is paralleled by an increase in AEA levels. AEA induces LTD in young rats demonstrating that CB1R receptors are present and functional at these synapses by the early stages of postnatal development. Modulating 2-AG levels using THL has no effect on the HFS-induced expression of CB1R receptor-mediated LTD, supporting the evidence that AEA mediates HFS-induced LTD (Ade and Lovinger 2007). Down-state iLTD was enhanced by FAAH inhibitor, but this treatment had no effect on up-state iLTD. Subthreshold stimulation induced down-state iLTD only in FAAH null mutant mouse. It had no effect in wild-type mice. Subthreshold

stimulation also had no effect on up-state iLTD in either wild-type or FAAH null mice (Mathur et al. 2013). A FAAH inhibitor enhances subthreshold stimulation to allow induction of LTD (Kreitzer and Malenka 2007). On the other hand, LFS (20 Hz)-induced eCB-LTD in indirect MSNs is 2-AG mediated (Lerner and Kreitzer 2012). An A2A antagonist blocks both this form of eCB-LTD and HFS-induced eCB-LTD, but A2A inhibition can also increase MFS-induced eCB-LTD in indirect MSNs, and this is blocked by THL, also suggesting 2-AG dependence (Lerner et al. 2010; Lerner and Kreitzer 2012). Up-state iLTD is mediated by 2-AG and mGluR activation. DGL inhibition blocked up-state iLTD, demonstrating it is mediated by 2-AG. MGL inhibition enhanced subthreshold LFS to induce iLTD in the up-state, but had no effect in the down-state (Mathur et al. 2013). Furthermore, inhibiting 2-AG production blocks the expression of DSE and DSI in striatal MSNs as well as mGluR1/5- and mAChR-induced inhibition of excitatory and inhibitory transmission (Uchigashima et al. 2007).

Following their production, eCBs are released from a neuron presumably via a membrane transporter, such as the as yet molecularly unidentified AMT. In support of this, intracellular, but not extracellular, application of an AMT blocker prevents eCB-LTD, and AEA and 2-AG loading-induced plasticity require AMT (Ronesi et al. 2004). L-type VGCC activator eCB-LTD and eCB-iLTD are prevented by an AMT blocker as well (Adermark et al. 2009). However, the AMT also can serve to limit the spread of endocannabinoids by cellular uptake (Day et al. 2001; Deutsch et al. 2001; Hillard et al. 1997; Hillard and Jarrahian 2000). AMT inhibitors therefore can also induce eCB-LTD on their own (Gerdeman et al. 2002; Robbe et al. 2002b). Since the AMT operates by facilitated diffusion, pharmacological manipulations of the AMT must be considered in terms of the concentration gradient of endocannabinoids. MFS-induced (5 min 10 Hz stimulation) LTD in the dorsal striatum appears to be independent of the AMT (Ronesi and Lovinger 2005).

3.2 Presynaptic Expression of Cannabinoid Receptor-Mediated Plasticity

It is clear that as a presynaptically localized Gi/o-coupled GPCR, CB1R is in a prime location to inhibit neurotransmitter release (Fig. 3). For example, CB1R inhibits neurotransmitter release through inhibition of presynaptic VGCCs. CB1R can inhibit N-type and P/Q-type VGCCs, the types that are typically found presynaptically (Caulfield and Brown 1992; Kushmerick et al. 2004; Mackie et al. 1993, 1995; Mackie and Hille 1992; Shen and Thayer 1998; Twitchell et al. 1997). Indeed, in the dorsal striatum, the N-type VGCC blocker conotoxin prevents the inhibitory effect of WIN55,212-2 (Huang et al. 2001). CB1-mediated LTD in the amygdala is mediated via P/Q-type VGCC inhibition (Huang et al. 2003) as is mGluR2/3-mediated LTD in the nucleus accumbens (Robbe et al. 2002a); thus it is

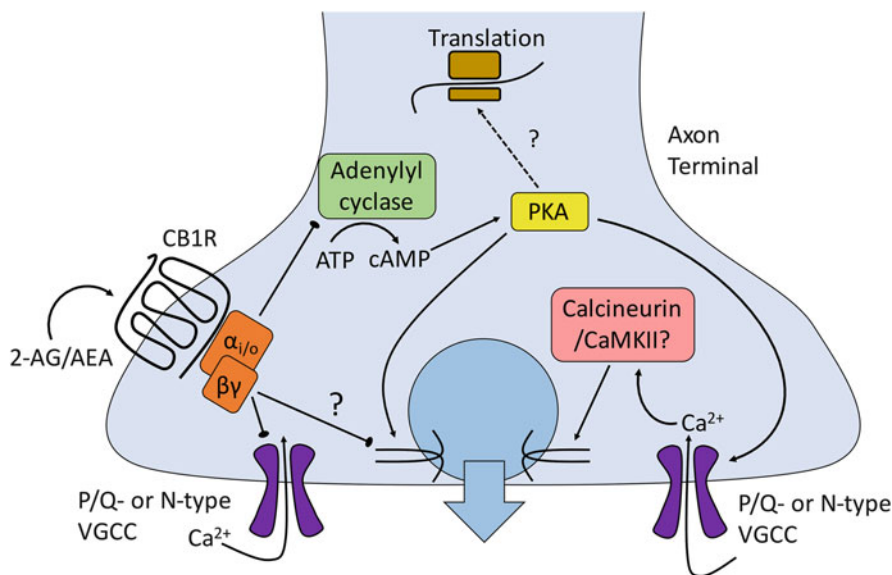


Fig. 3 Mechanisms of presynaptic CB1 cannabinoid receptor-mediated inhibition of neurotransmitter release. CB1 activation by the endocannabinoids 2-AG and AEA results in activation of Gi/o-type G proteins. G protein beta-gamma subunits have an inhibitory impact on presynaptic voltage-gated calcium channels (VGCCs) and release machinery, reducing neurotransmitter release. Signaling through the $G_{\alpha i}$ subunit results in inhibition of adenylyl cyclase and subsequent reduction in cAMP levels and reduced protein kinase A (PKA) signaling. Decreased PKA signaling impacts release machinery and VGCC function, altering neurotransmitter release probabilities. Decreases in calcium entry likely alter the function of calcium-sensitive kinases or phosphatases that may further impact neurotransmission. These changes may also underlie CB1-mediated long-term synaptic depression. Presynaptic protein translation may also play a role in this long-term depression

conceivable that CB1R could do likewise in the nucleus accumbens and possibly in the dorsal striatum. However Robbe and colleagues (Robbe et al. 2001) provided evidence that presynaptically localized, CB1R agonist-mediated synaptic depression occurred via K^+ channel modulation and not via L-, N-, or P/Q-type VGCCs in nucleus accumbens. However, these findings must be interpreted with caution, as manipulations of presynaptic potassium channels usually alter presynaptic calcium levels. There is currently no evidence in the dorsal striatum that CB1R affects neurotransmitter release via coupling to K^+ channels. CB1R coupling to VGCCs and/or GIRKs is the likely mechanism for CB1-mediated STD, such as DSE and DSI (Narushima et al. 2006a, b; Uchigashima et al. 2007). There are multiple possible routes that connect CB1R activation with LTD of synaptic transmission (Atwood et al. 2014b). Presynaptic calcium appears to play some role in this process as decreasing extracellular Ca^{2+} blocks direct CB1R-agonist-induced LTD, whereas chelating postsynaptic intracellular Ca^{2+} has no effect (Singla et al. 2007). Reducing EPSC amplitude by 95% using ionotropic glutamate receptor

antagonists also does not prevent this form of LTD. Thus postsynaptic Ca^{2+} is not needed for LTD mediated strictly by CB1R activation. What is required is sustained presynaptic activity (Adermark and Lovinger 2007b, 2009; Adermark et al. 2009; Singla et al. 2007). As indicated above, CB1R-mediated inhibition of excitatory transmission by WIN55,212-2 in NAc can be prevented by K^+ channel blockers, but not VGCC blockers (Robbe et al. 2001). However, MFS-induced eCB-LTD in nucleus accumbens is dependent on P/Q VGCCs, but interestingly not N-type VGCCs or even K^+ channels (Mato et al. 2008). Another source of presynaptic calcium that could determine the expression of LTD is NMDARs. These receptors are important for some forms of presynaptically expressed LTD (Rodriguez-Moreno et al. 2010). However, they do not appear necessary for any form of definitive eCB-LTD described thus far (Calabresi et al. 1992b; Chepkova et al. 2009; Fino et al. 2010; Grueter et al. 2010; Kreitzer and Malenka 2005; Lovinger et al. 1993; Robbe et al. 2002b; Ronesi and Lovinger 2005; Walsh 1993). eCB-LTD induced by mGluR1/5 activation can be reduced by NMDAR antagonists, but it is not dependent on them (Sergeeva et al. 2007). Interestingly in juvenile mice, mGluR1/5-driven LTD is NMDAR-dependent and CB1R-independent and becomes NMDAR-independent and CB1-dependent in adolescence (Chepkova et al. 2009). The change is likely due to an increased CB1R expression in the striatum during development. Alterations in presynaptic calcium levels can alter the function of presynaptic calcium-sensitive proteins such as calcineurin, calmodulin, and calmodulin-dependent protein kinase II, proteins that may be involved in Gi/o-coupled GPCR-mediated LTD in other brain regions (Atwood et al. 2014b; Heifets et al. 2008; Kobayashi et al. 1999; Li et al. 2002; Stanton and Gage 1996). It is unknown whether these proteins play a role in striatal CB1R-mediated LTD.

Another clear pathway downstream from CB1R activation is the cAMP/PKA pathway. Increasing cAMP levels with forskolin or inhibiting PKA blocks the effect of a CB1R agonist in the dorsal striatum (Huang et al. 2002). In addition, Beta2-adrenergic receptor activation that increases adenylyl cyclase activity reduces the inhibitory effect of a CB1R agonist (Huang et al. 2002). The authors of this study also found that a likely target of PKA itself is CB1R as forskolin increased CB1R phosphorylation, although the link with this and synaptic depression is not clear. CB1R-mediated STD of excitatory transmission in the nucleus accumbens occurs independently from cAMP signaling; however eCB-LTD and the mutually occlusive mGluR2/3 LTD are mediated by cAMP/PKA signaling in addition to the P/Q VGCC discussed above (Mato et al. 2005, 2008; Robbe et al. 2001). It is conceivable therefore that eCB-LTD is expressed as an alteration of PKA-mediated phosphorylation of P/Q type of VGCCs. 5-HT1bR-mediated LTD in the dorsal striatum is cAMP/PKA pathway dependent, and this form of LTD is also mutually occlusive with eCB-LTD suggesting that this signaling is also important for cannabinoid-mediated LTD. Early work on corticostriatal LTD found that protein kinase inhibitors prevent eCB-LTD in the striatum, although it is not clear if these inhibitors affected presynaptic kinases (Calabresi et al. 1994). Another target of presynaptic cAMP/PKA signaling is the vesicular release machinery itself (Atwood et al. 2014b). One potential member of this machinery that may

be involved in eCB-LTD is RIM1. RIM1 has been shown to be involved in eCB-LTD in other brain regions. A RIM1 null mutant mouse has reduced LFS and mGluR1/5-induced LTD in nucleus accumbens in indirect MSNs (Grueter et al. 2010). It is also possible that cAMP/PKA signaling may alter local presynaptic protein synthesis and this could determine the expression of CB1R-mediated LTD. Extracellular application of protein translation inhibitors blocks eCB-LTD, but intracellular application of translation and transcription inhibitors does not. Translation inhibitors also prevent mGluR1/5-induced LTD. Protein synthesis inhibitors do not prevent inhibition mediated by direct CB1R activation. Translation occurring in the presynaptic terminal is required for the maintenance, but not the induction of CB1-mediated LTD (Yin et al. 2006). AEA loading-mediated static LTD of inhibitory synapses in the dorsal striatum requires protein synthesis as does L-type VGCC activator-induced eCB-LTD (Adermark and Lovinger 2009; Adermark et al. 2009). It is abundantly clear that further work is needed to solidify a link between CB1R activation and the molecular mechanisms involved in presynaptic expression of LTD.

4 In Vivo Functions of Striatal Cannabinoid Signaling

4.1 Role of the Striatal Endocannabinoid System in Behavior

The striatum participates in a variety of behaviors involving action control, reward- and reinforcement-based learning, and addiction. Investigators have just begun to explore the roles in behavioral control of eCBs and CB1 receptors on the different striatal cellular elements.

It has long been known that activation of striatal CB1 receptors via local THC injection can produce akinesia and catalepsy in rodents (Gough and Olley 1978). The extent of this effect depends on the drug dose. Thus, some subpopulation of striatal CB1 receptors can exert strong control over movement initiation. Severe locomotor reductions are observed at doses that produce ~60% occupancy of striatal receptors (Dhawan et al. 2006). Catalepsy is one of the “tetrad” behaviors used to characterize in vivo effects of CB1 receptor activation. A recent study indicates that the CB1 receptors expressed by MSNs are not the subpopulation involved in catalepsy. Naydenov et al. (2014) have re-expressed CB1 receptors in knockout mice, using a GPR88 Cre mouse to obtain expression in MSNs. This strategy did not rescue the loss of agonist-induced catalepsy. It is possible that developmental effects or agonist effects on parallel circuits or other parts of the circuitry (e.g., globus pallidus, Pertwee and Wickens 1991) can produce catalepsy independent of direct actions within the striatum.

Effects of cannabinoid drugs, as well as the role of eCBs and CB1 in striatal-based learning, have been examined in some studies (for an excellent review, see Goodman and Packard 2015). Stimulus-response learning in the T-maze and similar

paradigms is dependent on striatal circuitry (Packard and McGaugh 1996). Jarbe and Henriksson showed that delta9-THC slows task acquisition and reversal of T-maze learning when given prior to training (Henriksson and Järbe 1972; Jarbe and Henriksson 1973). However, the use of the striatal-dependent response strategy was not assessed in this study, and thus the effects of peripherally delivered drug may involve brain regions other than the striatum. In a more specific assay of response learning, Goodman and Packard (2014) found that post-training injection of the CB1 agonist WIN 55,212-2 impaired memory for the correct turn in a water plus-maze task. Thus, activation of CB1 receptors impairs response learning and could involve the striatum and associated basal ganglia circuitry. Additional studies are needed to assess the anatomical locus of the receptors that mediate this impairment.

Endocannabinoid actions on CB1 receptors are implicated in the flexibility of stimulus-response learning in a reversal learning phase of the T-maze paradigm. When the CB1 antagonist AM251 was injected into DLS following reversal training, it impaired consolidation of the reversal. Injection of the antagonist into the dorsal hippocampus actually facilitated reversal consolidation, consistent with long-standing evidence that the hippocampus mediates spatial learning in this task that can compete with DLS-mediated S-R learning (Goodman and Packard 2014).

The Morris water maze has also been used to assess the effect of cannabinoids on striatal-based S-R learning. While the “hidden platform” variant of this task is well known to depend on circuitry that includes the hippocampus, this circuitry is not required for performance of the “cued” water maze version in which the platform location is marked by a visible flag or other cue. Instead, this cued version requires an intact striatum (Packard and McGaugh 1996). Consolidation of memory in this water maze task variant is impaired by either peripheral or intra-DLS post-training injection of WIN 55,212-2 (Goodman and Packard 2014). This finding supports the T-maze data in suggesting that strong activation of CB1 receptors in DLS has S-R memory impairing actions. This is certainly relevant to our understanding of cognitive impairment during cannabinoid intoxication.

Endocannabinoids appear to play a role in extinction of striatal-based learning. Instrumental learning, the acquisition of new actions initially driven by reward, involves two prominent corticostriatal circuits. The associative circuit that includes prefrontal cortices and the dorsomedial striatum (caudate nucleus in primates) is involved early in learning and controls behavior when it is goal-directed (i.e., sensitive to reward). The sensorimotor circuit that contains motor cortices and the dorsolateral striatum (putamen nucleus in primates) controls behavior when it becomes habitual (i.e., insensitive to reward). Activation of CB1 receptors appears to be necessary for habit learning and action control (Hilario et al. 2007). Synapses between the orbitofrontal cortex and the ventral portion of the dorsomedial striatum are one location where eCBs and CB1 have important roles in habit learning. Knocking out CB1 receptors at these synapses prevents mice from developing habitual instrumental learning in a lever pressing task (Gremel et al. 2016). This effect is probably due to suppression of information about outcome value that is important for supporting goal-directed actions. The idea that presynaptic

suppression of OFC-DMS inputs is important for establishment and maintenance of habitual behaviors is supported by experiments in which a Gi/o-coupled designer receptor activated by a designer drug (DREADD) construct is expressed in OFC. Activation of this receptor suppresses OFC output and promotes habitual behavior even under conditions where it is normally goal-directed (Gremel and Costa 2013; Gremel et al. 2016). These findings highlight the important role of eCBs and CB1 receptors in maintaining the proper balance of circuit function in the constant competition for goal-directed versus habitual control of behavior.

There is also evidence that altering eCB signaling affects learning-related neuronal activity in the nucleus accumbens/ventral striatum. Treatment with a CB1 antagonist suppresses food-rewarded operant responding, and firing of putative MSNs encoding predictive cue presentation and reward delivery was altered by this antagonist treatment (Hernandez and Cheer 2012). Activation of CB1 receptors by eCBs has also been implicated in dendritic spine changes produced by operant responding for highly palatable food (Guegan et al. 2013).

4.2 *Striatal Endocannabinoids in Drug Abuse and Addiction*

There is growing evidence of roles for eCBs and CB1 receptors in response to drugs of abuse, drug use disorders, and addiction. The role of CB1 in cannabis drug intoxication is clear, as the major psychoactive component of *Cannabis sativa*, THC, is a CB1 partial agonist. Activation of CB1 induces a behavioral “tetrad” that is associated with the cannabis high (Martin et al. 1991). As mentioned above, catalepsy is one of these behaviors and may well involve CB1 receptors in the striatum (Naydenov et al. 2014). Single (Mato et al. 2004), and prolonged exposure to THC reduces actions of other cannabinoid agonists and endocannabinoids on striatal receptors (Hoffman et al. 2004; Mato et al. 2005; Nazzaro et al. 2012), as mentioned above. While there has been some debate as to whether THC is “addictive,” there are clear signs of dependence following chronic exposure, and cannabis can be abused with clear signs of craving (e.g., Hart 2005; Haughey et al. 2008; Marshall et al. 2014). In animal models, THC appears to have rewarding effects, as it lowers the threshold for rewarding brain stimulation (reviewed in Gardner 2002), is self-administered intravenously (Tanda et al. 2000), and can induce a place preference (Valjent and Maldonado 2000). The role of striatal CB1 receptors in cannabis dependence is still being determined, but animals will operantly respond for direct injection of THC into the NAC shell region, indicating a role for this part of the ventral striatum in the rewarding or reinforcing effects of the drug (Zangen et al. 2006). Rats will also self-administer 2-AG, and this is associated with increased DA in the NAC shell (De Luca et al. 2014). In the striatum of human chronic marijuana users, positron emission tomography (PET) reveals a blunting of drug-induced dopamine release (Volkow et al. 2014). Decreases in CB1 receptor availability are also observed in the ventral striatum of chronic cannabis-using humans, as measured with PET (Ceccarini et al. 2015). Thus, there is increasing

evidence that chronic THC exposure produces signs of addiction in both experimental animals and humans, which can be accompanied by changes in striatal neurochemistry.

There is also evidence that drugs of abuse alter striatal endocannabinoid levels and striatal-based reward and habit learning. Single (Mato et al. 2004) and chronic exposure to THC is known to impair CB1 agonist-induced and endocannabinoid-mediated synaptic depression in a variety of brain regions including the dorsal and ventral striatum (Hoffman et al. 2004; Mato et al. 2005; Nazzaro et al. 2012). Habit learning in an instrumental nose-poke task is enhanced following 7 days of THC exposure, with concomitant loss of eCB-dependent corticostriatal LTD (Nazzaro et al. 2012). Interestingly, the deficit in LTD and the behavioral changes could be restored by boosting MSN excitability with a blocker of small-conductance potassium channels. This finding indicates that loss of eCB signaling is the result of deficient eCB production by MSNs.

Endocannabinoids in the ventral striatum have also been implicated in the rewarding and/or motivational effects of abused drugs. Blocking CB1 receptors reduces nicotine self-administration, and this is associated with decreased nicotine-induced increase in DA release in the NAc (Cohen et al. 2002). CB1 blockade in the NAc also reduces intravenous heroin self-administration (Caillé and Parsons 2006) and cue-induced heroin-seeking behavior (Alvarez-Jaimes et al. 2008); reduces drug-primed reinstatement of cocaine seeking (Xi et al. 2006), the “breakpoint” for cocaine self-administration in a progressive ratio procedure after prolonged drug exposure (Orio et al. 2009); and reduces ethanol self-administration in alcohol-preferring rats (Malinen and Hyttiä 2008). Cocaine effects on mGluR activation of eCB signaling also play a role in synaptic transmission changes in the NAc that may contribute to cocaine self-administration. After a withdrawal period following long-term cocaine self-administration, Group I mGluR signaling is changed from mGluR5-stimulated eCB production to mGluR1-stimulated AMPA receptor subunit alteration (McCutcheon et al. 2011). Cocaine effects on NAc dopamine and impulsivity also appear to involve CB1 (Hernandez et al. 2014). More recent evidence also suggests a role in cocaine self-administration of accumbens CB2 receptors (Xi et al. 2011), but the localization of these receptors on cellular elements in this region remains to be determined. Acute exposure to ethanol inhibits the firing of NAc neurons when that firing is driven by activation of glutamatergic afferents from the basolateral amygdala (Perra et al. 2005). These alcohol effects are greatly reduced by inhibition of FAAH or application of a CB1 agonist.

Using a chronic intermittent ethanol (CIE) exposure procedure, DePoy et al. (2013) found increased 2-AG levels in the DLS. This increase was accompanied by a loss of eCB-dependent LTD at glutamatergic synapses. Similar results were obtained using chronic ethanol exposure being administered via drinking water (Adermark et al. 2011; Xia et al. 2006). CIE enhanced DLS-dependent learning on touchscreen discrimination and reversal and conditioned approach tasks (DePoy et al. 2013). Hypertrophy of MSN dendritic spines was also observed following the CIE exposure regimen. While it is not yet clear that all of these behavioral and

morphological changes are the result of alterations in endocannabinoid signaling, this is certainly a reasonable mechanism to explain some of these ethanol effects. There is growing evidence that the DLS is involved in ethanol promotion of habitual behavior and habitual alcohol seeking (Chen et al. 2011a; Corbit et al. 2012; Cuzon Carlson et al. 2011). It will be important to determine how endocannabinoids contribute to these behavioral changes.

Activation of striatal CB1 receptors has been implicated in psychostimulant-induced increases in locomotion and sensitization of these locomotor effects (Corbille et al. 2007; Thiemann et al. 2008). In addition to the alcohol effects on striatal eCB-LTD mentioned above, other drugs of abuse also impair this prominent form of synaptic plasticity. Cocaine self-administration alters glutamatergic synaptic transmission in the rat NAc shell, via an endocannabinoid mechanism (Ortinski et al. 2012). A single exposure to cocaine is sufficient to ablate eCB-LTD in NAc (Fourgeaud et al. 2004). A single *in vivo* exposure to the widely prescribed and abused opiate drug oxycodone prevents subsequent induction of eCB-LTD (Atwood et al. 2014a). This treatment also prevents opiate receptor-mediated LTD, in particular LTD mediated by the Mu opiate receptor. This loss of LTD persists for up to 4 days after drug treatment. Interestingly, eCB- and MOR-induced LTD are mutually occlusive and appear to share common mechanisms. These common mechanisms are a likely target of relatively long-lasting oxycodone impairment of normal synaptic plasticity.

5 Conclusion

It is clear that endocannabinoid signaling is critical to the function of the striatum. Components of the endocannabinoid system are found at numerous striatal synapses and participate in dictating synaptic transmission. The net effect of this signaling helps determine the strength of striatal output and thus basal ganglia-cortical circuit function and by extension behavioral output. As evidenced by the impact of cannabinoids (phytocannabinoids, synthetic cannabinoids, and modulators of endocannabinoid signaling) on striatum-dependent behaviors, it is clear that this signaling system indeed plays a crucial role in behavior. Of particular interest here, we have discussed how cannabinoids play a role in drug use and abuse. There is a bidirectional relationship between drugs of abuse and striatal endocannabinoid signaling. Drugs of abuse alter the function of the endogenous cannabinoid signaling players in the striatum, and endocannabinoid signaling may determine behavioral responding for drugs of abuse. It is conceivable that manipulating this relationship may reveal new and better therapeutics for drug abuse and addiction. Perhaps restoring endocannabinoid signaling altered by drugs of abuse will prove efficacious. As discussed here CB1 receptor antagonists can clearly impact behavioral responding for drugs of abuse. Perhaps targeting other members of the endocannabinoid system could better counter the behavioral impact of drugs of abuse. As we gain a better understanding of the role of the endocannabinoid system

in striatal function, we will approach new avenues for treating drug abuse and addiction.

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Lipid Mediators in the Regulation of Emotions, Memory, and Cognitive Functions

Beat Lutz

Abstract This chapter aims at highlighting the diverse roles of endocannabinoids in the coordination of balanced neuronal activities, which finally set the basis for the organism's characteristics to store and remember important and useful things, to forget non-useful things, and to cope with new challenges. Altogether, the fine-tuned regulation of these processes is crucial for optimal life and survival. The endocannabinoid system appears to be a central intrinsic homeostatic factor in the organism, modulating these processes. Receptors for (endo)cannabinoids are also targets for exogenous cannabinoids, putting also relevance of external substances in the interference with these processes. The genetic dissection of the endocannabinoid system together with the many pharmacological, biochemical, behavioral, and electrophysiological approaches has led to relevant insights into this lipid signaling system in the regulation of emotions, memory, and cognitive functions. Recent progresses are discussed in this chapter, particularly the question on cell type- and region-specific involvements of endocannabinoids and the CB1 receptor-dependent regulation of distinct neuronal pathways in the context of fear behaviors and stress-induced effects on memory formation.

1 Introduction

The very widespread and diversely acting lipid signaling class of endocannabinoids has gained an intensive interest both in basic and in applied medical sciences but also in societal aspects of the steadily discussed issue of *Cannabis* legalization (Bloomfield et al. 2016; Volkow et al. 2016). Numerous recent reviews have elaborated on various aspects of the endocannabinoids anandamide (AEA, arachidonoyl ethanolamide) and 2-arachidonoyl glycerol (2-AG), their corresponding receptors, cannabinoid type 1 (CB1) receptor and type 2 (CB2) receptor, and the synthesizing and degrading enzymes of endocannabinoids, comprising the endocannabinoid system (Soltesz et al. 2015; Maccarrone et al. 2014;

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Lutz et al. 2015). The intrinsic complexity of the endocannabinoid system and also the continuous technical advancements in analytical approaches, together with posing novel questions that require an open-minded and an unbiased view, have enabled to reveal surprising and highly interesting new insights into the functioning of this lipid signaling system. This chapter is not meant to be complete nor to be well balanced, but wishes to discuss some topics that may be relevant for the further progress in understanding this signaling system.

A recurrent theme in understanding a signaling system is how the signal is generated at a particular site; where exactly the signal is received, i.e., where the receptors are located; and, finally, how the signal is further propagated and then how it is terminated. The fact that endocannabinoids are lipids and cannot be detected by high-resolution methods and that both CB1 and CB2 receptor proteins are often expressed at extremely low levels (e.g., CB1 receptor in astrocytes, oligodendrocytes, mitochondria; CB2 receptor in neurons) and in different cellular compartments (e.g., pre- and post-synapse, soma, mitochondria, lysosomes), but nevertheless revealing detectable and physiologically relevant functions, gives the promise that we will continue to gain new and exciting insights, thus making (endo) cannabinoid research a rewarding endeavor. Studies on the endogenous functions of the endocannabinoid system will fuel the research on the mechanistic understandings on how the different pharmacologically active plant-derived compounds (e.g., from *Cannabis sativa*) mediate their effects.

2 The Regulation of Fear Memory and Extinction

Due to high relevance in psychiatric disorders, such as posttraumatic stress disorders, phobias, and anxiety disorders, the scientific community has shown great interest on the impact of psychological and physical stress on emotional memory and, specifically, on how memory of traumatic events is “burnt” into neural systems and how to relieve patients from these intruding memories (Singewald et al. 2015). Numerous works have investigated the role of the endocannabinoid system in fear memory formation and, in particular, in fear extinction (Lutz et al. 2015; Morena and Campolongo 2014).

The preferred behavioral paradigm used has been the auditory and contextual fear conditioning where both memory formation and fear extinction have been investigated. Broadly speaking, CB1 receptor is necessary for extinction of aversive memories and for reduction of fear responses and is involved in fear-coping strategies (Lutz et al. 2015). Specifically, complete loss both of CB1 receptor (Marsicano et al. 2002; Lutz et al. 2015) and of the 2-AG-synthesizing enzyme DAGL α (Jenniches et al. 2016), thereby interrupting 2-AG/CB1 receptor signaling, leads to impaired fear extinction and increased anxiety. On the other hand, increased AEA signaling by genetic alterations or pharmacological interventions of the AEA-degrading enzyme fatty acid amide hydrolase (FAAH) resulted in the facilitation of fear extinction (Dincheva et al. 2015; Gunduz-Cinar et al. 2013),

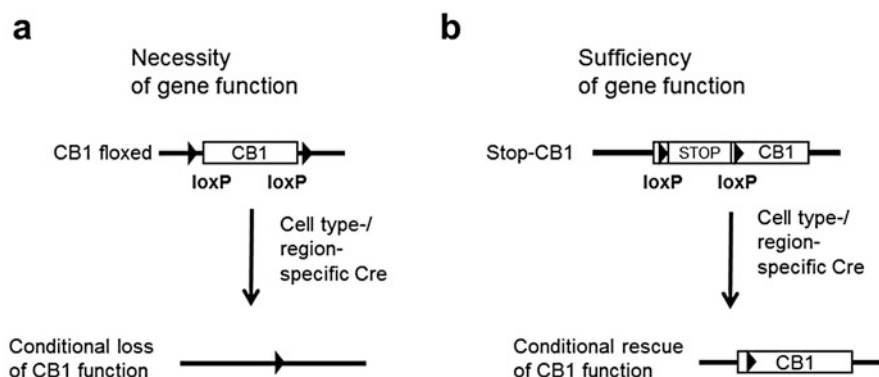


Fig. 1 Schematic representation of genetic analysis of CB1 receptor in mouse using the Cre/loxP system. **(a)** To assess necessary gene function, CB1-floxed mouse line, containing two loxP sites flanking an essential gene region (in this example, the coding region of the CB1 receptor gene) but retaining wild-type gene function, is crossed with a particular Cre recombinase-expressing transgenic mouse line or is stereotactically injected with virus expressing Cre recombinase in a region-/cell type-specific manner, leading to a cell type- and/or region-specific loss of gene function. **(b)** To assess sufficient gene function, Stop-CB1 mouse line contains a transcriptional stop cassette in the untranslated 5' region of the CB1 gene, flanked by two loxP sites, leading to the inactivation of CB1 gene function. Crossing of Stop-CB1 line with a particular Cre recombinase-expressing transgenic mouse line or stereotactically injected with virus expressing Cre recombinase in a region-/cell type-specific manner leads to a cell type- and/or region-specific reactivation of gene function, thus a rescue of gene function in a cell type- and/or region-specific manner

while genetic inactivation of the 2-AG-degrading enzyme monoacylglycerol lipase (MAGL) tended to show slower contextual fear extinction (Kishimoto et al. 2015).

To date, however, it is still unclear where exactly in the fear circuitry the CB1 receptor is centrally involved in order to regulate fear response, fear memory formation, and fear extinction. Conditional mutagenesis in mice using the Cre/loxP system has enabled the generation of CB1 receptor deficiency in particular cellular subpopulations or brain regions and thereby assessed the necessity of this receptor (Fig. 1a).

Using this strategy, the question is put forward under which circumstances the system “breaks” down and CB1 receptor-dependent behaviors are dysregulated. To this end, CB1 receptor expressed on GABAergic and glutamatergic neurons has been investigated in details (Dubreucq et al. 2012; Jacob et al. 2012; Lutz et al. 2015). Here, CB1 receptor expressed in dorsal telencephalic glutamatergic neurons is necessary for appropriate fear memory extinction, while CB1 receptor on fore-brain GABAergic neurons plays a minor role in the regulation of this behavior. CB1 receptor expressed in adrenergic/noradrenergic cells does not seem to play a role in fear expression and fear extinction (Busquets-Garcia et al. 2016). Most advanced insights into the role of CB1 receptor on a distinct projection with regard to a particular behavior were gained recently. Presynaptic CB1 receptor controls the expression of aversive memories by selectively modulating cholinergic transmission at medial habenular synapses projecting onto target postsynaptic

interpeduncular neurons (Soria-Gomez et al. 2015). Further elucidation of CB1 receptor function in these behaviors will require advanced genetic methods, whereby, e.g., the CB1 receptor gene is specifically inactivated only in a distinct neural cell type in a particular brain region. This requires the development of very specific Cre recombinase-expressing transgenic driver mouse lines (which are mostly not yet available), of newly developed viral constructs directing Cre recombinase expressing into distinct cell types, and possibly also the consideration of novel genetic methods, such as the CRISPR/Cas technique (Platt et al. 2014).

In the meantime, another genetic approach has been developed, aiming at shedding light onto the cell type-specific function of CB1 receptor in fear memory and anxiety (Ruehle et al. 2013). Based on the stringent genetic approaches and power in other model systems, e.g., yeast, *C. elegans*, and *Drosophila*, a complementary approach has been undertaken. Here, the aim is to reconstruct the “normal” behavior by reintroducing CB1 receptor only in distinct cell types in an otherwise CB1 receptor-deficient background, thereby investigating the sufficiency of CB1 receptor function for a distinct behavior. This approach is schematized in Fig. 1b.

By applying this approach, dorsal telencephalic glutamatergic CB1 receptor was sufficient for several CB1 receptor-dependent behaviors, including normal anxiety-like behavior, matching with the results obtained from experiments using conditional CB1 receptor deletion in this neuronal population (Ruehle et al. 2013). However, the major unexpected deviation was the freezing during cued fear extinction. Here, fear extinction behavior was worsened in the rescue mice as compared to mice with complete loss of CB1 receptor function. In continuation of this approach, mice were generated with a specific rescue of CB1 receptor in forebrain GABAergic interneurons and striatal GABAergic neurons. Again, while anxiety-like behavior was rescued, no substantial sufficiency of GABAergic CB1 receptor was observed for fear extinction (Remmers et al. 2017). While for the rescue of anxiety-like behaviors, both glutamatergic and GABAergic CB1 receptors, respectively, are sufficient, the pathways involved in fear extinction appear to be diverse and more complex. The currently known pathways (Tovote et al. 2015) will form a basis for further studies unraveling these CB1 receptor-containing pathways.

Figure 2 illustrates the two approaches of necessity and sufficiency in gene function, by using a simple representation and arguing here that a distinct sensory input (here, 8) will need a complex processing in neural networks, leading to the perception of the number 8 with all its meaning and implications. To address necessity, the loss of distinct component(s) in the information processing might lead to the perception of a wrong and misleading number (here, 5) or to information that might not be meaningful and interpretable. To address sufficiency, on a (genetic) null background, distinct components are reintroduced into the complex system, aiming at finding the minimal requirement for an information processing leading to the correct and discernable number 8. In this process, also other information might be generated (the inverted number 3).

However, considering the high complexity of brain neural networks and the high degree of self-organizing capacity and compensatory mechanisms of the brain after

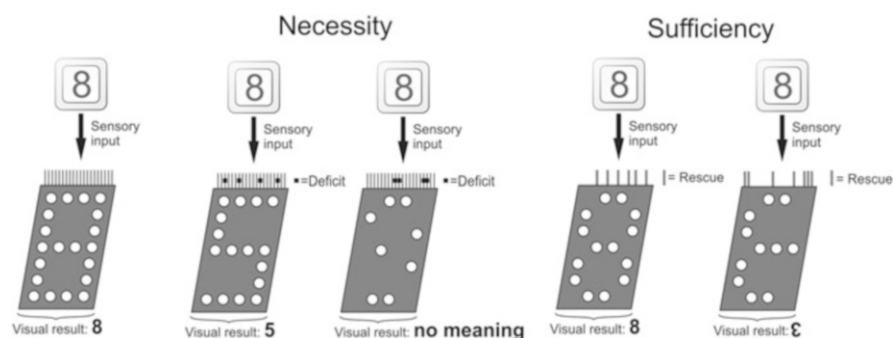


Fig. 2 Schematic representation to visualize the analyses of necessity (second and third panel) and sufficiency of gene function (fourth and fifth panel), using the example of information processing leading to the LED display of numbers in an electronic device. In order to test necessity, distinct inputs and processing are inactivated, leading to altered information (second and third panels, number 5 or complete loss of information). While the initial sensory information is always the same, sufficient quantity and quality of inputs and processing must be present, in order to retain the clear recognition of the number 8 (fourth panel). This approach can also lead to wrong and/or nonsense information (fifth panel, inverted 3)

distinct functional failures aiming at ensuring survival, if possible, the genetic approaches addressing necessity and sufficiency of gene function in distinct cell types and/or neuronal projections are very challenging and not without pitfalls. It might be argued that despite a correct rescue of the number 8, not the normally active and important networks were engaged but compensatory and self-organizing, and thereby rescuing processes have been activated. Therefore, the genetic approaches require the assistance and complementation by other methods, such as pharmacology, optogenetics, and electrophysiology. In this combination of methods, recent investigations have enabled novel insights into CB1 receptor functions, as detailed below in two examples.

3 Impact of Stress on Emotional Memory

It is well established that the emotional state effects memory consolidation processes (de Quervain et al. 2017; Luksys and Sandi 2011). Acute stressful encounters activate the sympathetic-adrenal system and the hypothalamic-pituitary-adrenal (HPA) axis, thereby initiating the peripheral production and release of hormones such as adrenaline, noradrenaline, and cortisol, to trigger both peripheral physiological responses, such as increase of blood pressure, and also responses in the central nervous system. Noradrenaline produced by the sympathetic nerve terminals, and adrenalin and cortisol in the adrenal glands, reaches the bloodstream. These hormones evoke pronounced effects on the cognitive capacity, by exerting their effects on different brain regions involved in the formation of memories (Gazarini et al. 2013; Singewald et al. 2015; Mizrachi Zer-Aviv et al. 2016). On

one hand, emotions can help us to remember important events of our life, but on the other hand, they can also be responsible for failures or exaggeration in “recording” of the memories.

The endocannabinoid system is a signaling system that helps to reduce the release of stress hormones when a stressful event triggers this response. In this process, the organism can recover to the conditions prior to the stress through the activation of the endocannabinoid system and tuning down the stress response (Morena et al. 2016).

It has been reported that the enhancement of AEA levels by pharmacological inhibition or genetic inactivation of the AEA-degrading enzyme FAAH relieves anxiety states induced, e.g., by chronic stressor exposure (Hill et al. 2013; Lomazzo et al. 2015) and alleviates impaired fear extinction after chronic stress (Laricchiuta et al. 2013). Two possible cellular processes are at play and may explain the beneficial effects of experimentally enhanced AEA levels in the alleviation of anxiety after chronic stress (Lutz et al. 2015). (i) It has been reported in rodents that CB1 receptor signaling is decreased after stress in several brain regions involved in emotional processing, including the hippocampus, nucleus accumbens, prefrontal cortex, and basolateral amygdala (BLA). (ii) Chronic stress increases FAAH activity and, thus, decreases AEA levels. The underlying mechanisms leading to decreased CB1 receptor signaling or increased FAAH activity after stress have not yet been elucidated. Recently, it was proposed that, in the BLA, postsynaptic activation of the corticotropin-releasing hormone receptor type 1 (CRHR1) initiates a pathway leading to the increased activity of FAAH, resulting in dysregulated synaptic function after chronic stress (Gray et al. 2015). CRHR1 in the amygdala seems also to be involved in the effect of stress regarding 2-AG signaling, which is dysfunctional after stress (Qin et al. 2015), and can be rescued by tyrosine phosphatase PTP1B inhibition or by glucocorticoid receptor antagonism.

4 Impact of Stress on Nonemotional Memory

The mechanisms underlying the influence of stress on the formation of nonemotional memories, such as episodic and autobiographic events and spatial information, show still a large gap in knowledge. Based on the involvement of the endocannabinoid system in the negative feedback of stress response, the question rises on the role of the CB1 receptor and the endocannabinoids in a behavioral paradigm of stress-induced impairment of consolidation of nonemotional memory. To this end, the novel object recognition test was applied, a behavioral paradigm where the hippocampus is centrally involved in (Busquets-Garcia et al. 2016). Mice were habituated to the V-maze, and after 24 h, mice were exposed for 9 min with two identical objects, each placed at the end of the arms. 20 min after this memory formation process, mice were acutely stressed by a foot shock. After 24 h, to assess memory consolidation, mice were placed into the same V-maze, where one of the

two objects was changed with a new, non-familiar object. Exploration for the new object in relation to the old, familiar object was expressed as a discrimination index, whereby high discrimination index means strong previously acquired memory consolidation. Using this behavioral paradigm, several interesting and unexpected observations were obtained. (i) While the acute stress after memory formation (day 1) did not impair short-term memory, when the discrimination index was determined 1 hour after memory formation, stress did impair memory consolidation in a CB1 receptor-dependent manner as measured on day 2. Remarkably, complete loss of CB1 receptor function led to alleviation of the stress-induced impairment of memory consolidation. The use of mutants with conditional CB1 receptor loss in forebrain GABAergic, in dorsal telencephalic glutamatergic, in forebrain GABAergic and dorsal telencephalic glutamatergic, and in central serotonergic neurons did not reveal CB1 receptor function in these cell types for this behavior. However, the loss in adrenergic/noradrenergic cells (both in the central and peripheral nervous system and in the adrenal gland) alleviated the stress-induced impairment of memory consolidation, similarly as the complete CB1 receptor-deficiency. (ii) Systemic pharmacological treatment with a peripherally restricted CB1 receptor antagonist (AM6545) prior to stress or local injection of CB1 receptor antagonist rimonabant (SR1) into the hippocampus led to the recovery of this memory impairment. (iii) Conditional rescue mutants expressing CB1 receptor only on adrenergic/noradrenergic cells regained the sensitivity to shock-induced impairment of memory consolidation, indicating that CB1 receptor expressed only in these cell types is sufficient for mediating stress-induced memory impairment. This is remarkable in the light of the complexity of the stress response and its effect on memory consolidation. (iv) Plasma levels of adrenalin and noradrenalin were increased at 90 min after memory formation in the AM6545 group as compared to vehicle group, suggesting that these increased levels in the phase of memory consolidation are important for prohibiting the stress-induced impairment of memory consolidation. (v) Sotalol, a peripherally restricted β -adrenergic receptor antagonist, was able to prevent the rescuing effect of rimonabant regarding stress-induced impairment of memory consolidation, pointing to a key role of peripheral β -adrenergic receptor signaling downstream of CB1 receptor blockade. Altogether, these data allow the proposition that only peripheral blockade of CB1 receptor can be beneficial for alleviation of stress-induced impairment of nonemotional memory and, furthermore, indicate an intriguing cross talk between peripheral and central mechanisms underlying the effect of stress on nonemotional memory consolidation and, in consequence, on potential new approaches in the treatment of cognitive interference on stress-related disorders. This is very attractive in the light that CB1 receptor blockade in the central nervous system may lead to serious side effects (Fig. 3).

Interestingly, another study showed that the anorexic and anxiogenic effects of the CB1 receptor antagonist rimonabant required the peripheral activation of the sympathetic system, as shown by the application of sotalol (Bellocchio et al. 2013). Both investigations strongly suggest the need of increasing the focus on the role of the endocannabinoid system in the interactions between peripheral and central

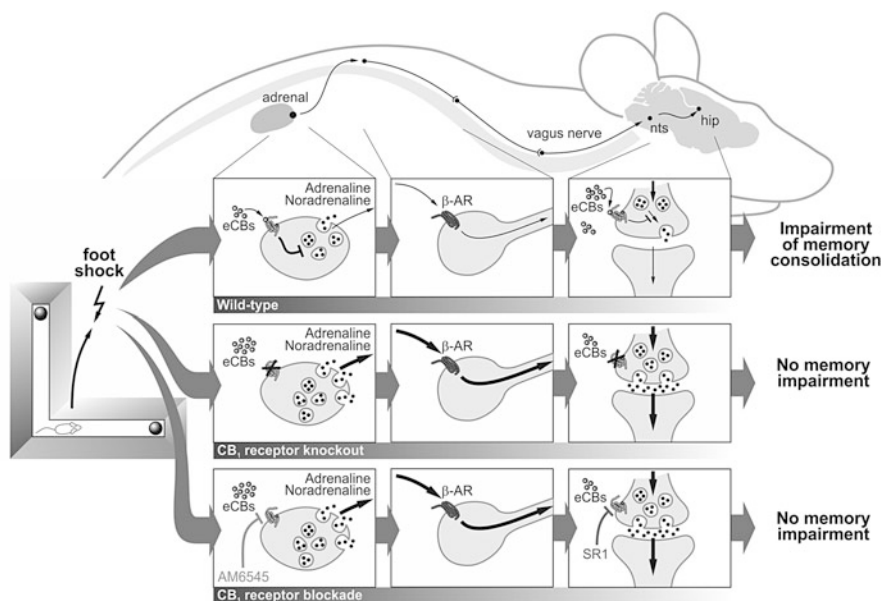


Fig. 3 Summary of the role of CB1 receptor and the endocannabinoid system in the mediation of stress-induced impairment of nonemotional memory consolidation. CB1 receptor both in hippocampal noradrenergic terminals and in the adrenal gland and sympathetic neuron is important for this effect, as both pharmacological blockade of peripheral CB1 receptor (using AM6545) and intrahippocampal (hip) blockade of CB1 receptor (using SR1) led to the alleviation of stress-induced memory impairment. In this memory consolidation-promoting effect, the peripheral β -adrenergic receptor (β -AR) must be functional. It is suggested that sustained adrenergic/noradrenergic signaling via peripheral nerves (e.g., vagus) and the nucleus of the tractus solitarius (nts) are involved

processes. It is obvious that the behavioral output (e.g., stress response, memory impairment, etc.) originates in the actions of central nervous system-guided processes, but they are strongly influenced by peripheral signaling systems. This has been recognized, of course, in the regulation and action of the HPA axis, where the central effects of cortisol and the interaction of the endocannabinoid system have been described in numerous studies. However, it remains to be investigated how the peripheral adrenergic/noradrenergic signals reach the brain and influence memory consolidation. Furthermore, acute stress enhances endocannabinoid levels in the hippocampus during the consolidation window in the object recognition task. Accordingly, intrahippocampal administration of rimonabant blocked the stress-induced impairment nonemotional memory both in wild-type mice and in mice re-expressing (rescuing) CB1 receptor only in the adrenergic/noradrenergic cell. Further experiments are needed to detail the function of CB1 receptor in these cell types and in the modulation of neurotransmitter release, e.g., in the hippocampus.

