CANNABINOID ACTION IN THE MOUSE OLFACTORY EPITHELIUM

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ABSTRACT

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Mammalian adult neurogenesis, i.e., the lifelong generation of new neurons through progenitor stem cells, contributes to the plasticity and repair potential of the nervous system. The peripheral olfactory epithelium exhibits natural replacement of olfactory sensory neurons through the proliferation and differentiation of a local basal progenitor cell population, rendering it an ideal system with which to investigate mechanisms of neurogenesis. The cannabinoid system can influence adult neurogenesis in the central nervous system. The goal of this thesis was to investigate the role of cannabinoid signaling in promoting neurogenesis in the mouse olfactory epithelium. Both exogenous and endogenous cannabinoid signaling leads to an increase in basal cell proliferation in the mouse olfactory epithelium. Furthermore, genetically deleting both cannabinoid receptor 1 and cannabinoid receptor 2 leads to an altered olfactory epithelium composition and dysfunctional proliferation. This body of work identifies a functional cannabinoid system in the mouse olfactory epithelium for the first time, and that cannabinoids can serve as trophic factors that contribute to the regulation of tissue homeostasis. It is essential to understand the complex mechanism of olfactory epithelium progenitor cell proliferation, which could lead to future regenerative strategies using human olfactory derived progenitor cell.

ACKNOWLEDGEMENTS

“Faith” is fine invention

By Emily Dickinson

“Faith” is a fine invention

For Gentlemen who *see!*

But Microscopes are prudent

In an Emergency!

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KEY TO ABBREVIATIONS

2-Arachidonylglycerol	2-AG
[N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]	AM251
5-bromo-2′-deoxyuridine	BrdU
C57BL/6	C57
Cyclic Adenosine Monophosphate	cAMP
Cannabinoid	CB
Cannabinoid 1	CB1
Cannabinoid 2	CB2
Cytokeratin 5	CK5
Diacylglycerol lipase	DAGL
4',6-diamidino-2-phenylindole	DAPI
Dentate Gyrus	DG
Dimethyl sulfoxide	DMSO
Extracellular Signal-Related Kinases	ERK
Fatty Acid Amide Hydrolase	FAAH
γ – Aminobutyric Acid	GABA
Globose Basal Cell	GBC
G-Protein Coupled Receptor	GPCR
Glycogen Synthase Kinase	GSK
Guanosine Triphosphate	GTP
Horizontal Basal Cell	HBC

Inositol Triphosphate Receptor 3	IP3R3
Immunoreactivity	IR
4-[*Bis*(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester	JZL 184
Knockout	KO
Monoacyl Glycerol	MAGL
Mammalian Achaete-Scute Homolog 1	Mash1
Mitogen-Activated Protein Kinases	MAPK
Metabotropic Glutamate Receptors	mGluR
Mammalian Target of Rapamycin Complex 1	mTOC1
N-acyltransferase	NAT
Olfactory Bulb	OB
Olfactory Epithelium	OE
Olfactory Marker Protein	OMP
Olfactory Sensory Neuron	OSN
Phosphate Buffered Saline	PBS
Phosphatidylinositol 3-Kinase	PI3K
Protein Kinase A	PKA
Phospholipase C	PLC
Peroxisome Proliferator-Activated Receptors	PPAR
Real-Time Polymerase Chain Reaction	RT-PCR
Region of Interested	ROI
Subventricular Zone	SVZ

Δ9 Tetrahydrocannabinol	THC
Terminal dUTP Nick End Labeling	TUNEL
Transient Receptor Potential Cation Channel Subfamily V, Member 1	TRPV1
Cyclohexylcarbamic acid 3'-(Aminocarbonyl)-[1,1'-biphenyl]-3-yl ester	URB 597
(R)-(+)-[2,3-Dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo [1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone methanesulfonate	WIN
Wildtype	WT

Chapter 1: Literature Review

A. Cannabinoid system and the central nervous system

Although cannabis has been a widely used illicit drug for centuries, cannabinoid chemistry and biological actions were not well defined until the recent discovery of cannabinoid receptors and isolation of endogenous ligands less than 25 years ago (Devane et al., 1988; Mechoulam, 1995; Munro et al., 1993). Since then recreational use of *Cannabis sativa* has been legalized in two states and medicinal use permitted in 20 states. Additionally, two synthetic cannabinoid pharmaceuticals are currently approved in the US drug discovery program focusing on therapeutic targets of nausea, analgesia, obesity, spasticity, neurodegenerative diseases, anti-tumor effects, mood elevation, insomnia, and substance abuse disorders (Pertwee, 2009). The cannabinoid system consists of cannabinoid receptors, all substrates that activate cannabinoid receptors including endogenous, phytocannabinoids (plant-derived), and synthetic ligands, and the metabolic enzymes involved in synthesis and/or degradation of those ligands. In the central nervous system, the endogenous cannabinoid system modulates several neural functions and behaviors, such as axonal growth and guidance during development, adult neurogenesis, learning and memory, food intake and metabolism, pain sensation, and inflammation. Additionally, deregulation of the endocannabinoid system is associated with several neurological dysfunctions such as Alzheimer's disease (Koppel et al., 2009), Huntington's disease (Pazos et al., 2008), schizophrenia (Ashton et al., 2005), epilepsy (Ludanyi et al., 2008), and mood disorders (Witkin et al., 2005).

Cannabinoid receptors

Two main cannabinoid (CB) receptor subtypes, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) have been characterized pharmacologically and molecularly. Consistent with their widespread distribution, CB receptors are involved in a variety of central and peripheral physiological functions, including neuronal development, modulation of neurotransmitter release, and energy metabolism as well as immune, cardiovascular, respiratory, and reproductive functions. CB receptors also modulate cellular functions during mammalian development and in adulthood, such as homeostasis, proliferation, motility, adhesion, and cell apoptosis.

CB receptors belong to the rhodopsin-like subgroup of the seven transmembrane domain G protein-coupled receptors (GPCRs) family. The hallmark of GPCRs is the ability to couple to an effector molecule by a guanine nucleotide-binding protein (G protein) (Figure 1.1). The effector is an enzyme that produces a diffusible second messenger, which in turn activates a biochemical cascade through phosphorylation by a protein kinase, mobilization of intracellular calcium, or ion channel regulation. G-proteins have three subunits, α, β, and γ. In the resting state, a guanosine diphosphate (GDP) molecule is bound to the Gα subunit forming a complex that resides in the inner surface of the membrane. When the G-protein complex associates with an appropriate activated metabotropic receptor, a conformation change in the Gα subunit causes the release of GDP in exchange for cytosolic guanosine triphosphate (GTP). The activated GTP-bound G-protein complex disassociates from the receptor into the GTP-bound Gα subunit, and the Gβγ complex, each able to influence its own effector proteins. The Gα subunit hydrolyses GTP into GDP through GTPase, thereby terminating its own activity.

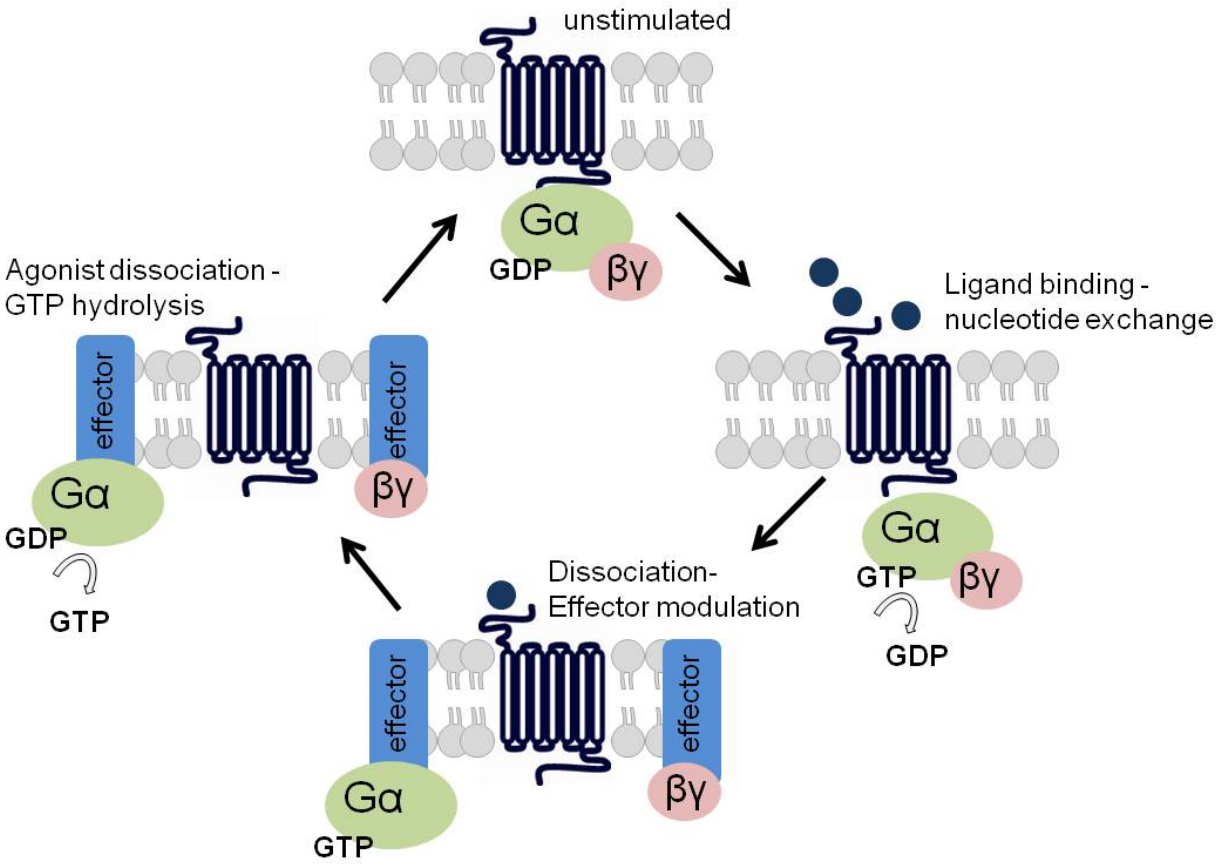

Figure 1.1 GPCR activation cycle. In the receptor inactive state (top),the α subunit and the βγ complex of the G protein are associated. Upon agonist binding (right), the receptor undergoes a conformational change and it catalyzes the exchange of GDP for GTP on the Gα subunit. GTP-bound Gα and the βγ complex dissociate and activate downstream effectors (bottom). Agonist dissociation and hydrolysis of GTP to GDP leads to reassociation of Gα and βγ subunits and termination of G protein signaling (left).