5 Fear Response to Unpredictable Threat

The fear reaction of a mammal to threat can be quick and rapidly dissipating or be more long-lasting and may turn into a pathological state in its extreme. While fear circuits activated by discrete stimuli (e.g., contextual and cued fear conditioning) have been understood in good details, the mechanisms underlying the shift from fear to an anxiety state have been barely understood (Tovote et al. 2015). Recent investigations proposed these two states to be operationalized as phasic and sustained fear in response to predictable and unpredictable threat, respectively (Davis et al. 2010; Seidenbecher et al. 2016). This gave an important entry point for translational research. Animals exposed to unpredictable, but not to predictable threats show behaviors resembling anxiety symptoms in humans, underlying the importance of unpredictability in anxiety disorders. Recent investigations suggest that the extended amygdala, including the central amygdala (CeA) and the bed nucleus of stria terminalis (BNST), is key in the control of these behaviors (Davis et al. 2010). The presence of a functional endocannabinoid system in CeA (Kamprath et al. 2011; Ramikie et al. 2014) and BNST (Puente et al. 2011) led to the motivation to investigate the role of endocannabinoids and CB1 receptor in a mouse model of sustained fear (Seidenbecher et al. 2016). Unpredictability of the threat and functional CB1 receptor in the BNST are required to develop sustained fear (Lange et al. 2016). Conditional genetic loss of function experiments, in combination with conditional genetic rescue experiments, and BNST-specific applications of CB1 receptor blocker, together with in vitro optogenetic and electrophysiological experiments, were able to propose a mechanism on how CB1 receptor regulates the emergence of sustained fear (Fig. 4).

Using this behavioral paradigm and advanced genetic interference methods should allow the further description of the CB1 receptor-containing projections from the amygdala to the alBNST and how the receiving neurons in the alBNST converge the two signals from BA and CeA, thus leading to output signals, which determine behavioral response of sustained fear. It appears that the integration of two signals in one neuron in the BNST requires time-dependent mechanisms, whereby the production and action of endocannabinoids have key roles.

6 CB2 Receptor and Emotions

In the very recent years, a possible involvement of CB2 receptor in neural functions has been proposed. CB2 receptor appears to be expressed in the hippocampus (Li and Kim 2015; Stempel et al. 2016), and genetic loss of CB1 receptor leads to reduced synaptic transmission and long-term potentiation (Li and Kim 2016b). Detailed studies on CB2 receptor function in the hippocampus led to the proposal that CB2 receptor is involved in a self-regulatory manner and regulates input/output functions and modulates gamma oscillations (Stempel et al. 2016). In fact, loss of

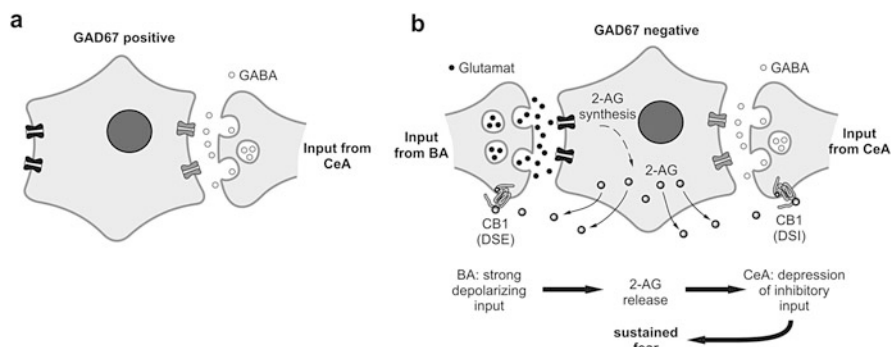


Fig. 4 Schematic drawing of CB1 receptor-regulated pathways from the basal amygdala (BA) and from the central amygdala (CeA) to the anterolateral bed nucleus of the stria terminalis (alBNST). (a) While in the alBNST, GABAergic neurons (identified as glutamic acid decarboxylase 67, GAD67-positive cells) do obtain GABAergic synaptic input from CeA, but in a CB1 receptor-independent manner, (b) GAD67-negative neurons (presumably glutamatergic neurons) receive glutamatergic inputs from BA and GABAergic inputs from CeA, both of them contain presynaptic CB1 receptor. Therefore, depolarization-induced suppression of excitation (DSE) and inhibition (DSI) can occur at both synapses. In its temporal sequence, BA input leads to activation of the alBNST GAD67-negative neuron; thereupon 2-AG is generated, which in turn suppresses the glutamatergic (from BA) and GABAergic inputs (from CeA), leading to the depression of the inhibitory input (a tonic disinhibition) and the slow activation in the alBNST neurons, mediating finally sustainment of fear response

CB2 impairs contextual fear memory, while cued fear conditioning was not altered (Li and Kim 2016a) and also reduces the consolidation of aversive memory as tested in the step-down inhibitory avoidance task (Garcia-Gutierrez et al. 2013). Further investigations using conditional CB2 receptor deletions will enable to uncover the underlying mechanisms involved in CB2 receptor-mediated functions in learning and memory.

Recently, the natural CB2 receptor agonist β -caryophyllene has been tested in a mouse model of Alzheimer disease in order to alleviate cognitive impairments. In fact, β -caryophyllene, given orally, prevented cognitive impairment in the Alzheimer mouse model APP/PS1, and this positive cognitive effect was associated with reduced β -amyloid burden in the hippocampus and cerebral cortex. Additionally, β -caryophyllene reduced astrogliosis and microglial activation as well as the levels of COX-2 protein and the mRNA levels of the proinflammatory cytokines tumor necrosis factor- α and interleukin-1 β in the cerebral cortex. The use of the CB2 antagonist AM630 or the PPAR γ antagonist GW9662 significantly reversed the protective effects of β -caryophyllene on APP/PS1 mice (Cheng et al. 2014). These are interesting results with high therapeutic potential, as inflammatory processes are central to many facets of brain dysregulation.

7 Therapeutic Interventions

The pharmacological intervention in order to alleviate fear expression or to promote fear extinction has attracted a long-standing interest. It was reported that at least in the rodent model system, the antidepressant drug fluoxetine promotes fear extinction (Karpova et al. 2011). A recent investigation showed that chronic fluoxetine treatment produced a significant and selective increase in levels of anandamide in the BLA and an associated decrease in activity of the AEA-catabolizing enzyme FAAH. Slice electrophysiological recordings showed that fluoxetine-induced increases in AEA were associated with the amplification of endocannabinoid-mediated tonic constraint of inhibitory, but not excitatory, transmission in the BLA. Behaviorally, chronic fluoxetine facilitated extinction retrieval in a manner that was prevented by systemic or BLA-specific blockade of CB1 receptor (Gunduz-Cinar et al. 2016). This fits to the proposition that enhanced AEA signaling in the amygdala beneficially affects fear extinction (Gunduz-Cinar et al. 2013). In contrast, pharmacological enhancement of 2-AG signaling with JZL184 leads to impairment of short-term fear extinction (Hartley et al. 2016) and even enhanced fear expression, presumably through CB1 receptor on GABAergic neurons (Llorente-Berzal et al. 2015).

Another drug to modulate fear memories in a beneficial manner is the natural component of *Cannabis sativa*, cannabidiol (CBD). Repeated microinjections of CBD into the infralimbic cortex (IL) facilitated fear extinction, as indicated by reduced levels of freezing during extinction test. Systemic administration of the CB1 receptor antagonist rimonabant blocked the effects of intra-IL CBD, suggesting that CBD acts through CB1 receptor to facilitate fear extinction (Do Monte et al. 2013). Another study showed that under conditions of strong fear conditioning, CBD reduced contextual fear memory expression both acutely during the extinction session and later at a fear retention test (Song et al. 2016). In contrast, when initial conditioning was weaker, CBD had an effect to increase freezing at the fear retention test relative to vehicle controls. This bidirectional effect of CBD may be related to stress levels induced by conditioning and evoked at retrieval during extinction, rather than the strength of the memory *per se*, implicating that the therapeutic application must be very thoughtfully considered. Mechanistic studies have recently demonstrated the ability of intra-nucleus accumbens injections of CBD to block the formation of conditioned freezing behaviors, which was dependent on intra-ventral tegmental GABAergic transmission (Norris et al. 2016). In summary, CBD seems to be an interesting drug for the treatment of dysregulation in fear and anxiety, though the underlying mechanisms have to be elucidated (Jurkus et al. 2016).

8 Conclusions

Due to the versatile roles of the endocannabinoid system in fear, anxiety, stress, and cognition and based on the data obtained in the recent years, further research on this lipid signaling system will enable us to obtain many novel insights into numerous central questions in biology, neuroscience, and even computational sciences. It will be a challenging task to uncover the precise roles of endocannabinoids and their receptors at distinct synapses and in the control of neural network regulation, finally regulating behavioral outputs. This knowledge can then be rationalized in translational neurosciences, aiming at, e.g., defining novel pharmacological interventions for fear and anxiety disorders and cognitive disabilities, where both drugs are specifically designed to target components of the endocannabinoid system, but also plant-derived compounds, which may have decreased specificity but overall may show high beneficial effects with low unwanted side effects.

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The Endocannabinoid System in Prefrontal Synaptopathies

Andrew F. Scheyer, Henry G.S. Martin, and Olivier J. Manzoni

Abstract Endocannabinoid (eCB) function in the prefrontal cortex (PFC) presents as a highly connected, widespread hub for the modulation of a vast array of synaptic functions. Given the known role of the PFC in a range of disorders from mental retardation, neurodegenerative diseases, and schizophrenia to drug addiction, stress, and anxiety, all of which are characterized by aberrant function at the synaptic compartment (here termed “synaptopathies”), it follows that eCB dysfunction is implicated as a significant causal factor in a plethora of disease states. Genetic and environmental manipulation of the eCB system in the PFC has thus been extensively studied with regard to many of these diseases and highlighted significant causal factors in their development and expression. Here, we examine the current body of literature as well as extensions of theoretical implications regarding the role of eCBs in the generation and presenting characteristics of a range of disorders. Elucidating the functions of eCBs in disease states will participate to the understanding of their etiology and the development of innovative pharmacotherapies.

1 Introduction

The eCB Hub System In mammals, the prefrontal cortex (PFC) is the most highly evolved brain region. The PFC is classically described as functioning in a manner essential to working memory, reasoning, action planning, cognitive flexibility, and emotionally guided behaviors (Goldman-Rakic 1990; Seamans et al. 1995). The power of the PFC with regard to regulation of our thoughts, actions, and emotions is guided by its complex internal circuit organization and extensive connectivity with other brain regions. Thus, a current hypothesis is that the PFC is a major functional network hub in the brain.

According to the hub theory of information processing, there is however a dark side to being a hub in a highly ordered network. Due to their high connectivity, compromised hubs are extremely deleterious to the integrity and information

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processing in the whole network. Thus it is unsurprising that PFC malfunctions are a common denominator in several neuropsychiatric diseases including (but not limited to) mental retardation, autism, schizophrenia, depression, and addiction (Goto et al. 2010).

At the synaptic level, the neural code is processed by postsynaptic molecular machines made of macromolecular complexes of interacting proteins organized into a scale-free network (Grant 2003). The “small-world” nature of multiprotein complexes implies highly organized interconnected structures where changes in one protein readily alter the functions of many others. Therefore, proteins with a higher number of connections, the so-called hub proteins, are of particular significance to network integrity, synaptic functions, and plasticity (Song and Singh 2013).

One of the most important signaling machines at central excitatory synapses is the endocannabinoid (eCB system). The “eCB hub system” is made of some of the most highly connected synaptic proteins (e.g., mGluR1/mGluR5, NMDAR, etc.; Melis and Pistis 2012). Logically, the “eCB hub system” participates in a wide array of physiological functions, many of which also implicate the PFC (e.g., memory, addiction, planning, etc.). This chapter will focus on the role of the “eCB hub system” in the etiology and expression of neuropsychiatric synaptic diseases (the so-called synaptopathies) implicating the eCB system within the PFC brain hub.

Prefrontal eCB System The eCB system is highly promiscuous; functional at inhibitory, excitatory, and neuromodulatory synapses throughout the central nervous system (Kano et al. 2009; Katona and Freund 2012). Similar to other brain regions, modulation of neurotransmission by eCBs in the PFC is common, but also restricted. CB1 receptors (CB1R) and other eCB molecular components show neuron-specific expression and selective subcellular localization, permitting the expression of homosynaptic and heterosynaptic modulation of neurotransmission (Katona and Freund 2012).

In the PFC, the highest concentrations of CB1R mRNA are found in interneurons, particularly those positive for cholecystokinin and calbindin in both deep and superficial cortical layers of the rat PFC (Marsicano and Lutz 1999). However the correlation between the degree of eCB modulation of synaptic transmission and the relative abundance of CB1R mRNA and protein is not necessarily direct. In both the hippocampus and somatosensory cortex where CB1R expression is also predominantly restricted to interneurons, it is equally excitatory synaptic transmission that is sensitive to CB1R knockout (Domenici et al. 2006; Monory et al. 2006). Furthermore, CB1R immunoreactivity is detected in fibers across the PFC surrounding pyramidal neurons in deep (layers 5/6) and superficial cortical (layer 2) layers suggesting that distant afferent connections may be equally modulated by eCBs (Egertová and Elphick 2000; Lafourcade et al. 2007). A similar pattern of CB1R immunohistochemistry is found in primate and human PFC tissue, where a strong CB1R immunoreactivity is also found in layer 4 (Eggan and Lewis 2007; Eggan et al. 2010; Long et al. 2012). However, direct laminar comparison between the primate and rat PFC is difficult due to distinct underlying cytoarchitecture

(Uylings et al. 2003). Electron microscopic studies of layer 5 synapses have identified CB1Rs at both symmetrical and asymmetrical synapses, with the receptor generally localized in the perisynaptic region of the presynapse (Eggen and Lewis 2007; Lafourcade et al. 2007). It is also worth noting that CB1Rs may be present in neuromodulatory afferents which are widely found in the PFC. Thus eCBs have the potential not only to modulate excitatory and inhibitory synaptic transmission in the PFC but also neuromodulatory tone (Katona and Freund 2012; Lee et al. 2015).

Other partners of the synaptic eCB system are broadly expressed in the PFC. mGluR5 shows dominant expression over mGluR1 and is found throughout the PFC layers (Hayashi et al. 1993; Lafourcade et al. 2007), where its activation triggers multiple forms of synaptic plasticity via the induction of eCB signaling (Lafourcade et al. 2007; Sheinin et al. 2008). Ultrastructural analysis of layer 5/6 asymmetrical synapses has identified co-localization of the 2-AG/mGluR5 signaling complex system (i.e., postsynaptic perisynaptic mGluR5 and DAGL α) with associated presynaptic CB1R (Jung et al. 2012). In contrast to some other brain regions, components of both the anandamide (AEA) and 2-arachidonylglycerol (2-AG) synthesis/degradation molecular machinery are found, including NAPE-PLD, MAGL α , and FAAH along with ABHD6 (Dinh et al. 2002; Egertová et al. 2003; Long et al. 2012; Marrs et al. 2010).

Notwithstanding the high expression of CB1R in PFC interneurons, eCB modulation of excitatory neurotransmission appears more prevalent at least in deep layers. Short-term plasticity of glutamatergic synapses (DSE) mediated by eCBs is inducible in layer 5 pyramidal rat neurons (Fortin and Levine 2007; Heng et al. 2011) (Fig. 1). The DSE is mGluR-independent and calcium sensitive; however, in

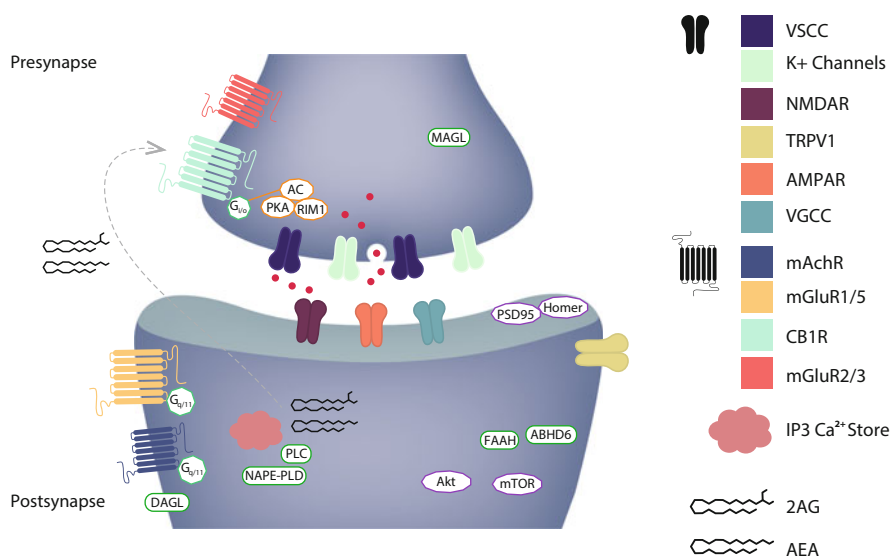


Fig. 1 Schematic representation of the principal components of the endocannabinoid-signaling complex at glutamatergic synapse of the CNS

contrast to the hippocampus, DSE does not appear to be universally expressed, and the success of induction may depend on pyramidal neuron subtype, species, developmental age, and layer specificity (Fortin and Levine 2007; Heng et al. 2011; Marrs et al. 2010). Notably, responses evoked in superficial layers are insensitive to CB1R agonists, in contrast to responses from basal dendrites, arguing for an input specificity (Fortin and Levine 2007). Activity-driven long-term depression, the phenomenon of lasting reduction in the efficacy of synaptic signaling following a specific pattern of activity, (eCB-LTD) is inducible in layer 5 pyramidal neurons in response to moderate frequency (10 Hz) stimulation, dependent on 2-AG signaling and mGluR5 activation (Lafourcade et al. 2007; Lovelace et al. 2014; Marrs et al. 2010). Alternatively, optogenetic-induced synaptic release of endogenous acetylcholine (ACh) and activation of M₁ muscarinic ACh receptors can also engage the eCB system at these synapses with concurrent synaptic transmission (Martin et al. 2015). In contrast eCB modulation of inhibitory transmission alone has not been reported in these neurons, and standard short-term plasticity (DSI; in which depolarization of the postsynaptic neuron induces a transient suppression of inhibitory transmission) protocols fail to produce transient depression (Fortin and Levine 2007; Marrs et al. 2010). However, a significant portion of inhibitory synapses (>30 %) co-express CB1R and D2R and appear sensitive to eCB modulation upon D2R co-activation (Chiu et al. 2010). Mechanistically, this reduction in inhibitory neurotransmission relies on postsynaptic mGluR5 activation. Thus, under conditions of increased DA drive, this may permit disinhibition of a subset of neurons receiving salient excitatory input.

eCB-mediated long-term plasticity has not been reported in superficial layers of the PFC; however, DSI is present. The DSI again appears limited to a subpopulation of GABA synapses, perhaps reflecting interneuron subtype, and is dependent on 2-AG/CB1R signaling (Yoshino et al. 2011). Furthermore, action potentials induced at natural firing frequencies may also induce short-term depression of inhibitory transmission. This may be mechanistically important in PFC-dependent functional working memory (Carter and Wang 2007). Although not strictly synaptic, an unusual form of eCB-mediated inhibition may also play a role in these neurons. Action potential firing is reported to activate a chloride current mediated by intracellular CB2R in these neurons, which in turn leads to a short-term depression of firing (den Boon et al. 2012, 2014). However, the role of CB2Rs in synaptic disorders of the PFC is not currently understood.

How eCB signaling modulates information processing in the PFC is currently unknown. Agonists of the eCB system enhance cortical up-states in the PFC, and antagonists increase down-states (Pava and Woodward 2014), likely via modulation of inhibitory neurotransmission in superficial layers. However, the role of the eCB system in the integration of PFC signaling remains an area of active research.

2 Drug Abuse and Addiction

The PFC and its synaptic connectivity with a variety of target brain structures has been identified as a crucial target for the development of rewarding behaviors including those which underlie drug abuse and addiction (Goldstein and Volkow 2011; Kalivas and Volkow 2005). Indeed, dysfunctional or abnormal activity in the PFC has been linked to control over drug-seeking behavior (for reviews, see George and Koob 2010; Goldstein and Volkow 2011; Lüscher and Malenka 2011).

Alcohol Density and reactivity of CB1R in the cortex, in addition to several other brain regions, are decreased following chronic ethanol exposure in animal models (Vinod et al. 2006). Whole-brain analyses suggest that this may be due to decreased CB1R mRNA induced by ethanol exposure (Basavarajappa and Hungund 1999). Similarly, human PET imaging following chronic ethanol abuse indicates long-term downregulation of CB1R surface expression (Hirvonen et al. 2013; Ceccarini et al. 2014). This phenomenon is accompanied by reduced activity of the AEA hydrolase, FAAH (Vinod and Hungund 2006).

Pharmacological manipulation of the eCB system has also been shown to alter rates and likelihood of ethanol consumption, suggesting a critical role for this system in the development or maintenance of addiction. Specifically, CB1R agonists consistently increase ethanol intake and motivation for consumption (Colombo et al. 2007; Gallate et al. 1999) as well as propensity toward relapse (Alen et al. 2008). Increasing eCB levels through FAAH inhibition mimicked this effect (Blednov et al. 2007; Hansson et al. 2007; Vinod et al. 2008). Conversely, CB1R antagonists or inverse agonists reduce intake (Arnone et al. 1997; Colombo et al. 2007; Freedland et al. 2001), a phenomenon that has been localized to the PFC (Hansson et al. 2007). Similarly, CB1R antagonist or inverse agonist administration to ethanol-preferring mice effectively attenuates sensitization (Marinho et al. 2015) in mice and presents a novel route toward the treatment of alcoholism in humans (Colombo et al. 2007; Maccioni et al. 2010; Vasiljevik et al. 2013).

Genetic evidence further suggests that eCB signaling in the PFC contributes to ethanol consumption/preference. Namely, ethanol-preferring rats exhibit decreased CB1R function, as well as altered levels of eCBs in the PFC (Hansson et al. 2007). Similarly, genetic changes leading to increased CB1R density in the PFC are correlated with enhanced ethanol-elicited subjective reward and brain activation in human subjects (Hutchison et al. 2008). Neuroimaging of alcoholic subjects confirms this as a functional deficit by revealing decreased CB1R receptor binding (Hirvonen et al. 2013), which may trigger the compensatory increase in CB1R density. Indeed, postmortem study of alcoholics reveals increased CB1R levels in the PFC (Erdozain et al. 2015) in addition to decreased AEA levels (Lehtonen et al. 2010). Additionally, downstream signaling assays reveal that CB1R activation is impaired in the PFC of alcoholics, suggesting a twofold reduction in function. This reduction is further confounded by reduced MAGL activity (Erdozain et al. 2015), which has also been observed in ethanol-preferring rats (Hansson et al. 2007). These results may further indicate compensatory changes in the eCB system, as

decreased CB1R signaling function is accompanied by decreased enzymatic degradation (and therefore likely increased circulating concentrations) of 2-AG.

Finally, converging evidence suggests a significant role for mGluR5 in the development or occurrence of alcohol dependence. Specifically, mGluR5-lacking mice exhibit an increased conditioned place preference at low doses of ethanol (Bird et al. 2008), in addition to increased rates of consumption during the dark cycle (Eisenhardt et al. 2015). Given the role of mGluR5 signaling in the production of on-demand eCBs, these data suggest that attenuated eCB signaling may contribute to the development of ethanol dependence. Clearly, the eCB system is an important and valuable target for the continued understanding and treatment of alcohol abuse and addiction, and continued research will be necessary to further elucidate the underlying mechanisms and progress toward potential targeted pharmacotherapeutic interventions for this disorder.

Cocaine The effects of cocaine on eCB signaling in the PFC are substantial and varied and depend largely upon the type or duration of administration as well as timing in relation to administration or consumption. During or immediately following cocaine self-administration, eCB-LTD in the PFC is suppressed or abolished (Kasanetz et al. 2013), an effect that is also seen in the nucleus accumbens following a single in vivo exposure (Fourgeaud et al. 2004). Intriguingly, while certain alterations in eCB function are induced directly as a result of cocaine exposure or self-administration, others may appear only during the transition to addiction, such as the suppression of mGluR2/3-LTD in the PFC (Kasanetz et al. 2013). Indeed, manipulation of altered eCB transmission may prove to be a valuable route toward the treatment of cocaine addiction, as evidenced by abolished reinstatement of cocaine seeking in rats following pretreatment with a CB1R antagonist (McReynolds et al. 2015).

Levels of 2-AG are increased in the PFC following withdrawal from self-administered or noncontingent cocaine exposure, while levels of AEA in the PFC are reduced following noncontingent but not self-administered cocaine (Bystrowska et al. 2014), further suggesting a role for alterations in the eCB system in the development of addiction-related behaviors. Additionally, both experimenter- and self-administered cocaine result in increases in *N*-acylethanolamines (Bystrowska et al. 2014) including the AEA function-enhancing fatty acid *N*-palmitoylethanolamine (Ho et al. 2008) and the putative eCB *N*-oleoylethanolamine (Overton et al. 2006).

Chronic exposure in both humans and mice has been shown to result in decreased surface expression of CB1R with increased cytosolic levels, suggesting a downregulation of function in the system. Furthermore, chronic cocaine exposure or intake decreases downstream signaling from the activated CB1R (Alv  ro-Bartolom   and Garc  a-Sevilla 2013). In this vein, the cocaine sensitization paradigm has further elucidated discrepancies between repeated and acute cocaine administration. While cocaine-sensitized mice exhibit increased levels of CB1R as well as both FAAH and MAGL in the PFC, acute cocaine only alters the ratios of eCB precursors and their respective enzymatic partners, suggesting that chronic

cocaine treatment induces either entirely different or more robust changes in the eCB system than acute exposure (Blanco et al. 2015). It is apparent, then, that cocaine self-administration in human addicts as well as animal models substantially impacts eCB function through manipulation of eCBs, their enzymatic pathways, and receptors.

Cannabis Cannabis abuse, especially during adolescence, has profound consequences on the functions of the eCB system in the PFC. Adults exhibit reduced or absent vulnerability to many of the metaplastic effects of cannabis exposure (Realini et al. 2011; Schneider and Koch 2003); however, the impact of adolescent exposure may prove relevant in the development of such aberrant behaviors as addiction and psychosis (for reviews, see Caballero and Tseng 2012; Hurd et al. 2014; Rubino and Parolaro 2008). PFC-dependent cognitive functions such as working memory are impaired in adulthood following cannabis exposure during development in animal models (Lovelace et al. 2015; Raver et al. 2013). Indeed, cannabis exposure during adolescence is positively correlated with the development of psychosis (see section: Schizophrenia) and increased risk of illicit drug use in humans (Ellgren et al. 2008; Fergusson and Horwood 2000; Yamaguchi and Kandel 1984; Lynskey et al. 2003; Argawal et al. 2004).

Interestingly, the impact of exposure to exogenous cannabinoids may be gender-specific (Lovelace et al. 2015; Realini et al. 2011; Rubino et al. 2008c, 2009). Reductions in CB1R expression in adulthood following adolescent exposure emerge differentially between genders, being more profound and widespread in female over male rats. Similarly, low PFC CREB activity is a consequence of adolescent THC exposure in adult female rats but not male (Rubino et al. 2008c). These differences are paralleled by the behavioral manifestations of such altered activity, including markers of depression (Rubino and Parolaro 2008) and addiction vulnerability (Higuera-Matas et al. 2008). Together, these results identify significant differences in the consequences of cannabinoid-induced deregulation of PFC function between genders.

In light of the cannabinoid-induced perturbations in PFC function, it is not surprising that repeated exposure to cannabinoid agonists during adolescence has profound and lasting effects on the function of the local eCB system. Animals exposed to a CB1R agonist subchronically during adolescence exhibit numerous deficits in eCB function as measured during adulthood, including desensitized CB1Rs, attenuation of the eCB-independent mGluR2/3-LTD, and a deficit in L2/3-L5 eCB-LTD (Lovelace et al. 2015). This adolescent exposure also results in aberrant development of other PFC proteins such as the μ -type opioid receptor that may also contribute to prefrontal synaptic dysfunction (Ellgren et al. 2008).

In humans, chronic cannabis use beginning during adolescence and early adulthood results in decreased CB1R expression as measured via PET imaging (Ceccarini et al. 2015), suggesting that cannabis use and sustained dysfunction in the eCB system is a common phenomenon. Thus, consumption of cannabis during adolescence results in persistent, substantial perturbations in eCB function that may

underlie many of the aforementioned behavioral abnormalities resultant of this exposure.

Other Drugs Various alterations in eCB function have been further noted with other drugs of abuse. Briefly, withdrawal following morphine sensitization is correlated with increased AEA levels as well as potentially impaired FAAH function in the PFC. Trends toward increased levels of AEA and 2-AG following acute exposure were also noted, suggesting a role for the eCB system in multiple stages of morphine abuse (Viganò et al. 2004). Significantly, there is overlap in CB1R and opioid receptor expression patterns (Rodriguez et al. 2001; Salio et al. 2001), and CB1R and mu-opioid receptors may form heterodimers (Rios et al. 2006; Hojo et al. 2008). Indeed, CB1R and opioid agonists exhibit significant cross-tolerance (Maldonado 2002; Thorat and Bhargava 1994; Viganò et al. 2005), suggesting further potential links between the development of opiate addiction and alterations in the eCB system.

Other drugs, such as methylphenidate, have been shown to induce lasting effects on synaptic plasticity in the PFC following cessation of use, including those previously exhibited to be eCB-dependent such as HFS-LTD (Burgos et al. 2015). Similarly, withdrawal from chronically administered nicotine induces increased levels of AEA in the PFC (Cippitelli et al. 2011). Interestingly, certain behavioral effects of nicotine withdrawal may be attenuated via inhibition of FAAH, further indicating a role for eCBs in nicotine abuse or withdrawal (Cippitelli et al. 2011). The involvement of the eCB system in the effects of such varied drugs of abuse is a clear indicator that further research must be conducted to elucidate the full role played by this molecular machinery and crucial synaptic modulator in drug abuse and addiction. Future research into the etiology and synaptic consequences of drug use and addiction must therefore consider the contributions and alterations of the eCB system in order to fully understand the cellular and molecular underpinnings of substance abuse disorders.

3 Alzheimer and Huntington Diseases

Cortical areas involved in cognitive flexibility, learning, memory formation, and storage are prime targets of the neurodegenerative and functional deficits accompanying Alzheimer disease (AD) and Huntington disease (HD). The complex multifactorial etiology of AD and HD and how the therapeutic opportunities provided by the fast-moving eCB pharmacology could help treat these neurodegenerative disorders are beyond the scope of this chapter. Excellent current updates can be found in Bedse et al. (Bedse et al. 2015) and Aso and Ferrer who noted:

Cannabinoids may target in parallel several processes that play key roles in AD, including A β and tau aberrant processing, chronic inflammatory responses, excitotoxicity, mitochondrial dysfunction and oxidative stress among others. Clinical data also reveal an

improvement in behavioral in patients with AD after treatment with cannabinoids. (Aso and Ferrer 2014; Fagan and Campbell 2014; Kluger et al. 2015)

Alzheimer Disease It is clear that the PFC eCB signaling system is not spared in AD, where the disease presents with such symptoms as a decline in executive function and memory and behaviors with a known dependence upon eCB function. In human patients, there is an increased [35 S]GTPS binding stimulated by the CB1R agonist WIN55,212-2 in PFC layers 5 and 6 of AD I–IV stages, while CB1R density is enhanced in layer 6 only at the later stages of the disease (Manuel et al. 2014). Accordingly, the binding of the CB1R radioligand [125I]SD7015 was found to be enhanced in the PFC of human patients (Farkas et al. 2012). Reminiscent of the human data, CB1R mRNA levels, but not protein levels, are augmented in the PFC of 3 x Tg-AD mice (Bedse et al. 2014). Furthermore, in the A β PPsw/PS1 Δ E9 mouse model of AD, there is higher CB1R-effector coupling compared to wild-type mice in the frontal cortex, but no difference in PFC-linked associative memory nor in lipidomic profiles (Maroof et al. 2014). In the same model, social recognition (a behavior where PFC contribution is likely) deficits were prevented by long-term cannabidiol (CBD) treatment (Cheng et al. 2014). Emphasizing the therapeutic potential of CBD in AD, substantial cognitive improvements were reported by a combination of THC and CBD (Aso et al. 2015).

In addition to CB1R, evidence points to a role for the CB2R in AD and associated neuroinflammation (Schmöle et al. 2015) (for a review, see Ahmed et al. 2015). CB2R mRNA and protein levels are increased in the cortex of human AD patients or A β PPsw/PS1 Δ E9 mice (Bedse et al. 2015), while increased CB2R expression in the PFC of later-stage AD patients correlates with amyloid A β levels (Solas et al. 2013).

These data indicate that targeting the eCB system is a viable therapeutic strategy in AD-related PFC deficits. Nonetheless, how AD impacts the synaptic functions of the eCB system in the PFC remains unknown.

Huntington Disease HD is a neurodegenerative disease caused by a CAG repeat expansion in the huntingtin gene. Executive dysfunction may precede clinical manifestation of the movement disorder. Reduced CB1R binding in the basal ganglia and substantia nigra is an early signature of HD (Glass et al. 1993, 2000; Richfield and Herkenham 1994), and in animal models of HD, both CB1R expression and eCB levels are reduced (Dowie et al. 2009; Glass 2001; Lastres-Becker et al. 2001, 2002). While evidence for a correlation between HD and the basal ganglia's eCB system is prevalent, the data linking HD progression to the eCB in the PFC are scarce (Maccarrone et al. 2007). In the cortex of presymptomatic R6/R2 mice, 2-AG levels are decreased by 25%, while in the cortex of symptomatic R6/2mice, a 28% decrease of 2-AG levels is combined with a 50% increase in AEA levels (Bari et al. 2013; Bisogno et al. 2008). In R6/2 mice, Centonze et al. (2005) showed a reduction in CB1R expression and CB1R-mediated synaptic responses at striatal GABAergic synapses. Based on the aforementioned biochemical evidences of PFC damages in HD and recent human PET data inversely correlating the disease burden with CB1R availability in the PFC (Van Laere et al. 2010), it is tempting to

hypothesize that executive deficits in HD implicate alterations of the synaptic functions of the eCB system in the PFC.

4 Schizophrenia

Schizophrenia is a severe synaptopathy characterized by disordered thought processes and both positive and negative cognitive maladies which often appear first during early adulthood and impact a wide array of cognitive and behavioral functions (Lewis and Lieberman 2000). Dysfunction in the PFC has been identified as a major contributing factor in the development and manifestation of this disease (Tsai and Coyle 2002), and dysregulation of the eCB system in particular has been identified as a possible cause (Tan et al. 2014; Ujike and Morita 2004; Volk and Lewis 2015). Here, we will examine evidence suggesting that dysfunction of eCB signaling in the PFC may be both a contributing factor to the development of schizophrenia as well as a point of vulnerability in those predisposed to the disease.

PFC eCB Dysfunction in Schizophrenia Schizophrenic humans show increased CB1R binding density in the PFC (Dalton et al. 2011; Dean et al. 2001; Jenko et al. 2012; Newell et al. 2006; Zavitsanou et al. 2004) as well as increased AEA levels in circulating CSF and blood (De Marchi et al. 2003; Giuffrida et al. 2004; Koethe et al. 2009; Leweke et al. 1999). PET studies utilizing a CB1R-selective ligand have confirmed that CB1R binding is elevated in schizophrenic patients and inversely correlated with negative symptoms (Wong et al. 2010). Furthermore, a link with CNR1, the gene encoding for the CB1R, has been noted as altered in schizophrenic patients (Leroy et al. 2001; Ujike et al. 2002). However, a more recent study identified a notable decrease in CB1R mRNA in the PFC of schizophrenic subjects (Eggan et al. 2008). This contradiction was attributed to possible allosteric binding of previously used radioligands and highlights a need for further, and more technically consistent, studies in determining this abnormality. Interestingly, a mutation in the CNR2 gene encoding for CB2R that reduces its function has also been found to be elevated in a postmortem study of schizophrenic brains (Ishiguro et al. 2010). In vitro modeling of this mutant CB2R showed decreased responding to 2-AG, and indeed in a model of schizophrenic symptoms induced by the administration of the NMDAR antagonist MK-801 (for a review, see Thornberg and Saklad 1996), the use of a CB2R inverse agonist exacerbated symptomology, confirming a potential role for CB2R dysfunction in schizophrenia.

While mRNA levels for some enzymes responsible for the synthesis and degradation of eCBs are unaltered in schizophrenics (Volk et al. 2010), recent evidence suggests that levels of the serine hydrolase α - β -hydrolase domain 6 (ABHD6) which regulates 2-AG signaling and metabolism in the PFC are increased in young schizophrenic patients compared to healthy controls (Volk et al. 2013). Interestingly, postmortem brain tissue analysis has revealed increased levels of 2-AG in the PFC of schizophrenic patients (Koethe et al. 2009), suggesting that this

elevated 2-AG metabolism may be a compensatory mechanism for the otherwise enhanced levels of circulating eCBs in schizophrenic individuals.

Animal models of schizophrenia similarly show significant alterations in eCB system function. Following chronic-intermittent phencyclidine (PCP) administration to model schizophrenic symptoms, CB1R-activated GTPyS binding is reduced, accompanied by increased levels of 2-AG in the PFC (Vigano et al. 2009). Not surprisingly, the symptoms of schizophrenia in this model are enhanced by THC administration, which is similarly observed in human schizophrenic subjects (Chakraborty et al. 2014; Negrete 1989; Turner and Tsuang 1990; Ujike and Morita 2004; Treffert 1978). Interestingly, certain aspects of the disease in a similar model of PCP-induced schizophrenic symptomology can be rescued using a systemically administered FAAH inhibitor or a direct CB1R agonist (Seillier et al. 2013). The contradictory findings between animal models and human studies may be due to compensatory changes in eCB function in response to the pharmacological generation of the disease model. Together, these results depict a multifaceted relationship between eCB function and schizophrenia, which may highlight avenues toward the identification of biomarkers of the disease. The sometimes contradictory and often complex interactions demonstrate a necessity for continued investigation.

Cannabis Use and Schizophrenia Enhanced deficits from cannabis use in schizophrenic subjects provide further evidence supporting a role for dysfunction in the eCB system. Compared to control individuals, schizophrenic humans show significantly increased responses to THC exposure with regard to multiple symptoms of the disease (D'Souza et al. 2005). Accordingly, the frequency and intensity of psychotic episodes in schizophrenic individuals is increased following cannabis use (Linzen et al. 1994; Negrete and Knapp 1986). This interaction is likely attributable to preexisting deficits in PFC function (Hambrecht and Häfner 2000; Knable and Weinberger 1997). Patients who continue to use cannabis show increased frequency and intensity of psychotic episodes (Foti et al. 2010), providing further evidence for an ongoing interaction between aberrant function in the eCB system and the course of the disease. Furthermore, gamma- and theta-band oscillations in the PFC are attenuated in rats exposed to a CB1R agonist (Hajós et al. 2008), where similar deficits have been identified in human schizophrenic subjects (Caballero and Tseng 2012; Spencer et al. 2003; Uhlhaas and Singer 2010). Together, these findings draw a clear correlation between altered eCB functions and the prevalence, timing, and severity of schizophrenia.

Cannabis consumption during adolescence, a critical period of development for the eCB system (particularly in the prefrontal cortex; Long et al. 2012), is positively correlated with an increased likelihood and severity of the emergence of schizophrenia (for reviews, see Arseneault et al. 2004; Casadio et al. 2011). While previous cannabis consumption is a significant predictor for the development of schizophrenia in predisposed populations (Compton et al. 2009), cannabis consumption prior to the age of 18 resulted in a more than doubled risk of schizophrenia precipitation in a large cohort of non-predisposed individuals, which is elevated to nearly sixfold with “heavy” use (Andréasson et al. 1987). The increased risk of

schizophrenia due to cannabis use is similar to so-called high-risk individuals (i.e., those with a strong family history of schizophrenia) which may cause as much as a sixfold increase in the likelihood of developing psychosis (Miller et al. 2001). Nevertheless, PCP-induced psychotic symptoms are enhanced in CB1R-KO mice (Haller et al. 2005). The available evidence clearly highlights a profound relationship between eCB function and schizophrenia, which warrants continued investigation both for elucidating the underlying mechanisms of disease development and potential pharmacotherapeutic interventions (Saito et al. 2013).

eCB-Based Pharmacotherapies Given the clear link between eCB dysfunction and schizophrenic symptomology, manipulation of the eCB system provides a clear and potentially valuable route toward the development of targeted pharmacotherapies for the treatment of schizophrenia. One existing candidate with such potential is CBD, a naturally occurring component of cannabis that acts as an indirect antagonist at both CBR1 and CB2R (for a review of pharmacological properties, see Mechoulam et al. 2007).

CBD administration attenuates several symptoms in animal models of schizophrenia including MK-801-induced hyperactivity as well as deficits in prepulse inhibition and social withdrawal (Gururajan et al. 2011; Long et al. 2006). Similarly, CBD has been shown to be equally efficacious as the atypical antipsychotic clozapine in attenuating chronic-MK801-induced deficits in social interaction and novel object recognition, two hallmarks of schizophrenic symptomology in animal models (Gomes et al. 2015b), possibly via reduced inflammation and inhibition of microglia in the PFC. These results are further supported by CBD's ability to attenuate MK-801-induced deficits in prepulse inhibition via reversal of elevated Delta-FosB and parvalbumin expression in the PFC (Gomes et al. 2015a).

In human subjects, CBD has been shown to attenuate the anxiety-inducing effects of THC (Crippa et al. 2009) as well as its psychotic symptoms as measured by the Positive and Negative Syndrome scale. These properties have been suggested to arise from the ability of CBD to disrupt connectivity between the amygdala and anterior cingulate (Fusar-Poli et al. 2009), in addition to reducing activation of the striatum, medial temporal cortex, and PFC (Bhattacharyya et al. 2012) in a manner opposite to THC.

Several clinical studies support the potential for CBD in the targeted pharmacotherapeutic treatment of schizophrenia (for a full review, see Iseger and Bossong 2015). Case studies of schizophrenic patients have shown promise in treatment via CBD (Zuardi et al. 1995), including treatment-resistant symptoms (Zuardi et al. 2006). Indeed, comparing the effects of CBD to the atypical antipsychotic amisulpride, Leweke and colleagues found no difference in efficacy in reducing psychotic symptomology. However, the therapeutic effects were accompanied by significantly reduced side effects (Leweke et al. 2012). Clearly, the evidence for preexisting or cannabis-induced eCB dysfunction in schizophrenia, combined with promising evidence for the treatment of its symptomology with CBD, indicates great promise in the development of eCB-targeting therapy.

However, further research is required to elucidate the mechanisms underlying these effects and develop more targeting approaches to pharmacological intervention.

5 Autism Spectrum Disorders

Autism spectrum disorders (ASDs) represent a group of both genetically and environmentally induced defects, which share behavioral phenotypes, notably deficits in social reciprocity and stereotyped behaviors. A common neurological nexus among these spectrum disorders is the PFC, which is linked genetically, anatomically, and functionally to ASD (Courchesne et al. 2011; Willsey et al. 2013). Yet, save Fragile X syndrome, there is surprisingly little clinical data on the eCB system in ASD and only indirect peripheral measurements of eCBs (Chakrabarti et al. 2015). Notwithstanding, there has been significant popular interest in the use of medical marijuana in the treatment of ASDs. Beyond the anecdotal however, there is limited current evidence supporting such an intervention (Hadland et al. 2015).

Fragile X Syndrome FXS is an X-chromosome-linked hereditary disorder characterized by intellectual disability and variable changes in social behavior often linked to autism (Garber et al. 2008). In most affected individuals, there is transcriptional silencing of the fragile X mental retardation gene (*FMR1*) due to expansion of a trinucleotide CCG repeat in the 5'-untranslated region and a corresponding loss or reduction in the FMR1 protein (FMRP; Penagarikano et al. 2007). Since FMRP is an RNA-binding protein, this in turn leads to the loss of regulated translation of a large subset of associated mRNAs notably rich in encoded synaptic proteins (Darnell et al. 2011). The large and diverse set of FMRP-associated mRNAs means that FXS probably has a highly complex developmentally linked etiology; however, an important and much explored hub in the expression and treatment of FXS has been the pathological enhancement of mGluR5 function (Darnell and Klann 2013). Treatment with mGluR5 antagonists in animal models of FXS recovers many of the associated physiological and behavioral deficits (Michalon et al. 2012). However, subsequent clinical trials have proven less promising, perhaps due to the difficulties in selectively targeting a FXS associated deficits without harming general mGluR5 function in the brain (Mullard 2015).

Many of the behavioral signatures of FXS are linked to PFC-directed actions: loss of executive control, deficits in working memory, and abnormal social interaction (Cornish et al. 2008). Likewise the *Fmr1* knockout mouse model shows alterations in both PFC-dependent learning and social behavior, although baseline and mGluR5 antagonist effects are rather inconsistent (Gantois et al. 2013; Kramvis et al. 2013; Krueger and Bear 2011; Liu and Smith 2009; Mao et al. 2013; Sidorov et al. 2014). Since eCB mobilization is a major downstream effector of mGluR5 signaling, it is unsurprising that there have been reports of changes in the eCB

system in FXS. In the hippocampus where enhanced mGluR5 function is most robustly reported, there is a parallel upregulation of the eCB signaling at both excitatory and inhibitory synapse (Tang and Alger 2015; Zhang and Alger 2010), and consequently drugs targeting the CB1R have proven successful in restoring hippocampal-based behavior in the *Fmr1* KO mouse model (Busquets-Garcia et al. 2013). In contrast, current evidence points to a deficit in the eCB signaling in the PFC. Similar to results in the nucleus accumbens, synaptic induction of eCB-LTD is impaired in layer 5 pyramidal neurons in adult *Fmr1* KO mice (Jung et al. 2012). This loss in eCB-LTD does not appear to be linked to a direct loss of CB1R function, but rather a deficit in coupling between mGluR5 and DAGL- α and thus the on-demand biosynthesis of 2-AG. Notably DAGL- α mRNA is the most prominent component of the eCB system thus far shown to be a FMRP target (Darnell et al. 2011). This opposite regulation in eCB signaling between the PFC and hippocampus appears to reflect a common phenomenon in FXS, where neuronal properties and signaling are changed in a region-specific manner (Contractor et al. 2015). Thus, pharmacological treatment of *Fmr1* KO animals with a drug that enhances eCB signaling, JZL184, has been shown to restore selective behavioral deficits (Jung et al. 2012). Therefore, although targeting the eCB system in the treatment of FXS may solve some of the global risks of directly targeting mGluR5, the heterogeneity in eCB deficits in FXS significantly complicates this approach.

Sodium Valproate Environmental insults leading to ASD have extensively been examined in the sodium valproate (VPA) rat model. Here, a single in utero exposure to the teratogen valproate results in offspring which exhibit several of the hallmarks of ASD (Rouillet et al. 2013). VPA pups show abnormal PFC connectivity and changes in pre- and postsynaptic function under basal conditions (Rinaldi et al. 2008). Furthermore, measurement of correlated activity in layer 5 neurons suggests a loss of muscarinic acetylcholine receptor (mAChR)-mediated decorrelation in these animals, indicating a deficit in attention microcircuitry (Luongo et al. 2015). Clinical studies have reported abnormalities in mAChR function in the autistic forebrain (Perry et al. 2001), and recent work has linked M₁ mAChR modulation of synaptic function to the eCB system in the PFC (Martin et al. 2015). Direct measurement of eCB synaptic plasticity, however, suggests that eCB-LTD is intact in the VPA rat at later developmental stages (Martin et al. 2015). These studies however focused on the function of the CB1R in the PFC. In contrast, direct measurement of eCBs and their associated receptors in the VPA pups have instead identified changes in other eCB receptors (GPR55, PPAR α) which may play an important role in ASD (Kerr et al. 2013). Notably, AEA, a putative agonist of GPR55 and PPAR, has recently shown to be mobilized by oxytocin receptors in cortical and subcortical regions linking pro-social signaling and the eCB system (Ninan 2011; Wei et al. 2015).

Neureligns The investigation of eCBs in genetic models of ASD, other than in the *Fmr1* knockout mouse, is similarly in its infancy. The established neuroligin 3 knockout mouse model and the neuroligin 3 mutant (arginine 451 $\rightarrow$ cysteine substitution) ASD gain of function mutation have been studied in the hippocampus.

In both models, deficits in interneuron GABA release linked to the eCB system are reported. Notably, at the cholecystokinin basket cell to CA1 pyramidal synapse, a selective loss in CB1R tonic signaling, but not phasic signaling, is found (Foldy et al. 2013). A similar enhancement in inhibitory drive is found in the somatosensory cortex which may also be linked to changes in tonic CB1R signaling in these same mice (Speed et al. 2015). Interestingly, a contrasting knockout of neuroligin partner β -neurexins led to an increase in CB1R tonic signaling at excitatory synapses and depressed glutamate release (Anderson et al. 2015). These data suggest that the eCB system may be integral to the changes in excitatory/inhibitory balance proposed to occur in ASD. However, currently neither the neuroligin nor nerexin model has been investigated in the PFC; thus, caution should be taken in the interpretation of these findings in the context of PFC physiology.

6 Intellectual Disability

The use of cannabis results in acute and chronic deficits in cognition. Perhaps unsurprisingly there is also evidence that mPFC deficits in eCB signaling are also linked to genetic intellectual disability (ID) disorders. As discussed above, one of the most consistent phenotypes in FXS is moderate ID. It appears that interventions which modulate mGluR5 signaling may normalize cognitive performance and learning in PFC-dependent tasks (Chen et al. 2014; Xu et al. 2012). However, whether this is linked to eCB signaling is unclear.

Down syndrome (DS), caused by a trisomy of chromosome 21, is the most common genetic cause of ID. Typical of other forms of ID, both dendritic and synaptic alterations in cortical neurons are found. In concert with these morphological changes, there are also DS-linked deficits in synaptic regulation (Garner and Wetmore 2012). An important DS-linked gene is DYRK1A. Increase of the genetic expression of Dyrk1A in mouse models recapitulates many of the synaptic deficits found in DS. In the mPFC of Dyrk1A mice, there is a loss of both LTP and eCB-LTD in deep layer 5 principal neurons (Thomazeau et al. 2014). This loss of eCB-LTD is not associated with reduced CB1R function, but instead involves reduced retrograde 2-AG signaling. Similar to findings in the Fmr1 KO mouse, enhancement of 2-AG with the MAGL inhibitor JZL184 effectively restores eCB-LTD. Promisingly, treatment of mice with JZL184 also restores some of the cognitive deficits found in the Ts65Dn mouse model of DS (Lysenko et al. 2014), suggesting the eCB system may in general be a therapeutic target in DS.

Currently it is unknown if eCB synaptic function is altered in other genetic disorders showing phenotypic ID (Rett syndrome, neurofibromatosis, tuberous sclerosis). However, they along with FXS and DS often share a commonality of deficits in postsynaptic protein expression and regulation (Castrén et al. 2012). Given that postsynaptic coupling to the eCB system in the PFC is defective in both FXS and DS, it will be interesting to see if the eCB system is likewise altered in other models of ID.

7 Stress and Anxiety

The involvement of the eCB system in the emotional states of stress and anxiety is readily apparent. While not identical, a great deal of overlap exists between these mental conditions and the current research addressing them. Therefore, stress and anxiety are presented here in tandem. Indeed, a great wealth of evidence points to a bidirectional relationship between eCB dysfunction and the intensity, duration, and frequency of such events as well as the impact of such states on eCB function itself. This is not surprising, given the dense distribution of CB1Rs in numerous regions of the brain involved in emotional states such as the PFC, amygdala, hippocampus, thalamus, and hypothalamus in both humans (Mato and Pazos 2004) and rodents (Herkenham 1992; Herkenham et al. 1990, 1991). Here, we will examine the evidence supporting a role for eCB dysfunction in anxious states as well as the impact of such states on the eCB system. There exists a significant overlap in research paradigms for animal models of chronic stress and those for depression, the former of which may precipitate symptomology resembling the latter (Willner 2005). As such, a further section on depression will focus primarily on animal models of depression and the results of chronic stress, while this section's primary focus is acute stress and anxiety. For a thorough review of the eCB system's role in stress, see chapter by Hill and McLaughlin, as well as a relevant review by McEwan et al. (2015).

eCB Dysfunction and Stress Phenotypes Dysfunctional eCB signaling has been linked to a variety of disordered emotional states in humans including elevated levels of anxiety. Recapitulation of this signaling dysfunction in animal models has yielded similar behavioral phenotypes, indicating a foundational relationship between the system-level aberration and the expression of elevated stress or anxiety. Indeed, genetic predisposition toward anxiety has been exhibited in animal models using a CB1R knockout mouse (Aso et al. 2008; Martin et al. 2002; Steiner et al. 2008). These CB1R knockout mice also show increased anxiety under basal conditions as compared to control mice, in addition to exhibiting exaggerated responses to subsequent stress (Hill et al. 2011a). Furthermore, genetic deletion of MAGL, which induces increased 2-AG levels in the mPFC, results in an anxiogenic phenotype through desensitized CB1R signaling (Imperatore et al. 2015), further supporting a correlation between attenuated eCB signaling and anxiety-like states. In humans, FAAH gene variation linked with decreased expression has been associated with reduced stress reactivity in line with that seen in animal models (Gunduz-Cinar et al. 2013). Finally, aberrant eCB signaling in animals fed a diet deficient in $n - 3$ polyunsaturated fats, which elevates levels of 2-AG (Watanabe et al. 2003) and induces a desensitized or uncoupled state in presynaptic CB1Rs (Lafourcade et al. 2011; Larrieu et al. 2012) in the PFC, has been shown to increase anxiety-like behaviors. Clearly, dysregulation of eCB function in the PFC significantly impacts the expression of anxiety as determined by a variety of animal models.

Pharmacological manipulation of eCB function can readily recapitulate the outcomes of the aforementioned dysfunctions and has led to a vastly increased understanding of the relationship between the eCB system and the emotional states of stress and anxiety. Indeed, while direct activation of CB1R has been repeatedly shown to produce anxiolytic effects in animals exposed to stressful conditions (Adamczyk et al. 2008; Bambico et al. 2007; Hill and Gorzalka 2005; Rutkowska and Jachimczuk 2004), CB1R antagonists induce an anxiogenic state which mirrors that of chronic stress in animals (Beyer et al. 2010) as well as humans (Hill and Gorzalka 2009; Nissen et al. 2008). Indeed, pretreatment with a CB1R antagonist significantly enhances stress-induced activation of neurons in the PFC (Patel et al. 2005), further supporting the observation that CB1R activation is inversely correlated with anxiogenic states in models of acute stress.

Conversely, direct activation of CB1Rs in the PFC induces a state that is functionally protective against stressful conditions (Rubino et al. 2008a, b). Additionally, enhancing CB1R function in the PFC via direct infusion of a CB1R agonist, FAAH inhibitor or reuptake inhibitor enhances the extinction of both learned and innate fear responses (Lin et al. 2009). Furthermore, enhancement of AEA levels through local infusion of a FAAH inhibitor reduces anxiety-like behaviors, while overexpression of FAAH conversely reduces AEA levels and therefore produces anxiogenic symptoms (Rubino et al. 2008b). Similar effects have been noted following direct infusion of an AEA reuptake inhibitor in the prelimbic PFC which reduces anxious responses in the elevated plus maze and Vogel conflict test, further supporting the notion that CB1 signaling in the PFC modulates both innate and learned fear behavior (Lisboa et al. 2015). These effects are mirrored following inhibition of AEA hydrolysis or reuptake prior to other stressful conditions such as the forced swim test (Adamczyk et al. 2008; Gobbi et al. 2005; Hill and Gorzalka 2005) and the tail suspension test (Naidu et al. 2007). More directly, local infusion of a CB1R agonist attenuates the stress response to the forced swim test. This effect was determined to act via a 5-HT-dependent mechanism (Bambico et al. 2007), lending support to the idea that eCB function in states of stress and anxiety is largely dependent upon modulation of 5-HT, GABA, or glucocorticoid signaling (Hill et al. 2011b). Aside from activation of CB1Rs, TRPV1 channel activation may contribute to the anxiety-modulating effects of local AEA signal enhancement, as TRPV1 blockade in the PFC has been shown to elicit an anxiolytic response (Aguiar et al. 2009). Given these consistent findings, it is clear that the eCB signaling and modulation of multiple neurotransmitter systems in the PFC is fundamentally tied to the emotional states of stress and anxiety, though further research is required to elucidate the exact mechanisms underlying this relationship.

Stress-Induced Modulation of eCB Function Stressful experience has been repeatedly shown to alter eCB signaling in the PFC. Chronic or repeated stress in rodents decreases AEA signaling, possibly through increased clearance of the molecule by its degrading enzyme FAAH (Bortolato et al. 2007; Rademacher et al. 2008). Interestingly, the same procedures result in increased 2-AG signaling, though

without any effect on CB1R receptor density (Rademacher et al. 2008). A similar increase in 2-AG signaling in the PFC is found following multiple, but not single, exposures to restraint stress (Patel et al. 2005). Furthermore, CB1R mRNA is downregulated in the PFC following predator threat stress exposure (Campos et al. 2013). An elegant study attempting to piece together the multiple changes in the eCB system during stress showed that exposure to stress increases 2-AG levels in the mPFC via a glucocorticoid-dependent mechanism as a means of regulating activation of the stress-mediating HPA axis (Hill et al. 2011b).

Taken together, the currently available data in the field indicate that the impact of stress on eCB functioning is largely negative (i.e., anxiogenic experiences decrease eCB signaling). However, the final outcome measures and resultant changes induced by stress provide an unclear picture of the multiple stages of regulation and compensatory changes that occur. In part, it is apparent that these discrepancies owe to the variance in protocol for induction, including the degree of predictability. Nonetheless, it is clear that stress-induced regulation of the eCB system is a complex and time-dependent phenomenon that requires increased and more consistent research before it is fully elucidated.

8 Depression

Humans have used cannabis for its multiple effects on mood for thousands of years. However, exposure to THC during critical development periods such as adolescence induces synaptic and behavioral abnormalities that suggest a depression-like state (Rubino et al. 2008c, 2009, 2015). The participation of CB1R in the physiological processing of emotions is well documented (Draycott et al. 2014; Martin et al. 2002; Rubino et al. 2015; Witkin et al. 2005), and a role for eCB dysfunction in major depression has long been postulated (Gorzalka and Hill 2011; Hillard and Liu 2014; Serra and Fratta 2007).

Individuals suffering from major depression exhibit a variety of abnormal functions in the PFC as determined by neuroimaging studies (for a review, see Rive et al. 2013). Notably, depressed suicide victims exhibit increased CB1R density, elevated levels of eCBs, and enhanced agonist-stimulated CB1R activity (Vinod and Hungund 2006). Similar postmortem studies have confirmed abnormal eCB function in the PFC of depressed subjects (Koethe et al. 2007), including elevated CB1R levels and enhanced CB1R-mediated signaling in the PFC of alcoholic suicide victims as compared to non-suicidal alcoholics (Vinod et al. 2005). This finding is further supported by elevated CB1R function in the brains of those diagnosed with depression (Choi et al. 2012) as well as increased levels of AEA and 2-AG found in the PFCs of depressed suicide victims (Hungund et al. 2004) and in the serum of depressed patients (Hill and Gorzalka 2009).

In addition to a wealth of measures in human depression subjects, early animal studies indicated a role for eCB signaling in mood disorders, notably the CB1R knockout mouse, which shows anxiety-like behaviors in such depression-linked

assays as the elevated plus-maze (EPM), open-field, and light-dark box (Haller et al. 2002; Maccarrone et al. 2002; Martin et al. 2002; Urigüen et al. 2002). These conditions are accompanied by aberrant neurochemical states known to be associated with mood disorders such as increased corticosterone responses and HPA axis activity (Urigüen et al. 2002). Similar effects are seen in nongenetically altered mice administered CB1R antagonists (Marsicano et al. 2002), while conversely, administration of Δ -9-THC in a model of depression in rats decreases depression-like symptomatology (Elbatsh et al. 2012). As detailed in depth below, these findings highlight a substantial role for the eCB system in the generation, expression, and treatment of mood disorders such as depression.

Animal Models Induction of a depression-like state in rodents achieved via chronic unpredictable stress leads to decreased levels of AEA in the PFC (Hill et al. 2008a). A similar effect is seen following the forced swim test, wherein the stressor induces a reduction in AEA levels in the PFC, which can be rescued (along with resultant depression-like symptomatology) via direct application of an FAAH inhibitor (McLaughlin and Gobbi 2012). Additionally, repeated depression-inducing stressors elevate CB1R expression and mRNA in the PFC (Bortolato et al. 2007; Lee and Hill 2013; McLaughlin et al. 2013; Zoppi et al. 2011). This elevation in CB1R expression is likely a compensatory mechanism in response to reduced AEA levels following the induction of stressful states (Hill and Gorzalka 2009; McLaughlin et al. 2014), indicating a dynamic response in PFC eCB function. These data may offer some explanation for competing or contradictory results in measurements of the eCB system in depression models. Interestingly, local CB1R blockade in the PFC increases immobility responses to the forced swim test following chronic unpredictable stress (McLaughlin et al. 2013), indicating that negative modulation of CB1R signaling in the PFC contributes strongly to the development of anxiogenic responding.

Pharmacological manipulation of eCB signaling has provided further insights into the role of eCBs in emotional behavior. Increasing levels of AEA via FAAH inhibition reduces immobility in the forced swim test, an indication of antidepressant-like activity (Adamczyk et al. 2008; Hill and Gorzalka 2005; Hill et al. 2006; Umathe et al. 2011). Similar dose-dependent antidepressant effects in this test are also seen following intra-PFC infusion of a CB1R agonist (Bambico et al. 2007) or FAAH inhibitor (McLaughlin et al. 2012). Conversely, genetic deletion of MAGL, leading to increased levels of 2-AG, increases anxiogenic responding (Imperatore et al. 2015). These findings further highlight the complex regulation of emotion by the eCB system, wherein low levels of cannabinoids inhibit glutamatergic transmission and thus induce an anxiolytic effect, while higher levels inhibit GABAergic transmission and generate anxiogenic behaviors (Rey et al. 2012).

Perhaps not surprisingly given the known alterations in eCB signaling during development, chronic stress differentially alters eCB signaling in the PFC in adolescent rats as compared to adult rats. Repeated stress in adult or adolescent rats causes increased CB1R binding in the PFC; however, while eCB signaling in

adult rats normalizes over a subsequent 40-day recovery period, adolescents exhibit sustained CB1R downregulation following stress exposure (Lee and Hill 2013). Indeed, adolescent rats exposed to chronic stress conditions exhibit lasting deficits in PFC function and exaggerated anxiogenic responses 30 days after the cessation of treatment, which could be ameliorated when the animals were treated with a CB1R agonist prior to stress sessions (Abush and Akirav 2013). The time course of impact for lasting consequences resultant of pharmacological enhancement or attenuation of the eCB system is clearly a topic requiring a substantial degree of additional research, especially considering the potential for eCB-targeting pharmacotherapies for targeting emotional states which appear during multiple stages of development.

CB1R-deficient mice are commonly employed as an animal model of depression (for a review, see Valverde and Torrens 2012). Such hallmarks of depressive behavior include passive coping (Aso et al. 2008; Choi et al. 2012; Mato et al. 2007; Steiner et al. 2008) and anhedonia (Martin et al. 2002; Sanchis-Segura et al. 2004). These mice also show such PFC-localized neurochemical signs of depression as increased extracellular levels of 5-HT (Aso et al. 2008), an effect which is also seen with the administration of a CB1R antagonist (Tzavara et al. 2003). Interestingly, activation of CB1Rs in the mPFC increases 5-HT neuron activity (Bambico et al. 2007; McLaughlin et al. 2012) and results in a hyperactive HPA axis (Hill et al. 2011b). This contradiction may be due to compensatory changes in signaling in CB1R-deficient models. The alteration of 5-HT levels in CB1R knock-out mice is further accompanied by dysfunction in 5-HT receptor signaling in the PFC (Mato et al. 2007). While further research is required to elucidate the exact mechanisms underlying these changes, the evidence clearly indicates a role for eCB signaling in the regulation of serotonergic neuronal communication in the PFC.

eCB-Targeting Pharmacotherapies While traditional pharmacotherapeutic approaches to the treatment of depression have focused on regulation of monoaminergic synaptic transmission, the role of eCBs in modulating serotonergic, dopaminergic, GABAergic, and noradrenergic neurotransmission has poised the eCB system as a logical and valuable target for treating the disorder (Wyrofsky et al. 2015). Indeed, CB1R antagonists exert antidepressant-like effects in animal models (Griebel et al. 2005; Shearman et al. 2003; Witkin et al. 2005) in a manner similar to traditional antidepressants, in that application of CB1R antagonists increases PFC levels of 5-HT, DA, and NA (Need et al. 2006; Tzavara et al. 2003). However, given the known complications with human administration of CB1R antagonists such as increased risk of anxiety, depressive mood disorders, and risk of self-harm (Christensen et al. 2007), this is unlikely to prove an applicable route for the treatment of depression.

Monoamine system-targeted antidepressants have been shown to similarly alter eCBs in the PFC (Hill et al. 2008a; Manna and Umathe 2012; Smaga et al. 2014). These effects are replicated in multiple classes of antidepressant, lending additional evidence to an interplay between eCB signaling and monoamine regulation. Indeed, altered CB1R transmission in the PFC in an animal model of depression is

effectively reversed following treatment with the 5-HT reuptake inhibitor (SSRI) fluoxetine (Rodriguez-Gaztelumendi et al. 2009), which has also been shown to increase CB1R signaling in the PFC (Mato et al. 2010). This finding is not surprising, given the previously demonstrated increase in CB1R density in other brain regions following treatment with an SSRI (Hill et al. 2006). Additionally, treatment with the MAOI tranylcypromine leads to decreased AEA levels and increased CB1R binding density in the PFC (Hill et al. 2008b). Preventative treatment with the tricyclic antidepressant imipramine during stress exposure prevents the normally observed increase in PFC CB1R density (Hill et al. 2008a). Finally, electroconvulsive shock therapy, which has been validated as a beneficial treatment for major depression, restores normal eCB function in the PFC (Hill et al. 2007). Together, these findings suggest significant, bidirectional interactivity between eCB signaling and monoamine regulation.

In humans, a number of studies utilizing direct CB1R modulators have been conducted for such mood disorders as seasonal affective disorder (Bergamaschi et al. 2011) and post-traumatic stress disorder (PTSD; Cameron et al. 2014; Fraser 2009; Passie et al. 2012; Roitman et al. 2014). Indeed, Δ -9-THC administration reduces negative responding and enhances positive association in a facial visualization task, suggesting an effective antidepressant-like activity in emotional processing (Bossong et al. 2013). The potential antidepressant effects of cannabinoids are not surprising given the preponderance of self-medication with cannabis in subjects diagnosed with generalized anxiety disorder, depression, and PTSD (Bowers and Ressler 2015), the latter of which is also associated with genetic risk factors related to abnormal eCB function (Dincheva et al. 2015; Gunduz-Cinar et al. 2013; Lu et al. 2008; Pardini et al. 2012). Less direct approaches to the treatment of mood disorders via eCB-targeting therapies such as inhibition of FAAH have been proposed and have shown significant promise in preclinical models of anxiety (for a full review, see Table 1 in Fowler 2015) and are currently under testing in a number of human clinical trials (Trials: NCT01665573, NCT01964651, NCT01650597, NCT02065739, NCT02169973).

In addition to CB1R activity, recent evidence has also illustrated a potential role for CB2Rs in depression. A CB2R gene polymorphism has been linked to depression in human subjects (Onaivi et al. 2008). Additionally, administration of a selective CB2 agonist has been shown to reduce depression-like immobility behavior in a rodent model (Hu et al. 2009). AEA signaling through TRPV1 receptors in the PFC has also been indicated in the generation of anxiolytic responses (Aguiar et al. 2009). Indeed, increasing concentrations of AEA in the PFC induces a shift from primarily CB1R-mediated anxiolytic response to TRPV1-mediated anxiogenic response (Rubino et al. 2008b), illuminating a delicate balance in targeting the eCB system with regard to the treatment of mood disorders. While TRPV1 knockout mice exhibit decreased immobility responses (You et al. 2012), direct TRPV1 activation has been shown to elicit antidepressant-like effects (Hayase 2011; Kasckow et al. 2004) which has been suggested as a possible mechanism of action contributing to the actions of such classical antidepressant drugs as fluoxetine (Manna and Umathe 2012). Clearly, regulation of eCB function

presents a valuable, though complicated, route toward the pharmacotherapeutic treatment of such mood disorders as depression.

9 Conclusion

In the PFC, the eCB system functions as a hub composed by highly organized synaptic proteins. The “eCB hub system” physiologically connects most, if not all, parts of the PFC circuitry to regulate synaptic transmission and ultimately participates to the expression of PFC-dependent behaviors. The central importance of the eCB hub system in PFC functions is highlighted by its covariance with synaptic and behavioral deficits in a wide array of etiologically diverse synaptopathies, from neurodegenerative to genetic and environmental. While immensely complicated, due in part to its widespread expression, the eCB hub system presents a relatively unexploited target of therapeutic opportunity for the recovery of synaptic function and plasticity in a wide range of disorders wherein restoring network integrity may ameliorate both the symptomology and, in many cases, underlying etiology of such maladies.

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Cannabinoids and Mitochondria

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Abstract Mitochondria are key organelles providing energy supply and many other vital functions to cells. Shortly after the discovery of plant-derived cannabinoid compounds, some studies indicated their impact onto mitochondrial functions. The later identification of cannabinoid receptors as classical seven-transmembrane G protein-coupled receptors suggested that these mitochondrial effects might be due to unspecific membrane-altering properties of cannabinoids. However, the recent discovery that brain mitochondria contain significant amounts of functional type-1 cannabinoid receptors (CB₁) shed new light on cannabinoid physiology and pharmacology. In this chapter, we will summarize historical and recent evidence of the cannabinoid impact on mitochondrial functions in peripheral and central organs of the body.

1 Introduction

Type-1 cannabinoid receptor CB₁ is a G protein-coupled receptor (GPCR), widely expressed in the brain. In the central nervous system, CB₁ receptor activation at both presynaptic and postsynaptic sites results in the regulation of neuronal excitability, neurotransmitter release, and synaptic plasticity. In turn, CB₁ receptor regulates numerous physiological and behavioral processes, including memory, pain perception, food intake, motor control, and others. However, the cellular

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mechanisms of CB₁-mediated control of these functions are only partially understood. Although CB₁ receptors primarily signal at the plasma membrane (pmCB₁), a portion of these receptors is also functionally present at mitochondrial membranes (mtCB₁). This chapter describes how cannabinoids and mtCB₁ receptor regulate brain mitochondrial functions and how these mechanisms might participate in the processing of neuronal information.

2 The Endocannabinoid System

The endocannabinoid system (ECS) has been identified as the target of the main psychoactive component of the plant *Cannabis sativa*, the Δ^9 -tetrahydrocannabinol, abbreviated as THC. Its structure was partially identified by Roger Adams and colleagues in the 1940s (Adams 1942) and then fully elucidated by Raphael Mechoulam and colleagues in the 1960s (Gaoni and Mechoulam 1964). In 1990, the first cannabinoid receptor was cloned, named CB₁ receptor and found to be highly expressed in the brain (Matsuda et al. 1990). The identification and the cloning of a cannabinoid receptor that responds to THC (Matsuda et al. 1990; Devane et al. 1992) gave the birth to an explosion of interest in the cannabinoid research that continues nowadays. The ECS is schematically composed by at least two receptors, the cannabinoid receptors 1 and 2 (CB₁ and CB₂, respectively), their endogenous ligands called endocannabinoids (eCBs), such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the enzymes involved in their synthesis and inactivation (Piomelli 2003; Di Marzo and De Petrocellis 2012). CB₁ receptors were initially identified mainly in the central nervous system (CNS) (Herkenham et al. 1990), whereas it is now clear that also many peripheral organs contain the receptor (Pagotto et al. 2006). CB₂ receptors are expressed primarily by immune and hematopoietic cells (Munro et al. 1993) and in microglial brain cells (Nunez et al. 2004), whereas their presence in neurons is still under scrutiny, due to the lack of suitable tools (Onaivi et al. 2006; Onaivi et al. 2012). However, it is now well established that most of central effects of cannabinoids in the brain are due to CB₁ receptor activation (Ledent et al. 1999; Zimmer et al. 1999).

CB₁ is a seven-transmembrane G protein-coupled receptor, and it is presently considered as the most abundant metabotropic receptor in the brain (Herkenham et al. 1991). The comprehension of the complex roles of the CB₁ protein in the physiological-behavioral effects of cannabinoids requires a precise mapping of their sites of action at the regional, cellular, and subcellular level. These receptors are particularly rich in certain areas of the CNS such as cerebellum and basal ganglia (Katona et al. 1999; Pettit et al. 1998; Tsou et al. 1998). CB₁ receptors are also dense in the hippocampus, cerebral cortex, hypothalamus, olfactory system, spinal cord, and other regions (Moldrich and Wenger 2000).

Despite the wide expression of CB₁ receptors in the CNS, their cellular distribution appears to be restricted to certain cell types. Within cortical regions, CB₁ is mainly found in GABAergic interneurons co-expressing glutamic acid decarboxylase and cholecystokinin (Marsicano and Lutz 1999). Cortical glutamatergic

neurons also contain CB₁ receptors but in lower amount as compared to GABAergic interneurons (Marsicano and Lutz 1999; Mailleux et al. 1992; Marsicano et al. 2003; Marsicano and Kuner 2008). Despite the minority of CB₁ receptors on glutamatergic cells, these receptors play significant roles in functions modulated by (endo)cannabinoids such as resistance to excitotoxic insults in the hippocampus (Monory et al. 2006), fear coping, stress and anxiety (Dubreucq et al. 2012; Metna-Laurent et al. 2012), and food intake (Bellocchio et al. 2010; Soria-Gomez et al. 2014). In addition, serotonin-releasing neurons projecting from the raphe nuclei to the basolateral amygdala and the hippocampal CA3 region also express CB₁ receptors (Haring et al. 2007). Despite its well-known neuronal localization, the low but functional presence of CB₁ receptors on astrocytes was recently revealed using pharmacological tools and conditional mutants (Bosier et al. 2013; Han et al. 2012; Navarrete and Araque 2008). Indeed, astroglial CB₁ receptors were shown to regulate astrocyte leptin signaling (Bosier et al. 2013) and to be responsible for the impairment in working memory performance induced by cannabinoids (Han et al. 2012). Thus, the anatomical and functional data shortly summarized above show that CB₁ receptors expressed in specific cell types play important physiological and pharmacological roles independently of the relative levels of expression. In this way, cellular localization is likely a better predictor of the functions of CB₁ receptors than their levels of expression (Marsicano and Kuner 2008).

CB₁ receptors are preferentially targeted to plasma membranes of presynaptic terminals and axons, where they likely mediate the well-known inhibitory actions on neurotransmitter release (Katona et al. 1999; Chevalleyre and Castillo 2003; Wilson and Nicoll 2001). Because of their presence within different neuronal types, CB₁ receptors modulate several major neurotransmitters such as glutamate, γ -aminobutyric acid (GABA), acetylcholine, noradrenaline, serotonin, and cholecystokinin, among others (Howlett 2002; Pertwee et al. 2002; Szabo and Schlicker 2005). In astroglial cells, CB₁ receptors were also shown to contribute to the regulation of synaptic transmission, possibly through the regulation of gliotransmitters (Navarrete and Araque 2008, 2010).

Besides their presynaptic distribution, CB₁ receptors were also observed in somatodendritic neuronal compartments in several brain regions, where they appear to be rather intracellular than located at plasma membranes (Ong and Mackie 1999; Pickel et al. 2004; Sierra et al. 2014; Wilson-Poe et al. 2012). The classical paradigm of GPCR functioning postulates that GPCRs are confined at the cell surface where, once activated by their agonists, they activate G proteins signaling and initiate various intracellular pathways. Their presence in intracellular compartments, such as the endoplasmic reticulum (ER), endosomes, or lysosomes, is therefore assimilated to a nonfunctional state corresponding either to their synthesis and trafficking toward cell surface or to their internalization (Ferguson et al. 2001). However, this idea was recently challenged by emerging evidence showing the functionality of different GPCRs at intracellular membranes, including ER, endosomes, nuclei, and mitochondria (Belous et al. 2004; Irannejad and von Zastrow 2014). CB₁ receptors were recently shown to share such unconventional

intracellular localization. Rozenfeld and colleagues showed that CB₁ receptors present in endosomal/lysosomal compartments are able to activate signaling (Rozenfeld and Devi 2008). Early studies in vitro showed the accumulation of cannabinoid-binding probes in subcellular fractions, including mitochondria (Colburn et al. 1974). More recent studies reported also anatomical evidence pointing to the presence of CB₁ receptor immunolabeling in intracellular compartments and in particular on mitochondria (Ong and Mackie 1999; Rodriguez et al. 2001). The mitochondrion was indeed described as one of the most commonly labeled organelles by CB₁-positive immunoparticles (Rodriguez et al. 2001).

3 Mitochondria

Although the brain represents only 2% of the body mass, it consumes 20% of the total body energy at rest (Erecinska and Silver 2001; Kety 1957; Rolfe and Brown 1997; Sokoloff 1960). Given that mitochondria are the main cellular converter of the energy contained in nutrients into a useable form, the adenosine triphosphate (ATP), mitochondria are essential for brain functions. Moreover, these organelles play several other crucial physiological processes important for brain physiology, including buffering of intracellular calcium (Ca²⁺), modulation of reactive oxygen species (ROS), production of several neurotransmitters, and metabolism of steroid. Accordingly, mitochondrial dysfunctions can contribute to the development of neurodegenerative diseases (Mattson et al. 2008).

3.1 Mitochondrial Structure

Mitochondria are double-membrane enclosed organelles consisting in an outer (OMM) and an inner mitochondrial membranes (IMM) (Frey and Mannella 2000). These membranes create two different mitochondrial compartments: the internal lumen called the matrix and a much narrower intermembrane space (IMS). The OMM appears similar to other cellular membranes, whereas the IMM is enriched in cardiolipin and is subdivided into two compartments: the inner-boundary membrane close to the OMM and the invaginations, called *cristae* (Herrmann and Riemer 2010). The inner-boundary membrane can be physically linked to the OMM. Several proteins, including the outer membrane voltage-dependent anion channel (VDAC), the inner membrane adenine nucleotide translocator (ANT), and the mitochondrial benzodiazepine receptor, form contact sites between the outer and the inner membranes, which are involved in mitochondria permeability transition and apoptosis (Brdiczka et al. 2006). Cristae are heavily enriched in complexes of the electron transport system (ETS; see below). Their structure, which can differ greatly according to cell types and physiological state, has important impact on mitochondrial functions such as apoptosis and ATP

production (Hackenbrock 1966; Scorrano et al. 2002). For instance, alterations of cristae structure is impaired in several human diseases including several neurodegenerative diseases (Trimmer et al. 2000; Moreira et al. 2001). Mitochondria are the only organelles to possess their own DNA (the mitochondrial DNA, or mtDNA), which is located in the matrix. mtDNA is the basis of the endosymbiotic hypothesis of mitochondria origin (Sagan 1967; Behnke 1977), and it encodes for 13 proteins that are subunit of the ETS (Tuppen et al. 2010; Taylor and Turnbull 2005). Not surprisingly, deleterious mtDNA mutations lead to several neuropathies and myopathies (Tuppen et al. 2010; Taylor and Turnbull 2005).

3.2 *ATP Production*

Mitochondria are considered as the bioenergetic powerhouses of cells because they are producing most of the ATP consumed by cells via the oxidative phosphorylation processes (OXPHOS). Briefly, proteins and carbohydrates are first oxidized in the cytosol, forming metabolites that translocate into mitochondria, whereas fatty acids are metabolized through β -oxidation directly in mitochondria. Here, these metabolites undergo sequential enzymatic reactions through the tricarboxylic acid (TCA) cycle allowing the production of electron donors. These reducing equivalents (NADH and FADH₂) feed in turn the ETS. This process consists of a series of four enzymatic complexes acting as electron carriers. Electrons enter in this respiratory chain either via the complex I (NADH-ubiquinone oxidoreductase) or complex II (succinate-ubiquinone oxidoreductase) and are subsequently transferred to complex III (ubiquinol-cytochrome *c* reductase) and finally to complex IV (cytochrome *c* oxidase) where they permit the reduction of oxygen (O₂) to form water (H₂O). The course of electrons through the complex I, III, and IV is coupled to the translocation of protons from the mitochondrial matrix to the IMS, inducing an electrochemical gradient ($\Delta\Psi$) across the mitochondrial IM. The complex V (F₀F₁ ATP synthase) exploits this mitochondrial membrane potential to produce ATP (Fig. 1; for a detailed description of OXPHOS process, please see (Navarro and Boveris 2007; Chance and Williams 1955; Mitchell 1961)). The ATP is then translocated out of mitochondria by the adenine nucleotide translocator (ANT, or ADP/ATP translocator) to ensure cellular processes. Given that the brain is a highly oxidative organ, most of the energy is derived from oxidative reactions (Cai et al. 2011). OXPHOS is therefore crucial for maintaining energy-dependent physiological processes such as axonal growth and branching, cytoskeletal remodeling, reversal of the ion influxes for the generation of action potentials and synaptic transmission (Harris et al. 2012), as well as other mitochondrial functions including ROS production and Ca²⁺ buffering.

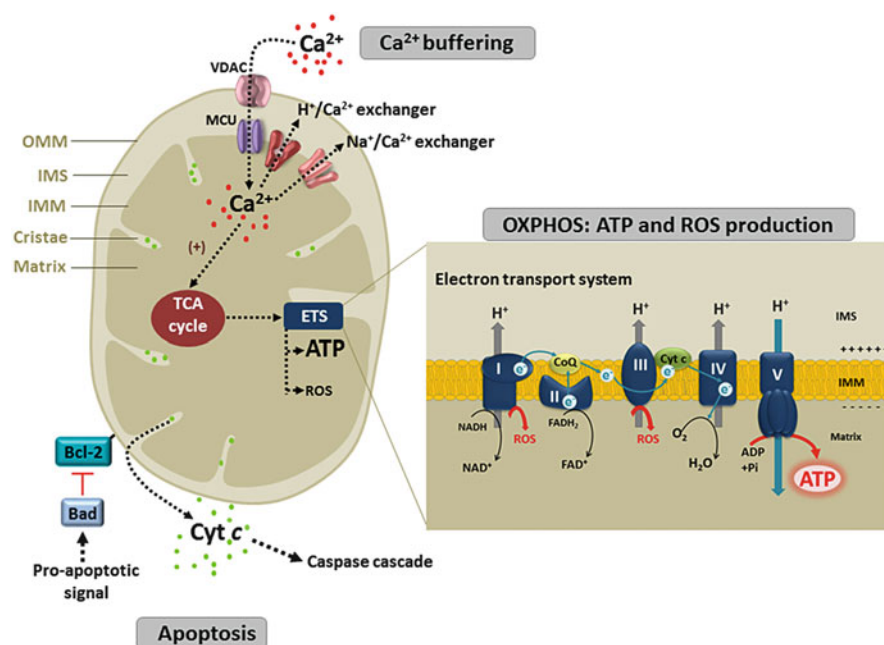


Fig. 1 Mitochondrial functions. Nutrients (carbohydrates, fatty acids, and amino acids) are oxidized in the cytosol and the TCA cycle which generates electron donors (NADH and FADH_2). These compounds then feed the electron transport system (ETS) which consists of a series of four enzymatic complexes which are enriched in mitochondria cristae. During the course of electrons across the complexes I, III, and IV, protons (H^+) are translocated from the matrix to the intermembrane space (IMS). This electrochemical gradient across the IMM allows the ATP synthase (complex V) producing ATP and represents a strong driving force for the import of Ca^{2+} into mitochondria. This process named oxidative phosphorylation (OXPHOS) generates most of the ATP consumed by cells and generates reactive oxygen species (ROS). Mitochondria regulate Ca^{2+} homeostasis. Ca^{2+} is imported across the outer mitochondrial membrane (OMM) through the large voltage-dependent anion channel (VDAC). Then, the mitochondrial Ca^{2+} uniporter (MCU) allows the import of Ca^{2+} across the inner mitochondrial membrane (IMM), whereas $\text{H}^+/\text{Ca}^{2+}$ and $\text{Na}^+/\text{Ca}^{2+}$ exchangers allow the extrusion of Ca^{2+} from mitochondrial matrix. Ca^{2+} increases the activity of three different dehydrogenases of the tricarboxylic acid (TCA) cycle, thereby regulating mitochondrial metabolism. Mitochondria are also key players in programmed cell death or apoptosis. Several pro-apoptotic proteins, including cytochrome c (cyt c), are located inside mitochondria. Upon apoptotic stimuli, the pro-apoptotic protein Bad translocates to mitochondria and inhibits the anti-apoptotic protein Bcl-2. This process leads to the release of cyt c into the cytosol, the activation of the nonspecific caspases and ultimately cell death

3.3 ROS Production

During mitochondrial respiration, significant amounts of ROS are formed, resulting from the incomplete reduction of the O_2 molecule into water (Fig. 1). Between 0.1 and 4% of O_2 is partly reduced by mitochondria to produce the anion superoxide (O_2^-). Mitochondria are considered as the major sites of ROS production in the cell

(Cadenas and Davies 2000). Much of the superoxide seems produced by complexes I and III (Fig. 1) and is then converted to hydrogen peroxide (H_2O_2) by the mitochondrial antioxidant manganese superoxide dismutase (MnSOD). H_2O_2 can also generate the highly reactive hydroxyl radical $\text{OH}^\cdot$. Excessive levels of ROS can damage all cellular components, including DNA, lipids, and proteins, leading to DNA mutagenesis, apoptosis, alteration of redox signaling, and accelerated aging (Marchi et al. 2012). Notably, high ROS production is linked to several neurodegenerative diseases (Hroudova and Fisar 2013). Besides their damaging impact, ROS produced by mitochondria are now considered as important signal transducers in physiological processes including synaptic plasticity (Lee et al. 2010; Knapp and Klann 2002; Ma et al. 2011), learning and memory (Olsen et al. 2013; Kishida and Klann 2007), and regulation of hypothalamic functions (Horvath et al. 2009).

3.4 Ca^{2+} Homeostasis

Beyond their role in generating ATP and ROS, mitochondria contribute to Ca^{2+} homeostasis (Fig. 1). Considering their capacity to remove Ca^{2+} from the cytoplasm and store it in their matrix, mitochondria are crucial regulators of intracellular Ca^{2+} dynamics and of several Ca^{2+} -dependent signaling processes such as synaptic transmission (Giorgi et al. 2012; Billups and Forsythe 2002). Mitochondrial Ca^{2+} uptake depends on the strong driving force created by the mitochondrial membrane potential across the IM that is built by the ETS (Bianchi et al. 2004). Ca^{2+} transport across the OMM occurs through VDAC. In contrast, the IMM contains different proteins able to modulate mitochondrial Ca^{2+} uptake and/or efflux, including the mitochondrial Ca^{2+} uniporter (MCU) which transfer Ca^{2+} into the mitochondrial matrix and $\text{Na}^+/\text{Ca}^{2+}$ and $\text{H}^+/\text{Ca}^{2+}$ antiporters that move Ca^{2+} out of the mitochondria (Giorgi et al. 2012) (Fig. 1). While Ca^{2+} uptake can stimulate the mitochondrial metabolism, excessive amounts of mitochondrial Ca^{2+} can be detrimental for the cell and lead to excitotoxicity and apoptosis (Benedict et al. 2012; Hajnoczky et al. 2006). A “normal” increase of Ca^{2+} in the mitochondrial matrix increases the production of ATP due to direct activation of three mitochondrial dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogenase, and α -ketoglutarate dehydrogenase) thereby linking increased activity of neurons to higher mitochondrial ATP production (Denton 2009; Gunter et al. 2004) (Fig. 1). More recently, it was shown that Ca^{2+} entry into the mitochondrion induces the upregulation of mitochondrial cAMP/PKA signaling through sAC, which results in higher ATP synthesis (Di Benedetto et al. 2013). It was suggested that Ca^{2+} could be one of the positive regulators of mitochondrial energy metabolism at nerve terminals by potentially acting as a feed-forward mechanism to boost ATP synthesis in order to prevent energy drop during synaptic activity (Rangaraju et al. 2014). However, mitochondrial Ca^{2+} overload lead to the release of Ca^{2+} out of mitochondria, swelling of the matrix, and rupture of the outer membrane (Nicholls 2005). The process, named permeability transition, was proposed as one mechanism to initiate the release of

cytochrome *c* and other pro-apoptotic protein and seems responsible for the neuronal death following pathological activation of NMDA-selective glutamate receptors (Nicholls 2005).

3.5 Apoptosis

Mitochondria are the main actors of programmed cell death or apoptosis (Fig. 1). Different pro-apoptotic proteins are indeed contained within mitochondria, including cytochrome *c*, the serine protease OMI/HtrA2, apoptosis-inducing factor, endonuclease G, and Smac/Diablo (Suen et al. 2008; Gorman et al. 2000). Upon apoptotic stimuli, pro-apoptotic proteins Bad, Bax, or Bid translocate to mitochondria and inhibit the action of anti-apoptotic Bcl-2 proteins, inducing the release of pro-apoptotic proteins from mitochondria, probably through the permeability transition pore (Czabotar et al. 2014) (Fig. 1). Once in the cytosol, cytochrome *c* interact with Apaf-1 to form the apoptosome and induce the activation of the nonspecific caspases and ultimately cell death (Fig. 1). In the nervous system, apoptotic mechanisms have been associated with various cellular processes, such as synaptic plasticity and neurodegenerative disorders (Mattson et al. 2008; Li and Sheng 2012). For instance, the mitochondrial apoptotic pathway is involved in long-term depression of synaptic transmission (LTD) (Li et al. 2010). The impaired synaptic plasticity observed in Alzheimer's disease was shown to involve the mitochondrial pathway of apoptosis (Olsen and Sheng 2012). Apoptosis deficiency was also proposed to cause brain overgrowth by controlling neural cell numbers in the developing brain (Kuan et al. 2000; Nonomura et al. 2013).

4 The Interplay Between Cannabinoids and Mitochondrial Functions

The identification of the chemical structures of cannabinoids (Adams 1942; Gaoni and Mechoulam 1964) rose a great interest to characterize the biological mechanisms underlying the effects of this class of plant-derived molecules. For instance, it was early proposed that THC could potentially alter the metabolism of monoamines (e.g., dopamine) in the brain by acting on the enzyme MAO, an oxidoreductase located in the mitochondrial OM and responsible for degradation of monoamine neurotransmitters (Shih et al. 1999). Once released into the synaptic cleft, monoamines' action is terminated by their reuptake into the presynaptic terminal, where they can be recycled into synaptic vesicles or degraded by MAO (Mukherjee and Yang 1999). Brain monoamines such as dopamine, serotonin, and norepinephrine play a key role in the regulation of brain functions, including motor control, mood, or cognitive functions (Koob and Le Moal 2001). As both MAO inhibitors and CB₁

receptor agonists exert antidepressant-like effect (Fiedorowicz and Swartz 2004; Hill and Gorzalka 2005), the interplay between MAO and cannabinoid system was tested. Early studies found that cannabinoid compounds inhibit the activity of MAO in isolated brain mitochondria (Schurr and Livne 1975, 1976; Schurr et al. 1978). The inhibitory effect of CB₁ receptor agonists (THC, WIN55,212-2 [WIN], and AEA) on MAO activity with serotonin as a substrate was confirmed in a crude mitochondrial fraction isolated from mammalian brain cortex (Fisar 2010).

Other cannabinoid-induced mitochondrial effects were early described. Already in the 1970s of the last century, different studies reported effects of cannabinoids on mitochondria, including decrease of complex I or V activities and changes of mitochondrial ultrastructure (Schurr and Livne 1975; Bartova and Birmingham 1976; Mahoney and Harris 1972; Chari-Bitron and Bino 1971; Bino et al. 1972). The impact of cannabinoids on OXPHOS was further described in the last 15 years, and nowadays it seems well accepted that most cannabinoids inhibit mitochondrial O₂ consumption, decrease ATP production, and disrupt mitochondrial membrane potential in different cell types and tissues (Badawy et al. 2009; Whyte et al. 2010; Zaccagnino et al. 2011; Athanasiou et al. 2007; Rossato et al. 2005; Sarafian et al. 2003). Moreover, blockade or activation of CB₁ receptors alters mitochondrial functions in peripheral adipocytes (Tedesco et al. 2008, 2010).

Different cannabinoids, including THC, cannabidiol, 2-AG, and AEA, are also known as apoptotic inducers in different types of cells, with mechanisms often involving ROS production, release of cytochrome *c*, and caspase activation (Shrivastava et al. 2011; Gallily et al. 2003; Pellerito et al. 2014; Massi et al. 2006; Lee et al. 2008; Szoke et al. 2002; Ligresti et al. 2006; Campbell 2001; Mato et al. 2010; Rimmerman et al. 2013; Ryan et al. 2009; Catanzaro et al. 2009; Siegmund et al. 2007).

Cannabinoids also participate in the regulation of mitochondrial transport along neuronal processes (Boesmans et al. 2009). The number of mitochondria transported in enteric neuronal fibers is decreased by CB₁ receptor agonists and conversely enhanced by CB₁ receptor inhibition, indicating a role of CB₁ receptors in slowing down mitochondrial trafficking (Boesmans et al. 2009) (Fig. 2).

CB₁ receptors activation can lead to robust feeding despite of the animal being sated (Bermudez-Silva et al. 2012). Interestingly, recent studies revealed that such cannabinoid-mediated feeding behavior involves changes in mitochondrial activity. Activation of CB₁ receptors by arachidonyl-2'-chloroethylamide (ACEA) alters hypothalamic mitochondrial respiration and increases ROS levels in hypothalamic pro-opiomelanocortin (POMC) neurons (Koch et al. 2015). The feeding effects induced by activation of CB₁ receptors were shown to depend on the mitochondrial uncoupling protein 2 (UCP2) (Koch et al. 2015).

Overall, these results emphasized the impact of cannabinoids on mitochondrial functions and regulation. But how do cannabinoids interact with mitochondrial functions? Several potential molecular mechanisms underlying this interaction were proposed during the last decades, all maintaining a certain degree of likelihood nowadays, depending on the experimental conditions (doses, modes of treatment, etc.), the types of cells, and/or the mitochondrial functions under scrutiny.