G-proteins can interact with the C-terminus (Nie and Lewis, 2001) and the third intracellular loop (Mukhopadhyay et al., 2000) of CB receptors.

GPCR superfamily classification is based on evolutionary sequence homology and functional similarity. Rhodopsin-like proteins are the largest GPCR family encompassing hormones and neurotransmitters including purine, neuropeptide, dopamine, eicosanoid, chemokine, and olfactory receptors. CB receptors reside on the cell membrane and contain an alkyl-binding domain responsible for the recognition of lipid ligands (Zhang and Xiong, 2009). CB1 receptors are expressed in neurons and are the most abundant GPCR in the mammalian brain (Herkenham et al., 1991; Howlett, 1998), therefore this thesis will focus on CB1 receptors. The importance of CB1 receptors in neuronal processing is supported by the observation that most behavioral effects seen after cannabinoid administration disappear after deletion of the gene encoding the CB1 receptor (Fang et al., 2009; Gandelman et al., 2010). Four behavioral hallmarks used to define cannabinoid intoxication in the rodent, hypothermia, rigid immobility, analgesia and decreased motor activity, are absent in CB1 receptor-deficient animals (Ledent et al., 1999; Zimmer et al., 1999). Additional functional consequences of CB1 activation are inhibition of excitatory and inhibitory neurotransmission and modulation of cognitive, memory, and motor functions (Maccarrone, 2008b).

CB1 receptor expression pattern is consistent with the psychoactive effects of cannabis. High levels of neuronal CB1 receptor density are found in brain regions implicated in the behavioral and cellular actions of cannabinoids, including the cortex, hippocampus, amygdala, basal ganglia, cerebellum, and brainstem (Herkenham et al., 1991). The majority of CB1 receptors detected through immunocytochemical and

electron microscopy studies are found on the plasma membrane of axon terminals, a site of neurotransmitter release (Katona et al., 1999; Nyiri et al., 2005). Specifically, the highest concentration of CB1 receptors is observed in presynaptic axons of both γ-aminobutyric acid (GABA) and glutamatergic neurons (Katona et al., 2000; Katona et al., 1999; Katona et al., 2006). Further studies have shown a reduction in GABA release after CB1 receptor stimulation (Katona et al., 1999). These data suggest that CB1 receptors participate in neuromodulation in a form of feedback inhibition of neurotransmitter release. CB1 receptors are also expressed on oligodendrocytes, astrocytes and microglia, although to a lesser extent than in neurons (Molina-Holgado et al., 2002; Ramirez et al., 2005).

CB2 receptors are primarily located on immune cells and are expressed in the central nervous system (CNS) by microglial cells. Given the presence of both CB1 and CB2 receptors on microglia, cannabinoids may be involved in neuroinflammatory responses (Holcomb et al., 1995a), and regulating cell survival (Guzman et al., 2001; Sanchez et al., 1998). However, recent studies suggest an increasing role of CB2 receptors in the CNS, given their presence in the brainstem, olfactory tubercle, cerebellum, hippocampus, and cortex, in addition to other CB1 receptor-positive brain regions. This implies a role for CB2 receptors in neural-cannabinoid interactions (Amstrup and Novak, 2003).

While CB1 receptors can couple with several different G-proteins, most often both CB receptors recruit the Gi/o subtype of GPCRs, which is supported by studies examining radiolabled GTPγS binding and pertussis toxin sensitivity of cannabinoid effects (Howlett, 1995; Pertwee, 1997). Recruitment of Gi/o proteins can lead to an

inhibition of adenylyl cyclase and reductions in cyclic AMP (cAMP) accumulation. In addition, both CB1 and CB2 receptors regulate the phosphorylation of various members of the family of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase-1 and -2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (Rueda et al., 2000; Valjent et al., 2005). Signaling through Gi/o proteins can also cause a reduction in gene expression through a decrease in protein kinase A (PKA) activity and cAMP response element activity. A reduction in PKA activity can lead to a decrease in the constitutive inhibitory phosphorylation of the protein c-Raf, thereby activating ERK1/2 (Davis et al., 2003; Derkinderen et al., 2003). Therefore, cannabinoid-mediated reduction in PKA activity may be directly correlated to an altered pattern of gene expression. CB receptor-mediated recruitment of both Gs and Gq/11 subunits has also been seen in animal models in which CB receptors are endogenously expressed (Bash et al., 2003; Glass and Felder, 1997). This alternative coupling appears to be physiologically relevant. This is supported by data showing that cannabinoid tolerance was associated with a molecular switch from Gi/o to Gs coupling in the striatum (Paquette et al., 2007), leading to a rise in intracellular calcium through phospholipase C (PLC) activity (Lander et al., 2009). Similarly, if Gi/o association is compromised by pertussis toxin treatment, interactions between CB1 receptors and Gq/11 or Gs α proteins are possible (Bonhaus et al., 1998; Felder et al., 1998). CB1 receptors can also induce elevation of intracellular free calcium through Gβγ-dependent activation of PLC in a neuroblastoma cell line (Sugiura et al., 1997). CB1 receptors also couple to ion channels, inhibiting voltage-gated calcium channels (Mackie and Hille, 1992; Twitchell et al., 1997) and activating inwardly rectifying potassium channels (Mackie et al., 1995).

Direct interaction of the Gi/o βγ subunit with voltage gated calcium channels (Wilson and Nicoll, 2002) may contribute to CB1 receptor-mediated depression of neurotransmitter release at GABA synapses in the hippocampus and glutamate synapses in the striatum (Hoffman and Lupica, 2000).

The amino acid sequence of the CB1 receptor is preserved across several species with high similarity to the human CB1 receptor (McPartland and Glass, 2003). There is a 44% overall homology between human CB1 and CB2 receptors (Munro et al., 1993). Some cannabinoid-like behaviors are seen following endocannabinoid administration in the CB1 receptor deficient mouse, suggesting additional cannabinoid targets in the CNS can contribute to cannabinoid actions (Di Marzo et al., 2000). Other possible cannabinoid targets include a group of GPCRs activated by endogenous lipid mediators, namely GPR55 (Sawzdargo et al., 1999), GPR119 (Overton et al., 2006) and GPR18 (Kohno et al., 2006). Cannabinoids can also activate members of the peroxisome proliferator-activated receptors (PPARs) family of nuclear receptors causing alterations in gene expression primarily involved in the regulation of metabolism and energy homeostasis, cell differentiation, and inflammation. However, most attention has been given to the transient receptor potential vanilloid type 1 receptor (TRPV1) as an alternative target of cannabinoids. TRP channels are non-selective cation channels that mediate nociception in both the central and peripheral nervous systems. TRPV1 receptors colocalize with CB1 and CB2 receptors in peripheral sensory neurons in the dorsal root ganglia and spinal cord (Ahluwalia et al., 2003; Anand et al., 2008; Price et al., 2004) and in central neurons (Cristino et al., 2006). The proximity of TRPV1 and CB receptors suggests possible intracellular cross-talk (Di Marzo and Cristino, 2008) and

dual activation by both synthetic or endogenous cannabinoid ligands has important functional consequences (Fioravanti et al., 2008; Hermann et al., 2003). For example,TRPV1 activation can lead to the production of the endocannabinoid anandamide (Ahluwalia et al., 2003; van der Stelt et al., 2005) which can further activate TRPV1 through its intracellular binding site (Jordt and Julius, 2002).

Cannabinoid ligands

Cannabinoid ligands are derived from both endogenous and exogenous sources. Exogenous cannabinoids include phytocannabinoids and synthetic molecules. The best known exogenous cannabinoid, Δ9 tetrahydrocannabinol (THC), is the psychoactive component of marijuana and is isolated from the plant *Cannabis sativa,* which has been used for over 8,000 years for both medical and recreational purposes. There are numerous plant-derived cannabinoid ligands including the Δ9THC precursor cannabidiol, and metabolite cannabinol, as well as cannabinoid acids. Synthetic cannabinoids are structurally diverse and can be classified as (1) classical, which are structurally similar to plant-derived cannabinoids, (2) nonclassical, which are not structurally similar to cannabinoids and encompass a wide range of synthetics (e.g., WIN55212-2), and (3) eicosanoids, which are structurally similar to endocannabinoids. Endocannabinoids are amides, esters, and ethers of long chain polyunsaturated fatty acids and are lipid messengers produced within the body that bind to CB receptors. Endocannabinoids are labile, released in small quantities, and are rapidly taken up or degraded. Although several endocannabinoids have been identified, the two most studied are *N*-arachidonoylethanolamide, later termed anandamide (AEA), and 2-

arachidonylglycerol (2-AG). Both are produced in the plasma membrane lipid bilayer from lipid precursors. Additional endogenous lipid signaling molecules proposed as members of the endocannabinoid family include (1) fatty acid AEA conjugates N-palmitoyl ethanolamide (PEA) and N-oleoyl ethanolamide (OEA), both activate the nuclear PPARs and cannabinoid-like GPCRs (O'Sullivan, 2007) and (2) 2-AG structural analog 2-oleoylglycerol (2-OG) which activates cannabinoid-like GPCRs (Syed et al., 2012).