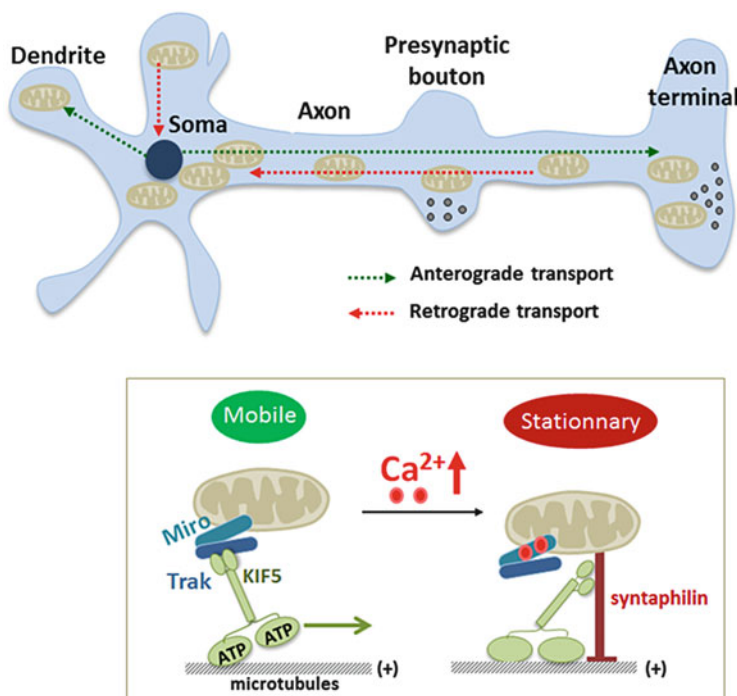


Fig. 2 Activity-dependent regulation of mitochondrial transport. The Miro–Trak adaptor complex mediates KIF5-driven mitochondrial transport. Ca^{2+} binding to Miro causes the release of KIF5 motors from mitochondria. Thus, Ca^{2+} influx after synaptic activity arrests mobile mitochondria at activated synapses. Additionally, a Miro- Ca^{2+} -sensing pathway triggers the binding switch of KIF5 motors from the Miro–Trak adaptor complex to anchoring protein syntrophin, which immobilizes axonal mitochondria via inhibiting motor ATPase activity

Cannabinoids were initially proposed to act on mitochondria because of their lipophilic structure, which could lead to alteration of mitochondrial membrane properties (Bartova and Birmingham 1976). To illustrate a receptor-independent mechanism, it was found that high doses of 2-AG induces cell death (Siegmund et al. 2007). Another cannabinoid, cannabidiol (CBD), which has low agonistic activity for CB_1 and CB_2 receptors also modulate mitochondrial functions (Shrivastava et al. 2011; Gallily et al. 2003; Massi et al. 2006; Lee et al. 2008; Mato et al. 2010; Rimmerman et al. 2013). The mechanisms underlying CBD interaction with mitochondria are not clarified. CBD impacts on mitochondrial Ca^{2+} homeostasis through its physical interaction with VDAC, decreasing its conductance and regulating intracellular Ca^{2+} levels (Shoshan-Barmatz et al. 2010). Therefore, it is possible that the antiepileptic and neuroprotective properties of CBD (Rimmerman et al. 2013; Cunha et al. 1980) involve this specific interaction of CBD and VDAC to limit increase of cytoplasmic Ca^{2+} levels.

After the identification of CB_1 receptors as typical plasma membrane GPCRs, it was proposed that cannabinoids might alter mitochondrial functions via indirect

CB₁ receptor-dependent signaling (Campbell 2001). For instance, the THC-induced apoptosis observed in cultured cortical neurons is blocked by the CB₁ receptor antagonist AM251 and pertussis toxin (PTX), suggesting that CB₁-dependent apoptosis via mitochondria involves receptor-mediated activation of the G-protein subtypes Gi/o (Campbell 2001). The THC-induced apoptosis in Jurkat cells seems to be mediated by downregulation of the Raf-1/mitogen-activated protein kinase/ERK kinase pathway and translocation of the pro-apoptotic Bad protein to mitochondria, and it is blocked by CB₁ and CB₂ antagonists (Jia et al. 2006). Moreover, anandamide induces receptor-dependent apoptosis involving ceramide synthesis and p38 mitogen-activated protein kinase activity (Fonseca et al. 2013). Activation of CB₁ receptors by ACEA activates p38 MAPK and reduces AMPK phosphorylation, which in turn decreases mitochondrial biogenesis in mouse white adipose tissue, muscle, and liver (Tedesco et al. 2010). These findings support the idea that cannabinoids might affect mitochondrial functions through pmCB₁ receptor-dependent modulation of cytoplasmic signaling pathways.

Therefore, until few years ago, cannabinoid effects on mitochondrial functions were fully ascribed to direct unspecific membrane disturbance induced by these lipid molecules or to indirect signal transduction originating from cannabinoid receptors located on plasma membranes, or both. Moreover, although mitochondrial staining with CB₁ receptor antisera was a common observation, it was considered as unspecific background for many years. However, recent results challenged this idea, indicating that CB₁ receptors are present at mitochondrial membranes in the periphery, such as in spermatozoa (Aquila et al. 2010), in skeletal muscles (Mendizabal-Zubiaga et al. 2016), and in the brain, where they directly regulate mitochondrial OXPHOS activity (Benard et al. 2012; Hebert-Chatelain et al. 2014a, 2016; Vallee et al. 2014).

5 The Functional Presence of Mitochondrial CB₁ Receptors in the Brain

In 2012, a study from our laboratory demonstrated the functional presence of CB₁ receptors on brain mitochondrial membranes, identifying mitochondrial CB₁ receptors (mtCB₁) as a direct modulator of bioenergetics cellular processes in the brain (Benard et al. 2012) (Fig. 3). Pharmacological activation of CB₁ receptors accompanied by rigorous controls using mutant mice fully lacking CB₁ expression (CB₁^{-/-} mice) showed that mtCB₁ receptors directly impact on endogenous respiration of brain mitochondria (Benard et al. 2012).

In more details, electron immunohistochemistry showed that approximately 10 to 15% of total CB₁ receptors in the CA1 hippocampal region are located in mitochondria of wild-type mice, largely above background levels quantified in tissues from CB₁^{-/-} mutants (Benard et al. 2012). MtCB₁ receptors are more densely present in GABAergic interneurons than on glutamatergic neurons in the

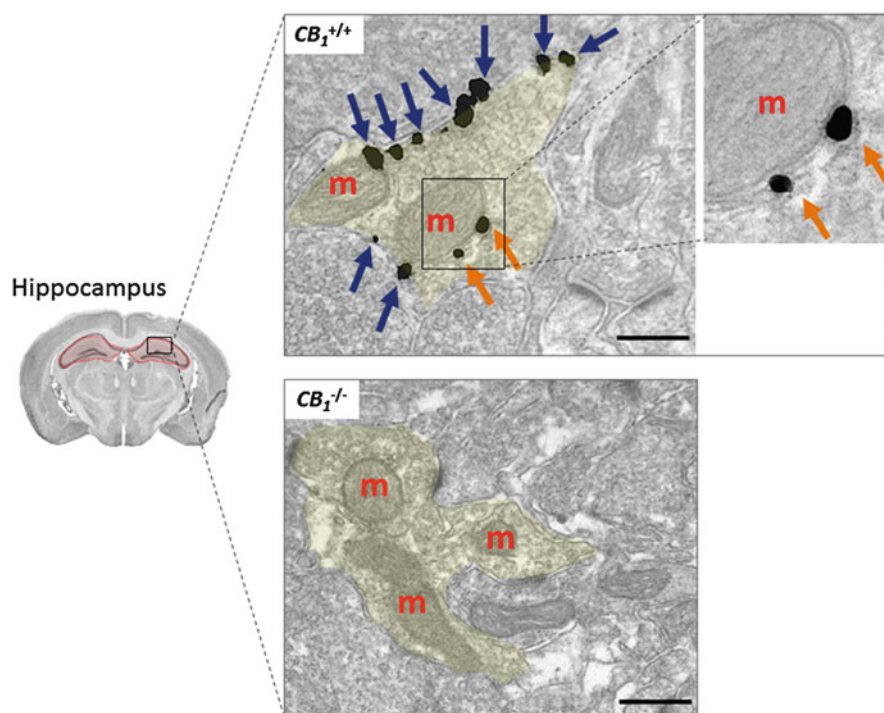


Fig. 3 CB₁ receptors in hippocampal mitochondria. Electron immunogold detection of CB₁ receptors within mitochondrial membranes of hippocampal cells in the CA1 region of wild-type $CB_1^{+/+}$ and knockout $CB_1^{-/-}$ mice. Inset, detail of single CB₁-positive mitochondria. Blue arrows, plasma membrane CB₁ receptors; orange arrows, mtCB₁ receptors. Scale bar, 0.25 μ m. Adapted from Benard et al. (2012)

hippocampus, which goes in parallel with the well-known higher densities of “GABAergic” CB₁ receptors as compared to “glutamatergic” ones. At the subcellular level, mtCB₁ receptors are equally distributed to both dendrites and axon terminals, making their localization different from the pool localized at plasma membranes (pmCB₁), almost exclusively found at presynaptic terminals (Benard et al. 2012). Further experiments suggested that mtCB₁ receptors are present at the level of OMMs and they have a topological orientation with a cytoplasmic protein N-terminus and the C-terminus facing the interior of the organelle (Benard et al. 2012), enabling the mtCB₁ receptor-dependent signaling to reach proteins within the mitochondrial matrix, including proteins regulated by the intramitochondrial cAMP-PKA pathway (see below). However, the precise location of mtCB₁ receptors within mitochondrial membranes (i.e., IMM, OMM, and/or IMM-OMM contact sites (Frey and Mannella 2000)) is being investigated but remains unknown. Interestingly, several G α_i proteins were observed in IMM-OMM contact sites in mitochondria of human placenta (Kuyznierewicz and Thomson 2002). Considering that mtCB₁ affects mitochondrial functions through these proteins

(Hebert-Chatelain et al. 2016), we can hypothesize that mtCB₁ receptors might be partially inserted within these contact sites. Since this first study, mtCB₁ receptors were also observed in different neuronal populations by other laboratories (Koch et al. 2015; Morozov et al. 2013; Ma et al. 2015). Nevertheless, it was shown that antibodies recognizing the CB₁ receptor could bind to nonspecific target such as the mitochondrial protein stomatin-like protein 2 (Morozov et al. 2013), indicating that identification of mtCB₁ receptors must be performed using CB₁^{-/-} tissues as negative controls and strict quantification procedures (see references, Hebert-Chatelain et al. (2014a, b), Morozov et al. (2014), for methodological discussions). Indeed, despite the general problems arisen from the use of antibodies, the adoption of appropriate methodological approaches has recently led to the agreement that mitochondrial localization of CB₁ receptors is present in the brain, including also hypothalamic cells (Koch et al. 2015).

The direct impact of mtCB₁ receptors on respiratory functions of mitochondria was tested in purified brain mitochondria from wild-type and CB₁^{-/-} mice, in CB₁-transfected primary mouse fibroblast (MFs) from CB₁^{-/-} mice, and in transfected HEK293 cells (Benard et al. 2012; Hebert-Chatelain et al. 2014a; Vallee et al. 2014). Exogenous application of the CB₁ receptor agonists THC, WIN, or HU210 decreases endogenous mitochondrial respiration, whereas no changes were observed in control-transfected cells or brain mitochondria from CB₁^{-/-} mice, indicating a direct regulation of respiration by cannabinoids through CB₁ receptors (Benard et al. 2012; Hebert-Chatelain et al. 2014a; Vallee et al. 2014). Similarly, a different laboratory observed that the CB₁ receptor antagonist AM251 is able to block the decrease of mitochondrial respiration induced by low doses of cannabinoids in brain mitochondria (Fisar et al. 2014). Our team also observed that treatment with synthetic cannabinoids HU210 and WIN decreases mobility of neuronal mitochondria (Hebert-Chatelain et al. 2016).

CB₁ receptor signaling is at the crossroad of the pharmacological effects of cannabinoids and the physiological roles of the ECS. Thus, an important question to address was whether the ECS physiologically modulates mitochondrial activity via mtCB₁ receptors. FAAH, the primary degradative enzyme for AEA, is densely present in the mitochondria (Benard et al. 2012), and we observed that purified mitochondria contain AEA- and 2-AG-degrading activity that is ascribed to FAAH and MAGL, representing approximately 18% and 12% from the total cellular enzyme activity, respectively (Benard et al. 2012). Pharmacological inhibition of MAGL in purified mitochondria increases 2-AG levels and decreases respiration (Benard et al. 2012). Interestingly, a strong inverse correlation was found between the levels of endogenous 2-AG in mitochondria and O₂ consumption (Benard et al. 2012), suggesting that endogenous mtCB₁ receptor signaling within mitochondria control the respiratory activity of the organelles.

To discriminate the respective influence of pmCB₁ versus intracellular CB₁ receptors on these effects, indirect pharmacological tools were developed. A biotinylated version of the lipophilic CB₁ receptor agonist HU210 (HU210-biotin, hereafter HU-biot), in which the presence of the hydrophilic biotin extension prevents the cell penetration, was synthesized and used in vitro (Fig 4a). Interestingly,

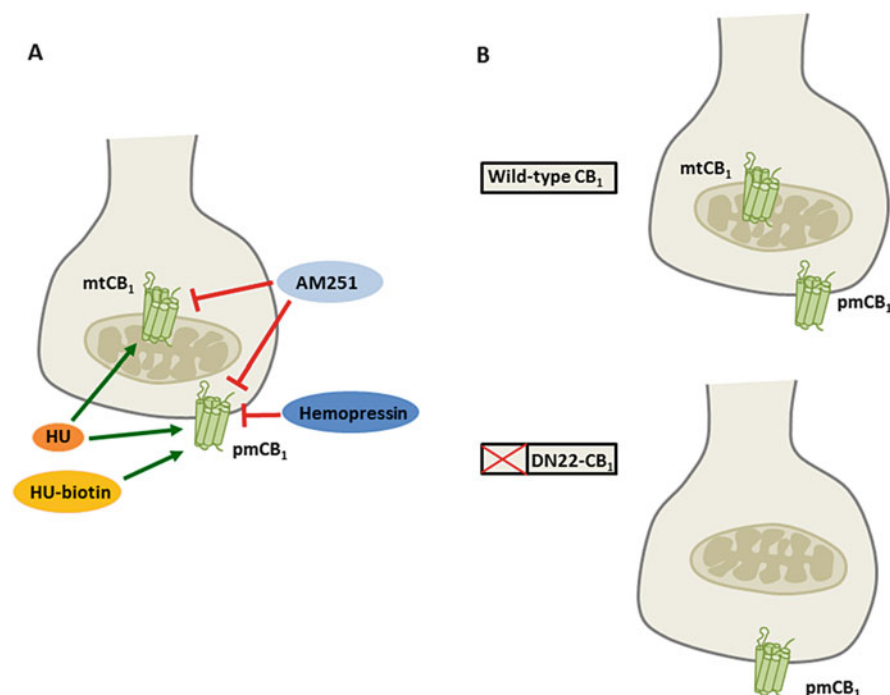


Fig. 4 Pharmacological and genetic tools to study the role of mtCB₁ receptors in brain physiology. (a) The CB₁ agonist HU210 (HU) is cell permeable and can activate both plasma membrane CB₁ (pmCB₁) and intracellular CB₁ receptors such as mtCB₁. The addition of hydrophilic biotin to HU210 (HU-biotin) blocks the capacity of this agonist to penetrate cell and activate mtCB₁. The CB₁ antagonist AM251 is cell permeant and can block the effect of both pmCB₁ and mtCB₁, whereas hemopressin (an antagonist of CB₁ that does not penetrate within the cell) only blocks the effect of pmCB₁. (b) The deletion of the 22 N-terminal amino acids of CB₁ (DN22-CB₁) excludes the receptor from mitochondria. These tools are then useful to describe the role of mtCB₁ on cannabinoid-dependent regulation of mitochondrial activity, brain physiology, and behavior

HU-biot treatment does not alter O₂ consumption in intact CB₁-expressing cells, whereas the effect appears when CB₁-MFs are permeabilized (Benard et al. 2012). Similarly, the cell-impermeant CB₁ antagonist hemopressin was not able to block the decrease of respiration induced by HU210 (Fig. 4a), unless cell was permeabilized (Benard et al. 2012). Altogether, the results demonstrate that reduction of the respiratory activity in living cells is limited to the action of intracellular CB₁ receptors and most likely through mtCB₁ receptors. Later, we developed genetic tools to target more specifically mtCB₁ receptors. We observed that deletion of its first 22 N-terminal amino acids (DN22-CB₁ mutant protein) excludes CB₁ receptors from mitochondria (Fig. 4b). Injection of an adeno-associated virus expressing wild-type CB₁ or DN22-CB₁ into the hippocampus of null CB₁^{-/-} mice induced the same total amount of total functional CB₁ protein (Hebert-Chatelain et al. 2016). However, the proportion of mtCB₁ receptors in CB₁^{-/-}

mice injected with viral DN22-CB₁ was equivalent to the amount of mtCB₁ observed in CB₁^{-/-} mice injected with GFP (Hebert-Chatelain et al. 2016), showing that the first 22 N-terminal amino acids of the CB₁ protein are essential to import the receptor to mitochondrial membranes. Cannabinoids are not able to impair mitochondrial respiration and mitochondrial trafficking in cells expressing the mutant DN22-CB₁ (Hebert-Chatelain et al. 2016), confirming that reduction of mitochondrial activity by cannabinoids occurs mainly through mtCB₁ receptors.

Our team recently characterized the molecular mechanisms linking activation of mtCB₁ receptors and decreased mitochondrial activity. Briefly, activated mtCB₁ receptors release Gα proteins, which then bind to the mitochondrial cAMP-generating enzyme soluble adenylyl cyclase (sAC). This interaction lowers mitochondrial cAMP levels and the activity of intramitochondrial PKA (Fig. 5a). Different inhibitors targeting the key steps of this biochemical cascade (i.e., the Gα_{i/o} inhibitor pertussis toxin, the sAC inhibitor KH7, and the PKA inhibitor H89) block the effect of cannabinoids on complex I activity, mitochondrial respiration, and mitochondrial mobility (Hebert-Chatelain et al. 2016). Therefore, activation of mtCB₁ receptors decreases mitochondrial activity through an intramitochondrial Gα-sAC-cAMP-PKA signaling pathway (Fig 5a). Considering that PKA can phosphorylate several mitochondrial proteins (Di Benedetto et al. 2013; Acin-Perez et al. 2009; Palmisano et al. 2007; Papa et al. 1996; Sardanelli et al. 2006; Technikova-Dobrova et al. 2001), we characterized the PKA-dependent mitochondrial phosphoproteome and observed that the complex I subunit NDUFS2 is less phosphorylated by PKA upon treatment with cannabinoids in an mtCB₁-dependent manner (Hebert-Chatelain et al. 2016). Strikingly, cannabinoids were not able to decrease mitochondrial activity in cells expressing a phospho-mimetic mutant of NDUFS2 (Hebert-Chatelain et al. 2016), indicating that mtCB₁ regulates mitochondrial respiration by decreasing the PKA-dependent phosphorylation of the OXPHOS component NDUFS2. Studying the impact of cannabinoids on the mitochondrial phosphoproteome will certainly benefit to our understanding of the processes by which cannabinoids impact on mitochondrial physiology and brain functions.

The idea that mtCB₁ receptors might participate in eCB-dependent modulation of synaptic transmission was further tested (Fig. 5b). Agonists of CB₁ receptors reduce excitatory synaptic transmission in hippocampal slices from wild-type animals, but not from CB₁^{-/-} mice (Kano et al. 2009). Activation of mtCB₁ receptors seems a key step in this process since cannabinoids are not able to reduce excitatory synaptic transmission in (1) hippocampal slices pretreated with KH7 that blocks sAC and mtCB₁-dependent signaling (see above) and (2) hippocampal slices from CB₁^{-/-} mice expressing viral DN22-CB₁ (Hebert-Chatelain et al. 2016). CB₁ receptors likely also participate in depolarization-induced suppression of inhibition (DSI), a form of short-term plasticity of inhibitory neurotransmission. Briefly, the depolarization of a postsynaptic cell leads to eCB mobilization, which retrogradely activates presynaptic CB₁ receptors and transiently decreases GABAergic inhibitory neurotransmission (Kano et al. 2009; Alger 2002). DSI levels are proportional to the duration of the depolarization step, with longer depolarization inducing

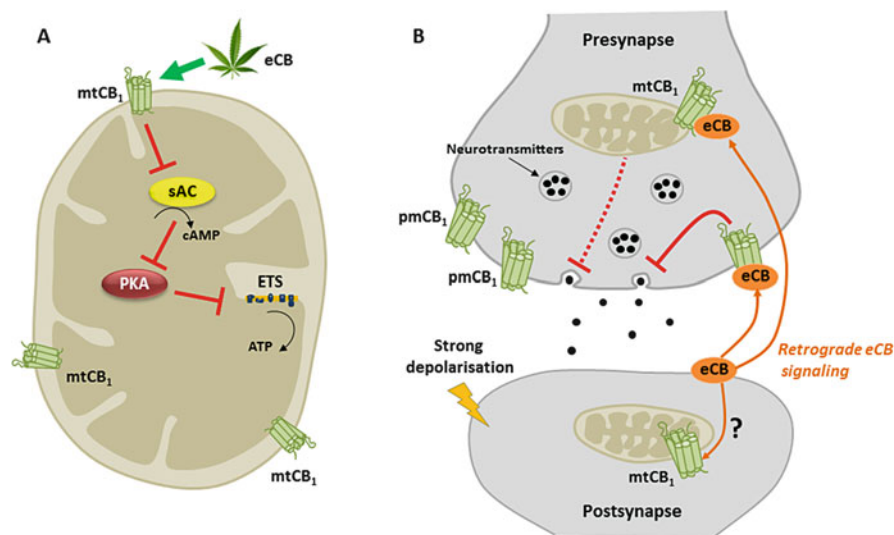


Fig. 5 Mitochondrial CB₁ receptor regulates oxidative phosphorylation and contributes to synaptic plasticity. (a) Possible molecular mechanism linking activation of mtCB₁ receptor and lower oxidative phosphorylation. Activation of mtCB₁ receptors by (endo)cannabinoids (eCB) decreases the activity of soluble adenylyl cyclase (sAC) and consequently reduces mitochondrial cAMP levels and PKA activity. This inhibition is associated with lower activity of the complex I of the electron transport system (ETS) and of oxidative phosphorylation process, suggesting that mtCB₁ receptors could control cellular bioenergetics through the mitochondrial cAMP-PKA pathway. (b) Intracellular CB₁ receptor, possibly mtCB₁, seems to participate in depolarization-induced suppression of inhibition (DSI), an endocannabinoid-dependent form of short-term plasticity of inhibitory transmission in the hippocampus. Briefly, a strong postsynaptic depolarization induces the release of eCBs which cross the synaptic cleft and activates presynaptic plasma membrane CB₁ receptors (pmCB₁). This process inhibits the release of the neurotransmitter GABA which results in reduced inhibitory neurotransmission. Results obtained in our laboratory suggest that activation of mtCB₁ receptor is involved in this process. For instance, the cell-impermeant CB₁ receptor agonist HU210-biotin only partially occluded DSI, whereas the inhibitor of the complex I of the ETS rotenone potentiated weak DSI (see text for a detailed description of these processes)

higher levels of presynaptic inhibition (Kano et al. 2009; Alger 2002). DSI is blocked by CB₁ receptor antagonists, but it is also prevented (occluded) by CB₁ receptor agonists, which occupy CB₁ receptors and impede further actions of endogenously mobilized eCBs (Kano et al. 2009; Alger 2002). In hippocampal slices, HU210 completely occluded “strong DSI” induced by 5 sec depolarization steps, whereas HU-biot at saturating doses had only a partial effect (approximately 50% occlusion). Similarly, the cell-impermeant CB₁ receptor antagonist, the peptide hemopressin (Heimann et al. 2007), partially reduced “strong DSI,” whereas the membrane-permeant CB₁ receptor antagonist AM251 abolished it. Finally, rotenone, a mitochondrial complex I inhibitor, was ineffective on “strong DSI,” but potentiated “weak DSI,” suggesting that mitochondrial processes participate in the expression of “strong DSI” (Fig. 5b). Altogether, these results suggest that mtCB₁ receptors are important in eCB-dependent synaptic plasticity. A recent study

showed that mtCB₁ receptors might be a potential target for the treatment of brain ischemic injury. This work showed that ACEA restored cell viability, inhibited generation of ROS, reduced apoptosis (both in vitro and in vivo), and improved OXPHOS enzyme activities after cerebral ischemia/reperfusion injury (Ma et al. 2015). Interestingly, these benefits were blocked by the cell-permeant AM251, but only partially reversed by the cell-impermeant hemopressin, suggesting that mtCB₁ receptors might be involved in the beneficial effects of ACEA during I/R (Ma et al. 2015).

Agonists of CB₁ receptors can impair high brain functions such as learning and memory. For instance, cannabinoids induce amnesia in the novel object recognition test in wild-type mice but not in CB₁^{-/-} mice (Puighermanal et al. 2009). The modulation of mitochondrial activity and synaptic transmission by mtCB₁ receptors suggest that cannabinoids could impact on high brain functions at least partly through mtCB₁ receptors. Indeed, we observed that the cannabinoid-induced impairment of memory performance was rescued in CB₁^{-/-} mice expressing hippocampal wild-type CB₁ but not DN22-CB₁ (Hebert-Chatelain et al. 2016). Modulation of mtCB₁-dependent signaling can also modify the impact of cannabinoids on memory performance. Indeed, intra-hippocampal injection of the sAC inhibitor KH7 or expression of a constitutively active mutant of PKA specifically targeted to mitochondria blocks the decrease of memory performance induced by systemic injection of WIN (Hebert-Chatelain et al. 2016). Similarly, viral expression of the phospho-mimetic mutant of NDUFS2 in mouse hippocampi also partly blocks the amnesic effect of WIN (Hebert-Chatelain et al. 2016). Altogether, these results indicate that cannabinoids modulate brain physiology and functions through mtCB₁-dependent regulation of mitochondrial activity. Future work will be needed to understand the role of mtCB₁ receptors in the numerous brain functions and behaviors that are modulated by (endo)cannabinoids.

Today, it appears that cannabinoids can have both receptor-dependent and receptor-independent effects on mitochondria. Considering that both pmCB₁ and mtCB₁ receptors impact on mitochondria, we can speculate that both receptors could cooperate to modulate mitochondrial functions. For instance, activation of pmCB₁ receptors could lead to long-term decrease of mitochondrial biogenesis and activity, as observed in white adipose tissue, muscle, and the liver (Tedesco et al. 2010), whereas activation of mtCB₁ receptors induces short-term and acute changes of mitochondrial activity (Benard et al. 2012; Hebert-Chatelain et al. 2014a). Further studies are needed to investigate the specific impact of pmCB₁ and mtCB₁ receptors on synaptic transmission, brain functions, and behavior.

6 Conclusion

It is well established that bioenergetics processes, including mitochondrial respiration, support neurotransmission and brain functions. Brain mtCB₁ receptor activation directly regulates mitochondrial respiration and ATP production. This function

seems to be regulated through an intramitochondrial $G\alpha$ -sAC-cAMP-PKA signaling pathway. Thus, mtCB₁ receptors might participate in brain functions by acutely altering mitochondrial respiration, thereby modulating energy supply, which is a key limiting factor of neuronal signaling. The correct functioning of mitochondria is fundamental for the generation of most cellular ATP, and its long-term impairment has been implicated in many neurological and psychiatric diseases. Further studies are needed to investigate the role of acute regulation of mitochondrial activity through mtCB₁ receptors in brain physiological and behavioral effects of cannabinoids. To this aim, the design and development of powerful tools that functionally discriminate between the functions played by mtCB₁ from the ones played by CB₁ receptors at other cellular locations (such as DN22-CB₁) are necessary. Finally, cannabinoid drugs are endowed with several therapeutic potentials via activation of CB₁ receptors, unfortunately limited by important side effects (Piomelli 2003). The selective targeting of specific subcellular populations of CB₁ receptors in the brain might become a very interesting way to obtain safer and more efficient therapeutic means against several brain disorders.

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Susceptibility to Psychiatric Diseases After Cannabis Abuse in Adolescence: Animal Models

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Abstract Epidemiological evidence suggests that adolescent exposure to delta-9-tetrahydrocannabinol, the psychoactive component of *Cannabis*, confers an increased risk for developing psychiatric disorders later in life. However, epidemiological studies have a correlative nature and are limited by ethical problems which can be circumvented by animal models.

After a preliminary section describing the dynamic changes that occur in the endocannabinoid system during adolescence, in the following sections, the more relevant animal data on the long-lasting consequences of adolescent exposure to cannabinoids is reported. Behavioral evidence in terms of impairment in cognition, vulnerability to mood disorders, schizophrenia, and subsequent drug abuse as well as the underlying neurobiological aspects are summarized.

The arising picture seems to support the hypothesis that adolescent exposure to cannabinoids might represent a risk factor for the development of psychiatric-like symptoms in adulthood since the external stimulation of the endocannabinoid system can profoundly alter its physiological role, thus triggering alterations in the maturational events occurring in the adolescent brain.

Quantifying the relative adverse and beneficial effects of cannabis and its constituent cannabinoids is becoming a priority particularly considering that several countries are legalizing its use. Although it is still a matter of debate, there is evidence suggesting that chronic adolescent marijuana exposure may be associated with a higher risk for neuropsychiatric diseases, including schizophrenia (Rubino and Parolaro 2015; Renard et al. 2014). During adolescence, the endocannabinoid system is highly active, driving the central nervous system maturation. Thus,

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adolescent exposure to exogenous cannabinoids might interfere with normal cortical maturation, causing neurobiological changes that impair brain function. This chapter will describe the behavioral and cellular evidence of enhanced susceptibility to neuropsychiatric disorders after cannabinoid abuse in adolescence obtained using validated animal models. Moreover, a preliminary description of the maturational processes occurring in the endocannabinoid system during adolescence will be provided.

1 The Endocannabinoid System in Adolescence

Several physical, neural, and behavioral changes occur simultaneously during adolescence. Indeed, the brain of adolescents is subjected to extensive synaptic and neurochemical remodeling in areas involved in emotions, learning, decision-making, and reward-motivated behaviors. These complex changes may make the human brain more vulnerable, and transient imbalances, primarily among developmental trajectories of corticolimbic structures, can leave an individual susceptible to mental illness. The endocannabinoid system (eCB) is a lipid signaling system consisting of specific receptors (cannabinoid CB1 and CB2 receptors), endogenous ligands (mainly anandamide and 2-arachidonoylglycerol), and a battery of enzymes responsible for the synthesis and degradation of these ligands. The eCB system plays a major role in neurodevelopmental processes which are particularly active during adolescence, and several studies have highlighted its active and dynamic nature during this age.

Rodriguez de Fonseca et al. (1993) showed that CB1 receptor (CB1R) binding in the rat brain is highest just prior to the onset of adolescence (postnatal day (PND) 25–29), followed by a general linear reduction to adult levels within limbic, striatal, and cortical structures. Accordingly, recent findings from different laboratories demonstrated that prefrontal cortex (PFC) CB1R expression in male rats declines in pre- to early adolescence with a region-specific rate (Ellgren et al. 2008; Heng et al. 2011). Indeed there is a gradual decline of the CB1R expression in limbic/associative regions, whereas major changes in sensorimotor regions are not evident until mid- to late adolescence, with the functionality of these receptors following the same developmental pattern (Heng et al. 2011). Accordingly, Rubino et al. (2015) showed that in female rodents, CB1Rs increase from mid- to late adolescence (PND 60) and decline by adulthood (PND 75; Rubino et al. 2015). The efficacy of CB1 receptor coupling with G proteins through adolescence does not show significant alteration, at least in the PFC, implying that CB1Rs seem to be more efficient during adolescence (Rubino et al. 2015). Recently, an interesting paper was published by Schneider et al. (2015) in which the introduction of a gain-of-function mutation in the gene that encodes for the CB1R generated rats with sustaining features of adolescent behavior in adulthood. Indeed, adult mutant rats exhibited typical high risk/novelty seeking, increased peer interaction, enhanced impulsivity, and augmented reward sensitivity for drug and nondrug reward when

compared to wild-type littermates. Partial inhibition of CB1R activity normalized mutant rats' behavior.

These observations in adult mutant rats together with the above cited reports on enhanced CB1R signaling in adolescence highlight that the activity state and functionality of the CB1R are critical for mediating adolescent behavior. Interestingly no differences were observed between the genotypes for the two main endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG).

The picture regarding changes in the level of the two main endocannabinoids, AEA and 2-AG, during adolescence is still at the beginning, and the few data available are somewhat divergent, probably for the difference in the strain and sex of the used animals.

In male rats, a continuous increase in PFC AEA levels throughout the adolescent period was reported, anandamide being almost three times higher in later adolescence. However, 2-arachidonoylglycerol concentrations in the same brain area were highest very early in adolescence (PND 29), decreased by PND 38, and increased again in late adolescence (PND 50; Ellgren et al. 2008). Similarly, Rubino et al. (2015), in the PFC of female rats, observed that AEA levels increased from mid- to late adolescence and then decreased into adulthood, while 2-AG levels first decreased and subsequently increased. Interestingly, despite these dynamic changes, the activity of the two degrading enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase, did not show any variation throughout the developmental window, suggesting a more likely involvement of the synthetic enzymes in regulating endocannabinoid levels.

Concerning other brain areas, it was reported that hypothalamic AEA content in female rats increases immediately preceding vaginal opening (as a physical marker of pubertal onset; Wenger et al. 2002), whereas in male rats the pattern of the AEA adolescent fluctuation in the amygdala, hippocampus, and hypothalamus (PND 25–70) was similar as in the PFC (Lee et al. 2013). Moreover, two other related N-acylethanolamines hydrolyzed by FAAH, oleylethanolamine and palmitoylethanolamine, exhibit the same temporal-specific pattern as AEA, indicating that the corticolimbic AEA fluctuations are at least partly due to corresponding changes in FAAH activity (Lee et al. 2013).

Concluding the eCB system during adolescence shows a high instability and participates actively to the final brain's refinement that occur in this period. External stimulation of this system can profoundly alter its physiological role triggering altered brain maturation.

2 Behavioral Consequences of Cannabinoid Abuse in Adolescence

2.1 Emotional Aspects

Chronic administration of cannabinoid compounds in adolescent animals has been reported to lead to altered emotional reactivity later in life.

The picture emerging from studies evaluating the effect on anxiety behavior is quite complex, since different results have been obtained depending on the specific component of anxiety under study and the tests used to monitor it. The most difficult interpretation regards data on general anxiety, monitored in the elevated plus maze test or the open field test. Indeed, adult animals that were exposed to cannabinoids during adolescence have been reported to exhibit no changes in their behavior (Rubino et al. 2008; Higuera-Matas et al. 2009; Bambico et al. 2010; O'Tuathaigh et al. 2010; Mateos et al. 2011; Bortolato et al. 2014; Cadoni et al. 2015), an anxiolytic-like response (Biscaia et al. 2003; Wegener and Koch 2009; Cadoni et al. 2015), or even anxiety (Llorente-Berzal et al. 2013; Stopponi et al. 2014; O'Tuathaigh et al. 2010; Abboussi et al. 2015; but see also Renard et al. 2016 who used the light–dark box test). This lack of consistency might be due to differences in several experimental parameters such as the cannabinoid compound used (synthetic versus natural), the precise developmental period of exposure, and the strain of animals used. For example, it is well known that synthetic cannabinoids are full CB1 receptor agonists, whereas the natural ingredient of *Cannabis*, delta-9 tetrahydrocannabinol (THC), is a partial agonist. This could greatly affect the adaptive changes occurring in the brain in response to chronic exposure. Also the strain of animals used in the experimentation could play a fundamental role in the response to cannabinoid treatment, due to a possible different genetic background. This is the case, for example, in Cadoni's study (Cadoni et al. 2015), where it was reported an anxiolytic-like effect in adult Lewis rats exposed to THC during adolescence and no changes in anxiety behavior in the Fisher344 ones. In the same line, O'Tuathaigh et al. (2010) reported that only mice KO for the catechol-O-methyltransferase (COMT) gene were able to develop an anxiety-like behavior at adulthood after adolescent exposure to THC, while wild-type ones did not.

These different experimental parameters seem to play a minor role when social anxiety has been investigated. All the researchers who monitored this aspect reported a decrease in social interaction in adult rats after adolescent cannabinoid exposure. This was demonstrated with both synthetic and natural cannabinoid agonists, in male and female animals (Leweke and Schneider 2011; O'Shea et al. 2004, 2006; Quinn et al. 2008; Realini et al. 2011; Zamberletti et al. 2014; Renard et al. 2016). The only exception is the Gleason's paper (2012) performed in mice, even if a trend to a decreased social interaction might be seen after the adolescent treatment. The most likely reason for the lack of the effect in Gleason's work may reside in the fact that the behavior was monitored long after the end of the treatment (nearly 3 months later), whereas in all the other studies it was checked within

2 months. This suggests that at least some of the altered behaviors induced by the adolescent cannabinoid exposure might be normalized with time. Although reduction in social interaction has been considered an anxiogenic behavior in rodents (File and Hyde 1978), it is also well known that a significant and pervasive impairment in social functioning is associated with major depressive disorder (Hirschfeld et al. 2000). According to this view, impaired social behavior might also be a sign present in a depressive-like phenotype. Besides reduced social behavior, two other features of depression-like reactivity in animals are behavioral despair/passive coping strategy (measured in the forced swim test) and anhedonia (assessed as sucrose preference or palatable food consumption). Accumulating literature provides evidence that adolescent exposure to THC or WIN 55,212-2 induces passive coping strategy in the forced swim test and a reduction in the sucrose preference or palatable food consumption (Rubino et al. 2008; Realini et al. 2011; Bambico et al. 2010; Zamberletti et al. 2014). This suggests the presence of dysfunction in motivational processes, and, intriguingly, these effects appear to be stronger in female rats (Rubino et al. 2008). In line with this, Chadwick et al. (2011) reported that adolescent exposure to CP-55,940 decreased sexual motivation in adult female rats.

As a whole, these findings suggest the presence of altered emotional reactivity and hedonic processes after adolescent cannabinoid exposure, especially in female animals.

2.2 Cognition

Most literature on adolescent cannabinoid exposure in animal models agrees on the presence of long-lasting impairments in learning and memory.

Impairments in episodic memory tested in the classical or spatial version of the novel object recognition test or in the social recognition test have been reported after adolescent exposure to synthetic or natural cannabinoid agonists in adult rats and mice and in both sexes (Abush and Akirav 2012; O'Shea et al. 2004, 2006; Quinn et al. 2008; Realini et al. 2011; Renard et al. 2013, 2016; Schneider and Koch 2003; Zamberletti et al. 2014; Abboussi et al. 2015; Lovelace et al. 2015). Few exceptions to this rule are represented by papers where adolescent exposure was performed for very short periods of time (3 days, Cadoni et al. 2015) or where the cognitive effect was monitored much longer than in other studies after the end of the treatment (Higuera-Matas et al. 2009). The former exception might suggest that a longer period of exposure is needed to elicit the cognitive deficit, whereas the latter suggests, again, the possibility that some signs might spontaneously return to control value with time. Spatial working memory deficits have been observed after adolescent THC exposure in adult rats (Rubino et al. 2009a, b, 2015), mice (O'Tuathaigh et al. 2010), and monkeys (Verrico et al. 2014), as well as impairments in cognitive flexibility assessed through the attentional set-shifting task (Gomes et al. 2015). When other forms of memory were considered, no lasting

effects were observed. This was the case for aversive memory (Rubino et al. 2009a, b) or “pure” spatial learning in the Morris water maze (Abush and Akirav 2012; Cha et al. 2006, 2007; Higuera-Matas et al. 2009).

These data point toward a task-specific effect of adolescent cannabinoid exposure on cognition, suggesting that the exposure might more likely affect the forms of memory where both PFC and hippocampus play a role and the integrity of their interaction is needed to perform the task.

2.3 Psychosis

Epidemiological studies suggest that cannabis use during adolescence confers an increased risk for developing psychotic symptoms later in life (D’Souza et al. 2009; Evins et al. 2012).

Schizophrenia represents a complex disorder associated with various disturbances in motivational, social, emotional, and cognitive processing. Some of these features are uniquely human (i.e., hallucination and delusions) and cannot be reproduced in animals. Differently, several experimental protocols can reproduce impairment in cognition and altered emotionality present in schizophrenia, and the consequences of cannabis in adolescence on these signs has been already examined in the previous chapters. Thus, in the present chapter, only the effect of adolescent cannabis use on two other paradigms considered as valid translational models of schizophrenia will be reported: the pre-pulse inhibition of startle (PPI—a measure of sensorimotor gating, the ability of an organism to attain information and process it correctly) and drug-induced hyperactivity (Lodge and Grace 2009; Powell and Miyakawa 2006).

2.3.1 Pre-pulse Inhibition

Chronic adolescent treatment with the cannabinoid agonist WIN 55,212-2 provoked impairment in PPI in rats and mice that was still present long after the treatment withdrawal (Gleason et al. 2012; Schneider and Koch 2003; Wegener and Koch 2009). In contrast, Llorente-Berzal et al. (2011) and O’Tuathaigh et al. (2012) did not show alterations in this behavior after adolescent exposure to synthetic cannabinoid agonists (CP55940, 0.4 mg/kg, or WIN 55,212-2, 1 or 2.5 mg/kg, respectively). The reason for this discrepancy is quite difficult to find. Data for both evidence were obtained in mice and rats, using a synthetic cannabinoid agonist. The only speculation we may put forward is that the last two groups performed a longer treatment (15 or 21 days) with the same dose of agonist (thus triggering a deep state of tolerance), while the former ones performed a shorter treatment (10 days) or used an irregular protocol of injections (none, one, or two daily injections for 25 days), thus reducing the risk to develop profound tolerance. More recently, Renard et al. (2016) showed that adult rats pretreated with THC in adolescence displayed PPI

deficits at both the pre-pulse intensity levels of 76 and 80 dB compared with control rats. Interestingly the same protocols in adult rats did not affect PPI.

2.3.2 Locomotor Hyperactivity

Finally, locomotor hyperactivity in rodents may have some face validity for certain components of the positive symptoms of schizophrenia, such as psychotic agitation (Van Den Buuse 2010). However, despite its ease of quantification in rodents, very few data are available about the long-term effect of adolescent cannabinoid exposure on this behavioral sign. A significant increase in locomotor activity in the open field was observed in adult rats exposed to WIN 55,212-2 during adolescence (Wegener and Koch 2009). Differently, other groups showed no significant alterations in the open field recordings (Biscaia et al. 2003; Rubino et al. 2008), and some others found that young adult rats chronically treated with CP55,940 showed reduced baseline locomotor activity 2 weeks after the treatment's cessation (Klug and Van Den Buuse 2013). Our group recently observed that adolescent exposure to THC increased the locomotor activating effect of phencyclidine (PCP) when this was administered acutely in adulthood (Zamberletti et al. 2014). In fact, the low dose of PCP injected (2.5 mg/kg) did not induced locomotor activation in vehicle-treated animals but significantly increased locomotion in THC-treated rats. Moreover, stereotyped behaviors were observed in vehicle-treated animals following acute PCP injection, but this effect was significantly enhanced in THC-treated rats.

2.3.3 Two-Hit Hypothesis

Schizophrenia is a multifactorial disease characterized by the combined action of multiple genes of small effect size (Owen et al. 2005) and a number of environmental risk factors (McGrath et al. 2003), which causes the development of this mental disorder (Mackay-Sim et al. 2004). This is conceptualized in the “two-hit hypothesis” of schizophrenia, which predicts that genetic and environmental risk factors interactively ($G \times E$ interaction) cause the development of the disorder (Bayer et al. 1999; Caspi and Moffitt 2006).

Genetic factors may confer vulnerability to psychosis outcomes following exposure to cannabis, i.e., a gene-environment interaction. In specific, the genes encoding for COMT, neuregulin (Nrg1), brain-derived neurotrophic factor (BDNF), and disrupted in schizophrenia 1 (DISC1) have been implicated in conferring such vulnerability.

COMT

In one of the first studies that drew attention to gene $\times$ environment interactions, Caspi et al. (2005) reported that the COMT gene moderated the risk of psychotic

disorder with adolescent cannabis exposure. The enzyme COMT plays a critical role in the breakdown of dopamine (DA) in the PFC (Papaleo et al. 2008), in contrast to the striatum where DA is cleared by a transporter. The COMT gene has a common polymorphism in humans where valine (Val) is substituted for methionine at the 158/108 locus, and this results in 40% higher enzymatic activity and thus more rapid degradation of DA. Lower cortical DA levels in individuals homozygous for the Val (158) polymorphism are associated with, among other things, poorer cognitive performance and inefficient pre-cortical functioning (Tunbridge et al. 2006). Accordingly, COMT knockout (KO) mice were more vulnerable than wild types (WT) to the disruptive effects of WIN55212 adolescent treatment on PPI. Moreover, acute pharmacological inhibition of COMT in mice modified acute cannabinoid effects on startle reactivity, as well as PPI. COMT KO mice also demonstrated differential effects of adolescent cannabinoid administration on sociability and anxiety-related behavior, confirming and extending earlier reports of COMT×cannabinoid effects on the expression of schizophrenia-related endophenotypes (O’Tuathaigh et al. 2012).

Neuregulin

Nrg1, a leading schizophrenia susceptibility gene, is relevant to several schizophrenia-related neurodevelopmental processes due to its involvement in axonal guidance, myelination, and GABAergic and glutamatergic neurotransmission (Mei and Xiong 2008). Heterozygous deletion of Nrg1 results in increased sensitivity of mice to schizophrenia-like symptoms induced by THC, especially under stressful conditions (Boucher et al. 2007). Long’s lab team exposed adolescent WT and Nrg1 heterozygous (HET) mice to chronic THC during adolescence (Long et al. 2013). Surprisingly, Nrg1 mutants appeared less susceptible to THC-induced suppression of investigative social behaviors than control mice. However, adolescent THC exacerbated the hyperlocomotive phenotype characteristic for adult Nrg1 mutant mice (Karl et al. 2007; Long et al. 2013). Nrg1 deficiency also modulated the effects of adolescent THC on neurotransmitter systems involved in the pathophysiology of schizophrenia. Genotype-specific THC effects on CB1 expression in the substantia nigra were found: reduced CB1 level was present in Nrg1 HET drug-free mice, but its level increased in Nrg1 mice post THC challenge. Lower CB1 expression levels in the substantia nigra might be responsible for the observed decreased susceptibility of adolescent Nrg1 mutant mice. Nrg1 also conferred opposing effects of THC on serotonin 2A receptor expression in the insular cortex, and NMDA receptor binding was selectively increased in the hippocampus and cingulate cortex of Nrg1 HET mice (Long et al. 2013) (for a better mechanistic understanding of Nrg1-THC interaction on NMDA receptor expression in the hippocampus, see Spencer et al. 2013).

Concluding Nrg1 modulated the behavioral sensitivity of mice to cannabinoids during adolescence providing evidence for a role of Nrg1-cannabis interactions in schizophrenia.

BDNF

Reduced BDNF signaling has been shown in the frontal cortex and hippocampus in schizophrenia. Young BDNF HET mice and wild-type controls were chronically treated with the cannabinoid receptor agonist, CP55,940. Two weeks later, baseline PPI was lower but average startle was increased in BDNF HET compared to wild-type controls. Acute CP55,940 administration before the PPI session increased PPI only in male HET mice. In females, only small increases of PPI in all groups upon acute CP55,940 administration were observed. Acute CP55,940 administration furthermore reduced startle, and this effect was greater in HET mice irrespective of chronic CP55,940 pretreatment. Moreover, male “two-hit” mice, but not females, were hypersensitive to the effect of acute CP55,940 on sensorimotor gating. These effects may be related to a selective upregulation of CB1 receptor density in the nucleus accumbens (Klug and Van Den Buuse 2013).

DISC1

Recent research evaluated the interaction between cannabis in adolescence and another genetic risk factor, DISC1 (Brandon and Sawa 2011; Kamiya et al. 2012). Ballinger et al. (2015) demonstrated that a perturbation in DISC1 exacerbates the response to adolescent THC exposure in adult mice, such as deficits in fear-associated memory. Moreover, downregulation of the expression of CB1Rs in the PFC, hippocampus, and amygdala was induced by either expression of dominant-negative mutant of DISC1 (DN-DISC1) or adolescent THC treatment. A synergistic reduction of c-Fos expression induced by cue-dependent fear memory retrieval in DN-DISC1 with adolescent THC exposure was also found. These results suggest that alteration of CB1R-mediated signaling in DN-DISC1 mice may underlie susceptibility to detrimental effects of adolescent cannabis exposure on adult behaviors.