Endogenous cannabinoid ligands, endocannabinoids

AEA is an eicosanoid derivative and was the first endogenous cannabinoid identified. AEA is a partial agonist at both CB1 and CB2 receptors (Sugiura and Waku, 2002). Although AEA binds and activates the CB1 receptor *in vitro*, this compound induces only weak and transient cannabinoid behavioral effects *in vivo*, possibly as a result of its rapid catabolism (Crawley et al., 1993; Piomelli et al., 2000). AEA is capable of interacting with other receptors, such as TRPV1 (Zygmunt et al., 1999), members of the PPAR family of receptors (Bouaboula et al., 2005), and cannabinoid-like receptors such as GPR55, GPR119, and GPR18 (McHugh et al., 2010) However, activation of TRPV1 by AEA is 5-20 fold less than its average efficacy at CB1 receptors (Zygmunt et al., 1999). AEA is synthesized from lipid precursors present in the plasma membrane through enzyme activation by multiple pathways (Figure 1.2). The major route of biosynthesis is the formation from N- arachidonoyl phosphatidylethanolamine through the action of a phosphodiesterase. Postsynaptic depolarization and intracellular calcium influx support AEA production, but how this occurs is not fully understood (Di Marzo, 2011).

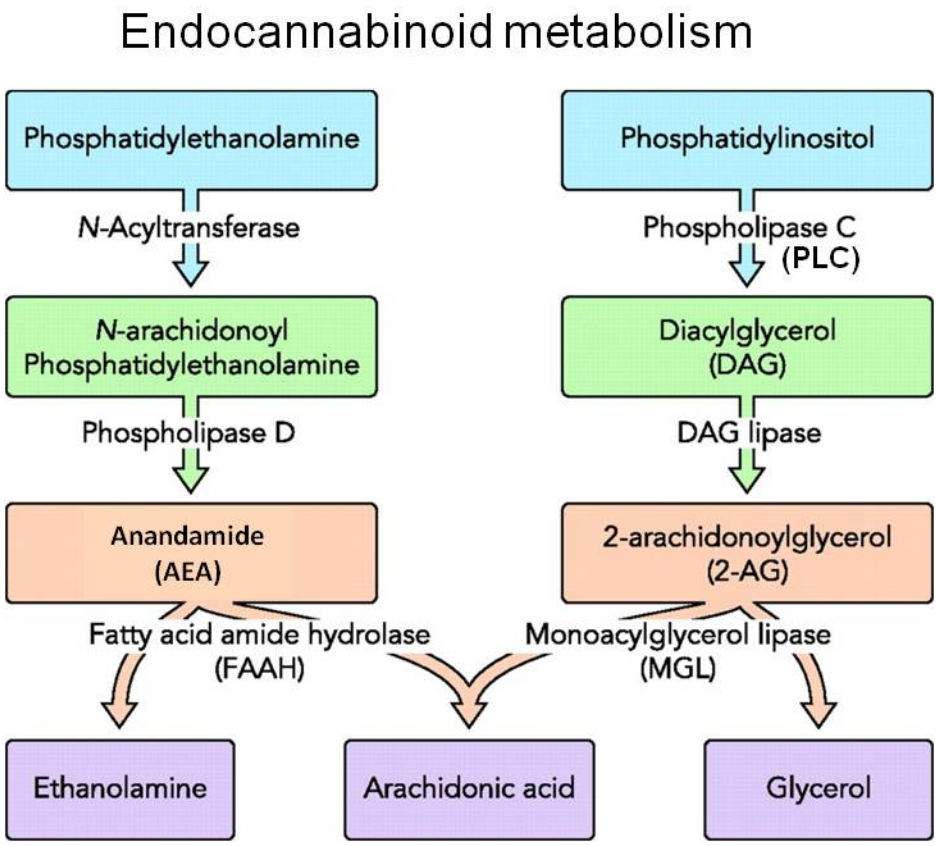

Figure 1.2 Pathways of endocannabinoid synthesis and degradation. The major route of AEA biosynthesis (left) is from the formation from N- arachidonoyl phosphatidylethanolamine through the action of phosphodiesterase, phospholipase D. AEA is metabolized within the cell through fatty acid amide hydrolase (FAAH) into ethanolamine and arachidonic acid. 2-AG synthesis (right) is through phospholipase C (PLC) action to generate diacylglycerol (DAG) before hydrolysis by diacylglycerol lipase (DAG) to 2-AG. 2-AG metabolism is through the action of monoacylglycerol lipase (MAG) which leaves arachidonic acid and glycerol.

Stimulus-dependent cleavage of phospholipid precursor N-arachidonoyl-PE, mediated by phospholipase D, produces AEA and phosphatidic acid. Neurons contain small quantities of N-arachidonoyl-PE, which are replenished by the enzyme *N*-acyltransferase (NAT). Intracellular calcium and cyclic AMP, which may be generated from cell depolarization or mobilization of calcium stores, control NAT activity (Cadas et al., 1996b). Calcium is required to engage NAT, which is inactive in its absence, whereas cAMP works through PKA-initiated phosphorylation to enhance NAT activity (Cadas et al., 1996a). Calcium-dependent AEA synthesis can be prevented by chelating extracellular calcium (Cadas et al., 1996a; Di Marzo et al., 1994). In addition, there is evidence that GPCRs can also trigger AEA synthesis (Ferrer et al., 2003; Giuffrida et al., 1999). Contrary to the classical notion of endocannabinoids produced mainly "on demand" by stimulus-dependent cleavage of membrane phospholipid precursors, AEA can be stored inside the cell (Oddi et al., 2008), and may contribute to tonic endocannabinoid receptor activation in the hippocampus (Kim and Alger, 2010), potentially through autocrine signaling (Bacci et al., 2004).

Endocannabinoids are inactivated by cellular uptake through a partially characterized endocannabinoid membrane transporter (Beltramo et al., 1997) and intracellular hydrolysis. Fatty acid amide hydrolase (FAAH) is an intracellular membrane-bound hydrolase that breaks down AEA into arachidonic acid and ethanolamine. FAAH is widely distributed in the rodent brain, where it is expressed at high concentration in cell bodies and dendrites of principal neurons (Egertova et al., 2003; Tsou et al., 1998). In the hippocampus, cortex, and cerebellum, FAAH-positive cell bodies mirror axon terminals that contain CB1 receptors, indicating FAAH

participates in the inactivation of neural AEA post-synaptically (Cravatt et al., 2001). FAAH-null mice can yield a 15-fold increase in basal AEA levels that can lead to an increase in activation of CB1-dependent responses and reduced pain sensation (Cravatt et al., 2001). AEA is produced in picomolar concentrations in regions of both rodent and human brain that contain a high density of CB1 receptors (e.g., hippocampus, cerebellum, and striatum) and regions with strong CB2 presence (e.g., brain stem) (Gong et al., 2006).

2-AG is present in the brain at nanomolar concentrations (Sugiura and Waku, 2002), which may imply that a significant portion of brain 2-AG is engaged in housekeeping functions in addition to acting as a signaling molecule. Similar to AEA, the highest neural 2-AG concentrations are located in the brainstem, medulla, striatum, and hippocampus and the lowest in the cortex, hypothalamus, and cerebellum (Buczynski and Parsons, 2010). 2-AG is a full agonist to both CB1 and CB2 receptors, while also activating PPARγ and GPR55 but not TRPV1 channels (Bouaboula et al., 2005). Neuronal 2-AG production can be initiated by an increase in the concentration of intracellular calcium, and is therefore activity dependent. A major route of 2-AG synthesis (Figure 1.2) is through phospholipase-mediated formation of diacylglycerol lipase (DAGL) (Stella et al., 1997). The enzyme phospholipase C (PLCβ) hydrolyzes phosphatidylinositol to generate DAG, which is converted to 2-AG by DAGLα and DAGLβ. Inhibitors of PLC and DAGL block calcium-dependent 2-AG accumulation in rat cortical neurons, suggesting primary involvement of this pathway in 2-AG synthesis (Stella et al., 1997). Both DAGLα and DAGLβ contribute to the steady state levels of 2-AG, although with unequal efficacy. DAGLα is also located post-synaptically (Katona et

al., 2006; Lafourcade et al., 2007; Yoshida et al., 2006). Studies involving genetic depletion of DAGLα have shown an 80% decrease in 2-AG production and that DAGLα is required for calcium dependent 2-AG biosynthesis (Gao et al., 2010b). Endocannabinoid-mediated retrograde signaling to presynaptic CB1 receptors is also absent in DAGLα-deficient mice, suggesting a primary role for 2-AG signaling in the synapse (Gao et al., 2010a; Tanimura et al., 2010). Additionally, glutamate release on group 1 metabotropic glutamate receptors (mGluRs) can generate 2-AG release by activating PLCβ (Maccarrone et al., 2008; Maejima et al., 2001; Varma et al., 2001). mGluRs are Gq/11 coupled receptors, and their activation is followed by the PLCβ-mediated cleavage of phosphatidylinositol bisphosphate into IP3 and DAG. Both PLCβ activity through post-synaptic calcium and GPCR signaling through mGluR activation can trigger long- and short-term plasticity.

2-AG is primarily metabolized by presynaptic monoacylglycerol lipase (MAGL), which accounts for nearly 85% of 2-AG hydrolysis in the mouse brain (Blankman et al., 2007; Gulyas et al., 2004; Saario et al., 2005). MAGL-selective inhibition increases the levels of brain 2-AG 8-fold (Long et al., 2009), and MAGL-deficient mice have an altered profile of endogenous 2-AG hydrolase activity and a dramatic increase in 2-AG levels eventually leading to desensitization of brain CB1 receptors (Chanda et al., 2010). MAGL also contributes to the duration and magnitude of 2-AG mediated synaptic plasticity (Hashimotodani et al., 2007; Pan et al., 2011; Schlosburg et al., 2010). Endocannabinoids can also be oxidized by cyclooxygenase-2, several lipoxygenase isozymes, and cytochrome P450s (Rouzer and Marnett, 2011).