Environment × Environment Interaction

Some evidence has been recently accumulated highlighting the presence of environment × environment interaction in cannabis vulnerability in adolescence. Neonatal rodents subjected to prefrontal cortex lesioning showed impairment in various forms of social behavior and in object recognition memory after WIN 55,212-2 exposure in adolescence (Schneider and Koch 2007). Similarly, exposure to THC in adolescence produced a larger disruption of PPI in rats reared in social isolation (Malone and Taylor 2006), and a greater cognitive impairment was shown after THC in rats preexposed to PCP (Vigano et al. 2009). Interestingly stressful events early in life (maternal deprivation/separation) associated with adolescent exposure to natural or synthetic cannabinoids provoked different behavioral results when compared with non-stressed control animals. Indeed no effect, increased

cannabinoid-induced effect, or even decreased cannabinoid-induced effect was observed, depending on the sex of the animals and the considered behavior (Llorente-Berzal et al. 2011; Zamberletti et al. 2012; Klug and Van Den Buuse 2012). Recently, Gomes et al. (2015) showed that WIN 55212-2 administration in adolescence did not exacerbate the behavioral and electrophysiological changes (increased locomotor response to amphetamine administration and increased number of spontaneously active dopamine neurons in the ventral tegmental area) present in the methylazoxymethanol acetate (MAM) developmental disruption model of schizophrenia. WIN 55212-2 treatment attenuated the locomotor response to amphetamine in MAM rats without affecting dopamine neuron activity.

To conclude, the interaction between a previous hit (genetic or environmental) and cannabinoid exposure in adolescence warrants further investigations, and the different outcomes seem to be dependent by the considered gene profile, the nature and the time of the environmental factors, and the sex.

3 The Gateway Hypothesis

The possibility that cannabis use during adolescence increases the vulnerability to drug abuse disorders later in life, the so-called gateway hypothesis, has been heavily debated over the last decades due to contrasting results obtained with epidemiological studies. The definitive causal link between cannabis use and subsequent abuse of other illicit drugs should come from the use of experimental animal models. However, despite the use of these models, no conclusive findings are available, due to the existence of contrasting results even under these controlled conditions.

The most consistent data have been collected in studies considering cannabinoid-opioid interaction. Male, but not female, rats presented significant increases in the acquisition of both morphine and heroin self-administration after adolescent exposure to natural or synthetic cannabinoid agonists (Ellgren et al. 2007; Biscaia et al. 2008). Similarly, chronic cannabinoids in adolescence increased the sensitivity to morphine or heroin conditioned place preference (CPP) (Morel et al. 2009; Cadoni et al. 2015). Conversely, the same authors under different conditions or other authors reported that adolescent THC exposure neither affected heroin CPP (Cadoni et al. 2015) nor heroin self-administration (Stopponi et al. 2014). However Stopponi et al. (2014) found that adolescent THC pretreatment enhanced subsequent vulnerability to relapse to heroin seeking caused by yohimbine administration (as a pharmacological stressor). These discrepancies might be explained by differences in experimental parameters such as the strain of animals under investigation and the different heroin doses used for self-administration.

More contrasting results have been found regarding the interaction with psychostimulant drugs. Increased acquisition of cocaine self-administration was reported in adult female, but not male, rats pretreated with cannabinoids in

adolescence (Higuera-Matas et al. 2008). However, the locomotor activating effects of cocaine are enhanced after adolescent THC exposure (Dow-Edwards and Izenwasser 2012). Conversely, pretreatment with natural or synthetic cannabinoid agonists during early adolescence did not alter locomotor responses to amphetamine (Ellgren et al. 2004). Finally, exposure to cannabinoids during adolescence enhanced the acquisition and reinstatement of 3,4-methylenedioxymetamphetamine hydrochloride-induced CPP in mice (Rodríguez-Arias et al. 2010).

Very recently, data on cannabinoid–cannabinoid interaction have been reported (Scherma et al. 2015). These authors demonstrated that adolescent exposure to THC significantly increases WIN 55212-2 self-administration in adulthood. Indeed, THC-exposed rats acquired cannabinoid self-administration more rapidly than controls and showed higher rates of consumption when self-administration behavior reached asymptote.

In conclusion, the picture is still quite unclear regarding the role of Cannabis as a possible gateway drug to subsequent abuse of other illicit compounds. Maybe a more complex theory taking into account also influences of individual (personality traits) and environmental (substance availability, peer influence) characteristics will better describe this complex phenomenon.

4 Cellular Mechanisms Underlying Adolescent Brain Vulnerability

Despite their paucity, the available data seem to support the hypothesis that cannabis in adolescence might exert developmental interference on the eCB system. Accordingly, adolescent THC exposure in male and female rats induced lasting CB1 receptor downregulation and desensitization in different cerebral areas, females being more sensitive than males (Rubino et al. 2008, 2015; Burston et al. 2010). Moreover, in the PFC of THC-exposed female animals, the significant decrease of CB1R binding, still present in adulthood, was paralleled by a significant decrease of AEA levels (Rubino et al. 2015). Mice exposed to WIN 55,212-2 during adolescence exhibited at adulthood increased MGL and FAAH levels, suggesting increases in endocannabinoid degradation (Gleason et al. 2012). Recently, Ortega-Alvaro et al. (2015) showed that CB1R deletion induces a pre-attentional deficit and CB1 KO mice have significantly lower PPI values than WT mice. Moreover, treatment with the CB1R antagonist AM251 produces an impairment of PPI in WT mice, and typical and atypical antipsychotics did not ameliorate PPI deficit in CB1 KO mice. These data emphasize the important role of CB1R in sensorimotor gating modulation and suggest that the long-lasting downregulation of CB1R observed after cannabinoid in adolescence can play a role in the PPI impairment.

Collectively strong cannabis exposure in adolescence can profoundly affect the developmental trajectories of the different components of the eCB system such as

receptors (CB1 and CB2) and endocannabinoid levels (AEA), thus provoking its dysregulation and indirectly interfering with the final brain connectivity. These data strengthen the hypothesis that the alteration in the dynamic changes present in the eCB system during adolescence provoked by cannabis could profoundly affect the neurodevelopmental processes in which this system is involved.

For example, working memory and decision-making are refined during adolescence and require the functional maturation of the PFC. Interestingly, the eCB tone seems to play a fundamental role in some maturational processes occurring in the adolescent PFC. Indeed, adolescent THC exposure induced alterations in the maturational fluctuations of NMDA and AMPA subunits in this brain area, leading to larger amounts of *gluN2B* and *gluA1* at adulthood (Rubino et al. 2015). Moreover, due to the fact that NMDA receptors play a critical role in regulating adolescent maturation of GABAergic networks in the PFC (Thomases et al. 2013), adolescent cannabinoid exposure might also affect the GABAergic system. Accordingly, Cass et al. (2014) showed that WIN55,212-2 exposure during early or mid-adolescence, but not later in life, caused a functional downregulation of GABAergic transmission in the PFC. Similarly, Zamberletti et al. (2014) demonstrated that adolescent THC exposure resulted in reduced *GAD67* and basal GABA levels in the same brain area. These data suggest that adolescent cannabinoid exposure impacts not only the endocannabinoid system but also the glutamatergic and the GABAergic ones. These three systems are important in shaping cortical oscillations, a neural network activity in the neocortex (Uhlhaas et al. 2009) that is implicated in cognitive and sensory processing (Buzsaki and Draguhn 2004; Wang 2010). Chronic exposure to cannabinoids during adolescence permanently suppresses pharmacologically evoked cortical oscillations (Raver et al. 2013), and this effect seems to be mediated partly by CB1 receptors, but there is also some evidence of the involvement of CB2 and other non-cannabinoid receptors (Raver and Keller 2014). Moreover, the functional downregulation of GABAergic transmission in the PFC induced by adolescent cannabinoid exposure might impact the functionality of PFC excitatory descending projections. According to this hypothesis, increased ventral tegmental area (VTA) DA neuronal firing frequencies and relative proportion of spikes firing in burst have been described in adult rats chronically exposed to THC during adolescence (Renard et al. 2016). Similarly, an increased number of spontaneously active VTA dopaminergic neurons were seen in adult rats treated with WIN55,212-2 during puberty (Gomes et al. 2015). This VTA hyper-DAergic activity impacts molecular and neuronal activity parameters in the VTA DAergic output targets, such as the PFC and NAc. Indeed, an increased dopamine turnover was observed in the ventral striatum of adult rats chronically treated with WIN55,212-2 during adolescence (Bortolato et al. 2014), as well as altered amounts of D1 and D2 receptors in PFC and NAc (Higuera-Matas et al. 2010; Zamberletti et al. 2012). Consistent with this state of hyper-DAergic function in the mesocorticolimbic system, acute administration of the D3 receptor antagonists U-99194A partially restored the cognitive performances in the novel object recognition test disrupted by chronic cannabinoid exposure during adolescence (Abboussi et al. 2015).

Besides the impact on the maturation of different neurotransmitter systems, the endocannabinoid system has been recently suggested to affect the process of synaptic pruning (Rubino et al. 2015). Alterations in this event can lead to structural changes in the adult brain that might play a part in the dysfunctionality triggered by adolescent cannabinoid exposure. In male rats, adolescent exposure to WIN55,212-2 significantly decreased spine density in the nucleus accumbens immediately after treatment (Carvalho et al. 2016). More interestingly, long after the end of treatment, adolescent cannabinoid exposure has been shown to reduce spine density in the dentate gyrus of the hippocampus at adulthood, paralleled by a significant decrease in dendrite length and number (Rubino et al. 2009b) as well as a significant decrease in the basal dendritic arborization of pyramidal neurons in layer II/III of the PFC (Rubino et al. 2015).

As the brain is composed of many different cell types, including, but not limited to, neurons, it is only logical that adolescent cannabinoid exposure could also affect the functionality of other cell types besides neurons. According to this view, adolescent WIN55,212-2 treatment has been shown to induce an increase in the survival of oligodendroglia precursors in the striatum and PFC immediately after treatment (Bortolato et al. 2014). The role of this alteration in cannabinoid-induced effects is still not known, but pave the way to the increasing importance for brain function of the interaction between glial cells and neurons. More recently, Zamberletti et al. (2015) reported that adolescent THC administration induces a persistent neuroinflammatory state specifically localized within the adult PFC, characterized by increased expression of pro-inflammatory markers (TNF- α , iNOS, and COX-2), and reduction of the anti-inflammatory cytokine IL-10. Moreover, this neuroinflammatory phenotype was associated with downregulation of CB1 receptor on neuronal cells and upregulation of CB2 on microglia cells. Interestingly, blocking microglia activation with ibudilast during THC treatment significantly attenuated short-term memory impairment in adulthood. This was paralleled by the prevention of the increases in TNF- α , iNOS, and COX-2 levels as well as of the upregulation of CB2 receptors on microglia cells.

5 Conclusions

In conclusion, this review shows that cannabinoid administration in adolescence can profoundly affect the maturation of the endocannabinoid system, thus triggering important dysregulation in the final remodeling of other neurotransmitter pathways (glutamate, GABA, and dopamine). These dysfunctions represent the neurobiological substrate of the behavioral abnormalities resembling psychiatric diseases that become evident at adulthood. Moreover, recent evidence suggests that besides neuron also glial cells are affected by cannabinoid in adolescence, thus underlying the presence of a more generalized dysfunctional picture involving all the cellular elements of the central nervous system. Finally, no conclusive findings

are available for the gateway hypothesis due to the presence of limited and contrasting data mainly focused on opioids and psychostimulants.

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Endocannabinoid Signaling in Reward and Addiction: From Homeostasis to Pathology

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Abstract The endogenous cannabinoid system is an important regulatory system involved in physiological homeostasis. Endocannabinoid signaling is known to modulate neural development, immune function, metabolism, synaptic plasticity, and emotional state. Accumulating evidence also implicates brain endocannabinoid signaling in the processing of natural and drug-induced reward states and dysregulated endocannabinoid signaling in the etiology of aberrant reward function and drug addiction. In this chapter, we discuss the influence of endocannabinoid signaling on the rewarding and motivational effects of natural rewards such as food, sex, and social interaction, as well as evidence demonstrating an endocannabinoid influence in the rewarding effects of abused drugs. The effects of long-term drug consumption on endocannabinoid signaling are discussed, along with evidence that the resultant dysregulation of endocannabinoid function contributes to various aspects of drug dependence and addiction including physical symptoms of drug withdrawal, increased stress responsivity, negative affective states, dysregulated synaptic plasticity, dysregulated extinction of drug-related memories, relapse to drug taking, and impaired cognitive function. Lastly, consideration is given to the role for dysregulated endocannabinoid signaling in pathological food reward and eating disorders.

Present knowledge of the endogenous cannabinoid system (ECS) derives from decades of research demonstrating that the effects of *Cannabis* are mediated by cannabinoid receptors in the brain and the subsequent identification and characterization of the endogenous ligands for these receptors. Although our understanding of the nature and breadth of endocannabinoid (eCB) influences is constantly expanding, it is now acknowledged that the brain ECS plays a prominent role in modulating brain reward function and the maintenance of emotional homeostasis. This chapter examines the evidence for an eCB influence in the positive reinforcing effects of natural rewards and drugs of abuse, as well as dysregulation of ECS function that may influence or contribute to aberrant synaptic plasticity, negative

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emotional states, and impaired learning and memory processes resulting from long-term drug exposure that sustains compulsive drug consumption characteristic of the addicted state.

1 Neurobiology of Reward

The brain reward system plays an important role in our survival as a species. In addition to homeostatic factors that drive behavior, the hedonic or pleasurable effects produced by eating, exercise, and sexual activity provide important motivational effects that increase the likelihood of future engagement in these critical activities (known as positive reinforcement). In a similar manner, though less commonly considered, the reward system participates in negative hedonic responses in which aversive or unpleasant events (sickness, bodily harm) increase the likelihood of behaviors that will relieve or avoid these negative states (negative reinforcement). Accordingly, proper reward system function is critical for the maintenance of behaviors that promote survival.

Our understanding of the neurobiological substrates for reward and reinforcement is derived from studies in the 1950s by Olds and Milner who demonstrated that rodents will work to receive electrical stimulation in various brain regions (Olds and Milner 1954). Although stimulation of many brain sites supports operant behavior, the most sensitive sites involve the trajectory of the medial forebrain bundle that connects the ventral tegmental area (VTA) to the basal forebrain, including the nucleus accumbens (NAc) in the ventral striatum. Substantial subsequent evidence has demonstrated a critical involvement of mesocorticolimbic dopamine (DA) signaling in the mediation of reward and reinforcement, suggesting that activation of this system induces general behavioral activation (Le Moal and Simon 1991), promotes goal-directed behavior (Salamone et al. 2007), and increases incentive salience to environmental stimuli (Robinson and Berridge 1993). Functional neuroimaging studies in humans demonstrate that the mesocorticolimbic system is activated by natural rewards including the sight of appetizing food and orgasm (Komisaruk et al. 2004; Volkow et al. 2011), and rodent studies demonstrate that food consumption, sex, and exercise each increase NAc DA signaling (Bassareo and Di Chiara 1999a; Fiorino et al. 1997; Freed and Yamamoto 1985; Hattori et al. 1994). PET studies in humans have shown that psychostimulants, nicotine, alcohol, and marijuana each increase DA in the dorsal and ventral striatum and that this is associated with the subjective rewarding effects of the drugs (Tomasi and Volkow 2013). Similarly, rodent studies demonstrate that all addictive drugs possess the ability to increase mesocorticolimbic DA and that this contributes to the positive reinforcing effects of these substances (particularly psychostimulants and nicotine) (Di Chiara and Bassareo 2007; Koob and Volkow 2010). Substantial evidence suggests that DA-independent signaling in the mesolimbic system also substantially impacts reward processing, including cholinergic, opioid, glutamatergic, and GABAergic signaling (Koob 1992; Nestler 2005).

The NAc receives input from the amygdala, frontal cortex, and hippocampus that may be converted to motivational actions through its connections with the extra-pyramidal motor system. In particular, the central nucleus of the amygdala (CeA) appears to play a role in mediating/modulating the positive reinforcing effects of abused drugs (Koob and Volkow 2010), and the ventral pallidum/substantia innominata, which receives a major innervation from the NAc, is also critically involved in modulating the positive reinforcing effects of both natural and drug rewards (Koob and Volkow 2010).

In general, NAc DA levels are decreased by many aversive conditions such as unavoidable shock, medial hypothalamic stimulation, chronic pain, and certain patterns of over- or undereating (Umberg and Pothos 2011). Numerous studies have also demonstrated deficiencies in NAc DA during withdrawal from addictive drugs (Umberg and Pothos 2011; Koob and Volkow 2010). Further, dysregulation of key neurochemical elements involved in stress responses mediated by the CeA, bed nucleus of the stria terminalis (BNST), frontal cortex, and medial shell of the NAc plays a major role in the negative reinforcing effects of withdrawal from highly palatable food and drugs of abuse (Koob and Volkow 2010; Koob et al. 2014; Blasio et al. 2013; Iemolo et al. 2013). Signaling systems involved in these processes include dysregulation of both pro-stress (corticotropin-releasing factor (CRF), dynorphin) and anti-stress (NPY, nociceptin) systems.

Thus, reward processing is mediated in large part through an interconnected network of structures that includes the VTA, NAc, ventral pallidum, CeA, BNST, and prefrontal cortex (PFC). In addition to the well-known involvement of mesocorticolimbic DA, reward processing is also heavily influenced by many other systems including glutamate, GABA, opioid peptides, and stress-related signaling systems.

2 The Endogenous Cannabinoid System (ECS)

Endocannabinoids are neuroactive lipids that participate in a range of physiological processes including reward, motivation, emotional homeostasis, pain processing, neuroprotection, and synaptic plasticity contributing to learning and memory. For the purposes of this chapter, a superficial overview of the main ECS components is provided below, and readers are referred to more in-depth coverage of this topic provided by several excellent recently published reviews (Lu and Mackie 2015; Hillard 2015; Mechoulam and Parker 2013).

In addition to the endocannabinoid lipid moieties themselves, the ECS is comprised of G protein-coupled receptors and metabolic enzymes for the synthesis and degradation of the ligands. Two major types of cannabinoid receptor have been characterized and cloned: CB₁ and CB₂. CB₁ receptors (CB₁Rs) are the most abundant G protein-coupled receptor expressed in the adult brain, with particularly dense expression in each of the interconnected structures involved in reward (Glass et al. 1997; Wang et al. 2003b; Herkenham et al. 1991) where they exert widespread

modulatory influences on excitatory and inhibitory signaling in a manner that influences reward processing (Sidhpura and Parsons 2011; Panagis et al. 2014). In particular, eCBs play a prominent role in fine-tuning the activity of the VTA-NAc DA projection and its influence on approach and avoidance behaviors that govern reward acquisition (Hernandez and Cheer 2015). CB₂ receptors (CB₂Rs) are mainly expressed by immune cells with recent evidence also suggesting CB₂R expression in neurons, glia, and endothelial cells in the brain (Atwood and Mackie 2010). CB₁R and CB₂R are coupled to similar transduction systems primarily through G_i or G_o proteins. CB₁Rs directly inhibit the release of GABA, glutamate, and acetylcholine that produce widespread effects on neural signaling across many neurotransmitter systems.

At present the best characterized endocannabinoid ligands are N-arachidonylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). Due to their lipid nature, AEA and 2-AG are not stored in vesicles but are synthesized on an “on demand” basis by cleavage from membrane precursors and immediate release through Ca²⁺-dependent mechanisms. AEA is derived from the phospholipid precursor N-arachidonoyl phosphatidylethanolamine (NAPE), and while the precise mechanisms for AEA formation are not known, a very likely candidate is through N-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD). 2-AG derives primarily from the hydrolytic metabolism of 1,2-diacylglycerol (DAG) by sn-1-selective DAG lipases (DAGL α , DAGL β). AEA is primarily catabolized through fatty acid amide hydrolase 1 (FAAH1), and 2-AG is catabolized through monoacylglycerol lipase (MAGL) and to a lesser extent α , β -hydrolase 6 (ABHD6), cyclooxygenase-2 (COX-2), and FAAH1. The eCB catabolic enzymes have distinct cellular anatomical locations with MAGL localized predominantly in presynaptic terminals and FAAH1 to the postsynaptic domain of neurons. AEA and 2-AG each exert agonist activity at CB₁R and CB₂R. AEA binds with slightly higher affinity to CB₁R vs. CB₂R, and like Δ 9-tetrahydrocannabinol (Δ 9-THC, the main psychoactive component of the *Cannabis* plant), AEA exhibits low efficacy as an agonist at both receptors, producing submaximal signaling upon binding. 2-AG binds with essentially equal affinity at CB₁R and CB₂R and exhibits greater agonist efficacy than AEA. AEA and 2-AG also exhibit agonist properties at several secondary receptors including peroxisome proliferator-activated receptors (PPARs), GPR55, and GPR119, and AEA exerts potent agonist effects at transient receptor potential ion channels including TRPV1.

3 Rewarding Effects of Altered eCB Signaling

Both exogenous AEA and exogenous 2-AG increase extracellular DA levels in the NAc in a CB₁-dependent manner (Solinas et al. 2006), and substantial evidence demonstrates a strong eCB influence in fine-tuning the activity of midbrain DA cells (Melis and Pistis 2012). Given this influence, and the rewarding effects produced by natural and synthetic CB₁ agonists, there has been considerable

interest in the influence of eCB signaling in the modulation of brain reward function.

Several preclinical studies have investigated whether enhanced eCB signaling itself produces rewarding effects, and much of this has compared eCB-induced behaviors with those produced by exogenous CB₁ agonists. The metabolically stable AEA analogs (R)-methanandamide, O-1812, and AM1346 each fully generalize to Δ^9 -THC in drug discrimination tests (Burkey and Nation 1997; Jarbe et al. 2006; Alici and Appel 2004; Solinas et al. 2007), and exogenous AEA itself also produces Δ^9 -THC-like discriminative effects when administered following FAAH inhibition (Solinas et al. 2007; Vann et al. 2009; Wiley et al. 2014). Because neither AEA nor FAAH inhibition produces Δ^9 -THC-like responding in their own right (Burkey and Nation 1997; Wiley et al. 1997, 2014; Gobbi et al. 2005; Solinas et al. 2007), these findings suggest that administration of exogenous AEA produces Δ^9 -THC-like interoceptive effects only under conditions of reduced hydrolytic clearance. In the absence of MAGL inhibition, exogenous 2-AG also does not substitute for Δ^9 -THC in discrimination tests (Wiley et al. 2014), though interestingly the MAGL inhibitor JZL184 produces partial generalization to Δ^9 -THC even without exogenously administered 2-AG (Wiley et al. 2014; Long et al. 2009b). However, these effects of JZL184 may result from concurrent MAGL and FAAH inhibition based on evidence that dual inhibition of MAGL and FAAH fully substitutes for Δ^9 -THC in discrimination tests (Wiley et al. 2014; Long et al. 2009b), and the more selective MAGL inhibitor KML29 does not generalize to Δ^9 -THC (Ignatowska-Jankowska et al. 2014). The Δ^9 -THC-like discriminative properties of enhanced eCB signaling are all blocked by CB₁ antagonism. Collectively, these findings indicate that systemic administration of eCBs produces CB₁-dependent Δ^9 -THC-like discriminative stimulus effects, though combined enhancement of AEA and 2-AG signaling is most effective for producing Δ^9 -THC-like effects. Neither exogenously administered AEA nor selective FAAH, MAGL, or dual FAAH/MAGL inhibition produces rewarding effects in the conditioned place preference model (Mallet and Beninger 1998; Gobbi et al. 2005; Gamage et al. 2015), and FAAH inhibitors and putative eCB transport inhibitors do not alter brain stimulation reward thresholds (Vlachou et al. 2006). Drugs that inhibit eCB hydrolysis do not support operant self-administration behavior in rodents, though exogenous AEA, the metabolically stable AEA analog methanandamide, and exogenous 2-AG do support operant self-administration behavior by squirrel monkeys through a CB₁R-reliant mechanism (Justinova et al. 2005, 2008a, 2011). Moreover, the eCB clearance inhibitors URB597, AM404, and VDM11 support operant self-administration behavior by squirrel monkeys with a prior history of nicotine, cocaine, or AEA self-administration (Justinova et al. 2015; Schindler et al. 2016). However, unlike drugs with prominent abuse liability, these eCB clearance inhibitors do not increase mesolimbic DA release (Solinas et al. 2007; Murillo-Rodriguez et al. 2013; Justinova et al. 2015), and it remains to be determined whether these compounds will be self-administered by drug-naïve monkeys or other species. Collectively, these findings suggest that administration of exogenous eCBs can produce positive reinforcing effects in a variety of paradigms indexing

brain reward, though most evidence suggests that pharmacological compounds that enhance eCB tone (e.g., selective FAAH or MAGL clearance inhibitors) do not produce substantial rewarding effects.

4 Endocannabinoid Influence on Natural Rewards

4.1 Food Reward

It is well known that palatable foods can lead people to eat, even when satiated. As such, eating can be motivated not only by hunger or homeostatic processes but also by hedonics. The ECS plays a prominent homeostatic role in controlling metabolic functions such as energy balance and food intake (Silvestri and Di Marzo 2013), and growing evidence also demonstrates an important ECS influence in the hedonic or rewarding properties of food intake. Both exogenous and endogenous CB₁ agonists reduce the latency to feed in pre-satiated or free-feeding animals, increase the amount of food consumed, and increase an animal's willingness to work for food reward (Kirkham and Williams 2001; Farrimond et al. 2011; Gallate and McGregor 1999; Kirkham et al. 2002; Williams and Kirkham 1999; Martinez-Gonzalez et al. 2004; Solinas and Goldberg 2005). Manipulations of CB₁ tone appear to preferentially influence the motivation for highly palatable rewards compared with standard isocaloric chow or neutral or mildly aversive rewards (Guegan et al. 2013; Dipatrizio and Simansky 2008; Shinohara et al. 2009). For example, studies on the microstructure of fluid consumption and taste reactivity demonstrate that CB₁ receptor agonists increase the hedonic effects of palatable rewards such as sucrose (Higgs et al. 2003; Jarrett et al. 2005, 2007; Mahler et al. 2007). Conversely, CB₁ receptor antagonism preferentially reduces the consumption of palatable and/or high-fat foods vs. standard chow (Simiand et al. 1998; South et al. 2007; Mathes et al. 2008), reduces the conditioned reinforcing effects produced by palatable substances such as sucrose or chocolate (Chaperon et al. 1998), and reduces the motivation for palatable foods indexed under progressive ratio schedules of reinforcement (Gallate and McGregor 1999; Gallate et al. 1999; Solinas and Goldberg 2005).

The neural pathways contributing to the hedonic aspects of feeding include the mesocorticolimbic system (Kalivas and Volkow 2005; Nestler 2005). Food-related visual or olfactory cues increase mesolimbic DA signaling in both humans (Macht and Mueller 2007) and laboratory animals (Hajnal et al. 2004; Rada et al. 2005; Scalfani et al. 1998) in part through CB₁-reliant mechanisms (Melis et al. 2007). The mesocorticolimbic system influences feeding behavior through interconnections with hypothalamic nuclei (Berridge et al. 2010), and many of the hypothalamic signaling molecules that modulate homeostatic feeding behavior are also present in mesocorticolimbic structures such as the NAc, VTA, and PFC (Horvath and Diano 2004; Kelley et al. 2005). In fact, an influence of feeding-related

hormones on eCB production (Di Marzo et al. 2001; Bermudez-Silva et al. 2012) and signaling (Edwards and Abizaid 2016) has been described, and this mechanism may play a prominent role in the regulation of consummatory behaviors. Fasting increases AEA and 2-AG levels in the limbic forebrain and hypothalamus of rodents, an effect that is ameliorated following feeding (Kirkham et al. 2002; Hanus et al. 2003). Doses of CB₁ agonists that induce food consumption in satiated rodents activate the corticostriatal-hypothalamic pathway (including the NAc) (Dodd et al. 2009), and food consumption is increased by direct infusion of eCBs or FAAH inhibitors into the hypothalamic nuclei (Anderson-Baker et al. 1979; Jamshidi and Taylor 2001; Verty et al. 2005) or NAc shell (Kirkham et al. 2002; Soria-Gomez et al. 2007). In particular, the NAc shell has been identified as a critical locus for the eCB modulation of the hedonic properties of food (Berridge et al. 2010; Mahler et al. 2007), and intra-NAc eCB infusions increase neural activity in hypothalamic nuclei through a CB₁-dependent mechanism (Soria-Gomez et al. 2007). Furthermore, transgenic mice overexpressing MAGL in the forebrain (MGL-Tg mice) do not form a conditioned place preference for high-fat foods, indicating that reductions in 2-AG may impede high-fat food reward (Wei et al. 2016). Collectively, these findings demonstrate that eCB signaling in the CNS plays a role in the motivation for and hedonic response to food consumption. Interestingly, there is emerging evidence that in addition to sweet taste receptors (T1R2/T1R3), sweet-sensitive taste cells in the peripheral system also express CB₁ receptors (Jyotaki et al. 2010), and both AEA and 2-AG increase gustatory nerve responses to sweet solutions (Yoshida et al. 2010). Thus, the ECS modulates food palatability via both central and peripheral pathways.

4.2 Sexual Reward

CB₁ receptors are densely expressed in the neural circuits that regulate sexual behavior, and substantial evidence indicates that both exogenous and endogenous cannabinoids can alter sexual behavior, though the effects are often highly divergent in males and females (Gorzalka et al. 2010; Lopez 2010). With regard to the hedonic aspects of sexual behavior, the majority of studies indicate a facilitatory effect of *Cannabis* on subjective indices of sexual arousal in women including desire, orgasmic function, and pleasure/enjoyment/satisfaction (Koff 1974; Dawley et al. 1979; Halikas et al. 1982; Klein et al. 2012). Similarly, low-dose *Cannabis* exposure increases sexual desire in males though simultaneously hindering erectile functioning. Rodent studies have revealed both facilitatory and inhibitory effects of cannabinoid agonists and eCB clearance inhibition (e.g., FAAH inhibitors) on female sexual receptivity and proceptivity and a general inhibitory effect on male sexual performance (Gorzalka et al. 2010; Lopez 2010; Canseco-Alba and Rodriguez-Manzo 2016), though the relative influence of these manipulations on the motivational vs. performance aspects of sexual behavior is not clear. In the most direct evaluation of an eCB influence on sexual motivation, Klein and colleagues

observed that physiological indices of sexual arousal in women are associated with decreased serum AEA levels, and subjective indices of arousal are associated with decreases in serum levels of both AEA and 2-AG (Klein et al. 2012). However, while these indices reflect an ECS involvement in the anticipatory/motivational effects produced by erotic visual stimuli, it remains unclear whether endocannabinoid signaling is altered by sexual activity itself and, if so, whether this results in a facilitation or attenuation of brain reward processing. Endocannabinoid signaling may also influence sexual reward indirectly through modulation of emotional state and/or sensation perception, though these possibilities have not been explored.

4.3 Social Interaction

Cooperative playing and social interaction are rewarding activities in both humans and rodents (Tabibnia and Lieberman 2007; Douglas et al. 2004). In rodents, social play is enhanced following inhibition of FAAH or putative eCB transporters (with VDM11) but diminished following administration of exogenous CB₁ agonists such as WIN 55,212-2 (Trezza and Vanderschuren 2008a, b, 2009). This highlights the distinct effects produced by selectively enhancing the effects of eCB signaling in selected synapses, wherein eCB formation is evoked (e.g., eCB clearance inhibition) vs. widespread CB₁ activation by exogenous agonists that engage CB₁ signaling in circuits not normally activated by a given behavior (Trezza et al. 2010). The hedonic aspects of social play are mediated through both opioid (mu opioid in particular) and CB₁ mechanisms, and the CB₁ influence appears to involve subsequent increases in DA signaling (Trezza et al. 2010). Interestingly, social reward is influenced by opioid and cannabinoid interactions in a manner similar to drug and food reward (Fattore et al. 2004; Solinas and Goldberg 2005).

5 eCB Influence on the Acute Rewarding Effects of Drugs of Abuse

There is substantial evidence implicating the ECS in the motivational effects produced by several classes of abused drugs including ethanol, nicotine, opiates, and psychostimulants. Much of this is based on the influence of CB₁ receptor signaling on drug-induced behaviors, though some studies have evaluated manipulations of eCB tone on drug reinforcement.

In general, CB₁ receptors exert a facilitatory influence on the rewarding effects of several classes of abused drugs (Table 1). Rodent studies employing the conditioned place preference and operant self-administration paradigms demonstrate that CB₁ agonists increase the motivational and rewarding effects of alcohol (Colombo

Table 1 Summary of CB₁R influence on motivation-related effects of abused drugs

Genetic or pharmacological manipulation	Ethanol	Nicotine	Opiates	Psychostimulants
CB ₁ R Knockout	↓ CPP ^a ↓ Operant SA ↓ EtOH-induced NAc DA	↓ CPP ^b ↓ Operant SA	↓ CPP ^c ↓ Operant SA	No change in CPP ^d No change in operant SA
CB ₁ R Antagonist	↓ Preference ^e ↓ Operant SA ↓ EtOH-induced NAc DA	↓ CPP ^f ↓ Operant SA ↓ Nic-induced NAc DA	↓ CPP ^g ↓ Operant SA	↑↑ Operant SA ^h ↑↑ Cocaine effects on ICSS
CB ₁ R Agonist	↑ Operant SA ⁱ ↑ Motivation for EtOH	↑ CPP ^j	↑ CPP ^k ↑ Motivation for heroin	↓ Operant SA ^l ↓ Cocaine effects on ICSS

^aHouchi et al. (2005), Thanos et al. (2005), Hungund et al. (2003), Naassila et al. (2004), Wang et al. (2003a)

^bCossu et al. (2001)

^cMartin et al. (2000), Ledent et al. (1999), Cossu et al. (2001)

^dMartin et al. (2000), Houchi et al. (2005), Cossu et al. (2001), Soria et al. (2005), Li et al. (2009)

^eArnone et al. (1997), Freedland et al. (2001), Gallate and McGregor (1999), Colombo et al. (1998), Gessa et al. (2005), Malinen and Hyttia (2008), Alvarez-Jaimes and Parsons (2009), Caille et al. (2007), Wang et al. (2003a), Perra et al. (2005), Cheer et al. (2007)

^fCohen et al. (2002), Shoaib (2008), Simonnet et al. (2013), Cheer et al. (2007)

^gCaille and Parsons (2003, 2006), De Vries et al. (2003), Solinas et al. (2003), Singh et al. (2004), Chaperon et al. (1998), Navarro et al. (2001)

^hFilip et al. (2006), Caille and Parsons (2006), Xi et al. (2008), Vinklerova et al. (2002), Chaperon et al. (1998), Cheer et al. (2007), Soria et al. (2005), Vlachou et al. (2003), Fattore et al. (1999), Tanda et al. (2000), De Vries et al. (2001), Caille et al. (2007), Lesscher et al. (2005)

ⁱColombo et al. (2002), Wang et al. (2003a), Gallate et al. (1999), Malinen and Hyttia (2008), Getachew et al. (2011)

^jValjent et al. (2002), Gamaledin et al. (2012b)

^kManzanedo et al. (2004), Solinas et al. (2005)

^lVlachou et al. (2003), Fattore et al. (1999)

et al. 2002; Wang et al. 2003a; Gallate et al. 1999; Malinen and Hyttia 2008; Getachew et al. 2011), nicotine (Valjent et al. 2002; Gamaledin et al. 2012b), and opiates (such as morphine and heroin) (Manzanedo et al. 2004; Solinas et al. 2005). In contrast, diminished CB₁ receptor signaling (either genetic receptor deletion or pharmacological inhibition) attenuates the motivational and rewarding effects of alcohol (Houchi et al. 2005; Thanos et al. 2005; Hungund et al. 2003; Naassila et al. 2004; Arnone et al. 1997; Wang et al. 2003a; Freedland et al. 2001; Gallate and McGregor 1999; Colombo et al. 1998; Gessa et al. 2005), nicotine (Cohen et al. 2002; Shoaib 2008; Cossu et al. 2001; Simonnet et al. 2013), and opiates (Chaperon et al. 1998; Navarro et al. 2001; Singh et al. 2004; Martin et al. 2000; Ledent et al. 1999; Cossu et al. 2001; Caille and Parsons 2003; De Vries et al. 2003; Solinas et al. 2003). The CB₁ influence on alcohol and nicotine reward is mediated in part,

through modulation of the mesolimbic DA response to these drugs as CB₁ receptor antagonism blocks the excitation of VTA DA cells produced by alcohol and nicotine, thereby attenuating the effects of these drugs on NAc DA release (Cohen et al. 2002; Hungund et al. 2003; Perra et al. 2005; Cheer et al. 2007). Blockade of CB₁ receptors in the VTA decreases both alcohol and nicotine self-administration (Malinen and Hyytiä 2008; Alvarez-Jaimes and Parsons 2009; Caille et al. 2007; Simonnet et al. 2013), and antagonism of NAc CB₁ receptors similarly reduces alcohol consumption (Alvarez-Jaimes and Parsons 2009; Caille et al. 2007). However, while nicotine reward is critically dependent on the mesolimbic DA system (D'Souza and Markou 2011), the motivational and rewarding effects of alcohol and opiates are less DA dependent (Koob 2013; Shippenberg and Elmer 1998; Pettit et al. 1984; Gerrits and Van Ree 1996; Platt et al. 2001) with CB₁R modulation of the rewarding effects of these drugs likely involving non-dopaminergic mechanisms. Indeed, although CB₁ receptor deletion results in attenuated opiate-induced increases in NAc DA (Mascia et al. 1999), acute CB₁ receptor blockade does not alter opiate-induced increases in NAc DA (Caille and Parsons 2003, 2006; Tanda et al. 1997). Rather, CB₁ receptor antagonism may modulate opiate reward through DA-independent attenuation of opiate-induced reductions in ventral pallidal GABA signaling (Caille and Parsons 2006).

The effects of CB₁ receptor manipulations on psychostimulant reward are less consistent. In contrast to the facilitation of ethanol, nicotine, and opiate reward, CB₁ agonists reduce both cocaine-induced facilitation of brain stimulation reward and cocaine self-administration by rats (Vlachou et al. 2003; Fattore et al. 1999). Further, CB₁ receptor inactivation does not affect psychostimulant-induced place conditioning (Martin et al. 2000; Houchi et al. 2005), psychostimulant self-administration (Cossu et al. 2001; Tanda et al. 2000; Fattore et al. 1999; De Vries et al. 2001; Caille and Parsons 2006; Filip et al. 2006; Caille et al. 2007; Lesscher et al. 2005), cocaine-induced increases in nucleus accumbens dopamine (Caille and Parsons 2003; Soria et al. 2005), or cocaine-induced enhancement of brain stimulation reward (Vlachou et al. 2003; Xi et al. 2008). However, some reports indicate that CB₁ inactivation attenuates cocaine and amphetamine self-administration (Soria et al. 2005; Xi et al. 2008; Vinklerova et al. 2002), blocks cocaine-induced increases in NAc DA signaling (Li et al. 2009; Cheer et al. 2007), and decreases cocaine-induced enhancement of brain stimulation reward sensitivity (Xi et al. 2008). In general, the inconsistent and generally subtle effects reported suggest that CB₁ receptors exert a relatively modest influence over psychostimulant-induced reward.

Recent evidence in mice also implicates CB₂Rs in the modulation of drug reward. CB₂R agonists reduce cocaine-induced CPP and cocaine self-administration in wild-type mice but not CB₂R knockout mice (Xi et al. 2011; Ignatowska-Jankowska et al. 2013; Zhang et al. 2014a), and cocaine self-administration is also reduced by CB₂R overexpression in the brain (Aracil-Fernandez et al. 2012). CB₂R agonists also reduce alcohol-induced CPP and alcohol consumption (Al Mansouri et al. 2014). Mouse VTA DA neurons express CB₂R mRNA and receptor immunostaining, and CB₂R agonists decrease the

activity of these neurons in wild-type but not CB₂R knockout mice (Zhang et al. 2014b). Surprisingly, CB₂R agonists facilitate nicotine-induced CPP (Ignatowska-Jankowska et al. 2013), and CB₂R inactivation reduces both nicotine-induced CPP and nicotine self-administration in mice (Navarrete et al. 2013; Ignatowska-Jankowska et al. 2013). In contrast, CB₂R antagonism does not alter cocaine or nicotine self-administration in rats but attenuates cocaine-induced and conditioned locomotion and reinstatement of cocaine-seeking behavior (without altering nicotine-induced drug seeking) (Blanco-Calvo et al. 2014; Adamczyk et al. 2012b; Gamaledin et al. 2012b). Further, a recent study reported the CB₂R agonism reduces cocaine consumption by rats, but not mice, and produces opposite effects on the motivation for cocaine in these species (Zhang et al. 2014a). These distinctions may result from species differences in the splicing and expression of CB₂R genes, possibly conferring distinct CB₂R structure, function, or pharmacology (Zhang et al. 2014a).

6 Evidence that Drugs of Abuse Increase Brain eCB Levels

The inhibitory influence of CB₁ receptor antagonism on drug reward has led to the hypothesis that abused drugs increase brain eCB formation possibly resulting in aberrant eCB signaling following long-term drug exposure. Several observations support these hypotheses.

Early studies reported that chronic alcohol exposure increases 2-AG and AEA formation in human neuroblastoma cells and cultured rodent neurons (Basavarajappa and Hungund 1999; Basavarajappa et al. 2000, 2003). Subsequent evaluations of postmortem brain tissue eCB content clearly demonstrate alcohol-induced alterations in 2-AG and AEA production, though substantial inconsistencies among studies make it difficult to draw clear conclusions on the direction of change and regional nature of the effects (Vinod et al. 2006, 2012; Gonzalez et al. 2002, 2004b; Malinen et al. 2009; Rubio et al. 2007). In vivo microdialysis studies in rats demonstrate that voluntary alcohol consumption robustly increases nucleus accumbens 2-AG but not AEA levels (Caille et al. 2007), while forced alcohol administration decreases AEA levels and induces more modest increases in 2-AG (Ferrer et al. 2007; Alvarez-Jaimes et al. 2009). This provides initial evidence that the volitional nature of drug exposure (e.g., voluntary vs. forced administration) may differentially impact eCB responses. The effects of alcohol on brain eCB production may also be region specific as alcohol-induced disruptions of extracellular eCB levels and mRNA expression of their associated enzymes are consistently observed in striatal regions (Caille et al. 2007; Ceccarini et al. 2013; Ferrer et al. 2007; Alvarez-Jaimes et al. 2009; Henricks et al. 2016) but not frontal cortical areas (Alvarez-Jaimes and Parsons 2009), and ethanol self-administration is reduced by localized antagonism of CB₁ receptors in the VTA and NAc shell but not PFC of outbred rats (Malinen and Hyttia 2008; Alvarez-Jaimes and Parsons 2009; Caille et al. 2007) (though disruptions in eCB processing have been observed in cortical

regions of rats selectively bred for high alcohol consumption (Hansson et al. 2007) and in mice following long-term alcohol exposure (Vinod et al. 2006)).

$\Delta 9$ -THC and synthetic CB₁ agonists also induce region-specific alterations in brain tissue eCB content. For example, chronic $\Delta 9$ -THC increases AEA (but not 2-AG) in limbic forebrain tissue and increases 2-AG (but not AEA) in the hippocampus, brainstem, and cerebellar tissue (Di Marzo et al. 2000; Gonzalez et al. 2004a; Castelli et al. 2007). In vivo microdialysis studies demonstrate that acute CB₁ agonist exposure increases 2-AG but decreases AEA levels in the rat hypothalamus (Bequet et al. 2007), and preliminary evidence from human clinical research studies also points to cannabinoid-induced increases in 2-AG and decrements in AEA production (Leweke et al. 2007; Morgan et al. 2013).

Chronic nicotine exposure also induces region-specific disruptions in brain tissue eCB content, with increased AEA (but not 2-AG) levels evident in limbic forebrain and caudate but decreased AEA and 2-AG levels in the cortex (Gonzalez et al. 2002). In vivo microdialysis studies demonstrate nicotine-induced increases in both 2-AG and AEA in the VTA and a sensitization of nicotine-induced 2-AG, but not AEA, formation results from chronic nicotine exposure (Buczynski et al. 2013). This enhancement of nicotine-induced VTA 2-AG formation results in diminished inhibitory constraint of VTA cell excitability, thereby enhancing nicotine-induced elevations in mesolimbic DA levels (Buczynski et al. 2016). Interestingly, while VTA 2-AG levels are increased by both voluntary self-administration and response-independent forced nicotine administration, VTA AEA levels are increased only by voluntary self-administration. Together with evidence of distinct eCB responses to self-administered vs. forced alcohol exposure (Caille et al. 2007; Ferrer et al. 2007; Alvarez-Jaimes et al. 2009), these observations suggest that brain eCB production can be influenced by activity of neural systems involved in the motivation for drug consumption, in addition to drug-related pharmacological effects.

Opiates also induce differential effects on brain 2-AG and AEA levels. Chronic morphine dose dependently reduces tissue 2-AG content in the striatum, cortex, hippocampus, hypothalamus, and limbic forebrain, while increased AEA content is observed in these regions (Vigano et al. 2003, 2004). Consistently, in vivo microdialysis studies demonstrate that heroin self-administration significantly increases interstitial AEA levels but decreases interstitial 2-AG levels in the nucleus accumbens (Caille et al. 2007).

In general, psychostimulants induce more subtle alterations in brain eCB levels as compared with the drugs described above (Zlebnik and Cheer 2016). Early studies reported that acute high-dose cocaine administration induces a subtle but significant increase in forebrain 2-AG content in mice (Patel et al. 2003), while chronic cocaine induces comparably subtle decreases in forebrain 2-AG content in rats (Gonzalez et al. 2002), though neither 2-AG nor AEA content is altered in other regions following acute or chronic cocaine exposure (Patel et al. 2003; Gonzalez et al. 2002). Similarly, cocaine self-administration does not alter either 2-AG or AEA content in nucleus accumbens microdialysates (Caille et al. 2007). However, recent studies provide some evidence of psychostimulant-induced alterations in eCB levels and processing. For example, cocaine self-administration is reported to

induce increased cerebellar AEA content; decreased 2-AG content in the frontal cortex, hippocampus, and cerebellum; and an increased ratio of DAGLa/MAGL expression in the hippocampus (Bystrowska et al. 2014; Rivera et al. 2013). Further, sensitization of the motor-activating effects of cocaine is associated with altered eCB metabolic enzyme expression in the cerebellum (Palomino et al. 2014), and detoxified cocaine addicts present significant increases in plasma AEA and decreases in plasma 2-AG content (Pavon et al. 2013).

These collective findings indicate that cannabinoids, ethanol, nicotine, and opiates can alter brain eCB content, consistent with substantial evidence of a CB₁ receptor influence on the behavioral effects produced by these drugs. Although there is relatively less information on psychostimulant-induced alterations in brain eCB content, the modest effects that have been reported are consistent with the subtle disruptions in psychostimulant-induced behaviors observed following CB₁ receptor antagonism.

eCBs are rapidly degraded, and thus strategies that reduce eCB clearance have been employed as a means to further investigate the eCB influence on drug reward. Most investigations have focused on the effects of FAAH inhibition because selective tools for inhibiting MAGL and other eCB clearance enzymes were not available until recently. Such studies have shed light on important species differences that confound the overall conclusions that can be made from existing data. For example, FAAH inhibition in mice increases nicotine reward in the CPP paradigm (Merritt et al. 2008; Muldoon et al. 2013), though in rats, FAAH inhibition prevents nicotine-induced CPP, diminishes nicotine self-administration, and blunts nicotine-induced increases in NAc DA release (Scherma et al. 2008). The potentiation of nicotine reward in mice by FAAH inhibition is CB₁R mediated, whereas the reduction in nicotine reward in rats results from activation of PPAR- α by non-cannabinoid lipids such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) that are hydrolytically cleared by FAAH (Melis and Pistis 2014). FAAH inhibition also produces distinct species-related alterations in alcohol consumption, with increased intake observed in mice but not rats (Blednov et al. 2007; Vinod et al. 2008; Hansson et al. 2007; Cippitelli et al. 2008). The mechanisms underlying these differences are not understood. Brain region-specific disruptions in FAAH activity may be an important factor in regard to alcohol reward as inhibition of FAAH activity specifically in the PFC results in increased alcohol consumption, and rats selectively bred for high alcohol intake and preference are characterized by reduced FAAH activity specifically in the PFC (Hansson et al. 2007; Cippitelli et al. 2008). The effects of FAAH inhibition on opiate and psychostimulant reward have primarily been studied in rats. FAAH inhibition does not alter morphine- or cocaine-induced disruptions in VTA DA cell firing or the self-administration of either drug (Justinova et al. 2008a; Luchicchi et al. 2010). However, FAAH inhibition diminishes cocaine-induced alterations in NAc medium-spiny neuron activity (Luchicchi et al. 2010), and this may contribute to enhanced sensitization of both cocaine-induced motor activity and mesolimbic dopamine responses following repeated cocaine exposure (Mereu et al. 2013). Other studies have investigated the effects of putative eCB transport inhibitors

such as AM404 and VDM11, and the findings thus far suggest that these compounds produce subtle and inconsistent effects on nicotine and cocaine reward (Scherma et al. 2012; Gamaledin et al. 2011, 2013; Vlachou et al. 2008).

While growing evidence implicates ECS influences in the modulation of acute drug reward, additional efforts are needed to further clarify the nature of eCB disruptions caused by different classes of abused drugs and the neural mechanisms through which these eCB influences are mediated. Selective inhibitors of 2-AG clearance have recently been developed, but studies are still in their infancy, so there are presently no published reports on the effects of enhanced 2-AG tone on drug reward and related physiological events. As such there remains a substantial gap of knowledge given the prominent 2-AG influence on neural signaling and plasticity related to both drug and natural rewards. Nevertheless, the role of the CB₁R in drug reward is unequivocal. Although there is evident complexity related to the effects produced by eCB clearance inhibition (producing discrete modulation of eCB tone in specific synapses/circuits as compared with broad CB₁R activation by exogenous CB₁R agonists), the extant evidence strongly supports an eCB influence on the sensitivity to and motivation for several drugs of abuse.

7 Overview of Drug Dependence, Addiction, and Withdrawal

The transition from intermittent and controlled drug use to compulsive forms of drug seeking and drug taking that characterize addiction is influenced by a number of factors. Substantial evidence implicates genetic influences in the development of substance use disorders and pathological forms of eating, sexual behavior, and gambling (Ducci and Goldman 2012). A crucial role for epigenetic mechanisms driving lasting changes in addiction-related gene expression is also increasingly recognized (Nestler 2014). Additionally, long-term drug exposure induces lasting neuroadaptations that alter the motivational mechanisms that propel drug seeking and use. Although initial drug use is motivated by the hedonic effects of drug consumption, prolonged drug exposure results in progressive blunting of reward system function that can motivate consumption of increasingly larger amounts of drug. Evidence suggests this results in part from decreased function of the mesolimbic DA system and its output to the ventral pallidum. Escalated frequency and amount of drug consumption lead to a dependent state wherein negative affective symptoms emerge during drug abstinence (e.g., dysphoria, anxiety, irritability). These negative emotional states arise from the recruitment of stress-signaling systems within the extended amygdala (such as corticotropin-releasing factor and dynorphin) and dysregulation of systems that constrains these responses (such as neuropeptide Y and nociceptin) (Koob and Volkow 2010). Renewed drug consumption alleviates these negative affective states, and this is conceptualized to contribute to compulsive drug use through negative reinforcement mechanisms

(Koob and Volkow 2010). Superimposed on these processes is a dysregulation of corticostriatal mechanisms contributing to stimulus response learning, conditioned reinforcement, and incentive motivation resulting in a narrowed focus on drug seeking at the expense of natural rewards (Kalivas and Volkow 2005; Everitt et al. 2008). eCBs exert prominent modulatory influence in the extended amygdala and corticostriatal circuits implicated in the etiology of addiction, and as described below, increasing evidence suggests that preexisting genetic influences on the ECS and/or drug-induced dysregulation of eCB function may participate in the development and maintenance of addiction. The following sections consider the consequences of chronic drug exposure on eCB signaling within the reward circuitry and related disruptions in synaptic plasticity, affective state, and learning and memory mechanisms related to extinction and relapse.

8 Evidence of Altered ECS Function Following Chronic Drug Exposure

It stands to reason that chronic *Cannabis* use induces dysregulation of brain cannabinoid receptors' availability and function. In vivo PET imaging studies in humans have revealed downregulation of brain CB₁R binding that correlates with the duration of prior *Cannabis* use, with particularly robust decrements observed in the temporal lobe, anterior and posterior cingulate cortex, and nucleus accumbens (Hirvonen et al. 2013; Ceccarini et al. 2015). These decrements in CB₁R availability appear to progressively recover with prolonged *Cannabis* abstinence. A similar profile of decreased brain CB₁R function and posttreatment recovery has been consistently reported in rodents given chronic CB₁R agonist exposure (Breivogel et al. 1999; Sim et al. 1996). Recent experiments employing stochastic optical reconstruction microscopy (STORM) demonstrate that chronic exposure to clinically relevant doses of Δ^9 -THC results in a startling loss of CB₁R on terminals of perisomatically projecting GABA interneurons in the mouse hippocampus and internalization of the remaining CB₁R (Dudok et al. 2015). The resulting deficits in inhibitory CB₁R control over hippocampal GABA release persisted during several weeks of Δ^9 -THC abstinence, and this may underlie the enduring loss of hippocampal LTP in rodents and memory deficits in humans evident following chronic cannabinoid exposure (Puighermanal et al. 2012). Surprisingly little is known of the effect of chronic cannabinoid exposure on other facets of ECS function. Chronic cannabinoid exposure increases enzymatic clearance of AEA and reduces brain tissue AEA content in rodents (Di Marzo et al. 2000; Schlosburg et al. 2009), and frequent *Cannabis* smokers present decreased AEA and increased 2-AG levels in the blood (Leweke et al. 2007; Morgan et al. 2013), though increased serum AEA levels are evident following at least 6 months of *Cannabis* abstinence (Muhl et al. 2014). The contribution of these disruptions to *Cannabis* use disorder and related physiological and behavioral disruptions is presently

unexplored. However, as discussed below, eCBs provide important homeostatic constraint over emotional state (Lutz 2009) and sleep function (Murillo-Rodriguez et al. 2011), and it's conceivable that Δ^9 -THC-induced impairment of eCB signaling contributes to negative emotional states and sleep disturbances present during protracted *Cannabis* abstinence (Budney et al. 2001; Gates et al. 2015).

Chronic exposure to non-cannabinoid drugs also disrupts eCB signaling and processing. Chronic alcohol exposure in rodents alters eCB-related gene expression in a manner sensitive to the intermittent nature of alcohol exposure and post-alcohol abstinence period (Serrano et al. 2012) and downregulates CB₁R expression and function (Mitrirattanakul et al. 2007; Ceccarini et al. 2013). Postmortem studies of alcohol-dependent humans also demonstrate disrupted CB₁R expression in the ventral striatum and cortical regions (Vinod et al. 2010), and in vivo imaging studies demonstrate decreased CB₁R availability in heavy drinking alcoholics that persist for at least 1 month of abstinence (Hirvonen et al. 2013; Ceccarini et al. 2014) (but see Neumeister et al. (2012)). Although a potential contribution of *CNR1* gene variants to these observations cannot be excluded, a common interpretation based on animal studies is that these CB₁R adaptations in alcoholic humans are a consequence of prolonged alcohol-induced increases in brain eCB levels. This is supported by evidence of transient recovery (and perhaps eventual upregulation) of CB₁R function in humans during protracted alcohol abstinence (Vinod et al. 2006; Mitrirattanakul et al. 2007). In rodents, chronic nicotine exposure induces distinct age-related disruptions in CB₁R binding, with increased levels evident in the PFC, VTA, and hippocampus of adolescent but not adult rats (Werling et al. 2009), and increased hippocampal and decreased striatal CB₁R binding in adult rats during protracted nicotine abstinence (Marco et al. 2007). Few studies have investigated altered CB₁R binding following chronic opiate or psychostimulant exposure, but findings in rodents implicate impaired CB₁R function in the development and expression of opiate dependence (Rubino et al. 1997; Cichewicz et al. 2001) and demonstrate that chronic cocaine increases CB₁R binding in dorsal striatum, NAc, and cortical areas (Adamczyk et al. 2012a). Interestingly, detoxified cocaine addicts present significant increases in plasma AEA and decreases in plasma 2-AG content (Pavon et al. 2013), but the functional consequence of these disturbances is not known. Overall, accruing data suggests that long-term exposure to a variety of drug classes compromises eCB processing and CB₁R expression and function. As discussed below, these perturbations may contribute to aberrant neural signaling during acute and protracted drug abstinence.

9 eCB Influence on Physical Withdrawal Symptoms

In dependent individuals, drug abstinence is associated with transient physical or somatic symptoms that can persist for several days. The intensity of these symptoms varies between drug classes, with severe somatic withdrawal signs evident during abstinence from opiates and alcohol and substantially less severe symptoms

during abstinence from nicotine, cocaine, and cannabinoids (West and Gossop 1994). Early conceptualizations of addiction are focused on the relief from somatic withdrawal symptoms as the motivational basis for drug use by dependent individuals (Wikler 1948; Dole 1965; Dole et al. 1966), though more recent evidence suggests that alleviation of somatic withdrawal is not a major factor contributing to relapse (Heilig et al. 2010; Hershon 1977). In fact the greatest susceptibility to drug relapse typically occurs well after the abatement of somatic withdrawal symptoms. Nonetheless, there is value in understanding the mechanisms contributing to somatic withdrawal symptoms for the development of palliative therapies for dependent individuals and avoidance of medically serious conditions associated with acute drug detoxification.

Chronic alcohol exposure results in significant downregulation of CB₁ receptor expression and function in many brain regions (Basavarajappa et al. 1998; Basavarajappa and Hungund 1999; Vinod et al. 2006, 2010; Moranta et al. 2006; Mitrirattanakul et al. 2007; Ceccarini et al. 2013, 2014; Hirvonen et al. 2013; Neumeister et al. 2012) and leads to disruptions in brain tissue AEA and 2-AG content (Vinod et al. 2006, 2012; Gonzalez et al. 2002, 2004b; Malinen et al. 2009; Rubio et al. 2007). Clinical research has revealed a correlation between the severity of alcohol withdrawal symptoms and polymorphisms in the gene encoding CB₁ receptors (Schmidt et al. 2002), and alcohol withdrawal severity is increased in CB₁ receptor knockout mice (Naassila et al. 2004). Accordingly, it is possible that impaired CB₁ signaling contributes to the intensity of physical symptoms of alcohol withdrawal. In this regard, it is notable that FAAH knockout mice exhibit significantly diminished alcohol withdrawal-related seizures relative to wild-type mice (Vinod et al. 2008; Blednov et al. 2007). The somatic symptoms of alcohol withdrawal, including seizure activity, result primarily from excessive excitatory glutamate signaling (De Witte et al. 2003). Interestingly, AEA modulates NMDA receptor function (Hampson et al. 1998) and reduces NMDA- and electroshock-induced seizures (Hayase et al. 2001; Wallace et al. 2002). Therefore, enhancement of AEA tone may provide protection against alcohol withdrawal-related seizures, though more research is necessary to support this possibility.

Chronic nicotine exposure does not appear to alter brain CB₁ receptor expression (Gonzalez et al. 2002), and precipitated somatic withdrawal signs in nicotine-dependent mice are not altered by CB₁ receptor deletion or moderate doses of the CB₁ receptor antagonist SR141716A (Castane et al. 2002, 2005; Cossu et al. 2001; Balerio et al. 2004; Merritt et al. 2008). In contrast, the Manzanares group observed that somatic indices of nicotine withdrawal are absent in CB₂ knockout mice and are attenuated in WT mice by treatment with the CB₂ antagonist AM630 (Navarrete et al. 2013), though no genotypic differences in somatic withdrawal were evident following a shorter duration of nicotine exposure (7 days vs. 14 days) (Ignatowska-Jankowska et al. 2013). This suggests a potential CB₂ influence in the somatic symptoms of withdrawal following long-term nicotine exposure. Acute Δ^9 -THC administration decreases somatic symptoms of nicotine withdrawal (Balerio et al. 2004, 2006). Moreover, nicotine-dependent MAGL knockout mice exhibit diminished precipitated withdrawal signs, and the MAGL inhibitor JZL184

dose-dependently reduces somatic and aversive signs of precipitated nicotine withdrawal in dependent mice through a CB₁ receptor-dependent mechanism (Muldoon et al. 2015). This same study also points to an association between MAGL gene polymorphisms and the severity of nicotine withdrawal symptoms in humans. In contrast, acute FAAH inhibition does not diminish somatic nicotine withdrawal symptoms in rats (Cippitelli et al. 2011), while somatic symptoms of withdrawal are worsened in mice by FAAH inhibition, though this may occur through non-CB₁ mechanisms (Merritt et al. 2008). Collectively these findings suggest that enhanced CB₁ receptor signaling resulting from MAGL inhibition may provide therapeutic benefit for the somatic symptoms of nicotine withdrawal.

Chronic opiate exposure alters CB₁ receptor expression and function (Rubino et al. 1997; Cichewicz et al. 2001; Smith et al. 2007), and this appears to play a role in the development and expression of opiate dependence. For example, the somatic symptoms of opiate withdrawal are significantly reduced in CB₁ knockout mice or WT mice receiving chronic CB₁ antagonist treatment concurrent with chronic morphine administration (Ledent et al. 1999; Lichtman et al. 2001; Mas-Nieto et al. 2001; Rubino et al. 2000), and it has long been recognized that Δ^9 -THC effectively reduces the intensity of somatic symptoms of opiate withdrawal (Bhargava 1976; Hine et al. 1975). These findings suggest that chronic opiate exposure dysregulates CB₁ receptor or eCB function in a manner that contributes to elicitation of somatic symptoms during drug withdrawal. Consistent with this hypothesis, administration of Δ^9 -THC, exogenous AEA, or exogenous 2-AG attenuates the intensity of somatic withdrawal symptoms (Vela et al. 1995; Yamaguchi et al. 2001; Gamage et al. 2015), and high doses of the selective MAGL inhibitor JZL184 significantly reduce all indices of somatic withdrawal in opiate-dependent mice, while the selective FAAH inhibitor PF-3845 reduces only a subset of withdrawal responses (Ramesh et al. 2011, 2013; Gamage et al. 2015). Perhaps more importantly, a novel dual inhibitor exhibiting >100-fold greater potency at FAAH vs. MAGL (SA-57) (Niphakis et al. 2012) significantly reduces somatic indices of precipitated withdrawal (Gamage et al. 2015). This is an important observation given that high-dose JZL184 exposure elicits some cannabimimetic effects (Long et al. 2009a), and repeated high-dose JZL184 administration induces functional CB₁ receptor tolerance and results in cannabinoid dependence (Schlosburg et al. 2010); these effects may limit the clinical viability of selective MAGL inhibition for treatment of opiate withdrawal. In contrast, SA-57 doses that efficaciously reduce opiate withdrawal do not produce cannabimimetic effects (Ramesh et al. 2013), and the moderate MAGL inhibition induced by this compound is not likely to induce CB₁ receptor downregulation or cannabinoid dependence (Kinsey et al. 2013; Ghosh et al. 2013). However, a caveat to these findings is that while SA-57 attenuates somatic aspects of withdrawal, its administration does not prevent the aversive aspects of withdrawal as measured by the conditioned place aversion assay in mice (Gamage et al. 2015). Nonetheless, these collective findings suggest that modest pharmacological enhancement of eCB signaling may provide palliative effects for the physical symptoms of opiate withdrawal.

10 Stress Responsivity

Substantial clinical and preclinical data indicate that various forms of stress are involved in the etiology and maintenance of addiction. High levels of stress often precede the development of substance use disorders (Jose et al. 2000; Richman et al. 1996; Rospenda et al. 2000), and stress-related increases in glucocorticoid signaling increases the acquisition of drug self-administration by rodents (Goeders 2002; Goeders and Guerin 1996; Koob and Kreek 2007; Vengeliene et al. 2003). Consistently, evidence suggests that both stress- and drug-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis sensitizes the function of reward pathways through glucocorticoid mechanisms (Piazza and Le Moal 1998; Rouge-Pont et al. 1995, 1998; Tidey and Miczek 1997). However, long-term drug use results in a blunting of reward system function, increased influence of CNS stress systems, and dysregulation of the HPA axis that is theorized to induce an allostatic shift in motivational influence that propels an escalation of drug consumption (Koob and Kreek 2007; Le Moal 2009; Koob and Le Moal 1997).

It is now well established that eCB signaling serves a homeostatic role in the constraint of HPA axis activation (Cota 2008; Gorzalka et al. 2008; Morena et al. 2016; also see chapter “Endocannabinoids, Stress, and Negative Affect” that details this function in greater depth). Stress-induced glucocorticoid secretion increases eCB production in several regions implicated in drug dependence and addiction including the amygdala, hippocampus, PFC, hypothalamic nuclei, and periaqueductal gray (Di et al. 2003, 2005, 2009; Hill et al. 2010a; Hohmann et al. 2005; Patel et al. 2004, 2005). Strong evidence demonstrates that eCB-mediated CB₁ activation in these and other regions constrains stress-induced HPA axis activation and resultant increases in glucocorticoid secretion (Barna et al. 2004; Cota et al. 2007; Di et al. 2003; Manzanares et al. 1999; Patel et al. 2004; Uriguen et al. 2004; Wade et al. 2006). In contrast, stress-induced reductions in AEA content are consistently observed in the amygdala, PFC, and hippocampus (Hill et al. 2008, 2009; Patel et al. 2005; Rademacher and Hillard 2007), and this is theorized to contribute to stress responsivity through a release of tonic HPA axis inhibition (Patel et al. 2004). Regardless of the specific mechanism that prevails, it is clear that eCB signaling participates in the constraint of HPA axis activation and may contribute to the termination of stress responses.

In this regard, it is conceivable that disrupted eCB-mediated plasticity induced by long-term drug exposure contributes to a persistent dysregulation of HPA axis function and increases stress sensitivity associated with addiction (Koob and Kreek 2007; Le Moal 2009; Koob and Le Moal 1997). Because stress exposure is implicated in the development of addiction (Goeders 2002; Goeders and Guerin 1996; Jose et al. 2000; Koob and Kreek 2007; Richman et al. 1996; Rospenda et al. 2000; Vengeliene et al. 2003), it is notable that stress can alter eCB-mediated plasticity in addiction-related brain regions (Natividad et al. 2017). For example, prolonged stress exposure impairs eCB-mediated DSI, LTD, and fEPSPs in the NAc (Wang et al. 2010), attenuates eCB-mediated DSE and DSI in the PVN

(Wamsteeker et al. 2010), and enhances 2-AG-mediated DSI in the basolateral amygdala (Patel et al. 2009). Similarly, establishment of conditioned fear increases the efficacy of DSE and DSI in the central nucleus of the amygdala (Kamprath et al. 2011).

11 Affective Disruptions

In addition to transient somatic symptoms (West and Gossop 1994), withdrawal from most drugs of abuse is associated with increased negative affective symptoms such as anxiety and depression (Alling et al. 1982; Coffey et al. 2000; Janiri et al. 2005; Nunes and Levin 2004; Nunes et al. 2004). Withdrawal-related negative affective states can persist for months and in some cases years during protracted abstinence, and the severity of these symptoms is closely associated with susceptibility to relapse (Annis et al. 1998; Miller and Harris 2000). Moreover, there is a prevalent comorbidity between affective disorders and substance use disorders, and affective disruption may be an antecedent to addiction (Bruijnzeel et al. 2004; Castle 2008; Conway et al. 2006; Pani et al. 2010; Schuckit 2006). As discussed in detail in chapter “Lipid Mediators in the Regulation of Emotions, Memory and Cognitive Functions” of this book, substantial evidence implicates the ECS in the regulation of affective state, and dysfunctional eCB signaling is associated with increased anxiety and depression. In light of the evidence for drug-induced changes in brain eCB levels and eCB-mediated synaptic regulation, it is conceivable that dysregulated eCB function contributes to affective disturbances associated with drug dependence and protracted withdrawal. Several studies have begun to investigate an involvement of eCB signaling in withdrawal-related affective disruptions (or the efficacy of eCB manipulations as treatments for these disruptions), and as described below, these studies have predominantly focused on anxiety-like behavior that accompanies withdrawal from chronic exposure to drugs of abuse and highly palatable food.

In tobacco-dependent individuals, smoking cessation leads to withdrawal symptoms that persist for several months (Hughes 2007; Hughes et al. 1991; Markou 2008). Symptoms such as dysphoria/aversion, anxiety, and irritability contribute to negative reinforcement mechanisms that perpetuate nicotine addiction (Hughes et al. 1991; Koob and Volkow 2010; Piper et al. 2011; Allen et al. 2008; Rose et al. 2010), and the severity of these symptoms is a predictor of increased relapse risk (Piasecki et al. 2003a, b, c; al’Absi et al. 2004). Because eCB signaling is implicated in the initiation and maintenance of nicotine self-administration, it is possible that persistent disruptions in eCB signaling contribute to the negative affective states of nicotine withdrawal. However, few studies have investigated this possibility.

The Maldonado group reported that CB₁ knockout mice exhibit more robust anxiety-like behavior during nicotine withdrawal than similarly treated wild-type mice do (Bura et al. 2010). However, nicotine-naïve CB₁ knockout mice also

exhibit greater anxiety-like behavior than wild types (Bura et al. 2010; Haller et al. 2002; Martin et al. 2002; Uriguen et al. 2004), and this innate phenotype clouds the determination as to whether or not withdrawal-related anxiety-like behavior is exacerbated by CB₁ receptor deletion. In rats, significant increases in anxiety-like behavior are evident after 36 h of spontaneous nicotine withdrawal, and this is dose-dependently reversed by treatment with the FAAH inhibitor URB597 at doses that do not alter anxiety-like behavior in nicotine-naïve rats (Cippitelli et al. 2011). This is temporally correlated with significant increases in AEA content in the amygdala, hypothalamus, and prefrontal cortical tissue, though no significant alterations in tissue 2-AG content are evident regardless of region analyzed (Cippitelli et al. 2011). It is somewhat surprising that FAAH inhibition ameliorates anxiety-like behavior at a time when AEA levels appear to be increased in brain regions known to modulate anxiety-like behaviors. However, while increased AEA content in these regions may reflect a compensatory response to withdrawal, it is possible the effect magnitude is insufficient to fully constrain withdrawal-related disturbances underlying increased anxiety. Thus, the bolstering of this response through FAAH inhibition provides more efficacious reversal of withdrawal-related neural dysfunction (Cippitelli et al. 2011). It is worth noting, however, that disruptions in postmortem tissue AEA content may not provide a precise index of signaling-competent AEA levels in vivo (Buczynski and Parsons 2010), and a more clear in vivo view of withdrawal-related disruptions in regional eCBs may require further study. Nonetheless, these findings provide compelling evidence that acute FAAH inhibition ameliorates increased anxiety-like states during protracted nicotine withdrawal.

The putative eCB uptake inhibitor AM404 has been found to attenuate nicotine withdrawal-related increases in immobility in the Porsolt forced swim test (Mannucci et al. 2011). Increased immobility in this test is often interpreted as a form of despair or hopelessness, and this paradigm is commonly used as a screen for antidepressant efficacy. Accordingly, withdrawal-related increases in immobility in the Porsolt test may reflect a form of depressive-like behavior, and the effects of AM404 may thus reflect a beneficial effect of enhanced eCB signaling on withdrawal-related depressive-like states (Mannucci et al. 2011). However, AM404 also dose-dependently reduced immobility in nicotine-naïve mice in this study, and as such a preferential influence of this eCB clearance inhibitor on withdrawal-related behaviors is unclear. Moreover, modeling depressive-like behavior in rodents is best accomplished through the use of several paradigms (Abelaira et al. 2013; Yan et al. 2010), and further work is clearly needed to evaluate possible eCB influences on nicotine withdrawal-related depression-like behavior.

Post-traumatic stress disorder (PTSD) is an anxiety disorder with particularly high prevalence among individuals with alcohol use disorders (Kessler et al. 1995). The fear-potentiated startle (FPS) paradigm is often used to model PTSD-like behaviors (Grillon 2002; Hijzen et al. 1995). Rodent lines selectively bred for high alcohol consumption exhibit significantly greater FPS than corollary lines bred for low alcohol consumption (McKinzie et al. 2000; Barrenha and Chester

2007), and these rodent lines may thus provide models for characterizing mechanisms contributing to the anxiety, alcohol use disorders, and intersection of these pathologies. In this regard, the nonselective FAAH inhibitor LY2183240 selectively reduces FPS in high alcohol-preferring mice but not low alcohol-preferring mice (Powers et al. 2010). However, LY2183240 effects on FPS were evident only following repeated FPS testing and not upon first exposure to the FPS paradigm, suggesting that FAAH inhibition facilitates the extinction of learned fear responses in high alcohol-preferring mice, consistent with substantial evidence that FAAH inhibition generally accelerates extinction of aversive memories (Gunduz-Cinar et al. 2013). LY2183240 also enhanced the expression of alcohol-induced conditioned place preference without altering alcohol consumption itself. This suggests that FAAH inhibition influences memory-related processes regulating the expression of conditioned fear and conditioned alcohol reward in animals genetically predisposed toward high alcohol consumption. Recent evidence also points to the efficacy of the more selective FAAH inhibitor URB597 for attenuating anxiety-like behavior evident in rats following acute, high-dose alcohol administration (Cippitelli et al. 2008). In this study, URB597 did not alter alcohol self-administration, cue-induced alcohol-seeking, or stress-induced alcohol-seeking behavior. Collectively, these findings suggest that FAAH inhibition may be beneficial for reducing alcohol-related anxiety-like behavior, though further investigation is warranted, particularly evaluations of persistent anxiety-like behavior associated with protracted withdrawal in alcohol-dependent subjects.

In humans, the chronic use of the NMDA glutamate receptor antagonist phencyclidine (PCP) evokes both positive and negative symptoms of schizophrenia and many of the characteristic cognitive deficits associated with the disease (Murray 2002). In rats, withdrawal from sub-chronic PCP exposure is associated with reduced social interaction (Lee et al. 2005; Seillier and Giuffrida 2009; Seillier et al. 2013), a phenotype often interpreted as an anxiety-like behavior (Lapiz-Bluhm et al. 2008) and reminiscent of negative symptoms of schizophrenia. PCP withdrawal is associated with reduced AEA content in the amygdala and prefrontal cortex (Seillier and Giuffrida 2009; Seillier et al. 2013), and withdrawal-related reductions in social interaction are reversed by the FAAH inhibitor URB597 through a CB₁-reliant mechanism (Seillier and Giuffrida 2009; Seillier et al. 2013).

Similar to drug addiction, eating disorders and pathological obesity are conceptualized as chronic relapsing conditions with alternating periods of abstinence (e.g., dieting) and relapse (compulsive overeating) (Corwin and Grigson 2009; Cottone et al. 2009a; Epstein and Shaham 2010; Johnson and Kenny 2010; Parylak et al. 2011). Rats given scheduled intermittent access to highly palatable sucrose food exhibit a withdrawal state upon forced abstinence from the palatable food, including increased anxiety-like behavior and decreased motivation for standard lab food (Cottone et al. 2008, 2009a, b). These effects are most evident during early abstinence and abate within 2–3 days. Moreover, these animals exhibit robust excessive food consumption of the palatable food when given renewed access. The negative affective state during abstinence and the excessive consumption upon renewed access to palatable food are each mediated by increased CRF1 signaling in

the amygdala (Iemolo et al. 2013), similar to withdrawal-related negative affective states and compulsive drug consumption evident in drug-dependent subjects (Koob et al. 2014; Koob 2010). This suggests that dysregulation of amygdalar signaling is a common factor contributing to pathological motivation for and consumption of food and drugs. Interestingly, 2-AG content is significantly increased in the CeA following 4 days' abstinence from palatable food, and this occurs in conjunction with increased CeA CB₁ mRNA and CB₁ protein expression (Blasio et al. 2013). Moreover, either systemic or intra-CeA administration of the CB₁ antagonist rimonabant precipitates an anxiogenic-like state in animals with a history of palatable food consumption but not in rats given only with standard lab chow. This suggests that increased 2-AG-mediated CB₁ signaling in CeA counteracts a withdrawal-like state in rats during abstinence from palatable food (Blasio et al. 2013).

12 Synaptic Plasticity

Substantial evidence demonstrates that exposure to most drugs of abuse dysregulates synaptic plasticity mechanisms in a variety of brain regions involved in the evolution of addiction (Hyman et al. 2006; Luscher and Malenka 2011). Acute exposure to alcohol, nicotine, morphine, or cocaine induces transient loss of LTP at inhibitory GABAergic synapses in the VTA (Niehaus et al. 2010; Liu et al. 2005; Melis et al. 2002; Nugent et al. 2007) and enhanced excitatory strength onto VTA DA neurons (Borgland et al. 2004; Faleiro et al. 2004; Saal et al. 2003). This tandem potentiation of excitatory transmission and loss of inhibitory LTP at VTA synapses serves to heighten midbrain DA cell excitability, increasing dopaminergic signaling throughout the mesocorticolimbic system.

As described in detail in the chapter titled "Endocannabinoid-Dependent Synaptic Plasticity in Striatum", eCBs and CB₁ receptors are implicated in several forms of synaptic plasticity involving presynaptic expression mechanisms (Lovinger 2008; Heifets and Castillo 2009). Both short- and long-term forms of eCB-mediated synaptic plasticity occur in many brain regions critically involved in various stages of the addiction cycle including the NAc, VTA, amygdala, prefrontal cortex, hippocampus, and dorsal striatum. Accordingly, drug-induced disruption of these mechanisms may contribute to aberrations in learning, memory, and affective state that propel addiction. Although a full description of these processes is provided in "Endocannabinoid-Dependent Synaptic Plasticity in Striatum", it is worth noting here that exposure to several classes of abused drugs dysregulates eCB-mediated forms of synaptic plasticity. For example, eCB-mediated LTD of excitatory and inhibitory signaling in the rodent NAc and hippocampus is abolished following a 7-day treatment with either Δ^9 -THC or the synthetic CB₁ agonist WIN 55,212-2 (Hoffman et al. 2003; Mato et al. 2004), correlating with decreased sensitivity to CB₁ inhibition of both excitatory and inhibitory signaling.

Both acute and chronic alcohol exposure substantially reduces a CB₁-dependent form of plasticity that results in long-lasting disinhibition of striatal output neurons (Clarke and Adermark 2010; Adermark et al. 2011) and reduces eCB-mediated LTD at inhibitory striatal synapses (though alcohol-induced disruption of eCB-LTD at excitatory synapses has not been observed) (Clarke and Adermark 2010). Because the dorsal striatum is involved in reward-guided learning and habitual behavior (Volkow et al. 2007; Yin et al. 2008), it is possible that these alcohol-induced disruptions in eCB-LTD contribute to maladaptive habitual behavior that contributes to addiction. Consistent with this hypothesis, recent work by the Lovinger lab demonstrates that chronic and intermittent alcohol exposure increases 2-AG levels in the dorsolateral striatum (DLS), leading to a compensatory downregulation of CB₁ receptor signaling and loss of CB₁-mediated LTD at excitatory synapses (DePoy et al. 2013). This was associated with enhancement of DLS-mediated learning processes that may contribute to habitual behaviors.

Cocaine exposure results in the loss of eCB-LTD of evoked excitatory transmission in the NAc (Fourgeaud et al. 2004) and facilitated eCB-LTD of GABAergic signaling at VTA DA synapses (Pan et al. 2008; Fourgeaud et al. 2004; Liu et al. 2005). Collectively, these cocaine-induced disruptions in eCB-LTD result in imbalanced mesolimbic DA function characterized by diminished inhibitory control over VTA DA cell bodies and heightened excitatory signaling in the NAc. Chronic cocaine exposure also results in disruption of eCB-LTD of excitatory transmission in the bed nucleus of the stria terminalis (BNST) (Grueter et al. 2006), a stress-responsive structure wherein excitatory transmission is critically involved in mediating stress-reward interactions and anxiety-like behavior (Delfs et al. 2000; McElligott and Winder 2009). The BNST sends substantial projections to the VTA, and accordingly disruption of BNST plasticity likely influences motivational responses to stress.

Little is known regarding the potential influence of opiate or nicotine exposure on eCB-mediated forms of synaptic plasticity. However, a recent report demonstrated that a history of nicotine self-administration induces CB₁-dependent LTP at synapses of infralimbic cortex afferents to the BNST (Reisiger et al. 2014). This perturbation may have relevance to many consequences of chronic nicotine exposure, and clear evidence is provided that this neuroplastic change contributes to cue-induced reinstatement of nicotine-seeking behavior (in an animal model of relapse).

As noted above, stress is implicated in the development of addiction (Koob and Kreek 2007), and it is therefore notable that stress can alter eCB-mediated plasticity in addiction-related brain regions. Prolonged exposure to unpredictable stress impairs eCB-mediated DSI, LTD, and fEPSPs in the NAc (Wang et al. 2010), while repeated restraint stress reduces eCB-mediated DSE and DSI in the rat PVN (Wamsteeker et al. 2010) and increases 2-AG-mediated DSI in the mouse basolateral amygdala (Patel et al. 2009). Similarly, establishment of conditioned fear increases the efficacy of eCB-mediated DSE and DSI in the central nucleus of the amygdala (Kamprath et al. 2011). Further, it has recently been shown that acute restraint stress induces a switch from eCB-mediated LTD to eCB-mediated LTP at

synapses of mPFC afferents to the BNST (Glangetas et al. 2013). As previously discussed, the ECS participates in a negative feedback system that limits the expression of anxiety under stressful circumstances and contributes to the suppression of aversive memories. These processes are mediated in part through eCB-mediated synaptic plasticity in the amygdalar nuclei (Kamprath et al. 2011; Lafenetre et al. 2007; Viveros et al. 2007), and disruptions of this eCB-mediated plasticity may contribute to its dysregulated affect (including increased anxiety) associated with protracted drug abstinence.

13 Extinction and Relapse

The ECS plays a prominent role in learning and memory processes (Hashimoto et al. 2007; Heifets and Castillo 2009; Marsicano and Lafenetre 2009), and because eCB signaling is disrupted by most drugs of abuse (see above sections), a role for the ECS in learning and memory components of addiction may be hypothesized. As previously reviewed, CB₁ receptors play an important role in the conditioned rewarding effects of alcohol (Houchi et al. 2005; Hungund et al. 2003; Thanos et al. 2005), opiates (Chaperon et al. 1998; Martin et al. 2000; Navarro et al. 2001; Singh et al. 2004), and nicotine (Castane et al. 2002; Forget et al. 2005, 2006; Le Foll and Goldberg 2004; Merritt et al. 2008), with a lesser CB₁ influence reported for the conditioning effects of psychostimulants (Houchi et al. 2005; Martin et al. 2000). Although these behaviors are generally interpreted in the context of drug reward, a CB₁ receptor influence on the associative learning aspects of drug exposure is also likely, which as discussed below may have relevance to the persistent reactivity to drug-related memories that characterize addiction.

13.1 Drug Seeking (Relapse)

Drug exposure produces powerful interoceptive effects, and memory of these effects increases the likelihood of continued drug use. With continued drug use, these interoceptive effects become associated with environmental cues to the extent where these drug-associated cues alone can induce craving and thereby propel drug use. This form of drug-related memory is also causal in relapse to drug use following periods of abstinence (Carter and Tiffany 1999; McLellan et al. 2000). Several factors are believed to be causal in drug relapse including craving induced by environments or situations previously associated with drug use (e.g., conditioning factors), acute exposure to the drug itself or a pharmacologically related agent during abstinence (e.g., drug priming), and stressful events.

Recently developed animal models of drug seeking demonstrate an important influence of cannabinoid signaling in the reinstatement of extinguished drug-seeking and drug-taking behaviors. For example, Δ^9 -THC and various synthetic

CB₁ agonists reinstate drug seeking for cannabinoids (Justinova et al. 2008b; Spano et al. 2004), opioids (De Vries et al. 2003; Fattore et al. 2003, 2005), ethanol (Lopez-Moreno et al. 2004; McGregor et al. 2005), nicotine (Biala and Budzynska 2008; Gamaledin et al. 2012a), and cocaine (De Vries et al. 2001). Conversely, CB₁ receptor antagonism attenuates drug-seeking behavior associated with a variety of abused substances. Rimobant (SR141716A) significantly reduces operant responding for conditioned cues previously associated with Δ^9 -THC (Justinova et al. 2008b), heroin (De Vries et al. 2003; Fattore et al. 2003, 2005), nicotine (Cohen et al. 2005; De Vries et al. 2005; Diergaarde et al. 2008), and ethanol (Cippitelli et al. 2005; Economidou et al. 2006). CB₁ antagonism also reduces excessive ethanol intake exhibited following periods of abstinence (an animal model of relapse-like alcohol consumption) (Gessa et al. 2005), and the novel CB₁ antagonist SLV330 reduces both drug- and cue-induced reinstatement of ethanol- and nicotine-seeking behavior (de Bruin et al. 2011). Studies employing site-specific antagonist infusions have uncovered important contributions of CB₁ receptors in the PFC and NAc shell in cue-induced reinstatement of both heroin- and nicotine-seeking behavior, and CB₁ receptors in the BLA appear to contribute to cue-induced nicotine-seeking, but not heroin-seeking, behavior (Alvarez-Jaimes et al. 2008; Kudas et al. 2007). Although CB₁ receptor inactivation produces subtle and inconsistent effects on psychostimulant self-administration as compared with other drugs of abuse, substantial evidence indicates a strong CB₁ receptor influence on the reinstatement of psychostimulant-seeking behavior. CB₁ antagonism attenuates both drug-primed and cue-induced reinstatement of cocaine-seeking (De Vries et al. 2001; Filip et al. 2006; Xi et al. 2006; Adamczyk et al. 2012b) and methamphetamine-seeking behavior in rats (Anggadiredja et al. 2004; Boctor et al. 2007). Further, both drug- and cue-induced reinstatement of cocaine and methamphetamine seeking is attenuated by the negative allosteric CB₁ modulator ORG27569 (Jing et al. 2014). Interestingly, although footshock-induced reinstatement of cocaine seeking is not blocked by either rimobant or the CB₁ antagonist AM251 (De Vries et al. 2001; Kupferschmidt et al. 2012a), AM251 blocks the reinstatement of cocaine seeking induced by forced swim stress and exogenous CRF administration (Vaughn et al. 2012; Kupferschmidt et al. 2012a), suggesting a CB₁ influence on some forms of stress-induced reinstatement of drug seeking. These latter findings may relate specifically to an AM251-induced modulation of the effects produced by the stress peptide CRF, given that AM251 also attenuates the anxiogenic effects of both cocaine withdrawal (an effect mediated by increased CRF signaling (DeVries and Pert 1998)) and exogenously administered CRF (Kupferschmidt et al. 2012b). However, the mechanisms for this putative interaction are not clear, and these findings are at odds with evidence that CB₁ signaling generally suppresses the physiological and behavioral responses to stress (see discussion below).

Thus, CB₁ receptor signaling is implicated in drug-seeking behavior for a variety of abused substances that differ substantially in pharmacodynamic mechanisms including cannabinoids, opioids, alcohol, nicotine, and psychostimulants. Moreover, CB₁ receptor antagonism blocks both cue- and “prime”-induced

reinstatements of seeking behavior for nondrug rewards such as sucrose and corn oil ((De Vries et al. 2005; Ward et al. 2009), but see Xi et al. (2006)). Accordingly, CB₁ receptor signaling appears to participate in the modulation of conditioned reward in general.

Few studies have characterized the influence of eCB signaling on drug-seeking behavior through more direct manipulations of eCB processing such as the inhibition of hydrolytic clearance mechanisms. Three studies have found that both drug-primed and cue-induced nicotine- and cocaine-seeking behaviors are reduced by URB597 and the less selective FAAH inhibitor PMSF (Forget et al. 2009; Scherma et al. 2008; Adamczyk et al. 2009). This is somewhat surprising given that CB₁ receptor stimulation enhances both nicotine- and cocaine-seeking behavior (Biala and Budzynska 2008; De Vries et al. 2001). However, because tonic eCB production is believed to be low, the inhibition of eCB clearance is anticipated to preferentially amplify eCB signaling in circuits/synapses activated by a given stimulus (in this case, drug-seeking behavior), rather than producing more widespread indiscriminate CB₁ activation as is produced by exogenous CB₁ agonists. Moreover, FAAH hydrolyzes a large variety of fatty acid moieties (Ahn et al. 2008), and it is conceivable that the effects of FAAH inhibition on drug seeking are mediated through actions of non-cannabinoid lipids. In this regard, it's notable that the putative eCB uptake inhibitor VDM11 also attenuates both nicotine- and cue-induced nicotine-seeking behavior (Gamaledin et al. 2011). This latter finding is of interest as unlike URB597, VDM11 elevates AEA levels with reduced effects on non-cannabinoid FAAH substrates that may influence behavior through PPAR α signaling (De Petrocellis et al. 2000; van der Stelt et al. 2006). Similar to the effects of VDM11, the putative eCB uptake inhibitor AM404 dose-dependently attenuates both nicotine- and cue-induced nicotine-seeking behavior, though AM404 did not alter nicotine self-administration itself (Gamaledin et al. 2013). In contrast to the effects on nicotine- and cocaine-seeking behavior, neither URB597 nor AM404 alters either cue- or stress-induced reinstatement of alcohol-seeking behavior (Cipitelli et al. 2007; 2008).

Recent data demonstrate that detoxified alcoholics present significantly lower baseline plasma AEA levels as compared with nondependent social drinkers (Mangieri et al. 2009). Further, a significant negative relationship between resting plasma AEA levels and the severity of cue-induced craving was evident in social drinkers but not alcoholics. In social drinkers, alcohol-related cues significantly increased both craving and plasma AEA levels, and the relative magnitude of cue-induced increases in AEA was significantly correlated with the severity of craving. However, cue-induced changes in AEA were indexed by a percentage change from baseline, and thus this index is substantially influenced by baseline AEA levels (e.g., the same absolute change in AEA content will be reflected as a greater percentage change in subjects with lower vs. higher baseline AEA levels). Accordingly, the positive correlation between cue-induced changes in plasma AEA and craving intensity further supports the importance of baseline AEA levels in cue-induced craving in social drinkers. Interestingly, although alcohol-related cues elicited more intense craving in alcoholics vs. social drinkers, alcoholics did not

present significant cue-induced increases in plasma AEA. Based on these observations, it was concluded that cue-induced increases in plasma AEA contributes to interoceptive signaling that moderates the desire for alcohol in social drinkers. It may also be concluded that deficits in plasma AEA levels confer increased susceptibility to cue-induced alcohol craving. While cue-induced elevations in plasma AEA may contribute to craving responses, it is curious that elevations in AEA were not evident in alcoholics who also presented the greatest level of cue-induced craving. It is conceivable that cue-induced increases in plasma AEA reflect an adaptive response, and its absence in alcoholics somehow contributes to excessive craving responses to alcohol-related cues. Further evaluations in humans are clearly needed to investigate these issues.

13.2 Extinction Learning

The potent motivational effects of drug-related cues create substantial difficulties during periods of attempted drug abstinence and are causal in the reinstatement of drug intake (e.g., relapse) (Carter and Tiffany 1999). One approach for lessening the motivational impact of drug-associated cues is through extinction learning, wherein a subject learns that a drug-associated cue no longer has predictive value. However, extinction therapy for addiction is generally ineffective for reducing relapse in both humans (Conklin and Tiffany 2002) and rodents (Crombag and Shaham 2002), and it is conceivable that this results from disruptions in the learning mechanisms that override the original association memory (Bouton 2004; Rescorla 1996). The ECS plays a prominent role in memory extinction, particularly the extinction of aversive memory. Substantial evidence demonstrates that CB₁ signaling blockade results in impaired extinction of cued fear memory, contextual fear memory, and fear-potentiated startle and spatial memory under aversive mildly stressful conditions (Marsicano et al. 2002; Suzuki et al. 2004; Chhatwal et al. 2005, 2009; Niyuhire et al. 2007; Varvel et al. 2005; Varvel and Lichtman 2002; Pamplona et al. 2006, 2008). Moreover, recent findings demonstrate that a line of mice selectively bred for high alcohol consumption exhibits excessive fear-potentiated startle responses and that the nonselective FAAH inhibitor LY2183240 facilitates extinction of fear-potentiated startle in these mice but not mice bred for low alcohol consumption (Powers et al. 2010). This suggests that FAAH inhibition influences memory-related processes regulating the expression of conditioned fear in animals genetically predisposed toward high alcohol consumption. However, it has consistently been reported that CB₁ inactivation does not alter the extinction of behaviors motivated by appetitive and non-aversive memories (Holter et al. 2005; Niyuhire et al. 2007; Ward et al. 2007; Harloe et al. 2008; Hernandez and Cheer 2011). Because aversive memory may be involved in relapse to drug taking (Kaplan et al. 2011; Quirk and Gehlert 2003; Stewart and Kushner 2001), deficient eCB signaling following long-term drug exposure may contribute to the limited efficacy of extinction therapy for addiction.

13.3 Amygdala: Cortical Communication as a Possible Mechanism for eCB Modulation of Drug-Seeking Behavior

Considerable evidence highlights the importance of functional communication between the basolateral amygdala and prefrontal cortex (BLA->PFC) as a mediator of emotionally salient learning, memory, and synaptic plasticity. Distinct subpopulations of neurons in the PFC control the processing of aversive fear-related memory (Laviolette et al. 2005; Lauzon et al. 2009; Laviolette and Grace 2006) and rewarding and appetitive memory (Bishop et al. 2011; Sun et al. 2011), and both of these subpopulations are influenced by CB₁ modulation of BLA inputs (Tan et al. 2014). Growing evidence demonstrates that excessive activation of CB₁ receptors in either the BLA or PFC leads to amplification of aversive emotional information, while deficient CB₁ signaling in these areas blunts the salience of normally aversive events while enhancing the salience of sub-reward threshold stimuli (Tan et al. 2010, 2014; Laviolette and Grace 2006). These processes appear to be mediated through direct interactions with subcortical DAergic motivational systems arising from the VTA, and through these interactions, cortical CB₁ receptors are capable of modulating reward processing, even the switching of emotional valence from normally rewarding conditioned stimuli to aversive stimuli (Ahmad et al. 2013). The specific contribution of these mechanisms in the pathologically elevated motivational salience to drug-predictive cues and/or motivational effects of aversive memory contributing to drug seeking and relapse remains to be characterized (Tan et al. 2014). However, the potential involvement of these mechanisms not only in addiction but also in abnormal emotional learning and memory in other neuropsychiatric conditions is worth noting.