In addition to neurons, microglia can also synthesize both AEA and 2-AG through an ATP-mediated process (Carrier et al., 2004; Walter et al., 2003). This mechanism involves the activation of purinergic P2X7 ionotropic receptors, which are highly permeable to calcium, and the induction of sustained rises in intracellular calcium that directly increases DAGL activity while inhibiting MAGL activity (Witting et al., 2004). Microglia likely constitute a main cellular source of endocannabinoids under neuroinflammatory conditions (Walter et al., 2002; Walter et al., 2003; Witting et al., 2004). Microglia also inactivate both AEA and 2-AG through FAAH and MAGL (Witting et al., 2004).

Cannabinoid signaling in the brain

Unlike classical neurotransmitters, such as amino acids, amines, or neuropeptides, lipophilic endocannabinoids are not stored in vesicles, but synthesized and released phasically upon stimulation of post-synaptic neurons, leading to the concept that endocannabinoids are mobilized as needed or “on demand” (Marsicano et al., 2003; Piomelli, 2003). However, recent data also suggest tonic release of endocannabinoids can occur under basal conditions (Long et al., 2009; Marrs et al., 2010). Endocannabinoids are degraded by metabolic enzymes into molecules that can be recycled and used to synthesize new endocannabinoids, or may be converted into biologically active metabolites. Activation of CB1 receptors can have different outcomes *in vivo* depending on the endocannabinoid ligand. AEA signaling contributes to pain sensation (Calignano et al., 1998), inflammation (Cravatt and Lichtman, 2004), depression (Gobbi et al., 2005), and improved decision making and cognitive

performance (Fagundo et al., 2013), while having less effect on hypothermia, movement, or food intake (Kathuria et al., 2003). In contrast, 2-AG signaling contributes to hypothermia, movement, analgesia, and retrograde signaling (Long et al., 2009). Behavioral variations between 2-AG and AEA activation may be explained by differences in biosynthetic pathways and metabolic products or relative concentrations in specific brain regions. However, separating functional contributions of AEA from 2-AG is difficult because both biosynthetic pathways involve arachidonic acid metabolism. Furthermore, neurons have developed mechanisms by which AEA and 2-AG can reciprocally influence their production possibly as a way of refining endocannabinoid tone (Di Marzo, 2011; Maccarrone, 2008b; Starowicz et al., 2007).

Retrograde synaptic signaling

Retrograde signaling is the predominate mode by which endocannabinoids mediate synaptic functions at both inhibitory and excitatory synapses (Wilson and Nicoll, 2002). Retrograde signaling occurs when stimulus-dependent synthesis of endocannabinoids in the postsynaptic neuron is followed by released and backward movement across the synapse to bind to presynaptic CB receptors causing alterations in neurotransmitter release. Stimulus-dependent endocannabinoid release can be induced by (1) postsynaptic depolarization, (2) activation of postsynaptic Gq-coupled receptors, (3) combined Gq activation and postsynaptic depolarization, or (4) repetitive synaptic activation. Postsynaptic neuronal depolarization or Gq protein coupling elevates intracellular calcium through voltage-gated calcium channels and elicits endocannabinoid production. Hydrophobic endocannabinoid molecules are not packed into conventional synaptic vesicles but rather, are synthesized within cell membranes

and released upon physiological stimuli (Piomelli et al., 2007). The precise mechanism by which endocannabinoids cross the synaptic cleft to reach their targets is unknown, but extracellular lipid-binding proteins may be involved (Hillard et al., 1999; Hurst et al., 2010). Facilitated diffusion driven by a carrier-protein for reuptake of endocannabinoids has been partially characterized, and termed the endocannabinoid membrane transporter (Beltramo et al., 1997). The neuronal presynaptic molecular target of endocannabinoids is most often the CB1, and the signal is terminated through hydrolysis or oxidation.

One of the most studied functions of retrograde endocannabinoid-mediated activity is synaptic plasticity (Gerdeman and Lovinger, 2003). Endocannabinoid-mediated synaptic plasticity serves either to attenuate or enhance excitability, depending on the release of an excitatory or inhibitory neurotransmitter in the presynaptic neuron. The general outcome of retrograde endocannabinoid signaling is a decrease in synaptic transmission, but the time course of activation separates this process into short- and long-term plasticity (Chevaleyre et al., 2006). Short-term plasticity has a rapid but transient onset whereby CB1 receptors inhibit presynaptic calcium influx through voltage-gated calcium channels, likely via direct interaction with the βγ subunit (Wilson et al., 2001). Long-term plasticity requires a longer induction, but is sustained for up to several hours and requires inhibition of adenylyl cyclase and downregulation of cAMP/PKA pathway via Gi/o α subunit (Chevaleyre et al., 2006; Heifets and Castillo, 2009). Reductions in protein kinase A activity and increased calcium-dependent activity of the protein phosphatase calcineurin that can control neurotransmitter release by regulating synaptic vesicle dynamics via Rab3-interacting

molecule-1α (Wilson et al., 2001). The effects of endocannabinoid-mediated synaptic plasticity are seen in the striatum (Levenes et al., 1998), pyramidal neurons in the hippocampus,(Sullivan, 2000) and nucleus accumbens (Robbe et al., 2002).

Other signaling mechanisms

Alternatively, endocannabinoids can also signal in a non-retrograde manner, in which they modulate neuronal function and synaptic transmission by activating non CB receptors located on or within neuronal, epithelial, or glial cells. The TRPV1 receptor has an intracellular binding site making it possible for AEA to activate this receptor before being released, thereby regulating calcium homeostasis as an intracellular messenger (van der Stelt et al., 2005). Endocannabinoids, their metabolites, and similar lipid messengers (AEA, 2-AG, OEP, and PEA) can also stimulate the PPAR family of nuclear receptors directly, indirectly through metabolic products, or through cell surface receptor activation and consequent intracellular signaling activation (O'Sullivan, 2007). Endocannabinoid-mediated actions at PPARs can lead to altered gene expression, regulation of feeding and body weight (Fu et al., 2003), and reductions in pain and inflammation without directly acting on CB receptors (LoVerme et al., 2005).

Endocannabinoids are involved in functions for which on demand signaling may not be possible or appropriate, including intercellular signaling, growth-related or metabolic actions, and housekeeping or homeostatic roles (Piomelli, 2003). These actions typically occur over longer time periods and imply tonic or weakly stimulated release mechanisms. Indeed, there is evidence for tonic release of AEA onto peripheral trigeminal fibers (Akerman et al., 2004) and onto a subset of interneurons in the hippocampus that express CB1 receptors (Alger and Tang, 2010; Ali and Todorova,

2010; Kim and Alger, 2010). Not all 2-AG is produced and therefore released on demand, suggesting involvement of endocannabinoids in homeostatic or growth-related roles during adulthood and development. Basal 2-AG is found in high levels in many rodent brain regions, including the hippocampus, amygdala, and cortex (Buczynski and Parsons, 2010; Stella et al., 1997), therefore 2-AG may be present in unstimulated resting neurons possibly acting as an intracellular signaling messenger (Blankman et al., 2007; Piomelli, 2003). The reported high levels of basal 2-AG are not completely released into the extracellular space (Hentges et al., 2005; Oliet et al., 2007), suggesting 2-AG may be stored within the cell (Alger and Kim, 2011). 2-AG-mediated retrograde signaling is absent in DAGLα null mice, yet basal 2-AG levels are decreased by 80%, suggesting that the remaining 20% of 2-AG is derived from another precursor and plays no role in retrograde signaling. An endocannabinoid system is also present in neurogenic niches of the embryonic and adult brain. The regulatory role and signaling mechanisms of endocannabinoids in proliferation in the embryonic brain is preserved in areas of ongoing adult neurogenesis, and will be discussed in a later section.

B. Adult neurogenesis

Stem cells

Stem cell biology has rapidly expanded since the identification of hematopoietic stem cells and adult neurogenesis in the 1960's (Altman, 1969; Becker et al., 1963). Stem cells are undifferentiated cells that have the potential to develop into many differentiated cell types. In mammals, there are two main types of stem cells: embryonic stem cells, which are isolated from the blastocytes, and adult stem cells, which are

found in various tissue including in the central and peripheral nervous systems (Hipp and Atala, 2008). Stem cells are able to go through numerous cycles of cell division while maintaining the undifferentiated state. During embryogenesis the zygote is totipotent since it has the ability to divide and produce an entire organism. Embryonic stem cells are pluripotent, and have the ability to differentiate into any of the three germ layers. These layers then produce multipotent progenitor cells that have the potential to give rise to cells from multiple, albeit limited, lineages (Figure 1.3) (Hipp and Atala, 2008). Until recently, it was thought only certain tissue areas had the ability to renew, but it is becoming more evident that many adult tissues have a small percentage of progenitor or stem cells generally acting to replace damaged cells (Sartipy, 2013). Therefore, understanding the molecular mechanisms responsible for progenitor cell generation and differentiation is required knowledge to further therapeutic applications.

Adult neurogenesis plays an active and important role in maintaining normal homeostatic processes and contributes to the plasticity of the central nervous system (Lledo and Gheusi, 2006; Ming and Song, 2011). Adult neurogenesis involves the proliferation, migration, differentiation, integration and survival of newly generated cells (Zhao et al., 2008). It was previously thought that generation of neuronal cells in mammals was limited to pre-natal development, and the adult brain lacked stem cells. Early reports of neurogenesis in the adult brain of rodents (Altman, 1969; Bayer, 1982; Kaplan and Hinds, 1977) were finally confirmed due to improved neurogenic markers. Later it was discovered that both humans and non-human primates are also capable of postnatal neurogenesis (Eriksson et al., 1998; Gould et al., 1999; Reynolds and Weiss, 1992). Both adult and embryonic mammalian nervous systems contain neural stem

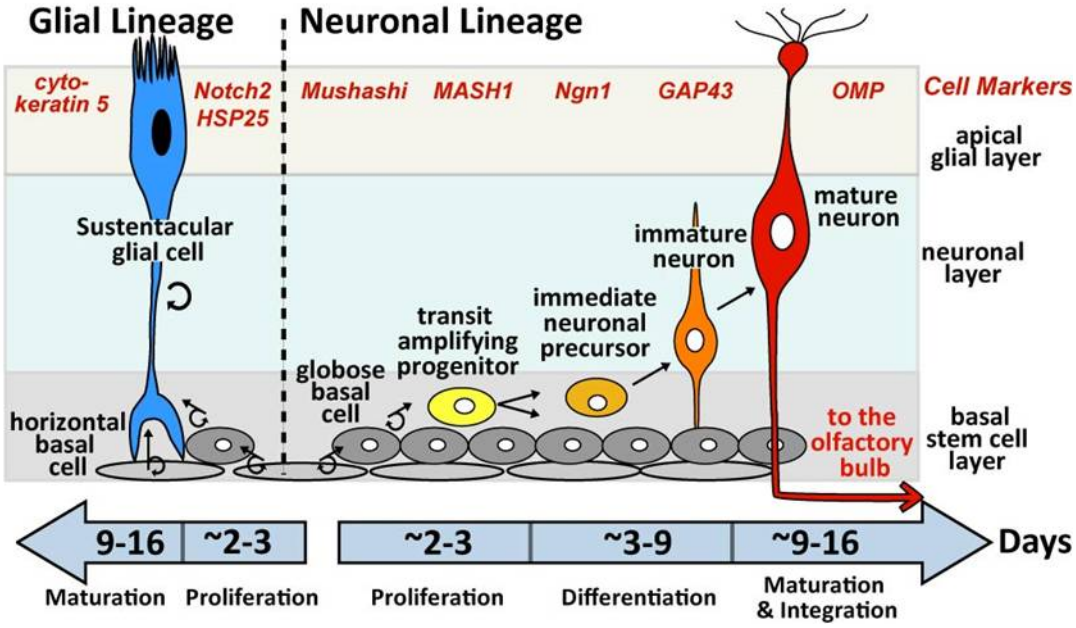

Figure 1.3 Olfactory epithelium cell types and neurogenesis.

Proliferation of horizontal or globose basal stem cells (grey) give rise to transit amplifying progenitor cells (yellow) and neuronal precursors that differentiate into immature neurons (orange) and migrate into the neuronal layer where they integrate, mature (red) and send processes to the OB after 2 weeks. Various cell markers used for immunohistochemistry are shown in red. Basal cells also give rise to glial-like sustentacular cells (blue).

cells, and progenitor cells. Adult neural stem cells are defined by their ability to self-replicate and differentiate into multiple cell types found in the CNS, including neurons, astrocytes, and oligodendrocytes (Gage, 2000), while neural progenitor cells do not fully meet the attributes of a neural stem cells (Potten and Loeffler, 1990). Embryonic stem cells are isolated from the blastocyst stage of an embryo and have the potential to generate germ layers; adult neural stem cells have restricted neurogenic potential which could be due to a change in the microenvironment of the stem cell. Neurogenic regions consist of a resident population of neural stem cells and a microenvironment that not only physically supports stem cells, but also functionally controls their development (Li and Xie, 2005). Adult neurogenesis in humans and rodents have been most studied in two specialized neurogenic regions: (1) the subventricular zone of the lateral ventricles (SVZ) where new cells are generated and migrate into the olfactory bulb (OB), and (2) the subgranular zone of the dentate gyrus where new hippocampal cells are generated (Gage et al., 1995; Reynolds and Weiss, 1992). Neural progenitor cells are not limited to these neurogenic regions of the brain, and proliferation can be observed in many brain regions, especially after injury. However, in non-neurogenic regions it appears that neurogenesis is actively repressed by the local environment. Adult neurogenesis is considered important for regulation of cognition and mood (Zhao et al., 2008), and may contribute to hippocampal and olfactory learning and memory (Deng et al., 2010; Kitamura et al., 2009). Newborn neurons integrate in the hippocampus and OB of most mammals throughout adulthood. Recently, a human cell dating strategy utilizing atmospheric ^{14}C absorbed in plants, eaten by humans, and incorporated in duplicated genomic DNA has been used to confirm adult hippocampal neurogenesis. Using this

technique, 700 new neurons are added in the adult hippocampus each day, indicating that neurons are generated throughout adulthood and that the rates are comparable in middle aged humans and mice (Spalding et al., 2005).

Subventricular zone

Adult neurogenesis requires both a local population of neural stem cells and a supportive microenvironment to initiate and sustain proliferation. Astrocytes, in addition to providing structural support, express secreted and membrane-bound molecules, including cytokines, growth factors, and neurotransmitters in response to physiological and pathological stimuli (Lafon-Cazal et al., 2003). Growth factors such as epidermal growth factor and basic fibroblast growth factor act as key regulators of neural progenitor cell proliferation and differentiation (Temple, 2001), however the precise mechanisms involved in cell fate determination remain unknown. neural stem cells located in the SVZ are represented by a subset of slowly dividing radial-like astrocytic cells, or B cells, that are glial fibrillary acidic protein (GFAP) positive and that can differentiate into the major brain cellular lineages (Doetsch et al., 1999; Laywell et al., 2000; Seri et al., 2001). Type B cells can proliferate to generate rapidly dividing cells called transient amplifying progenitors, which are positive for the homeobox transcription factor, Dlx2. Transient amplifying progenitors then generate migrating neuroblasts, distinguished by neural cell adhesion molecule immunoreactivity (Bonfanti and Theodosis, 1994). These cells form chain-like cell aggregates and migrate along the rostral migratory stream to the OB and then travel radially to reach their final position. These cells differentiate into the two main classes of OB inhibitory interneurons, granule and periglomerular cells, around 30 days after birth (Alvarez-

Buylla, 1997; Lledo et al., 2008). The OB receives newly generated interneurons, at a high rate, throughout the lifespan of the rodent (Altman, 1969). After one month, almost half of the new neurons have been eliminated while the other half is integrated into the OB (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002). The SVZ neurogenic niche occurs in rodents and some primates and is altered in humans because of the pronounced enlargement of the frontal cortex in the human forebrain. (Rakic, 2004; Sanai et al., 2004). Therefore, most of the rostral caudate nucleus, SVZ, and frontal cortex are located at levels rostral to the olfactory tubercle, and the rostral migratory stream in the human brain must take a caudal path before entering the olfactory tract. A human ventriculo-olfactory neurogenic system containing the SVZ, the rostral migratory stream, the olfactory tract, and the OB has been identified and is organized around a lateral ventricular extension reaching the OB (Curtis et al., 2007).

Continuous cell influx from the SVZ affects both the morphology and function of the OB. Neural cell adhesion molecule -deficient mice, presumably without migrating neuroblasts, have significantly smaller OBs (Cremer et al., 1994; Tomasiewicz et al., 1993). Olfactory sensory deprivation decreases the survival of newly integrated cells (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002), while increasing OB activity with long-term exposure to high concentrations of various odorants increases cell survival (Bovetti et al., 2009; Rochefort et al., 2002). The functional implication of new neurons has been studied in models whereby inhibiting neurogenesis leads to impairments in multiple aspects of olfactory function. Such impairments include increased threshold of olfactory detection (Lazarini et al., 2009), decreased perceptual learning (Moreno et al., 2009), and deficits in long-term associative olfactory memory

(Lazarini et al., 2009; Sultan et al., 2010). The survival rate of newborn OB neurons may depend on the experience of the animal, as adult circuits can influence their morphology and functional properties through activity-dependent processes. Deprivation of olfactory sensory inputs delays maturation and impairs the survival of new OB interneurons (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002), while long-term odor exposure increases the survival of interneurons (Rochefort et al., 2002) and transiently improves odor memory (Rochefort and Lledo, 2005). New functional interneurons take several weeks to mature and integrate into OB circuitry, and once established play an essential role in shaping the information that reaches the olfactory cortex (Gheusi and Lledo, 2007). This suggests a role for adult neurogenesis in long-term olfactory learning and memory.

Subgranular zone

The hippocampus, more specifically the subgranular zone, is another center of adult neurogenesis in the brain. Quiescent neural stem cells in the subgranular zone are a population of radial astrocytic cells with processes that project through and along the granular cell layer. Neural stem cells in the subgranular zone express neural transcription factors Sox2 and nestin (Suh et al., 2007). Sox2 is a transcription factor that maintains the undifferentiated state of neural stem cells. Nestin is an intermediate filament protein expressed in neural stem cells considered in the early stages of development. Similar to SVZ cells, subgranular zone neural stem cells generate actively dividing nonradial transient amplifying progenitors that lose the GFAP marker before reaching a neuroblast stage. Subgranular zone cells are also doublecortin immunoreactive and migrate a short distance into the glutamatergic granule cell layer as

immature neurons (Mu et al., 2010). Immature neurons in the granule cell layer extend axonal projections along the mossy fiber pathway to the hippocampal pyramidal cell layer, while their dendrites project in the opposite direction (Cameron et al., 1993), continuing to mature and increase in complexity over several weeks (van Praag et al., 2002). At around two months, the basic structural and physiological properties of the adult-born cells are indistinguishable from those of mature dentate gyrus cells. Granule cells in the dentate gyrus are generated continuously in both rodents and humans (Ming and Song, 2005a). However, the molecular mechanisms underlying nerve growth and axon/dendrite guidance of adult-generated granular neurons are largely unknown.