14 Cognitive Function

Impairments in cognitive processing are hallmarks of addiction. The progression from initial, intermittent, controlled drug use to compulsive drug seeking and consumption in the addicted state is conceptualized as a progressive deterioration of executive control over behavior (primarily reliant on frontal-cortical and hippocampal function) and the emergence of habitual or compulsive behaviors that drive drug seeking and drug taking (mediated in large part through dorsal striatal signaling) (Everitt and Robbins 2005; Jentsch and Taylor 1999). Endocannabinoids exert prominent control of frontal-cortical, hippocampal, and striatal synaptic signaling, and growing evidence implicates dysregulated eCB signaling in both aberrant executive function and habit-based behaviors driven by dorsal striatal signaling.

Endocannabinoid signaling regulates neural activity, structural plasticity, and functional output of the medial prefrontal cortex (McLaughlin et al. 2014) and is implicated in several PFC-mediated cognitive functions including attentional set

shifting (Klugmann et al. 2011) and reversal learning in the Morris water maze (Varvel and Lichtman 2002; Lee et al. 2014). CB₁ receptor antagonism or deletion results in shorter and less complex dendrites of neurons in layer II/III of the prelimbic cortex (Hill et al. 2011; Lee et al. 2014) and impairments in cognitive flexibility that resemble behaviors in mPFC-lesioned animals (Lacroix et al. 2002; Lee et al. 2014). Conversely, genetic deletion or pharmacological inhibition of FAAH enhances the acquisition of the Barnes maze task (Wise et al. 2009), enhances memory acquisition in a passive avoidance task (Mazzola et al. 2009), and facilitates acquisition and extinction of a spatial memory task (Varvel et al. 2007). Physical activity increases circulating AEA levels in both humans and rodents (Sparling et al. 2003; Hill et al. 2010b; Raichlen et al. 2013; Heyman et al. 2012), and this may contribute to the beneficial effects of exercise on hippocampal cell proliferation, neurogenesis, and synaptic plasticity (Hill et al. 2010b; Wolf et al. 2010; Madronal et al. 2012). Treadmill running increases spatial working memory through a CB₁-reliant mechanism, and similar working memory enhancement is observed in sedentary animals treated with URB597 (Ferreira-Vieira et al. 2014).

Despite growing evidence for an eCB influence in cognitive functions mediated by the mPFC and hippocampus, few studies have investigated disruptions in these mechanisms following long-term drug exposure. Drug-induced impairments in recognition memory may result from altered hippocampal CB₁ receptor function, and blockade of CB₁ receptors during drug exposure has been shown to prevent deficits in recognition memory produced by chronic morphine, MDMA, and nicotine exposure (Nawata et al. 2010; Vaseghi et al. 2012, 2013; Saravia et al. 2016). Regarding drug-induced disruptions in frontal cortical function, the Woodward lab recently demonstrated that alcohol withdrawal is associated with deficient CB₁-mediated inhibition of GABAergic signaling in layers II/III of the PFC and possible increases in CB₁-mediated inhibition of GABAergic signaling in layers V/VI (Pava and Woodward 2014), suggesting a role for dysregulated CB₁ signaling in altered cortical network activity that may underlie impaired cognitive function in detoxified alcoholics.

15 Food Addiction and Eating Disorders

Food addiction is defined as “a loss of control over food intake,” and growing evidence demonstrates that food and drug addiction have overlapping neuroadaptations in the mesolimbic system (Lutter and Nestler 2009). Indeed, eating disorders such as binge eating disorder (BED), anorexia nervosa (AN), and bulimia nervosa (BN) are hypothesized to derive in part from aberrant brain reward function (Kaye 2008; Marco et al. 2012; Scherma et al. 2014; Stoving et al. 2009). Similar to drug addiction, food addiction is defined in part by compulsive eating, continued aberrant feeding behavior despite negative consequences, and unsuccessful attempts to “normalize” dysfunctional eating (Cassin and von Ranson 2007;

Table 2 Summary of ECS involvement in food intake and eating disorders

Pharmacological manipulation	Healthy animals	Binge eating disorder	Anorexia nervosa
Baseline ECS	↑↓ Energy balance ^a ↑↓ Food intake ↑↓ Hedonic aspects of feeding	↑ AEA levels in blood ^b	↑ AEA levels in blood ^c ↑ CB ₁ mRNA in blood ↑ CB ₁ binding in brain ↓ FAAH activity
CB ₁ R antagonist	↓ Motivation for food ^d ↓ Preference for palatable foods ↓ Hedonic effects of food	↓ Compulsive eating ^e ↑ Weight loss	
CB ₁ R agonist	↑ Motivation for food ^f ↑ Preference for palatable foods ↑ Hedonic effects of food		↑ Weight gain ^g

^aSilvestri and Di Marzo (2013), Melis et al. (2007), Di Marzo et al. (2001), Bermudez-Silva et al. (2012), Kirkham et al. (2002), Hanus et al. (2003), Berridge et al. (2010), Jyotaki et al. (2010), Yoshida et al. (2010), Cervino et al. (2009)

^bMonteleone et al. (2005)

^cMonteleone et al. (2005, 2009), Frieling et al. (2009), Gerard et al. (2011)

^dSimiand et al. (1998), South et al. (2007), Mathes et al. (2008), Chaperon et al. (1998), Gallate and McGregor (1999), Gallate et al. (1999), Solinas and Goldberg (2005), Jarrett et al. (2007), Melis et al. (2007)

^eScherma et al. (2013), Pataky et al. (2013)

^fKirkham and Williams (2001), Farrimond et al. (2011), Kirkham et al. (2002), Williams and Kirkham (1999), Martinez-Gonzalez et al. (2004), Solinas and Goldberg (2005), Guegan et al. (2013), Dipatrizio and Simansky (2008), Shinohara et al. (2009), Higgs et al. (2003), Jarrett et al. (2005, 2007), Mahler et al. (2007), Gallate et al. (1999), Dodd et al. (2009), Anderson-Baker et al. (1979), Jamshidi and Taylor (2001), Verty et al. (2005), Soria-Gomez et al. (2007)

^gAndries et al. (2014)

Gearhardt et al. 2012). The influence of cultural pressures and stress in these disorders is well documented, though there are also strong genetic contributions to these diseases (Kaye 2008; Klump et al. 2001). Furthermore, it is estimated that more than 50% of individuals with BED meet the criteria for food addiction (Cassin and von Ranson 2007; Gearhardt et al. 2012).

Several findings suggest an ECS involvement in eating disorders, including aberrant reward processing associated with these conditions (Table 2). For example, abnormal eating behaviors like self-starvation (in AN) or binge eating (in BN or BED) become rewarding in their own right, and this may be influenced by disrupted eCB function (Monteleone et al. 2012; Cervino et al. 2009). Patients with AN or BED (but not BN) present with increased blood AEA levels as compared with controls, though no changes in blood 2-AG levels are associated with these disorders (Monteleone et al. 2005). Moreover, blood AEA levels are significantly and inversely correlated with plasma leptin levels in women with AN, consistent with the inhibitory influence of leptin on AEA synthesis (Di Marzo et al. 2001) and

leptin deficiency in these eating disorders. It should be noted, however, that although plasma eCB levels provide a potential index of disrupted eCB processing, they do not directly reflect dynamic changes in eCB signaling in the brain or other tissues. A *CNR1* polymorphism is associated with vulnerability to AN (Siegfried et al. 2004) (but see Muller et al. (2008)), CB_1 mRNA levels are increased in the blood of AN and BN patients (Frieling et al. 2009), and increased brain CB_1 binding has been observed in patients with AN and BN (Gerard et al. 2011). Specifically, AN and BN patients exhibit increased CB_1 availability in the bilateral insular cortex (a region critically involved in reward and emotional processing as well as interoception), and increased CB_1 availability is evident in the inferior frontal and temporal cortices of AN patients (regions involved in emotional processing and executive function). An association between the *C385A* polymorphism in the gene encoding FAAH (leading to diminished FAAH activity) and the *CNR1* polymorphism rs1049353 has been observed in higher frequency in patients with AN and BN (Monteleone et al. 2009) with a synergistic effect of these polymorphisms evident in patients with AN. The *C385A* polymorphism is also significantly associated with obesity (Monteleone et al. 2008; Sipe et al. 2005), though the relative contributions of possible disruptions in metabolism and hedonic mechanisms have not been determined. An association between a nonsynonymous *CNR2* polymorphism and both AN and BN has also recently been reported (Ishiguro et al. 2010).

Limited research has been conducted on the effects of CB manipulations in animal models of eating disorders. Sub-chronic $\Delta 9$ -THC treatment (0.5–2 mg/kg/day) was found to increase food intake and reduce body weight loss in an activity-based rat model of anorexia nervosa (Verty et al. 2005), though these effects were not evident in similar studies conducted in mice (Lewis and Brett 2010). Conversely, CB_1 receptor antagonism selectively reduces binge-like vs. normal patterns of consumption for highly palatable sweet and high-fat foods (Scherma et al. 2013). It is possible this latter finding involves a mesolimbic DA mechanism in light of evidence that binge-like consumption of palatable or high-fat foods elicits increased NAc DA release upon repeated bouts of consumption (Rada et al. 2005; Liang et al. 2006), while the DAergic response to normal patterns of consumption abates upon repeated exposure to palatable foods (Bassareo and Di Chiara 1999b). Regarding clinical studies in humans, two small initial trials assessing the efficacy of $\Delta 9$ -THC treatments for AN failed to demonstrate significant benefits on weight gain, though some beneficial effects on depression and perfectionism were evident (for review see Stoving et al. 2009). However, a more recent and larger trial demonstrated that a 4-week treatment with dronabinol induced a small but significant weight gain in women suffering from severe-enduring AN (Andries et al. 2014). Further, a recent randomized, placebo-controlled, double-blind trial in 289 obese subjects with binge eating disorders demonstrated that a 6-month treatment with rimonabant significantly reduced binge eating and led to significant weight loss with modest presentation of adverse psychiatric side effects (Pataky et al. 2013).

16 Conclusions and Future Directions

Endocannabinoid signaling participates in the mediation and modulation of both natural reward (such as food consumption, exercise, sex, and social interaction) and drug-induced reward. Most natural rewards and drugs of abuse alter brain eCB levels, and there is a robust CB₁R influence on the motivation for both natural and drug rewards. Chronic drug use results in a neuroadaptive downregulation of CB₁R and/or CB₂R availability and function and may also lead to disruptions in eCB biosynthesis and clearance. The resulting deficit in eCB signaling may contribute to increased stress responsivity, increased negative affect, impaired extinction of drug-related memories, and drug seeking/craving that are known contributors to relapse. Recent animal studies suggest that eCB clearance inhibitors may have therapeutic potential for ameliorating these behavioral abnormalities related to addiction disorders. This therapeutic approach may present fewer unwanted behavioral effects than that produced by exogenous cannabinoids, given that eCBs are generally produced in a synapse-specific manner, and as such, eCB clearance inhibition may preferentially facilitate eCB signaling in specific circuits engaged by distinct stimuli (e.g., stress, drug-associated cues, etc.).

There are a number of notable gaps in our understanding of the eCB influence on reward and addiction. The ECS plays a prominent role in neuronal guidance and brain development (Maccarrone et al. 2014), and as such, disruptions in eCB function at an early age likely have substantial consequences for adult brain function. This is underscored by increasing evidence of the long-term consequences of prenatal or adolescent cannabinoid exposure (Hurd et al. 2014; Calvigioni et al. 2014). Though the effects of early-life exposure to non-cannabinoid drugs are well studied, the specific contributions of persistent drug-induced disruptions in eCB signaling on adult neural function and behavior are not understood. Robust bidirectional interactions between the ECS and sex hormones are now recognized (Gorzalka and Dang 2012), but few studies have characterized possible sex differences in the eCB influence on reward function, addiction, stress, and cognitive processing. There are also substantial limitations in the interpretation and replication of genetic analyses of the eCB influence in addiction due to heterogeneity of the populations studied, drug class, polysubstance use, and even drug use phenotypes examined. Large-scale future studies across different populations and drug classes will be critical to understand the relative impact and causal nature of ECS-related genetic mutations in the vulnerability to addictive disorders. Filling these gaps of knowledge is critical given the important need for scientific data to help guide current discussions and changes being made in marijuana legalization policies.

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Roles of *N*-Acylethanolamines in Brain Functions and Neuropsychiatric Diseases

Marco Pistis and Anna Lisa Muntoni

Abstract *N*-acylethanolamines (NAEs) are bioactive lipids, structural analogues to the endocannabinoid arachidonoyl ethanolamide (anandamide), whose functions and properties are being elucidated in recent years. By activating their receptors, specifically peroxisome proliferator-activated receptors (PPARs), these molecules exert a variety of physiological effects via genomic and rapid non-genomic mechanisms. Regulation of lipid metabolism, energy homeostasis, and anti-inflammation are among the best-characterized effects of PPAR activation. NAEs are abundant in the CNS and their receptors are widely expressed both in neurons and in glial cells, where they modulate brain functions and are involved in the pathophysiology of neurological and psychiatric disorders. In the brain, they participate in the regulation of feeding behavior, cognitive functions, mood, reward, and sleep-wake cycles, and evidence suggests that they might be therapeutically exploited as neuroprotective agents, “anti-addictive” medications, anticonvulsant, and antidepressant.

In this chapter, we will review the state of the art on these neuromodulators and their receptors in the brain and will discuss new hypotheses on their physiological and pathophysiological roles.

1 Introduction

Fatty acid ethanolamides, generally referred as *N*-acylethanolamines (NAEs), are a group of endogenous lipid molecules with long-chain fatty acids (Schmid et al. 1990; Hansen et al. 2000). It was long known that these molecules are ubiquitously found in animal tissues (Bachur et al. 1965), in both the periphery and the brain, and

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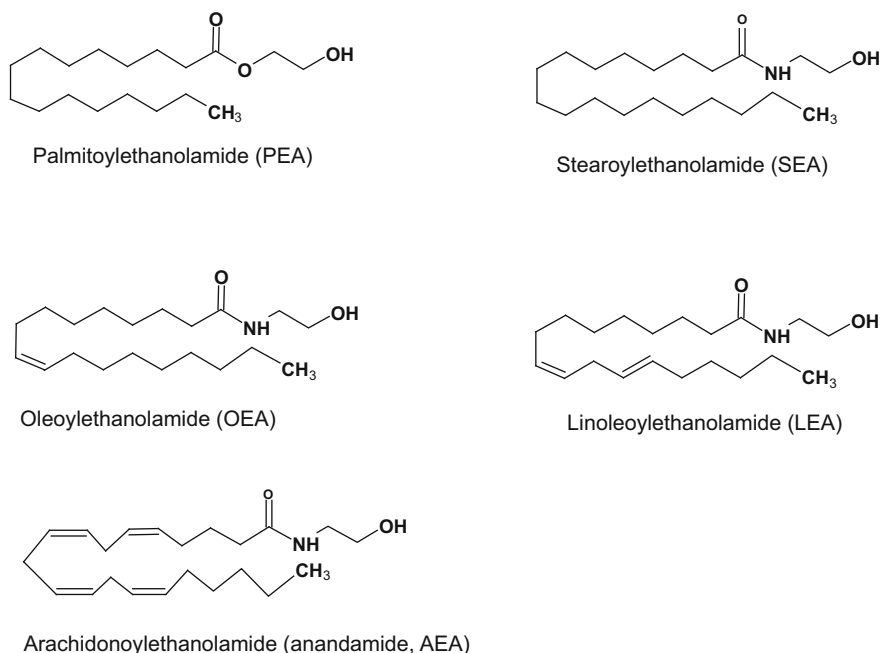


Fig. 1 Chemical structures of representative NAEs

have attracted attention in recent years as they possess a variety of biological activities.

Among NAEs, the most studied are arachidonoylethanolamide (anandamide, AEA), palmitoylethanolamide (PEA), and oleoylethanolamide (OEA); other congeners are stearoylethanolamide (SEA) and linoleoylethanolamide (LEA) (Fig. 1) (Hansen 2010; Rahman et al. 2014). Anandamide is the first endocannabinoid to be discovered (Devane et al. 1992) and the only NAE to bind to cannabinoid type 1 (CB1) and type 2 (CB2) receptors. Quantitatively, anandamide is a minor component in most animal tissues when compared with other NAEs such as PEA, SEA, OEA, and LEA (Hansen and Diep 2009). NAEs, although belonging to the same extended endocannabinoid-like family as the cannabinoid agonist AEA, exert a variety of biological effects through several other receptors, in particular peroxisome proliferator-activated receptor- α (PPAR α) (Hansen 2010; Lo Verme et al. 2005; Petrosino et al. 2010), but also G-protein-coupled receptors GPR55 (Baker et al. 2006) and GPR119 and transient receptor potential vanilloid type 1 (TRPV1) (Piomelli 2013).

Although these molecules were known from many years, the role of PEA and OEA, as well as of other NAEs, in the CNS has been elucidated only recently, when the discovery of AEA (Devane et al. 1992) fueled a renewed interest in these lipid messengers.

Specifically, functions and properties of PEA, a saturated fatty acid (palmitic acid) derivative, were discovered first in 1957, when this lipid (from soybeans, peanuts, and egg yolk) was found to exert anti-inflammatory activity in guinea pigs (Kuehl et al. 1957). High levels of PEA were measured in mammalian tissues, especially in the brain (Bachur et al. 1965). The finding that PEA is the most abundant NAE in the rat brain has been more recently substantiated by the use of lipidomics techniques (Kilaru et al. 2010).

The monounsaturated OEA is a potent anorectic lipid mediator (Fu et al. 2003, 2005; Rodriguez de Fonseca et al. 2001; Piomelli 2013) and shares this property with PEA, LEA (Hansen 2014; Hansen and Diep 2009), and SEA (Terrazzino et al. 2004). As anorexiant, however, PEA is significantly less potent than OEA in reducing food intake, and LEA is similar in potency to OEA (Rodriguez de Fonseca et al. 2001; Diep et al. 2011).

Besides their anti-inflammatory activity and their physiological functions as modulators of feeding behavior, NAEs, and specifically PEA and OEA, have been recently involved in pathophysiology of neurological and psychiatric disorders, ranging from addiction, neurodegenerative diseases, epilepsy, and mood disorders (Pistis and Melis 2010; Scherma et al. 2016; Melis and Pistis 2014). In this review, we will focus on physiology of non-cannabinoid NAEs in the CNS and on their relevance in neuropsychiatric disorders.

2 Synthesis and Catabolism

NAEs including AEA and the non-cannabinoid PEA and OEA are not stored in vesicles but produced “on demand”. Their endogenous levels are strictly regulated by enzymes responsible for their formation and degradation (Ueda et al. 2010a; Rahman et al. 2014). In fact, they are synthesized from glycerophospholipids via their corresponding *N*-acylphosphatidylethanolamines (NAPEs) (Okamoto et al. 2004; Rahman et al. 2014; Ueda et al. 2010b; Hansen 2010; Hansen et al. 2000). The classical pathway for NAE synthesis is a two-step process (Fig. 2). The first step is the generation of NAPE by a Ca^{2+} -dependent *N*-acyltransferase (NAT), which transfers the Sn-1 fatty acid to phosphatidylethanolamine from a donor phospholipid (Hansen et al. 2000; Hansen and Diep 2009). *N*-Acyltransferase is not specific for any fatty acids, since it catalyzes the transfer of any acyl group from the Sn-1 position of donor phospholipids. NAPE is then hydrolyzed by NAPE-hydrolyzing PLD (NAPE-PLD) with the generation of NAEs (Rahman et al. 2014). Human, mouse, and rat NAPE-PLD cloning allowed the functional characterization of NAE biosynthetic pathways (Okamoto et al. 2004). NAPE-PLD $^{-/-}$ mice (Leung et al. 2006) show a highly reduced Ca^{2+} -dependent conversion of NAPE to long-chain saturated and unsaturated NAEs in brain tissue. These mice display decreased levels of OEA and PEA, but similar levels of AEA compared with wild-type littermates (Leung et al. 2006). This indicates that AEA is formed by other

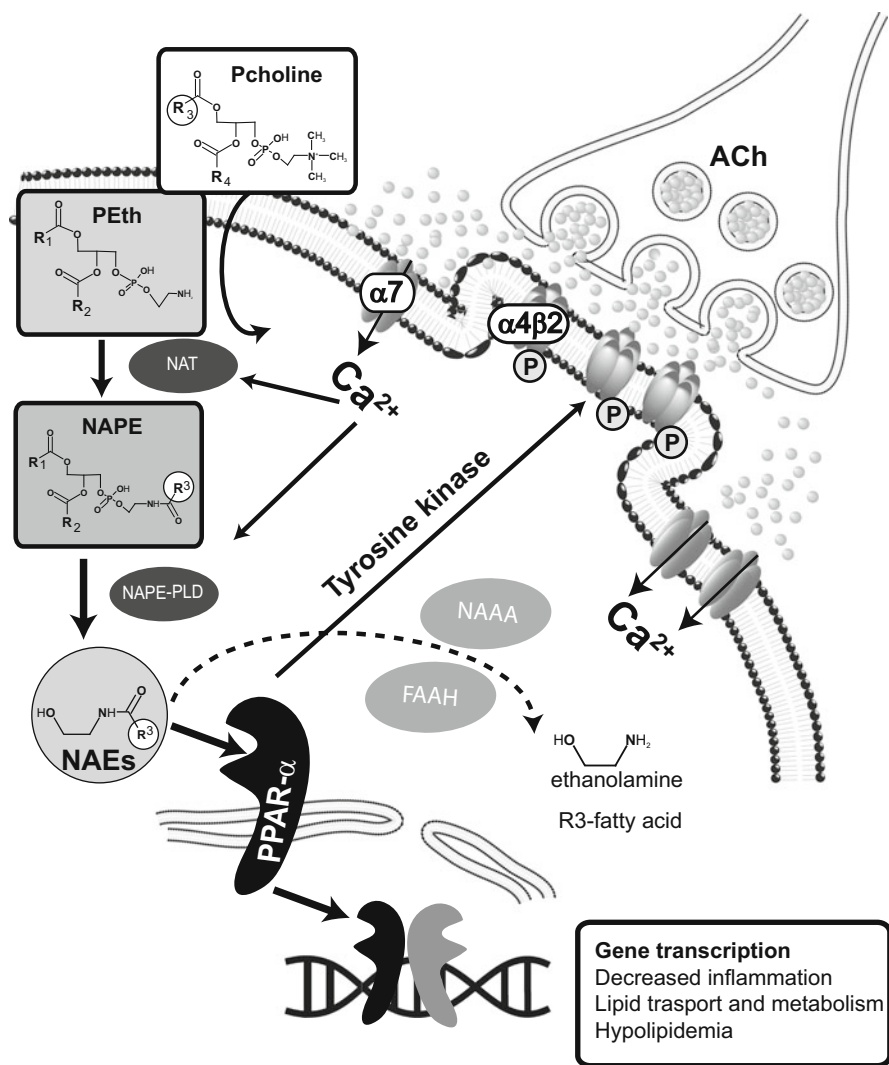


Fig. 2 Schematic diagram illustrating the canonical biosynthetic and catabolic pathways for NAE formation and their cellular mechanisms of actions through their receptor of PPAR α . Phosphatidylcholine (Pcholine) donates a fatty acid moiety from the sn-1 position (R_3) to a phosphatidylethanolamine (PEth). This reaction is catalyzed by N-acyltransferase (NAT). The resulting N-acylphosphatidylethanolamine (NAPE) is hydrolyzed by NAPE-PLD to the corresponding N-acylethanolamine (NAE). Activation of PPAR α by NAEs results in genomic effects (gene transcription) and in non-genomic actions, such as activation of a tyrosine kinase and phosphorylation of $\beta 2^*$ nAChRs (i.e., $\alpha 4\beta 2$). Ca^{2+} entry mediated by $\alpha 7$ -nAChRs activates NAS synthesis through the Ca^{2+} -dependent NAT and NAPE-PLD. Fatty acid amide hydrolase (FAAH) and NAE-hydrolyzing acid amidase (NAAA) are the major inactivating enzymes for OEA, PEA, and AEA which convert them into ethanolamine and corresponding fatty acids (oleic, palmitic, and arachidonic acids, respectively)

parallel pathways (Hansen and Diep 2009) and strongly suggests the existence of other NAE-forming enzymes within NAPE-PLD-independent pathways (Tsuboi et al. 2011; Simon and Cravatt 2010; Leung et al. 2006; Rahman et al. 2014). No gross phenotype in NAPE-PLD-deficient mice has been reported, whereas in humans a polymorphism of NAPE-PLD was associated with obesity in a Norwegian population (Wangensteen et al. 2010).

NAPE-PLD is expressed in many tissues, including the brain (Egertova et al. 2008; Morishita et al. 2005; Cristino et al. 2008; Suarez et al. 2008). The cellular localization of NAPE-PLD in the brain is informative on the functional significance of NAEs in the CNS. NAPE-PLD mRNA and immunoreactivity are located both presynaptically and postsynaptically and are intense in axons of the vomeronasal nerve projecting to the accessory olfactory bulb (Egertova et al. 2008) and in the hippocampus (Cristino et al. 2008; Egertova et al. 2008; Nyilas et al. 2008). Other brain areas with NAPE-PLD immunoreactivity are the cortex, thalamus, hypothalamus (Reguero et al. 2014; Egertova et al. 2008), and cerebellum (Suarez et al. 2008). The postsynaptic localization of NAPE-PLD indicates that NAEs may act as an autocrine or paracrine signal at receptors expressed in the same or neighboring cells. On the other hand, the axonal localization suggests that synthesized NAEs may be released to target postsynaptic neurons and regulate synaptic signaling.

The generated NAEs are then degraded to their corresponding free fatty acids and ethanolamine (Cravatt et al. 1996; Deutsch et al. 2002) (Fig. 2). This hydrolysis is catalyzed mainly by fatty acid amide hydrolase (FAAH) (Cravatt et al. 1996) and NAE-hydrolyzing acid amidase (NAAA) (Tsuboi et al. 2005). FAAH hydrolyzes all NAEs with high efficiency, and it is expressed in many different tissues and cell types, including in the brain.

NAAA has no sequence homology with FAAH and is expressed in lysosomes. NAAA displays same specificity toward unsaturated NAEs such as PEA to much greater extent than AEA (Tsuboi et al. 2007a). In the rat, NAAA is highly expressed in the lung, spleen, thymus, and intestine (Tsuboi et al. 2007a), very high in alveolar macrophages (Tsuboi et al. 2007b), but low in the brain.

Alternatively, AEA and other polyunsaturated NAEs are oxygenated in their polyunsaturated fatty acyl moieties and converted to prostamides by cyclooxygenase-2 (COX-2) (Yu et al. 1997; Kozak et al. 2002) or to other oxygenated metabolites by lipoxygenases (Hampson et al. 1995; Ueda et al. 1995) or by cytochrome P-450 (Bornheim et al. 1993). In addition to these enzymes, more recent studies revealed the involvement of new players in NAE metabolism (see Rahman et al. 2014 for a comprehensive review).

3 Receptors for NAEs

3.1 Peroxisome Proliferator-Activated Receptor- α (PPAR α)

PPARs belong to the large superfamily of transcription factors, accounting 48 members in the human genome (Germain et al. 2006). The PPAR subfamily, specifically, is composed of three isotypes: PPAR α , PPAR γ , and PPAR β/δ .

The denomination of these receptors dates back to the 1960s, when the lipid-lowering drug clofibrate was found to induce proliferation of peroxisomes in the liver and hepatomegaly in rodents (Thorp and Waring 1962; Hess et al. 1965). The receptor activated by clofibrate, other fibrates, and synthetic ligands was cloned and denominated peroxisome proliferator-activated receptor- α (Issemann and Green 1990). Although the other members of this subfamily (β/δ and γ) are structural homologs of PPAR α , they do not induce peroxisome proliferation (Graves et al. 1992; Dreyer et al. 1992; Schmidt et al. 1992). Nevertheless, the name has remained.

PPARs, like other nuclear receptors, are composed of four domains (Fidaleo et al. 2014), among which the DNA-binding domain and the ligand-binding domain, which are the most conserved portions of the protein across the different isotypes (Desvergne and Wahli 1999; Escriva et al. 1998; Laudet et al. 1992). The ligand-binding domain, in particular, is strikingly large in comparison with other nuclear receptors (Desvergne and Wahli 1999). This explains why PPARs are activated by a large number of diverse endogenous ligands as well as by synthetic agonists: saturated and unsaturated fatty acids (palmitic acid, oleic acid, linoleic acid, and arachidonic acid), NAEs, and eicosanoids (Desvergne and Wahli 1999).

Considerable evidence suggests that NAEs display most of their CNS activity through PPAR α . In HeLa cells stably expressing a luciferase reporter gene together with the ligand-binding domain of human PPAR α , OEA activates PPAR α with an EC₅₀ of 120 nM, whereas PEA displays lower potency (EC₅₀ of 3.1 μ M) (Lo Verme et al. 2005; Fu et al. 2003). Under identical conditions, SEA was reported to be ineffective (Lo Verme et al. 2005).

PPAR α has been cloned and characterized in several species, including humans (Sher et al. 1993). PPAR α expression is enriched in tissues with high fatty acid oxidation rates such as the liver, heart, skeletal muscle, brown adipose tissue, and kidney. It is also expressed in other tissues and cells including the intestine, vascular endothelium, smooth muscle, and immune cells such as monocytes, macrophages, and lymphocytes (Lefebvre et al. 2006; Chinetti et al. 1998) and in the CNS (Mandard et al. 2004; Galan-Rodriguez et al. 2009; Moreno et al. 2004; Braissant et al. 1996; Auboeuf et al. 1997). The highest PPAR α expression was found in the basal ganglia; some thalamic, mesencephalic, and cranial motor nuclei; the reticular formation; and the large motoneurons of the spinal cord (Fidaleo et al. 2014; Moreno et al. 2004). Besides neuronal localization, a specific distribution of PPAR α in ependymal and astroglial cells, but not in oligodendrocytes, was reported (Moreno et al. 2004).

Several structurally different classes of compounds bind to PPAR α , including the hypolipidemic fibrates. Saturated or monounsaturated NAEs are high-affinity ligands for PPAR α , particularly OEA which may be considered an endogenous ligand at concentrations normally achieved under physiological conditions (Fu et al. 2003).

3.2 *GPR119 and GPR55*

NAEs bind also to G-protein-coupled receptors 119 (GPR119) and 55 (GPR55). GPR119 is involved in insulin secretion, and, for this reason, it is emerging as a potential therapeutic target for type 2 diabetes, with beneficial effects on glucose homeostasis (Moran et al. 2014). There is no evidence of GPR119 expression in the CNS, therefore a detailed discussion of its functions is not within the aims of this chapter.

GPR55 is expressed in the CNS (Sawzdargo et al. 1999; Ryberg et al. 2007), as well as in peripheral tissues (intestine, bone marrow, immune and endothelial cells, spleen, and platelets) (Pietr et al. 2009; Balenga et al. 2011; Rowley et al. 2011; Cherif et al. 2015). GPR55 is a 319-amino acid protein that was cloned in 1999 and mapped to chromosome 2q37 in humans (Sawzdargo et al. 1999). GPR55 shows low amino acid homology with CB1 (13.5%) or CB2 (14.4%) (Ryberg et al. 2007; Kapur et al. 2009; Baker et al. 2006). Similarly to PPAR α , GPR55 is a receptor for small lipid mediators, and it is also activated by some synthetic cannabinoids and related molecules. The lipid lysophosphatidylinositol (LPI), which activates GPR55 but has no affinity for CB1 or CB2 receptors, was the first endogenous ligand identified for this receptor (Oka et al. 2007). Among lipids and NAEs, PEA binds with high affinity at GPR55 ($EC_{50} = 1\text{--}20$ nM), whereas OEA is less potent ($EC_{50} = 440$ nM) (Baker et al. 2006; Ryberg et al. 2007). Recent evidence suggests that GPR55 is involved in the regulation of axonal growth during development (Cherif et al. 2015). GPR55 participates also in central processing of neuropathic and inflammatory pain (Deliu et al. 2015) as it has been reported to play a pronociceptive role in the periaqueductal gray. Consistently, GPR55 $^{-/-}$ (knockout) mice have been shown to be protected in models of inflammatory and neuropathic pain. This suggests that GPR55 antagonists may have therapeutic potential as analgesics for both these pain types (Staton et al. 2008). It is not clear how the anti-inflammatory and anti-neuropathic effects of PEA can be reconciled with the functional role of GPR55 in pain and inflammation. As little is known on the physiological roles of GPR55, particularly in the CNS, further studies are needed to shed some lights into this receptor and its endogenous agonists.

4 Physiological Role of NAEs and PPAR

PPAR α is a transcriptional regulator of genes involved in peroxisomal and mitochondrial β -oxidation, fatty acid transport, and, in rodents, hepatic glucose production (Xu et al. 2002). PPAR α negatively regulates pro-inflammatory and acute phase response signaling pathways, as seen in rodent models of systemic inflammation (Gervois et al. 2004; Bensinger and Tontonoz 2008; Glass and Ogawa 2006). Consistently, PPAR $\alpha^{-/-}$ mice display longer inflammatory responses (Devchand et al. 1996), increased susceptibility to experimental colitis, and experimental autoimmune encephalitis (an experimental model of multiple sclerosis) (Straus and Glass 2007). PPAR α agonists reduce peripheral inflammation in a PPAR α -dependent manner (Sheu et al. 2002; Lo Verme et al. 2005).

The canonical mechanism of action downstream to PPAR α activation is regulation of gene transcription (Ferre 2004; Berger and Moller 2002; Moreno et al. 2004). Specifically, ligand binding promotes dissociation of corepressor proteins, association of coactivators, and coactivator proteins and heterodimerization with the retinoid X receptor (RXR). This dimer is then translocated into the nucleus and binds to specific regions on DNA termed peroxisome proliferator response elements (PPRE). The result is either an increase or a decrease of gene transcription, depending on the target gene. Besides this typical transduction mechanism, non-transcriptional actions by PPAR α have also been observed (Melis et al. 2008; Ropero et al. 2009; Gardner et al. 2005). These effects are rapid in onset (2–5 min). This short onset time rules out the possibility of a genomic mechanism of action and involves signaling pathways similar to those described for many other nuclear receptor ligands (Losel and Wehling 2003; Losel et al. 2003; Moraes et al. 2007; Ropero et al. 2009). Consistent with this scenario, PPAR α activation induced production of cytosolic effectors, such as reactive oxygen species (Ropero et al. 2009; Melis et al. 2008).

These non-transcriptional effects take place also in the CNS and have the potential to regulate neuronal functions on a short timescale. In fact, our recent studies show that in ventral tegmental area (VTA), dopamine neurons OEA and PEA bind to PPAR α within the cytosol and trigger a rapid non-genomic mechanism leading to increased endogenous hydrogen peroxide and consequent activation of tyrosine kinase(s) (Melis et al. 2008, 2010). In turn, these tyrosine kinases phosphorylate the $\beta 2$ subunits of the nicotinic acetylcholine receptors (nAChRs) (Melis et al. 2013b). Phosphorylation of nAChRs is an efficient mechanism to control receptor functions and results in a faster desensitization rate or a downregulation (Huganir and Greengard 1990). As cholinergic afferents control firing rate and burst firing of midbrain dopamine cells, the functional regulation of $\beta 2$ -containing nAChRs ($\beta 2^*\text{nAChR}$, the asterisk indicates the presence of other subunits) alters dopaminergic activity. Thus, NAEs as PPAR α ligands act as intrinsic modulators of cholinergic transmission and modify dopamine cell excitability, contributing to acetylcholine effects on dopamine system. Moreover, our studies revealed that PPAR α -induced regulation of $\beta 2^*\text{nAChR}$ abolished electrophysiological,

neurochemical, and behavioral effects of nicotine (Scherma et al. 2008; Melis and Pistis 2014; Melis et al. 2013a, b; Panlilio et al. 2012). Therefore, enhancing brain levels of PPAR α endogenous agonists or activating this receptor with synthetic ligands represents a novel strategy for anti-smoking medications (Melis and Pistis 2014) (see next section).

Noteworthy, this cellular mechanism of action explains why the effects of PPAR α activation are specific to nicotine, as it does not attenuate cocaine or cannabinoid-induced electrophysiological or behavioral effects (Justinova et al. 2008, 2015; Luchicchi et al. 2010).

As mentioned above, PPAR α activation can be achieved by preventing degradation of endogenous ligands or by promoting their synthesis (Melis et al. 2013a, b). NAE's synthesis is increased by elevating intracellular Ca²⁺, as NAPE-PLD is a Ca²⁺-dependent enzyme (Ueda et al. 2001). In fact, activation of α 7-nAChRs, a low-affinity Ca²⁺-permeable nAChR subunit combination, enhanced midbrain OEA and PEA levels and prevented nicotine-induced excitation of dopamine neurons both in vitro and in vivo (Melis et al. 2013b). Thus, these observations indicate that α 7-nAChRs function as sensors in dopamine neurons for excessive cholinergic transmission, which is attenuated by subsequent PPAR α activation by endogenous agonists and phosphorylation of β 2*nAChRs.

5 Role of NAEs and PPAR α in Neuropsychiatric Diseases

5.1 Addiction

In the past two decades, research advances have progressively sustained the idea of addiction as a brain disease (Volkow et al. 2016). In particular, addiction can be defined as a chronic, relapsing disorder of brain reward, motivation, cognition, affective functioning, memory, and related circuitry (American Society of Addiction Medicine, ASAM, 2011). Dysfunction in these pathways leads to a behavioral pathology characterized by compulsive drug seeking and use with progressive loss of control over consumption despite the emergence of significant negative or disadvantageous consequences (see Volkow et al. 2016 and references therein). In the fifth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) (2013), the term addiction is synonymous with the most severe stage of substance use disorder (see Volkow et al. 2016). Drug addiction represents the most prevalent neuropsychiatric disorder affecting modern society (United Nations Office on Drugs and Crime, 2015). The global health burden attributable to alcohol and illicit drug use amounts to 5.4% of the total burden of diseases (WHO 2003).

A better knowledge of the neurobiological basis underlying addiction is certainly crucial for developing more effective pharmacological and behavioral interventions to counteract harmful consequences of drug use disorders. In this respect, the evidence that NAEs such as OEA and PEA block nicotine-induced excitation of

midbrain dopamine neurons by acting on PPAR α (Melis et al. 2008) pinpointed their key role in the modulation of the brain reward system and, therefore, in the pathophysiology of nicotine addiction ((Melis et al. 2010); reviewed in (Pistis and Melis 2010)). In fact, since dopamine neurons projecting from the VTA to the nucleus accumbens (NAc) act as “reward sensors,” these results offered new perspectives in both understanding and treating tobacco dependence. Accordingly, a study by Scherma et al. (2008) unveiled that boosting brain levels of NAEs reverses abuse-related behavioral and neurochemical effects of nicotine in rats, including those predictive of relapse liability (Scherma et al. 2008). But how does URB597 exert its anti-addictive and anti-rewarding actions? Both the receptor and the mechanism responsible for the effect played by enhanced NAE levels against nicotine were identified soon after (Melis et al. 2008). In particular, it was demonstrated that URB597 counteracts nicotine-induced stimulation of the dopamine system by selectively enhancing intracellular levels of OEA and/or PEA within this circuit and by activating PPAR α (Luchicchi et al. 2010; Melis et al. 2008; Mascia et al. 2011). However, even though PPAR α was recognized as the receptor accounting for the “anti-nicotine” effect played by NAEs (Melis et al. 2008), a contribution of CB1 receptors activated by anandamide cannot be completely ruled out (Luchicchi et al. 2010; Melis et al. 2008; Mascia et al. 2011).

This wealth of experimental data revealed a novel property of endocannabinoid-like NAEs and suggested that increasing brain levels of OEA and PEA, or activating their receptors (PPAR α) with synthetic ligands, might represent a novel and effective approach for achieving smoking cessation (Melis et al. 2013a, b; Melis and Pistis 2014; Scherma et al. 2008). Hence, a new frontier for the treatment of tobacco addiction, the number one preventable cause of death in the developed world, was opened. In keeping with these observations, synthetic PPAR α ligands such as fibrates, long-standing clinically available hypolipidemic drugs, were found to suppress (i) nicotine-induced stimulation of dopamine neurons and increased extracellular dopamine levels in the shell of the NAc, which are key features of its acute rewarding properties (Panlilio et al. 2012), and (ii) nicotine intake and reinstatement of nicotine-seeking behavior after a period of prolonged abstinence (Panlilio et al. 2012). Thus, all this evidence has prompted the idea that targeting PPAR α represents a promising therapeutic strategy in nicotine relapse prevention in humans and, in the end, for quitting smoking.

So far, anti-addictive actions of PPAR α agonists seem to be almost exclusively limited to nicotine in laboratory animals (Justinova et al. 2008, 2015; Luchicchi et al. 2010). The fact that in dopamine neurons PPAR α affects the phosphorylation status of β 2*nAChRs by activating tyrosine kinase(s) (Melis et al. 2010, 2013b), therefore leading to a reduced excitation of dopamine neurons by nicotinic agonists, might provide a possible explanation for this specificity. Hence, it is not surprising that URB597 does not influence either self-administration of the main psychoactive ingredient of cannabis (i.e., Δ 9-tetrahydrocannabinol, Δ 9-THC) or cocaine in nonhuman primates (Justinova et al. 2008). Moreover, URB597 is unable to prevent dopamine neuronal responses to morphine or cocaine in rats (Luchicchi et al. 2010). Accordingly, in humans, it has been reported that a natural genetic variation in

FAAH, +385 A/A (P129T), is associated with an increased risk of illicit, but not licit (i.e., nicotine, alcohol), drug use problems (Sipe et al. 2002; Tyndale et al. 2007). Notably, this FAAH variant displays a reduced expression and activity in humans, thereby supporting a potential link between enhanced NAE circulating levels and illicit drug abuse and dependence (Chiang et al. 2004).

Most recently, a link of PPAR α (and γ) with both alcohol consumption in rodents and withdrawal and dependence in humans emerged (Haile and Kosten 2017; Blednov et al. 2015). Specifically, Blednov et al. (2015) investigated the effects of different classes of PPAR agonists on chronic alcohol intake and preference in mice with a genetic predisposition for high alcohol consumption and then examined human genome-wide association data for polymorphisms in PPAR genes in alcohol-dependent subjects. According to the authors, the observed reduction of alcohol intake in mice and the genetic association between alcohol dependence and withdrawal (according to DSM-5 criteria) in humans underscore the potential for reconsidering clinically approved PPAR α or PPAR γ agonists for the treatment of alcohol use disorders and alcoholism (Blednov et al. 2015). Consistently, administration of either OEA or synthetic PPAR α agonists was lately shown to block cue-induced reinstatement of alcohol-seeking behavior and reduce the severity of somatic withdrawal symptoms in alcohol-dependent rats (Bilbao et al. 2015; Haile and Kosten 2017; Blednov et al. 2016), thus supporting the intriguing possibility of using PPAR α as novel therapeutic tool also for alcoholism.

5.2 *Epilepsy and Other Neurological Disorders*

Epilepsy is a common and prevalent neurological disorder affecting 1–2% of the population worldwide (Thurman et al. 2011). As of 2015, nearly 50 million of people suffer from this disease, characterized by recurrent spontaneous seizures which negatively impact quality of life and respond to medication in about 70% of cases (see for a recent review Varvel et al. 2015). In addition, currently available antiepileptic drugs are mainly symptomatic and have numerous side effects. Thus, an urgent need exists to identify and exploit new, effective therapies. Unraveling the neurobiological mechanisms that subserve the generation of epilepsy will help the development of drugs to modify the disease outcome and, potentially, to prevent epileptogenesis. Intriguingly, the interplay between PPAR α and nAChRs shows relevance also for epilepsy and can be exploited as an innovative therapeutic target. Indeed, nAChRs are involved in the pathogenetic mechanisms underlying seizures and epilepsy. In particular, converging evidence from genetic studies in epileptic patients and animal models of seizures demonstrated that nAChR activity is increased in some types of epilepsy, including nocturnal frontal lobe epilepsy (De Fusco et al. 2000; Steinlein et al. 1997; Steinlein 2004; Sutor and Zolles 2001). From this standpoint, negative modulation of nAChRs exerted by endogenous NAEs, or by exogenous ligands of their receptors (PPAR α), might represent a potential disease-modifying strategy for epilepsies where nAChRs contribute to

neuronal excitation and synchronization. This is particularly relevant considering that, as above mentioned, current therapies, besides from being ineffective in the 30% of patients, are mainly symptomatic and do not necessarily affect the epileptogenic process or the disease progression.

Accordingly, several preclinical studies have shown that selective agonists of PPAR α raise seizure thresholds, thus supporting PPARs as potential drug targets for seizure control (Auvin [2012](#)). PEA, for example, proved to have antiepileptic effects in kindled rats (Sheerin et al. [2004](#)) and showed anticonvulsant activity in mice (Lambert et al. [2001](#)). Similarly, fenofibrate, a synthetic PPAR α ligand long used as hypolipidemic agent in clinical practice, has been reported to be effective as anticonvulsant in pentylenetetrazole (PTZ)-induced seizures and on latencies to the onset of status epilepticus induced by lithium-pilocarpine in adult rats (Porta et al. [2009](#)). Likewise, acute and chronic PPAR α agonists (e.g., fenofibrate) are protective against nicotine-induced seizures and abolished nicotine-induced generation of ictal activity and synchronization in the frontal cortex (Puligheddu et al. [2013](#)). Remarkably, these latter results were paralleled by an increased ratio of phosphorylated/dephosphorylated $\beta 2$ nAChR subunits in the frontal cortex following acute PPAR α ligand treatment, whereas the chronic regimen induced a threefold augment in OEA levels in the same brain region (Puligheddu et al., [2013](#)).

Notably, these observations can also be applied to other models of epilepsy, since PEA was shown to display antiepileptic actions, though via direct PPAR α and indirect CB1 activation, in a rat genetic model of absence epilepsy (Citraro et al. [2013](#)) and in DBA/2 mice (Citraro et al. [2016](#)). In addition, a recent study by Saha et al. ([2014](#)) reported the anti-kindling effect of the PPAR α agonist bezafibrate in PTZ-induced kindling seizure model. Importantly, and not surprisingly, bezafibrate also reduced the neuronal damage and apoptosis in hippocampal areas of the rat brain (Saha et al. [2014](#)).

In this scenario, it is worth to mention that ketogenic diet (KD) proved as efficacious as fenofibrate in exerting anticonvulsive properties in animal models of epilepsy (Porta et al. [2009](#)). The KD is a high-fat, adequate-protein, and low-carbohydrate diet long used as beneficial therapy for refractory epilepsy, especially in children (Bough and Rho [2007](#)). It has been proposed that KD, by increasing fast inhibition in the rat hippocampus dentate gyrus, might protect these neurons from excitotoxicity and, consequently, caused anticonvulsant and anti-epileptogenic effects (Bough et al. [2003](#)). However, even though the KD mechanism of action is still unclear and under investigation (Porta et al. [2009](#); Bough and Rho [2007](#)), it is tempting to hypothesize the existence of a direct, causal relationship between augmented levels of NAEs and activation of PPAR α following KD. In fact, both brain and peripheral tissue levels of NAEs change with high-fat diets (see Pistis and Melis, [2010](#) and references therein), thus suggesting that these lipid molecules would behave like PPAR α agonists (Cullingford [2008](#)) and dampen hippocampal neuronal excitability (Bough et al. [2003](#)). Moreover, circulating levels of different PPAR α endogenous ligands (e.g., fatty acids, oxysterols, hormones) might increase following KD and exert themselves the anticonvulsive properties ascribed to this diet (Pistis and Melis [2010](#)).