Newborn adult hippocampal neurons not only replace lost neurons, but contribute to the plasticity of the existing circuitry as plasticity can alter in responds to experiences encountered throughout adulthood (Ge et al., 2008). Neurogenesis in the adult hippocampus is important for higher-order cognitive function and learning and memory (Deng et al., 2010). Learning, exposure to enriched environment, and exercise, are major regulators of hippocampal neurogenesis (Gould et al., 1999). The hippocampus is especially important for episodic and spatial memory. Learning of hippocampus-dependent tasks but not hippocampus-independent tasks increases the number of adult-born dentate gyrus cells at around one week of age (Gould et al., 1999; Leuner et al., 2004). For example, special navigation tasks can promote the survival of dentate gyrus cells that were born within a critical period before training (Dupret et al., 2007). Mechanisms for memory formation and retention, (i.e, long term potentiation) have been shown to increase subgranular zone progenitor proliferation and promote survival of new neurons (Bruel-Jungerman et al., 2006). In rodents, voluntary wheel running

significantly increases the proliferation of neural progenitor cells in the subgranular zone and SVZ of both young and aged animals (van Praag et al., 1999; van Praag et al., 2005). Hippocampal neurogenesis is also affected by aging, seizures, and antidepressant treatments. For example, seizure activity induces proliferation of neural progenitors, and results in abnormal morphological development of granule cells and contributes to seizure-associated cognitive deficits (Overstreet-Wadiche and Westbrook, 2006; Parent, 2007). Antidepressants can also regulate adult hippocampal neurogenesis by increasing neural progenitor proliferation, accelerating synaptic integration, and enhancing survival of newborn neurons (Sahay and Hen, 2007; Warner-Schmidt and Duman, 2006). Furthermore, neurogenesis is compromised during the aging process. A drastic reduction of adult hippocampal neurogenesis is seen in aged animals, which is caused by a depletion of neural stem cells and changes in the hippocampal microenvironment (Fabel and Kempermann, 2008).

Cannabinoids and adult cell proliferation

It is well established that the cannabinoid system modulates progenitor cells throughout development (Diaz-Alonso et al., 2012). The endocannabinoid system has a cell specific and spatio-temporal distribution from very early stages of embryonic development, even before the appearance of the neural tube (Sun and Dey, 2009). Mammalian CB1 receptor expression during neural development is predominately present in white matter during neurogenesis and axonal migration prior to synaptic maturation and signaling. Not until postnatal stages and into adulthood are CB1 receptors expressed in grey matter (Berrendero et al., 1998), suggesting

neurodevelopmental CB1 receptor actions are likely to be independent of their neuromodulatory role at the mature neuronal synapse. The presence and function of the cannabinoid system in progenitor cells in the embryonic brain is preserved in the adult brain in neurogenic regions.

The cannabinoid system modulates many functions involved in adult neurogenesis including proliferation, differentiation, migration, and integration in functional synapses. CB1 and CB2 receptor activation can modulate neural stem cell proliferation *in vitro* models (i.e., neurosphere cultures). Neurospheres are non-adherent *in vitro* culture of neuronal stem cells that grow as free-floating clusters. Neurospheres from embryonic and postnatal development stages express CB receptors and metabolic endocannabinoid enzymes. Increases in their intracellular calcium concentration increase endocannabinoid production (Aguado et al., 2005; Molina-Holgado et al., 2007; Palazuelos et al., 2006). Neurosphere proliferation is regulated by both CB1 and CB2 receptor stimulation through the PI3K/Akt signaling pathway (Molina-Holgado et al., 2007). CB2 receptor activation *in vitro* promotes neural progenitor cell proliferation and neurosphere generation which can be decreased with CB2 receptor specific antagonists (Molina-Holgado et al., 2007).

In the central nervous system, CB1 and CB2 receptor activation can modulate neural stem cell proliferation *in vivo* (Molina-Holgado et al., 2007). Neural progenitor cells reside in the SVZ and DG of the hippocampus and in the OB. Embryonic as well as adult neural progenitor cells express a functional endocannabinoid system (Aguado et al., 2006), including CB1 and CB2 receptors, biosynthetic and degradative enzymes, and produce endocannabinoid ligands in response to increased intracellular calcium

concentrations (Jiang et al., 2005; Palazuelos et al., 2006). Pharmacological and genetic mouse models have shown that endocannabinoids stimulate hippocampal and SVZ neural progenitor cell proliferation via CB1 and CB2 receptors and modulate the self-renewal ability of neural progenitor cells (Aguado et al., 2007; Rubio-Araiz et al., 2008). Furthermore, CB1 receptors are present in dividing cells identified by 5-bromo-2′-deoxyuridine (BrdU) labeling and the expression of endogenous cell cycle marker Ki-67 (Aguado et al., 2005; Mulder et al., 2008). CB1 receptor knockout mice show nearly a 50% reduction in dividing BrdU-positive cells in the SVZ and DG (Jin et al., 2004). Indirectly increasing the endocannabinoid AEA, by inhibiting its degradation enzyme FAAH, induces progenitor cell proliferation in the DG (Aguado et al., 2005; Aguado et al., 2006). DAGLα and β contribute to 2-AG regulation of adult neurogenesis in the SVZ and DG (Gao et al., 2010b; Goncalves et al., 2008). Young adult mice show an almost 50% reduction in SVZ proliferation in mice lacking the biosynthesis enzyme DAGLα gene using Ki-67 and DCX immunoreactivity (Gao et al., 2010b). Progenitor proliferation was observed in CB2 receptor deficient mice in both physiological conditions as well as after excitotoxicity (Palazuelos et al., 2006). In young mice, CB2 receptor and DAGL antagonists inhibit cell proliferation in the SVZ, which is associated with a reduction in the appearance of new neurons in the OB (Goncalves et al., 2008). In contrast, CB2 receptor agonists can stimulate cell proliferation in the SVZ of older animals and increase the appearance of new neurons in the OB (Goncalves et al., 2008), suggesting that CB2 receptor signaling may counteract the natural decline in adult neurogenesis that is associated with aging (Goncalves et al., 2008; Marchalant et al., 2009b; Marchalant et al., 2008). Similarly, CB1 receptor-deficient mice show an early onset of

age-related memory deficits (Albayram et al., 2012). Additionally, the synthetic cannabinoid agonist WIN can partially restore proliferation and neurogenesis in the hippocampus of aged rats (Marchalant et al., 2008).

Endocannabinoids also regulate neural progenitor differentiation (Aguado et al., 2006; Guzman, 2003; Guzman et al., 2002). The cannabinoid system contributes to the regulation of neural precursors committed to a neuronal or glial lineage (Aguado et al., 2006). However, cannabinoid influence on cell lineage is not consistent and is dependent on location, age, pharmacology, pathophysiology, and signal bioavailability (e.g., locally generated endocannabinoids versus acute repeated injections of synthetic agonists). Endocannabinoid signaling promotes differentiation of cortical neuronal progenitor cells and of adult hippocampal progenitors in AEA degradation enzyme FAAH knockout mice (Aguado et al., 2006). Methandamide, a nonhydrolyzable AEA analog, significantly decreases neurogenesis measured by BrdU in the adult DG (Rueda et al., 2002b). Additionally, the synthetic agonist HU10 inhibited neuronal progenitor cell differentiation through reduction of the ERK pathway activation in embryonic cortical cells and human neural progenitor cells (Rueda et al., 2002b). CB1 and CB2 receptor activation increases oligodendrocyte numbers (Arevalo-Martin et al., 2007) through survival mechanisms involving (phosphoinositide-3 kinase) PI3K/Akt signaling (Molina-Holgado et al., 2002) (discussed further in Chapter 5). However, pharmacological stimulation of CB1 receptors may result in neurogenesis (Jiang et al., 2005). In SVZ cell cultures, both AEA and CB1 activation enhance neural stem cell differentiation into neurons, without affecting astrocyte or oligodendrocyte differentiation, while also upregulating the expression of pro-neural genes (Compagnucci et al., 2013;

Xapelli et al., 2013). Interestingly, a four-day treatment of AEA promotes glial differentiation, whereas a seven-day treatment promotes neuronal differentiation *in vivo* (Soltys et al., 2010), suggesting that signaling duration may also influence neural progenitor cell lineage. Cannabinoids can also oppose the anti-neurogenic effect of neuronal nitric oxide (Kim et al., 2006). Collectively, these data suggest the existence of an endogenous cannabinoid tone actively modulating neuronal/glial balance through the CB1 receptor.

The cannabinoid system can also influence post-differentiation processes such as migration, maturation, and cell survival. Mouse migratory neuroblasts express CB receptors and 2-AG synthesis and degradation enzymes. Inhibition of DAGL activity or CB receptors substantially decreases migration along the rostral migratory stream (Oudin et al., 2011). Similarly, direct activation of CB receptors or prevention of 2-AG breakdown increases neuroblast migration *in vivo* and *in vitro* (Oudin et al., 2011). CB1 activation also enhances the maturation of neural stem cells through long term inhibition of ERK1/2 pathway (Compagnucci et al., 2013). Cannabinoids can also take part in the control of neural cell fate, thereby modulating the balance between cell death and survival (Guzman, 2003; Mechoulam et al., 2002).

Cannabinoid receptor signaling mechanisms of cell proliferation

In contrast to the well-studied and understood signaling mechanism at neuronal synapses, endocannabinoid signaling mechanisms in progenitor cells remain to be fully understood. CB receptors present on progenitor cells are activated by endocannabinoid ligands potentially by surrounding neurons (Piomelli, 2003) in a paracrine or autocrine

manner or by progenitor cells themselves (Aguado et al., 2005) and ependymal cells (Oudin et al., 2011).

CB receptors are coupled to the activation of phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated protein kinase (ERK) pathways, which are classical routes mediating proliferation and cell survival (Galve-Roperh et al., 2007; Pertwee et al., 2010). The proliferative role of the PI3K/Akt cascade has been demonstrated in CB2 receptor-mediated progenitor cell proliferation (Palazuelos et al., 2012) and via CB1 receptors in cortical and cerebellar progenitors (Galve-Roperh et al., 2013; Trazzi et al., 2010). The mechanisms of CB1 receptor-mediated ERK activation are multiple and interconnected, thus providing a complex scenario. CB1 coupling to Gi/o proteins and the resultant reduction in cAMP levels partly mediate the activation of the ERK pathway by de-inhibiting ERK pathway by protein kinase A (Davis et al., 2003; Derkinderen et al., 2003). In addition, GPCR βγ subunit activation can stimulate ERK signaling in a PI3K manner (Dalton and Howlett, 2012; Hart et al., 2004). It is likely that ERK activation may occur by different mechanisms depending upon CB1 receptor activation at different time points. CB receptor signaling in neural cells may also involve activation of mammalian target of rapamycin complex 1 (mTORC1), a protein kinase that regulates cell growth, proliferation, and survival (Puighermanal et al., 2009). CB2 receptor activation can couple to mTORC1 activation in neural progenitor cells in the subgranular zone of the adult hippocampus leading to proliferation (Palazuelos et al., 2012). CB1 receptor stimulation in adult hippocampus can also activate mTORC1 signaling, possibly effecting protein synthesis and neuronal plasticity (Puighermanal et

al., 2012). The endocannabinoid system also modulates oligodendrocyte differentiation and cell survival partially through mTORC1 regulation (Gomez et al., 2011).

Aside from the canonical signaling pathway, CB1 receptors may also have indirect actions that promote neurogenesis via crosstalk with growth factor signaling pathways essential for progenitor cell proliferation (Williams et al., 2003). CB1 receptor activation is involved in the regulation of neurotrophin brain-derived neurotrophic factor (BDNF) levels. CB1 receptor-deficient mice have reduced hippocampal BDNF levels under basal circumstances, which may explain some of the neuronal plasticity and emotional alteration (i.e., anxiety, despair, memory deficits) in those animals (Aso et al., 2008; Marsicano et al., 2002a). Additionally, growth factors critical for the maintenance of adult neural stem cells, such as epidermal growth factor and fibroblast growth factor 2, are also regulated by CB signaling during neurodegenerative paradigms, such as hippocampal and striatal excitotoxicity (Aguado et al., 2007; De March et al., 2008; Marsicano et al., 2003). Reciprocally, extracellular signaling cues mobilize endocannabinoids. Fibroblast growth factor along with neural cell adhesion molecule increase 2-AG levels through DAGL and PLCγ activation (Williams et al., 2003).

CB1 receptor activation can also regulate many transcription factors involved in neural progenitor cell homeostasis, neuronal differentiation, and maturation (Bromberg et al., 2008). In neuroblastoma cells and SVZ progenitor cells, CB1 receptor activation modulates the transcription factor Pax6 (Bromberg et al., 2008), a paired box family member essential for the generation of glutamatergic neurons and cortical neurogenesis (Diaz-Alonso et al., 2012; Osumi et al., 2008). Pax6 is involved in neural progenitor cell proliferation and influences neuronal differentiation. CB1 receptor signaling via PI3K/Akt

activation can control Pax6 in neural progenitor cells (Galve-Roperh et al., 2013). CB1 activation regulates Pax6 pos-translationally by phosphorylation mediated by the PI3K/Akt pathway in differentiating neuroblastoma cells (Bromberg et al., 2008). CB receptor activation also promotes the expression of differentiation transcription factor Olig-2 through PI3K/Akt/mTORC1 signaling in oligodendrocyte progenitor cells (Diaz-Alonso et al., 2012). CB1 receptors may regulate cell fate decision by modulating growth factor, neurotrophin, and cytokine expression. CB receptors regulate PI3K/Akt signaling in neural progenitor cells through transcripts in cooperation with epidermal growth factor receptors (Sutterlin et al., 2013).

Measuring proliferation

Common markers for neural stem cells and neural progenitor cells such as nestin, BrdU, and DCX can also detect reactive astrocytes or cancer cells (Kaneko et al., 2000; Komitova and Eriksson, 2004; Lendahl et al., 1990). BrdU labeling in conjunction with cell specific markers is a common method of studying adult neurogenesis. BrdU is a thymidine analog that gets incorporated into DNA during the dividing phase, S phase, of the cell cycle. One drawback is that BrdU can also be incorporated during DNA synthesis associated with repair, gene duplication without cell division, and apoptosis (Taupin, 2007). It is also slightly toxic at doses above 50 mg/kg which can lead to negative effects on proliferation of neural progenitor cells *in vitro* (Cooper-Kuhn and Kuhn, 2002; Ross et al., 2008). In this dissertation, I did not use other proliferative markers such as Ki-67 or PCNA because they are not specific to the phase of the cell cycle, exhibit nonspecific immunoreactivity outside of proliferation, are subject to variability depending on fixation, and cannot be used for cell lineage analysis

(Ohta and Ichimura, 2000; Taupin, 2007). The loss of BrdU immunoreactivity following high doses of irradiation (Taupin, 2007) suggests that BrdU incorporation does not typically reflect DNA repair. BrdU incorporation occurs primarily in cells undergoing DNA synthesis. For example, in the OE following OB ablation in young adult rodents, apoptotic cells were positive for Terminal dUTP Nick End Labeling (TUNEL), a cell death marker, but rarely incorporated BrdU (Bauer and Patterson, 2005). This suggests that dying neurons do not re-enter the cell cycle following bulbectomy lesion and that BrdU incorporation is an appropriate experimental approach to monitor proliferation in the olfactory system (Brann and Firestein, 2010).

C. Proliferation in the mouse olfactory epithelium

The peripheral olfactory epithelium (OE) is an ideal system with which to investigate neuroproliferative factors, as neurogenesis continues to occur throughout life under both physiological and injury conditions. The OE lines the posterior dorsal part of the nasal cavity, and the axonal projection from the sensory neurons synapses onto the OB, the area of the brain that serves as the first CNS relay for olfactory information. Several features of the OE make it an ideal system with which to study proliferation: (1) the olfactory epithelium is the only system in which sensory neurons are in direct contact with the environment and therefore can be easily damaged by environmental pollutants (2) sense of smell is essential for survival through foraging, predator detection, and reproduction in many mammals and is fundamental to health and nutrition in humans, (3) olfactory sensory neurons provide a route for viruses, bacteria, and some environmental toxicants to enter the brain (4) life-long capacity to regenerate

neuronal and non-neuronal cell types (5) restoration of sensory function is possible through OE neurogenesis (6) its peripheral location makes the OE easy to access and isolate.

The OE is a pseudostratified neuroepithelium that contains several cell types (Figure 1.3). Bipolar olfactory sensory neurons (OSNs) have dendritic knobs that lie above the surface of the epithelium with cilia that extend from the knobs to the external environment where volatile chemical molecules come into contact with olfactory receptors on the cilia. Mature OSNs have a cell soma located in the middle third of the epithelium and an unmyelinated axon that projects to the OB. Non-neuronal microvillous and sustentacular cells have their cell bodies close to the apical surface of the epithelium. Microvillous cells are the primary source of NYP in the OE, and have been described variously as primary olfactory neurons, secondary chemosensory cells, or non-sensory cells. A second type of non-neuronal cell found in the OE is the glial-like sustentacular cell, which spans the height of the OE. Sustentacular cells have a large cell soma located in the upper third of the epithelium and a thin cytoplasmic extension that terminates in an endfoot process, occasionally around basal cells. Basal cells are multipotent progenitor cells that lie near the basal lamina, and can proliferate and differentiate into both neurons and sustentacular cells. Non-neuronal sustentacular cells can be self-derived or renewed from basal cells (Huard et al., 1998), while neurons are generated solely from basal cells.

Basal cells function as stem cells and multipotent progenitor cells under a variety of conditions including normal maintenance and regeneration of the OE after injury (Carter et al., 2004; Chen et al., 2004a; Leung et al., 2007a). Basal cells give the OE its

regenerative ability, making the neuroepithelium an ideal system with which to study proliferation (Caggiano et al., 1994). Chronic imaging of pre- and post-olfactory epithelial injury has shown that OSNs retain their ability to reestablish functional inputs to the OB, suggesting that the mechanism underlying developmental innervation persists in adulthood and allows neuronal restoration after massive sensory neuron loss (Cheung et al., 2014). Despite a high rate of proliferation of neural precursors, the olfactory epithelial thickness remains constant in the adult rat from two to twelve months (Weiler and Farbman, 1997b), suggesting a balance between cell proliferation and cell death and that many new cells don't fully mature and integrate into olfactory epithelial circuitry.

Basal cells

There are two populations of multipotent stem-like cells in the adult OE: globose basal cells (GBCs) which proliferate at a high rate and differentiate into both OSNs and sustentacular cells, and horizontal basal cells (HBCs) which are flat horizontal basal cells that lie adjacent to the basal lamina and have a lower mitotic index (Holbrook et al., 1995) GBCs are a heterogeneous population of stem and progenitor cells that emerges early in embryonic development. HBCs resemble a type of reserve population of quiescent cells that requires injury to be activated. In contrast, GBCs are always multipotent even in the absence of injury, and are responsible for the physiological level of ongoing proliferation in the OE. Multipotent horizontal and globose basal cells give successive rise to transit amplifying progenitors, immediate neuronal precursor cells, immature neurons, and mature neurons that extend axons that must find the proper synaptic targets in the OB (Figure 1.3) (Beites et al., 2005; Caggiano et al., 1994; Leung

et al., 2007a; Schwob et al., 1994). A heterogeneous GBC subpopulation can be identified by the expression of certain transcription factors. For example, Notch signaling directs multipotent GBCs to derive sustentacular cells, while transcription factor Sox2 regulates the proliferation of progenitors and neural differentiation (Guo et al., 2010). The GBC population that contributes to neuronal lineage sequentially expresses the proneural genes Ascl1 (also known as Mash1) and Neurog1 (Cau et al., 2002). Mash1 positive GBCs are the earliest neuronal precursors identified (Gordon et al., 1995). GBCs are mitotically active in normal mucosa, and a population of GBCs proliferates to an enhanced degree after various forms of damage that elicit either selective regeneration of the neuronal population or reconstitution of the OE (Graziadei and Graziadei, 1979; Huard et al., 1998). GBCs have a subpopulation that shows features of central stem cells such as the incorporation of BrdU, reentry into the mitotic cycle following lesion, and activation after injury (Chen et al., 2004a; Jang et al., 2014).