Whether or not NAEs, as well as KD, have anticonvulsants and anti-epileptogenic effects via activation of PPAR α , it is undeniable the physiological role played by these receptors in neuroinflammation and protection from excitotoxicity and, therefore, a link between these phenomena (Pistis and Melis 2010; Melis and Pistis 2014). Hence, considering their multifaceted pharmacological properties (neurotrophic/neuroprotective and anti-inflammatory), PPAR pathways are under evaluation as potential therapeutic targets as well as vulnerability factors in Parkinson's disease (PD), Alzheimer's disease (AD), and multiple sclerosis (MS) (Pistis and Melis 2010; Fidaleo et al. 2014). For example, NAEs seem to be directly implicated in the pathogenesis of AD (Pazos et al. 2004), the most common cause of dementia and one of the leading sources of morbidity and mortality in the aging population (Reitz et al. 2011). Accordingly, OEA, PEA, and the NAE-enhancing FAAH inhibitor URB597 increase memory acquisition and consolidation in naïve rats in a PPAR α -dependent manner (Mazzola et al. 2009; Campolongo et al. 2009), while PPAR γ improve both learning and memory not only in an experimental model of AD (Heneka et al. 2005) but also in AD patients (Risner et al. 2006; Watson et al. 2005; Landreth et al. 2008; Jiang et al. 2008). Importantly, activation of PPAR γ is also able to ameliorate AD-related signs by reducing both microglial activation and A β plaques (Heneka et al. 2005). In line with these observations, Scuderi et al. (2011, 2012) demonstrated that PEA anti-inflammatory properties are responsible for counteracting A β -induced astrogliosis and helped to identify the molecular apparatus by which PEA, via PPAR α activation, contributes to downregulate astroglial reaction, pro-inflammatory signal overproduction, and neuronal loss. Next, an elegant *in vivo* study by D'Agostino et al. (2012) substantiated the fundamental role of PPAR α for the neuroprotective and memory-rescuing effects of PEA in an AD animal model (see (Scuderi and Steardo 2013; Mattace Raso et al. 2014) for recent reviews).

On the other hand, MS subjects show reduced levels of NAEs (Di Filippo et al. 2008), thus suggesting the involvement of endocannabinoid system imbalances also in the pathogenesis of MS (Shohami and Mechoulam 2006), the most widespread disabling neurological condition of young adults around the world (see for a recent review Ascherio and Munger 2016). However, it should be pointed out that during MS clinical exacerbations, levels of OEA, PEA, and AEA increased, although being still lower than in controls. This evidence consolidates the hypothesis that NAEs can have a significant impact in reducing inflammation and, therefore, act as endogenous neuroprotective molecules (Di Filippo et al. 2008). In accordance with this, an increase of CNS PEA levels has been observed in a mouse chronic model of MS (Loria et al. 2008), which might be interpreted as an adaptive mechanism to preserve function and integrity during the development of neurodegenerative damage (Mattace Raso et al. 2014). Noteworthy, in the same MS chronic model, PEA administration induced, together with an anti-inflammatory effect, a reduction of motor disability (Loria et al. 2008). Concerning PD, which is among the most prevalent neurodegenerative conditions (Pringsheim et al. 2014), a protective/neurotrophic (and, therefore, potentially therapeutic) role of NAEs has also been demonstrated. In particular, it has been shown that OEA significantly decreased

behavioral PD symptoms and exerted neuroprotective effects on the nigrostriatal circuit in an experimental model of the disease through a PPAR α -dependent mechanism (Galan-Rodriguez et al. 2009; Gonzalez-Aparicio and Moratalla 2014; Avagliano et al. 2016). Similarly, systemic administration of PEA reduced 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced glial cell activation and protected against MPTP-related loss of tyrosine hydroxylase-positive neurons in the substantia nigra pars compacta, two effects that were blunted in PPAR α -/- mice. Moreover, chronic administration of PEA reversed MPTP-associated motor deficits, as revealed by the analysis of forepaw step width and percentage of faults (Esposito et al. 2012). The fact that PEA proved to be neuroprotective even when administered after the insult is of particular importance, as the lack of PD biomarkers and the difficulties of early diagnosis make the pharmacotherapy of PD possible only when dopamine neuronal loss is advanced and the first symptoms have appeared (Esposito et al. 2012). Likewise, PPAR γ ligands have been reported to arrest PD progression in preclinical settings (Randy and Guoying 2007; Carta 2013; Schintu et al. 2009; Pinto et al. 2016). However, it should be mentioned that a recent study published in *The Lancet Neurology* (NINDS Exploratory Trials in Parkinson Disease (NET-PD) FS-ZONE Investigators, 2015), which assessed the PPAR γ agonist pioglitazone for its disease-modifying potential in early-stage PD, does not support initiation of further trials with this drug (see also Brundin and Wyse 2015).

5.3 *Mood Disorders and Schizophrenia*

The magnitude, suffering, and burden of mental health disorders in terms of disability and costs for individuals, families, and societies are astonishing. According to WHO's Global Burden of Disease reports, four of the six primary causes of years lived with disability are due to neuropsychiatric disorders (depression, alcohol use disorders, schizophrenia, and bipolar disorder). In particular, unipolar depression alone leads to 12.15% of years lived with disability and ranks as the third most important contributor to the global burden of diseases (Whiteford et al. 2013 and references therein). Depression is indeed a very common illness worldwide, with an estimated 350 million people affected, and is one of the priority conditions covered by WHO's Mental Health Gap Action Programme (Reed et al. 2015). Current therapies for this disorder are ineffective in many patients or cause intolerable side effects, thus highlighting the importance of novel, improved therapeutic options. Mesolimbic dopamine system dysfunctions have been implicated in numerous brain disorders, including depression (reviewed in Nestler and Carlezon 2006; Russo and Nestler 2013). Apart from specific anti-inflammatory and neuroprotective mechanisms, it has been proposed that PPARs may affect mood by interfering with its neurobiological bases (Rolland et al. 2013). Thus, NAEs, as endogenous PPAR α ligands and modulators of midbrain dopamine neuronal activity, are well suited to regulate several dopamine-dependent brain

physiological and pathological processes. Specifically, NAEs regulate cholinergic input onto dopamine cells and dampen hypercholinergic drive (Fig. 2) and the consequent aberrant excitation of dopamine neurons. This intrinsic negative feedback mechanism might be exploited as a therapeutic strategy in depression, which is characterized also by an unbalance between dopamine and acetylcholine systems (Melis et al. 2013b). Accordingly, experimental evidence suggests that abnormal nicotinic signaling may contribute to depressive symptoms (Mineur and Picciotto 2010; Tizabi et al. 2000) and raises the possibility that $\beta 2^*$ nAChR availability could be a biomarker of depression, correlated with severity of the symptoms (see for a review Picciotto et al. 2015). Consistently, a recent human imaging study has revealed that ACh levels are elevated in patients who are actively depressed, as measured by occupancy of nAChRs throughout the brain, and remain high in patients who have a history of depression (Saricicek et al. 2012). In this context, the PPAR α agonist fenofibrate (Jiang et al. 2017; Scheggi et al. 2016) or WY-14643 (Yang et al. 2017), PEA (Yu et al. 2011), and FAAH inhibitors show antidepressant-like activity in rodents (Gobbi et al. 2005; Bortolato et al. 2007; Adamczyk et al. 2008; Umathe et al. 2011). In addition, OEA was also lately proven to be effective in ameliorating animal depression-like behaviors, even though through complex mechanisms (Yu et al. 2015; Jin et al. 2015). Similarly, PPAR γ agonists (i.e., pioglitazone, rosiglitazone) have displayed some antidepressant-like properties in preclinical settings (Eissa Ahmed and Al-Rasheed 2009; Sadaghiani et al. 2011). Even though it is still unclear whether the PPAR γ beneficial effect lays on the NO pathways (Sadaghiani et al. 2011) or whether it is NMDAR-related (Salehi-Sadaghiani et al. 2012), these results encouraged clinical research. In humans, few studies have been carried out so far, the first of which reporting a moderate improvement in two different depression scales in depressed (and insulin-resistant) patients treated with rosiglitazone (Rasgon et al. 2010). Subsequently, randomized controlled trials tested pioglitazone as adjunctive therapy (Sepanjnia et al. 2012) or as monotherapy (Colle et al. 2017) on subjects with a major depressive disorder and demonstrated a significant amelioration in early response, remission, and symptom attenuation for the pioglitazone-treated group. More recently, open-label administration of this PPAR γ agonist was found to be associated with improvement of depressive symptoms in bipolar disorder patients (Kemp et al. 2014). Remarkably, a positive correlation has been observed between depressive symptomatology score and change in interleukin (IL)-6, thus indicating that reduction in inflammation might contribute to the mechanism by which pioglitazone modulates mood (Kemp et al. 2014).

Noteworthy, NAEs can also play a role in the etiopathogenesis of schizophrenia (see for a review Rolland et al. 2013), in which substantial dopamine transmission dysfunctions are strongly involved. Briefly, schizophrenia is a severe mental disorder afflicting approximately 1% of the population around the world (which corresponds to more than 21 million people) (see for a recent review Owen et al. 2016). Clinically, schizophrenia is characterized by profound disruptions in thinking that affect language, perception, and the sense of self. The so-called psychotic experiences (or positive symptoms), such as hearing voices or delusions, are the

main target of current antipsychotic drugs which all block dopamine D2 receptors (Leucht et al. 2009). In line with this, and with the above discussed observations, PPAR α may exert antipsychotic effects by dampening dopamine neuron activity. Data from preclinical and clinical studies seem to support this hypothesis. For example, the PPAR α agonist fenofibrate reduces prepulse inhibition disruption in a neurodevelopmental model of schizophrenia, an effect that might be explained by a direct action of fenofibrate on dopaminergic transmission (Rolland et al. 2012). Consistently, PPAR α or $\alpha 7$ -nAChRs agonists have been demonstrated to be beneficial in animal models of schizophrenia (Zanaletti et al. 2012; Rolland et al. 2012; Kucinski et al. 2012; Pichat et al. 2007; Thomsen et al. 2012). Moreover, since the idea is emerging that schizophrenia displays a pro-inflammatory phenotype (e.g., pro-inflammatory cytokines IL-6 and TNF- α are upregulated in obesity, inflammation, and schizophrenia), a recent human genetic study measured expression of prototypic obesogenic and immunogenic genes in peripheral blood cells from controls and schizophrenic patients (Chase et al. 2015). Intriguingly, Chase et al. reported a profound dysregulation of genes relating to metabolic inflammation, including a significant decrease in PPAR α mRNA levels and increases in IL-6 and TNF- α , with additional BMI interactions. Finally, a recent (Croatian) population study aimed at determining whether a functional L162V polymorphism in PPAR α gene, extensively investigated in the etiology of abnormal lipid and glucose metabolism, was also linked to schizophrenia risk (Nadalin et al. 2014). While the PPAR α -L162V polymorphism did not show an association with schizophrenia risk, it impacts clinical expression of the illness and plasma lipid concentrations in female patients (Nadalin et al. 2014). According to the authors, these polymorphisms could have a possible protective effect toward negative symptom severity in female schizophrenic patients.

6 Concluding Remarks

The explosion of research in the field of the endocannabinoid system in the last two decades has fueled an increased interest in lipid messengers. NAEs have many similarities with endocannabinoids: they share synthetic and catabolic pathways but also the logic in signaling mechanism, as they are produced “on demand” and their diffusion and targets are not restricted to the prototypical anterograde signaling that characterizes classical neurotransmitters. Yet, peculiarities are emerging that might render NAEs and their receptors a new Pandora’s box for pharmacologists. NAE signaling in the CNS is engaged under physiological conditions but, more importantly, under several pathological circumstances. Thus, exploiting these targets with novel pharmacological tools will help us to better understand pathophysiological mechanisms in neuropsychiatric disorders and will pave new roads to therapeutic intervention.

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Mast Cells and Glia as Targets for the Anandamide Congener Palmitoylethanolamide: an Anti-inflammatory and Neuroprotective Lipid Signaling Molecule

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Abstract Glia and microglia in particular elaborate pro-inflammatory molecules which play key roles in central nervous system (CNS) disorders from neuropathic pain and epilepsy to neurodegenerative diseases. Microglia respond also to pro-inflammatory signals released from other nonneuronal cells, mainly those of immune origin such as mast cells. The latter can be found in most tissues, are CNS-resident, and traverse the blood–spinal cord and blood–brain barriers when barrier compromise results from CNS pathology. The existence of multiple lines of communication between mast cells and glia may provide new avenues for the development of therapies which target neuroinflammation by differentially modulating activation of those nonneuronal cell populations controlling neuronal sensitization—both peripherally and centrally. Mast cells and glia have “built in” homeostatic mechanisms/molecules that come into play as a result of tissue damage or stimulation of inflammatory responses. Such molecules include the N-acylethanolamine family. One such member, N-palmitoylethanolamine, is proposed to have a key role in the maintenance of cellular homeostasis in the face of external stressors provoking, for example, inflammation. N-Palmitoylethanolamine has been proven efficacious in mast cell-mediated experimental models of acute and neurogenic inflammation. This review will give an overview of current knowledge relating to the pathobiology of neuroinflammation, the role of microglia and mast cells, and the proposal that mast cell–microglia cross talk may exacerbate acute symptoms of chronic neurodegenerative disease and accelerate disease progression, as well as promote pain transmission pathways. We will conclude by considering the therapeutic potential of treating systemic inflammation or the blocking of signaling pathways from the periphery to the brain.

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1 Introduction

Inflammation is the body's attempt at self-protection to remove harmful stimuli (Nathan and Ding 2010) and is part of the immune response. Yet, there are settings when the inflammatory response itself damages host tissue and causes organ dysfunction, for example, when there is an overly robust acute or subacute inflammatory response to pathogens or debris from damaged host cells (Castellheim et al. 2009). In their recent review, Nathan and Ding (2010) clearly point out that the core problem with inflammation is not how often it starts but how often it fails to subside. In fact, few would dispute that non-resolving inflammation is one of the principal contributors to the medical burden in industrialized societies. Inflammation is particularly insidious where the peripheral and central nervous systems are involved ("neuroinflammation"), playing an important role in the pathogenesis of chronic pain and neuropathic pain (Myers et al. 2006), chronic neurodegenerative diseases (Freeman and Ting 2015; Iadecola and Anrather 2011; McGeer and McGeer 2013), neuropsychiatric illness (Castanon et al. 2015; Najjar et al. 2013; Theoharides et al. 2015a), and probably even autism spectrum disorder (Noriega and Savelkoul 2014; Theoharides et al. 2015b). It has even been suggested that neuroinflammation may raise the brain's sensitivity to stress (Rivat et al. 2010).

One of the most significant advances in the neurosciences over the past several decades has been the revelation that an extensive bidirectional communication exists between the immune system and the central nervous system (CNS). Pro-inflammatory cytokines occupy a key position in this highway, acting to regulate host responses to infection, inflammation, and reactions to stress or trauma. Principal protagonists in these actions are glia (astrocytes and microglia—the brain's resident macrophages) and mast cells. Both cell populations constitute important sources of inflammatory mediators and may have cardinal roles in conditions ranging from chronic pain to neurodegenerative diseases and neuropsychiatric disorders (Amor et al. 2014; Appel et al. 2011; Cunningham 2013; Harcha et al. 2015; Lull and Block 2010; Silver and Curley 2013; Skaper et al. 2014a, b; Thacker et al. 2007; Theoharides et al. 2015a, b). This review is intended to provide the reader with an overview of neuroinflammation's role in neurological diseases, followed by a consideration of approaches to counteract neuroinflammation based on endogenous defense mechanisms and lipid signaling molecules.

2 Glia and Mast Cells: Partners in Neuroinflammation

A number of cell types participate in neuroinflammation, including glia and immune system-derived cells, both tissue-resident and blood-borne. This review will focus on glia and mast cells, a choice supported by a large and ever-growing literature. Not only do glial cell activations contribute to neuropathology, but microglia and astrocytes also respond to pro-inflammatory signals released from

Table 1 Mast cells in a nutshell

Origin and classification
• Paul Ehrlich in 1878 first observed these cells on the basis of their unique staining characteristics and large cytoplasmic granules
• Very close to basophil granulocytes in blood; however, appear to be generated by different precursor cells in the bone marrow
• Thought to originate from bone marrow precursors expressing CD34; a distinct subset of mast cells can also be induced upon host responses to inflammation
• Hematopoietic lineage development of tissue mast cells is unique compared to other myeloid-derived cells because it is early lineage progenitors, undetectable by histochemistry, that leave the bone marrow to enter the circulation
• Immature lineage mast cells undergo transendothelial recruitment into peripheral tissues; appearance of secretory granules with a particular protease phenotype is regulated by the peripheral tissue
• Two types of mast cells are recognized, those from connective tissue and a distinct set of mucosal mast cells. Activities of the latter are dependent on T cells
• Present in most tissues close to blood vessels; are especially prominent near boundaries between the body’s external environment and the internal milieu, e.g., the skin, mucosa of lungs and digestive tract, as well as in mouth, conjunctiva, and nose
• Found within the nervous system, including the meninges, brain parenchyma, and peripheral nerves
Physiology
• Play a key role in the inflammatory process
• Activation leads to rapid release of granules into the interstitium
• Degranulation can be caused by direct injury (e.g., physical or chemical), cross-linking of IgE receptors, or activated complement proteins
• Capable of elaborating a vast array of important cytokines and other inflammatory mediators
• Express multiple “pattern recognition receptors” thought to be involved in recognizing broad classes of pathogens
• Granules “preloaded” with bioactive chemicals, proteoglycans, serine proteases, and neuropeptides; these can be transferred to adjacent cells of the immune system and neurons via transgranulation and their pseudopodia
Role in disease
• Allergic reactions
• Anaphylactic shock
• Chronic and neuropathic pain
• Acute and chronic neurodegenerative disorders

other cells of immune origin. Mast cells represent a potentially important—and still relatively underappreciated—peripheral immune signaling link to the brain in an inflammatory setting (Table 1). These effector cells of the innate immune system, once activated, secrete numerous vasoactive, neurosensitizing, and pro-inflammatory mediators, which include biogenic amines, cytokines, enzymes, lipid metabolites, ATP, neuropeptides, growth factors, and nitric oxide (Table 2) (Silver and Curley 2013). By nature of their immune regulatory role, mast cells participate in IgE switching by B cells (Gauchat et al. 1993) and the release of chemoattractants that recruit eosinophils (Wardlaw et al. 1986) and monocytes.

Table 2 Mast cell mediators

• Biogenic amines
– Biogenic amines (histamine (2–5 pg/cell), serotonin)
• Cytokines
– Interleukins 1, 4, 6
– Leukemia inhibitory factor
– Tumor necrosis factor- α
– Interferon- γ
– Transforming growth factor- β
– Granulocyte-macrophage colony-stimulating factor
• Enzymes
– Acid hydrolases
– Chymase
– Phospholipases
– Rat mast cell protease I and II
– Trypsin
• Lipid metabolites
– Prostaglandin D ₂
– Leukotriene C ₄
– Chemokines (CCXL8, CCL2)
– Platelet-activating factor
• Other bioactive molecules
– Neuropeptides (e.g., vasoactive intestinal peptide, substance P)
– Proteoglycans, mainly heparin (active as an anticoagulant)
– Nerve growth factor
– ATP
– Nitric oxide

Given their oftentimes proximity at sites of inflammation and propensity for reciprocal interaction, the following discussion will integrate glia and mast cells when describing neuropathologies.

2.1 Neuropathic Pain

Neuropathic pain results from damage, degeneration, or dysfunction of the sensory nervous system and represents a substantial and growing unmet medical need. Central neuropathic pain accompanies spinal cord injury, multiple sclerosis, and some strokes, while painful peripheral neuropathies frequently occur with diabetes and other metabolic conditions. Neuropathic pain can result also as a direct result of cancer on peripheral nerves or as a side effect of chemotherapy. The triggering and maintenance of neuropathic pain states depends on Schwann cells, spinal microglia, and astrocytes, together with elements of the peripheral immune system (DeLeo

and Yeziński 2001). Spinal microglia release interleukin-1 β (IL-1 β) which may induce phosphorylation of the glutamatergic N-methyl-D-aspartate receptor NR1 subunit to strengthen painful signal transmission (Wei et al. 2007). Under pathological conditions dorsal horn microglia become activated with the upregulation of purinergic receptors (Chessell et al. 2005; Tsuda et al. 2009) which participate in neuropathic pain (Chessell et al. 2005; Ballini et al. 2011).

Mast cells, upon degranulation, release molecules which activate or sensitize nociceptors, thereby contributing directly to neuropathic pain (Xanthos et al. 2011). Peripheral nerve mast cells constitute the first line of activation at the site of damage and promote the recruitment of neutrophils and macrophages (Zuo et al. 2003). Degranulation of mast cells activates also trigemino-cervical and lumbosacral pain pathways to elicit widespread tactile pain hypersensitivity (Levy et al. 2012), possibly mediated by a sensitizing effect of histamine on nociceptors. Further, the rapid release of nerve growth factor (NGF) by mast cells can sensitize nociceptors by engaging the latter's high-affinity TrkA receptors (and indirectly via other peripheral cell types) (Koda and Mizumura 2002). Mast cells themselves respond to NGF in a paracrine/autocrine manner (Leon et al. 1994). Collectively, these events facilitate T-cell recruitment to reinforce and maintain inflammatory reactions. The released mediators/factors can induce activity in axons and/or undergo retrograde transport to the cell body of spinal ganglion neurons and influence gene expression. In addition, mast cells may enhance recruitment of other key immune cell types which, in turn, release pro-nociceptive mediators, such as IL-6 (Leal-Berumen et al. 1994). Glucocorticoid therapy reduces pain and tumor necrosis factor- α (TNF- α)-positive mast cell numbers in rats with chronic constrictive nerve injury, strengthening a role for mast cells in chronic pain states (Hayashi et al. 2011). Mast cells are important mediators of chronic visceral pain, as well (Wood 2011).

2.2 *Acute Brain Injury*

Stroke and traumatic brain injury are characterized by an inflammatory response in which microglia activation and macrophage/neutrophil infiltration are well-recognized players (Wang et al. 2007). Left unchecked, this can ultimately lead to secondary injury. In certain instances, however, attenuating microglial activation can actually be beneficial (Hanisch and Kettenmann 2007). It is thus not surprising that a great deal of effort has been expended on strategies to inhibit the consequences of blood-borne neutrophil and phagocyte infiltration in ischemia. Consequently, brain-resident cell types able to mount an immediate host response in the cerebral parenchyma and meninges (e.g., mast cells) have received much less attention (Silver and Curley 2013). As with peripheral nerve damage—and contrary to long-held beliefs (Chew et al. 2006)—mast cell activation is clearly the “first responder” in this injury (Jin et al. 2009). While CNS microglia/macrophages and endothelial cells may produce TNF- α in response to stimuli, mast cells are

“primed” to initiate acute inflammation with their stores of *preformed* TNF- α (Gordon and Galli 1991). Mast cell-stabilizing agents limit brain damage caused by perinatal hypoxia–ischemia and transient focal ischemia (Jin et al. 2007, 2009; Strbian et al. 2007). With their complement of vasoactive and matrix-degrading components and proteases able to activate matrix metalloproteinases, mast cells are well-positioned as an early response element in the regulation of acute blood–brain barrier changes after cerebral ischemia and hemorrhage (Lindsberg et al. 2010). Cerebral mast cells, through their regulation of acute microvascular gelatinase activation, can effect blood–brain barrier disruption following transient cerebral ischemia (Mattila et al. 2011). For a discussion of microglia and mast cell involvement in chronic neurodegenerative diseases, see recent reviews by Skaper et al. (2012, 2014a, b).

3 Microglia and Mast Cells: The Other Side of the Story

Acute CNS injuries are characterized by a prolonged inflammatory response involving microglial activation and infiltration of macrophages and neutrophils. Conceivably, microglia accumulation at the lesion site and penumbra may serve a neuroprotective role. Genetic ablation of microglia aggravates infarct size after middle cerebral artery occlusion (Lalancette-Hébert et al. 2007). Injection of microglia into the bloodstream of Mongolian gerbils is “redirected” to an ischemic hippocampal lesion (probably aided by a compromised blood–brain barrier) and improves neuron cell survival (Imai et al. 2007). Microglia may protect hippocampal neurons from excitotoxicity (Vinet et al. 2012). Resting microglia respond/repair subclinical abnormalities of the brain without a complete activation transformation (Hanisch and Kettenmann 2007) and likely also have a key role in so-called developmental “synaptic pruning” (Paolicelli et al. 2011) as well as adult neurogenesis (Ribeiro Xavier et al. 2015).

Apart from well-recognized roles of activated microglia in disease progression (Kim and de Vellis 2005), they also deliver trophic factors (Kotter et al. 2005), remove myelin debris (Filbin 2003), and recruit oligodendrocyte precursor cells to the lesion site (Fan et al. 2007), thereby supporting myelin regeneration. In the case of Alzheimer disease, Fan et al. (2007) reported that anti-inflammatory drugs which suppress microglial inflammatory response attenuate neurological symptoms in a mouse model; however, other authors have found that reducing or ablating resident microglia fails to alter amyloid plaque load in these transgenic Alzheimer disease mouse models (Grathwohl et al. 2009). Deleting the microglial chemokine receptor CCR2 (which mediates the accumulation of mononuclear phagocytes at sites of inflammation) accelerated early disease progression and impaired microglial accumulation in an Alzheimer disease mouse model (El Khoury et al. 2007). Although Toll-like receptors (TLRs) are generally associated with inflammation, Song et al. (2011) claimed that microglia activation via TLR4 signaling partially reduced

amyloid β -peptide deposits and preserved cognitive functions otherwise compromised by amyloid β -peptide-mediated neurotoxicity.

In certain settings, mast cells may exert a beneficial action. For example, mast cells of human origin express and store angiogenin within their granules, which is released upon stimulation by Fc ϵ RI-mediated signals, Toll-like receptor ligands, and NGF (Kulka et al. 2009), with NGF being more efficacious compared with Fc ϵ RI cross-linking in the release of angiogenin. That human angiogenin is reported to be neuroprotective and to promote the survival and neurogenesis of motor neurons (Subramanian et al. 2008) is of interest, as recent studies associate angiogenin gene mutations with amyotrophic lateral sclerosis (Gellera et al. 2008) and suggest a possible disease link. Mast cells are a source of serotonin in the hippocampus, which can contribute to behavioral and physiological functions in the hippocampus (Nautiyal et al. 2012). Additionally, in cutaneous tissue mast cells may contribute to wound healing (Weller et al. 2006).

4 Microglia and Mast Cells: A Binary Act

The contribution of mast cells and microglia to neuroinflammation has the potential to be aggravated by the ability of these two cell types to interact with each other. This aspect of nonneuronal cell involvement in neuroinflammation has been discussed in detail earlier (Skaper et al. 2012); here we will only describe several representative examples of such interactions. Consider, for example, TLRs. The latter comprise one of the principal classes of pathogen-associated molecular patterns, that is, molecules associated with groups of pathogens recognized by innate immune system cells (such as microglia and mast cells). Ligand engagement of mast cell surface TLR2/TLR4 triggers cytokine release which recruits immune cells to the sites of injury; microglia recruitment depends on signaling pathways involving TLR2/TLR4 (Pietrzak et al. 2011). Mast cell activation upregulates also chemokine expression (including CCL5/RANTES); these chemokines can induce a pro-inflammatory response in microglia. Further, microglia-derived IL-6 and CCL5 may, in turn, affect mast cell expression of TLR2/TLR4. ATP, while generally thought of as an energy source, may act as an autocrine/paracrine factor for mast cells: ATP released from one mast cell (e.g., Fc ϵ R1 cross-linking, stress) is capable of diffusing several hundred micrometers to provoke a rise in Ca^{2+} in neighboring cells (Osipchuk and Cahalan 1992). ATP, by binding to P2 receptors, stimulates the release of IL-33 from microglia pre-activated (“primed”) with pathogen-associated molecular patterns (Chakraborty et al. 2010), which then induces mast cells to secrete IL-6, IL-13, and CCL2 which then modulate microglia activity. In effect, this creates a continual cycle. Mast cell tryptase cleavage/activation of proteinase-activated receptor 2 (Bushnell 2007) on microglia upregulates P2X₄ receptor and brain-derived neurotrophic factor release (Trang et al. 2009), while TNF- α and IL-6 from microglia upregulate proteinase-activated receptor 2 expression by mast cells, leading to mast cell activation and TNF- α release (Zhang et al. 2010a, b). Elements

Table 3 Potential avenues of mast cell–glia communication

Effector	Biological actions		References
	Microglia/astrocytes	Mast cells	
ATP/P2 receptors	ATP stimulates IL-33 release from microglia pre-activated with pathogen-associated molecular patterns via TLRs	IL-33 binds to its receptor on mast cells and induces secretion of IL-6, IL-13, and monocyte chemoattractant protein 1 which, in turn, could modulate microglia activity	Burnstock et al. (2011), Osipchuk and Cahalan (1992)
Proteinase-activated receptor 2 (PAR2)	Mast cell tryptase cleaves/activates PAR2 on microglia, resulting in P2X ₄ upregulation and release of brain-derived neurotrophic factor	IL-6 and TNF- α from microglia upregulate mast cell expression of PAR2, leading to mast cell activation and TNF- α release	Zhang et al. (2010a, b, 2012), Osipchuk and Cahalan (1992)
TLR2, TLR4	IL-6 and CCL5 released from microglia affect surface expression of TLR2/TLR4 on mast cells	Upregulation of cytokine/chemokine release; CCL5/RANTES induces pro-inflammatory profile in microglia; recruitment of immune cells to site of injury	Buchanan et al. (2010), Kim et al. (2007), Liu et al. (2012), Orinska et al. (2005), Pietrzak et al. (2011), Skuljec et al. (2011), Tanga et al. (2005)
CXCR4/CXCL12	Promotes microglia migration and activation; CXCR4 and CXCL12 are both upregulated in hypoxia/ischemia	CXCR4 acts as mast cell chemotaxin	Juremalm et al. (2000), Knerlich-Lukoschus et al. (2011), Yang et al. (2010)
C5a receptor (C5aR)	C5aR upregulated by microglia activation; C5a peptide released in neuroinflammation; cross talk between C5a and TLR4	C5aR upregulated upon activation; a strong mast cell chemoattractant signal toward C5a peptide; cross talk between C5a and TLR4	Gasque et al. (1997), Griffin et al. (2007), Soruri et al. (2008)
CD40/CD40L	Astrocyte-enhanced expression of CD40—cross talk with CD40L leads to production of inflammatory cytokines; astrocyte-derived cytokines/chemokines trigger mast cell degranulation	Enhanced surface expression of CD40L—cross talk with CD40 leads to production of inflammatory cytokines	Kim et al. (2011)

(continued)

Table 3 (continued)

Effector	Biological actions		References
	Microglia/astrocytes	Mast cells	
Translocator protein (TSPO)	Upregulated in retinal microglia in retinal inflammation/injury	The TSPO endogenous ligand diazepam-binding inhibitor (DBI) upregulated in microglia; DBI–TSPO signaling can promote microglia–microglia interactions which influence mast cell activity	Wang et al. (2014)

IL interleukin, *PAR* proteinase-activated receptor, *TLR* Toll-like receptor, *TNF-α* tumor necrosis factor-α

of microglia–mast cell cross talk appear to engage even the complement system. The chemoattractant C5a and its receptor are upregulated on reactive astrocytes and microglia in inflamed CNS tissue (Gasque et al. 1997), and C5a receptor on activated mast cells is a strong chemoattractant signal toward C5a peptide. Moreover, there is evidence to suggest mast cell–astrocyte communication at the level of CD40L/CD40 (Kim et al. 2011), as well as microglia–astrocyte interaction at the level of translocator protein, a marker of gliosis in neurodegeneration (Wang et al. 2014). Conceivably, these last findings propose an additional element whereby microglia and mast cells may work in concert to promote neuroinflammation (Table 3).

5 Microglia and Mast Cells as Therapeutic Targets for Neuroinflammation

Pharmacological attenuation of mast cell and microglial activation is viewed as an attractive therapeutic approach for neuropathic pain and other neuropathological conditions associated with inflammation (Gosselin et al. 2010; Mika 2008; Oliveira et al. 2011; Suk and Ock 2011; Xanthos et al. 2011). Attention has been directed also to therapeutic strategies aimed at inhibiting neurotoxic glial cell activation (Ralay Ranaivo et al. 2006).

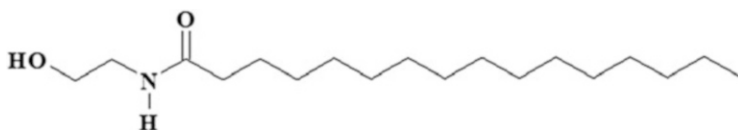


Fig. 1 Chemical structure of N-palmitoylethanolamine

5.1 Palmitoylethanolamide: A Wide-Acting Anti-inflammatory and Neuroprotective N-Acylethanolamine

In addition to more “traditional” medicinal chemistry approaches to develop molecules for controlling neuroinflammation, we should be aware of natural agents which are part and parcel of endogenous protective mechanisms activated following tissue damage or stimulation of inflammatory responses. Indeed, a number of molecules have so far been identified which take part in these protective mechanisms, whereby chronic inflammatory processes may trigger a program of resolution that encompasses the production of lipid mediators with the capacity to switch off inflammation (Buckley et al. 2013; Piomelli and Sasso 2014). One family of such molecules worth taking note of are the N-acylethanolamines, a class of naturally occurring lipid signaling molecules composed of a fatty acid and ethanolamine—the fatty acid ethanolamides (FAEs). Principal FAE family members include the endocannabinoid N-arachidonoylethanolamine (anandamide) and its congeners N-stearoylethanolamine, N-oleoylethanolamine, and N-palmitoylethanolamine (PEA) (chemical name: N-(2-hydroxyethyl)hexadecanamide) (Pacher et al. 2006). The PEA chemical structure is shown in Fig. 1. It is intriguing that, apart from its abundance in mammalian brain, PEA (and other NAEs) has been found even in some marine species (Bisogno et al. 1997b; Sepe et al. 1998) as well as in the CNS of the leech *Hirudo medicinalis* (Matias et al. 2001).

PEA and its congeners can be generated from N-acylated phosphatidylethanolamine (NAPE) by more than one enzymatic pathway (Rahman et al. 2014; Ueda et al. 2013), although this principally involves a membrane-associated NAPE-phospholipase D which produces the respective NAE and phosphatidic acid (Fig. 2) (Leung et al. 2006) and includes the conversion of N-palmitoyl-phosphatidylethanolamine into PEA. In the mammalian brain, NAEs are hydrolyzed by (1) an endoplasmic reticulum fatty acid amide hydrolase (FAAH) which breaks down NAEs into the corresponding fatty acid and ethanolamine (Cravatt et al. 1996) and (2) a lysosomal NAE-hydrolyzing acid amidase (NAAA) (Tsuboi et al. 2007) (Fig. 2). NAAA is mainly located in macrophages, where it hydrolyzes NAEs shorter than 18 carbon atoms like PEA. In contrast, FAAH hydrolyzes PEA, N-stearoylethanolamine, and N-oleoylethanolamine.

The idea that PEA plays a role in maintaining cellular homeostasis by acting as mediator of resolution of inflammatory processes draws support from a number of studies. For example, consider that PEA is produced/hydrolyzed by microglia and

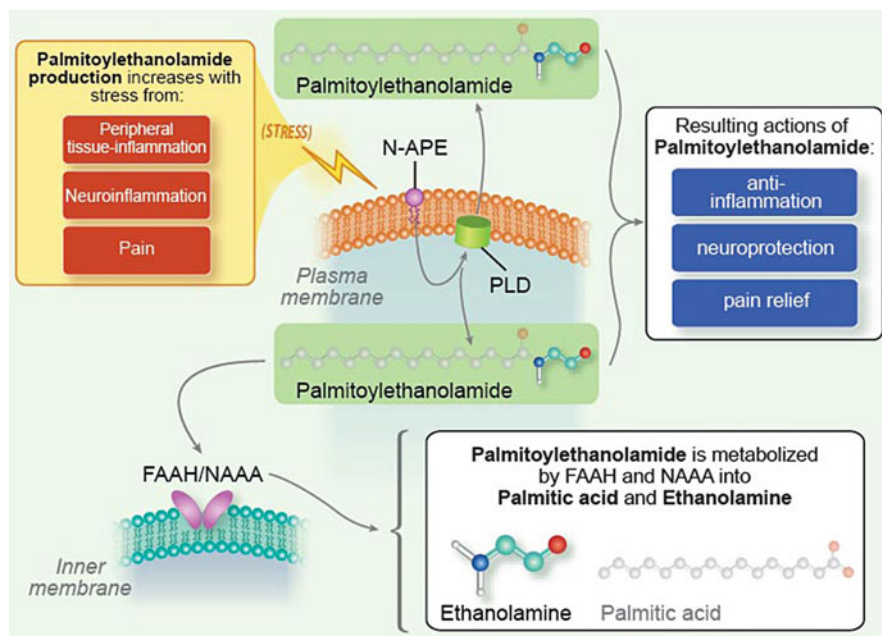


Fig. 2 Palmitoylethanolamide synthesis and metabolism. A plasma membrane-associated N-acylated phosphatidylethanolamine phospholipase D (PLD) converts N-palmitoyl-phosphatidylethanolamine (NAPE) into palmitoylethanolamide and phosphatidic acid. Palmitoylethanolamide is metabolized to palmitic acid and ethanolamine by both a relatively nonselective fatty acid amide hydrolase (FAAH) and the more selective N-acyl-ethanolamine-hydrolyzing acid amidase (NAAA). Tissue levels of palmitoylethanolamide increase in stressful settings such as peripheral tissue inflammation, neuroinflammation, and pain [Reprinted from *Immunology*, 141(3), S.D. Skaper, L. Facci, P. Giusti, Mast cells, glia and neuroinflammation: partners in crime?, 314–327 (Fig. 2). Copyright (2014), with permission from John Wiley and Sons]

mast cells (Bisogno et al. 1997a; Muccioli and Stella 2008), PEA down-modulates mast cell activation (Facci et al. 1995), and it controls microglial cell behaviors (Franklin et al. 2003; Luongo et al. 2013). Further, PEA levels are raised in brain areas involved in nociception and in spinal cord following induction of neuropathic pain (Petrosino et al. 2007), in conditions associated with pain development (Franklin et al. 2003; Jhaveri et al. 2008), in spinal cord of spastic mice with chronic relapsing experimental allergic encephalomyelitis (Baker et al. 2001), and in interstitium of trapezius muscle of women with chronic widespread pain and chronic neck–shoulder pain (Ghafouri et al. 2013).

The last two decades have seen an ever-expanding database dedicated to the anti-neuroinflammatory and neuroprotective actions of PEA at the preclinical level (Alhouayek and Muccioli 2014; Esposito et al. 2014; Fidaleo et al. 2014; Mattace Raso et al. 2014; Skaper et al. 1996). The principle findings reported in Table 4 clearly illustrate the diversity of models and neuropathological conditions where

Table 4 Preclinical studies demonstrating anti-neuroinflammatory and/or neuroprotective effects of palmitoylethanolamide

Model	Action	References
Chronic constriction injury in sciatic nerve	Anti-allodynic and anti-hyperalgesic effects Reduces mast cell activation Preservation of nerve structural integrity	Costa et al. (2008), De Filippis et al. (2011), Di Cesare et al. (2013)
Acute inflammation (formalin, dextran, carrageenan injection in rat hindpaw)	Reduces mast cell activation, tissue edema, inflammatory/mechanical hyperalgesia	Calignano et al. (1998), Costa et al. (2002), D'Agostino et al. (2007, 2009), Jaggar et al. (1998), Mazzari et al. (1996)
Rat model of endometriosis plus ureteral calculosis	Reduces viscerovisceral hyperalgesia	Iuvone et al. (2015)
Traumatic brain injury in mice	Reduces edema and infarct size Improves neurobehavior functions	Ahmad et al. (2012a)
MPTP mouse model of Parkinson disease	Protects against MPTP-induced neurotoxicity, microglial and astrocyte activation, and functional deficits	Esposito et al. (2012)
Stroke (middle cerebral artery occlusion in rats)	Reduces edema and infarct size Improves neurobehavior functions	Ahmad et al. (2012b)
β -Amyloid peptide injection in rat brain	Counteracts reactive gliosis Reduces behavior impairments	Scuderi et al. (2011)
β -Amyloid-induced astrogliosis	Regulates production of pro-angiogenic mediators	Cipriano et al. (2015)
WAG/Rij rat model of absence epilepsy	Antiepileptic action	Citraro et al. (2013)

MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

PEA was found to be pharmacologically efficacious. The case of chronic pelvic pain in women related to endometriosis is of particular interest, as this presents an example of what one might describe as “reverse” translational science: a newly published study by Iuvone et al. (2015) demonstrates the ability of ultramicronized PEA to significantly reduce viscerovisceral hyperalgesia in a rat model of endometriosis plus ureteral calculosis with mast cell involvement—in confirmation of clinical studies carried out several years before (Cobellis et al. 2011; Lo Monte et al. 2013).

The bulk of PEA studies in man have been conducted to address the molecule's effectiveness against chronic pain and neuropathic pain associated with a variety of etiopathogeneses. This review will not treat this aspect of PEA action, and the reader is referred instead to several recent reports (Hesselink and Hekker 2012; Paladini et al. 2016; Skaper et al. 2014a, b). In particular, the report by Paladini et al. (2016) presents a pooled data meta-analysis of 1484 patients with chronic

pain, 1188 of which were treated with micronized or ultramicronized PEA for periods of 21–60 days with daily doses ranging from 300 to 1200 mg. None of the clinical trials reported treatment-related adverse events.

Inhibiting PEA degradation by targeting its principal catabolic enzyme, NAE-hydrolyzing acid amidase (NAAA) represents a potential alternative route in the treatment of neuroinflammation. A chemically diverse range of selective NAAA inhibitors have been published (Solorzano et al. 2009; Saturnino et al. 2010; Duranti et al. 2012; Li et al. 2012; Yamano et al. 2012; Yang et al. 2015; Sasso et al. 2013; Vitale et al. 2014) including systemically active compounds which are able to modulate responses induced by inflammatory stimuli in vivo and in vitro (Solorzano et al. 2009; Li et al. 2012; Sasso et al. 2013; Ribeiro et al. 2015) and also elevate PEA levels (Solorzano et al. 2009; Li et al. 2012; Yang et al. 2015). The most recent NAAA inhibitors report single-digit nanomolar intracellular activity ($IC_{50} = 7$ nM) on both the rat and human enzyme (Sasso et al. 2013).

Pharmacological block of FAAH and/or NAAA with nonphysiological agents as a strategy to increase endogenous N-acyl ethanolamine levels, in particular PEA, may present certain drawbacks from a metabolic point of view. One needs to keep in mind that NAAA substrates such as PEA are produced on demand, rather than being constitutive. As such, enzymes like NAAA are probably intended to *modulate* substrate availability. Indeed, several studies suggest that genetic deletion or pharmacological block of FAAH may actually result in paradoxical effects (Siegmund et al. 2013; Hoyer et al. 2014), including β -amyloid peptide exacerbation of inflammation in astrocytes lacking FAAH (Benito et al. 2012) and altered neural proliferation, apoptosis, and gliosis in a brain region-dependent manner, in a negative energy context (Rivera et al. 2015). This could be a consequence of the fact that, in order to exert a regulatory effect on nonneuronal cells (e.g., glia and mast cells) implicated in neuroinflammation, PEA pleiotropic effects (Skaper and Facci 2012) should thus be tightly controlled by a mechanism allowing for their inactivation. Conceivably, protracted block of FAAH might also alter endocannabinoid system tone by reducing levels of 2-arachidonoylglycerol while increasing those of anandamide. We have thus proposed that pharmacologically modulating—and not blocking—the specific amidases for N-acylamides and in particular NAAA could be a viable strategy which preserves the PEA role in maintaining cellular homeostasis through its rapid on-demand synthesis and equally rapid degradation (Skaper et al. 2015).

PEA is an agonist for peroxisome proliferator-activated receptor (PPAR) α , a member of a group of lipid-activated nuclear receptor proteins that function as transcription factors exerting numerous functions in development and metabolism (Alhouayek and Muccioli 2014; Fidaleo et al. 2014; Lo Verme et al. 2005). PPAR α - and γ -isoforms especially appear to be associated with inflammatory events. PEA anti-inflammatory, antinociceptive/anti-neuropathic, and neuroprotective actions are either absent in PPAR α -null mice or blocked by PPAR α antagonists (Fidaleo et al. 2014; Skaper et al. 2014a, b). It has also been suggested that PEA exerts a so-called entourage effect, whereby the lipid mediator enhances the anti-inflammatory and antinociceptive activity of other endogenous compounds by

potentiating their affinity for a receptor or by inhibiting their metabolic degradation (Smart et al. 2002). In this regard anandamide is a candidate molecule, given its anti-inflammatory and antinociceptive effects. Anandamide and its congeners like PEA may act also through the transient receptor potential vanilloid type 1 (TRPV1) receptor, a nonselective cation channel expressed in small diameter sensory neurons which is activated by noxious heat, low pH, and capsaicin. Anandamide is a TRPV1 receptor agonist, while PEA enhances anandamide stimulation of the human TRPV1 receptor (De Petrocellis et al. 2001) in a cannabinoid CB2 receptor antagonist-sensitive fashion. Because PEA has no appreciable affinity for either CB1 or CB2 receptors, this latter effect could result from PEA acting indirectly by potentiating anandamide actions (Calignano et al. 1998). Mast cells (Bíró et al. 1998) and microglia (Kim et al. 2006) are reported to express TRPV1 receptors.

6 Concluding Remarks

Inflammatory signaling molecules derived from the innate and adaptive immune systems, together with CNS glia, can profoundly affect a wide range of CNS functions. Microglia, acting as sensors for disturbed brain tissue homeostasis, accumulate locally in response to neuronal cell injury or entry of foreign material into the brain. We still know relatively little about resident brain cell types capable of mounting immediate host responses in the brain and meninges. Mast cells, rather than microglia, represent the “first responders” to injury rather than microglia (Jin et al. 2009). Degranulation/mediator release by the former is very rapid, while longer-lasting activation elaborates *de novo*-formed mediators. In spite of research efforts to date, gaps remain in our knowledge of glial and mast cell changes in human chronic pain, especially a clear demonstration that glial and mast cell activation occurs in hypersensitized patients. We lack also systematic studies which provide a correlation between the magnitude of glial and/or mast cell markers in cerebrospinal fluid or spinal tissue and the intensity of pain in patients.

In spite of the fact that we have at our disposition a well-stocked “medicine cabinet” (antidepressants, anticonvulsants, sodium channel blockers, glutamate receptor antagonists, opioids) to combat chronic and neuropathic pain, these agents treat the symptoms rather than the underlying pathophysiology. Unfortunately, at best they provide transient relief in only a fraction of neuropathic pain patients and can lead to serious CNS side effects. Incomplete understanding of mechanisms underlying the induction and maintenance of neuropathic pain has hindered more effective treatments, including elucidating the role of mast cells and how they might interact with microglia.

Without doubt, we still have much to learn about signaling mechanisms that regulate neuroinflammation. Targeting endogenous regulators of neuroinflammation may be one strategy for impinging on a diverse array of nervous system disorders. In this context it may be useful also to investigate the role of mast cells in

inflammatory diseases as a network, which would call for a critical examination of tissue localization, function, and dynamic interaction with endogenous cells.

The production of PEA during inflammatory conditions supports its role as part of a complex homeostatic system controlling the basal threshold of both inflammation and pain. That selective inhibition of PEA degradation is anti-inflammatory further supports the view of PEA involvement in the control of pain and inflammation. One might also ask if we are not missing important therapeutic avenues by studying glia and mast cells as isolated players in neuroinflammation. PEA, its analogues, and its agents that inhibit its degradation are likely to further the development of novel therapeutic strategies for the treatment of neuropathological conditions.

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