HBCs function as a reserve population of multipotent progenitor cells in the adult OE that are activated to participate in reconstruction of the OE following injury (Leung et al., 2007a). HBCs are defined by their presence as a single-cell layer below the GBCs, but with specific expression of keratin 5 and keratin 14 and by their direct contact with the basal lamina (Holbrook et al., 1995). They rarely divide to self-renew *in vivo* (Mackay-Sim and Kittel, 1991) but can clonally expand *in vitro* to generate both neuronal and glial cell types (Carter et al., 2004). This was shown by ablating sustentacular cells, OSNs, and a substantial GBC population by methyl bromide gas, then tracking the lineage of HBCs using a genetic mouse model (Leung et al., 2007a). These cellular properties are similar to neural stem cells found in the SVZ and

hippocampus (Carter et al., 2004; Doetsch et al., 1999; Palmer et al., 1997). HBCs also give rise to Mash1 positive cells as intermediates in the terminal differentiation pathway (Leung et al., 2007a). HBC proliferation is stimulated by epidermal growth factor and tumor necrosis factor, while GBC proliferation is stimulated by fibroblast growth factor (Newman et al., 2000). Fibroblast growth factor and epidermal growth factor are important indicators of neural stem cell proliferation and are required to generate multipotent neurospheres from olfactory mucosa (Murrell et al., 2005; Murrell et al., 2008) and from adult rodent brain (Reynolds and Weiss, 1992). HBCs proliferate slowly, and self-renew (Mackay-Sim and Kittel, 1991) and are able to generate all OE cell types *in vivo* and *in vitro* (Carter et al., 2004; Leung et al., 2007a).

Transcription factors Sox2 and Pax6 both function at multiple levels in the developing and adult brains as well as the OE. Sox2 is one of the key factors in regulation of embryonic stem cell self-renewal and induction of somatic cells to become pluripotent stem cells (Pevny and Lovell-Badge, 1997; Takahashi and Yamanaka, 2006). Sox2 is expressed by neural progenitor cells and neural stem cells in the SVZ into adulthood (Pevny and Placzek, 2005), and regulates ongoing neurogenesis in the postnatal DG (Favaro et al., 2009). Pax6 regulates multipotent progenitor cells (Walther et al., 1991). Down-regulation of Pax6 is associated with the differentiation of radial glia in the SVZ (Gotz et al., 1998). In Pax6 null mice, the nose fails to form properly, indicating that Pax6 is involved in olfactory development (Collinson et al., 2003). Sox2 and Pax6 are expressed in adult OE HBCs, sustentacular cells, and a subset of GBCs that are multipotent and Mash1 positive (usually committed to neuronal lineage), but only a small subset of immediate neuronal progenitor cells (Chen et al., 2004a; Davis

and Reed, 1996; Guo et al., 2010). Therefore, Sox2 and Pax6 are expressed by GBCs with the characteristics of stem cells, but do not apparently commit to neuronal fate. Additionally, BDNF is also expressed in the OE. After extensive damage, mRNA expression of BDNF dramatically increases in the OE during the first seven days post lesion. This expression pattern suggests that BDNF plays a role in the regeneration of new OSNs in the OE, until axons project to the OB. BDNF immunoreactivity is also found in HBCs after bulbectomy (Buckland and Cunningham, 1999).

D. Cannabinoids and the olfactory system

Volatile chemical odorants are inhaled through the nose and are detected by odorant receptors on cilia that protrude from OSN knobs that extend into the OE mucosa. Olfactory receptors are GPCRs containing the Gαolf subunit, which induces an increase in adenylyl cyclase and cAMP leading to the opening of nucleotide-gated cation channels and depolarization generating an action potential. The signal then travels to the OB where OSNs first synapse within discrete anatomical areas called glomeruli. The axon of each OSN synapses in only one glomerulus. Each glomerulus contains mitral and tufted relay neurons, which project axons to the olfactory cortex, and periglomerular interneurons, which encircle the glomerulus. Additionally, granule cell interneurons provide negative feedback circuits and are located deeper within the bulb. Such interneurons in the OB process odors, yet their exact roles are not fully understood. Two types of inhibitory interneurons, periglomerular and granule cells, act at two distinct levels within the OB and coordinate the spiking of mitral cells, which are the principal output neurons of the OB. Periglomerular cells are $GABA_A$ergic (Hayar et

al., 2004; Kiyokage et al., 2010), and presynaptically inhibit OSNs signaling within the OB through GABAergic transmission (Aroniadou-Anderjaska et al., 2000; Murphy et al., 2005). Periglomerular cells receive input from the olfactory nerve or dendritic glutamatergic input from external tufted glutamatergic cells or mitral cells (Hayar and Ennis, 2007; Hayar et al., 2004).

The cannabinoid system is present in central and peripheral olfactory regions. CB receptors have been identified in rodent OB glomerular layer (Gong et al., 2006). Neurons in the glomerular layer are immunoreactive for enzymes that synthesize endocannabinoids (Okamoto et al., 2007; Piomelli, 2003). Periglomerular cells are likely candidates for direct effects of endocannabinoids since CB1 receptors are robustly expressed in the glomerular layer (Moldrich and Wenger, 2000). AEA displays inhibitory effect on firing rates periglomerular cells when hyperpolarized (Wang et al., 2012). The CB1 receptor antagonist AM251 reversibly increased periglomerular cell firing and depolarized the cells, while CB agonist WIN hyperpolarized the cells (Wang et al., 2012). In the presence of AM251 bath application of WIN failed to induce a decrease in firing rate or change in membrane potential (Wang et al., 2012). Electrophysiological studies in the OB have shown through trains of depolarizing voltage steps evoked suppression of inhibitions, suggesting that spontaneous rhythmic bursting of tufted cells triggers the release of endocannabinoids from tufted cells, that can reduce GABA release from periglomerular cells which in turn regulates the activity of periglomerular cell synaptic targets such as tufted cells (Wang et al., 2012). Burst firing of tufted cells triggers endocannabinoid release and retrograde inhibition of PG cells leading to a

decrease in GABA release, and subsequent transient decrease of PG cell inhibition of tufted cells (Wang et al., 2012).

A cannabinoid system has been identified in the peripheral OE of *Xenopus laevis (Czesnik et al., 2007)*. CB1 receptors are expressed on sensory neurons and 2-AG is synthesized in both OSNs, and glial-like sustentacular cells (Breunig et al., 2010b). This cannabinoid system modulates odor-evoked responses in OSNs in *Xenopus* (Czesnik et al., 2007). Although a cannabinoid system exists in the mammalian OB, no reports have described a functional cannabinoid system in the peripheral olfactory system of mammals.

E. Summary of dissertation experiments

The presence of an endocannabinoid system in neurogenic regions of the CNS and its involvement in proliferation, differentiation, and migration indicate a similar phenomenon may occur in peripheral neurogenic regions such as the OE. **The overall hypothesis of this dissertation is that cannabinoid receptor activation promotes neurogenesis in the mouse olfactory epithelium.** Behavioral, pharmacological, immunohistochemical, and whole animal studies were used to test the predictions of this hypothesis.

Chapter 2 is a description of experiments testing the hypothesis that the mouse OE contains an endocannabinoid system. The presence of CB1 and CB2 receptor protein and mRNA was examined, along with the subcellular distribution of CB1 receptors using immunohistochemistry. Additionally, functional activation of CB receptors was examined using calcium imaging.

Chapter 3 includes a description of experiments testing the hypothesis that CB1/CB2 receptor knockout mice will have impaired olfactory-mediated behaviors. First, OE morphology was compared between wildtype and CB1/CB2 receptor knockout (KO) mice. OE thickness was measured and basal cell and OSN numbers were counted. Olfactory-mediated behavioral tests including the buried food test and habituation/dishabituation test were performed on both wildtype and CB1/CB2 receptor KO mice.

Chapter 4 is a description of experiments testing the hypothesis that CB receptor activation increases neurogenesis in the mouse olfactory epithelium. Proliferation levels after exogenous and endogenous cannabinoid administration were measured in neonatal mouse OE. Adult proliferation levels were also measured in both wildtype and CB1/CB2 receptor KO mice after intranasal aspiration of an exogenous cannabinoid ligand, CB1 receptor specific antagonist, and endocannabinoid degradation enzyme inhibitors. Neurogenesis was measured through colocalization of neuronal and proliferation markers two weeks after cannabinoid manipulation in the wildtype mouse OE. Finally, cell death numbers were examined in both wildtype and CB1/CB2 receptor KO mice at several time points throughout the progression of neurogenesis.

Using this dissertation as a background, future experiments could examine the effects of chronic administration of cannabinoid agonists on basal cell proliferation and neurogenesis in the mouse OE. Additionally, it would be interesting to investigate the contribution of the cannabinoid system to the repopulation of OE cells after environmental toxic damage. Overall, this work has identified a functional cannabinoid system in the mouse OE for the first time and furthers our understanding of peripheral

progenitor cell proliferation and neurogenesis. It is essential to understand the complex mechanism of OE progenitor cell proliferation, which could lead to future regenerative strategies using human olfactory derived progenitor cells.

Chapter 2: Identification of a cannabinoid system in the mouse olfactory epithelium

Introduction

Cannabinoid system

Although cannabis has been a widely used illicit drug in many parts of the world, cannabinoid chemistry and biological actions were not well defined until the recent discovery of CB receptors and endogenous ligand less than 25 years ago (Devane et al., 1988; Mechoulam, 1995; Munro et al., 1993). The endocannabinoid system consists predominately of two main ligands, *N*-arachidonoylethanolamide (AEA or anandamide) and 2-arachidonylglycerol (2-AG), two G-protein coupled receptors (GPCR), cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), and the endogenous compounds involved in the synthesis and degradation of cannabinoid ligands. CB1 receptors are one of the most abundant G protein-coupled receptors in the brain, whereas CB2 receptors are localized on immune cells, yet recent attention has been given to the importance of CB2 receptors in central nervous system functions (Van Sickle et al., 2005). Classically, CB receptors preferentially recruits Gi/oα proteins (Mackie and Stella, 2006) resulting in inhibition of adenylyl cyclase and a decrease in cAMP levels. However, CB1 receptors have been reported to recruit to Gs or Gq/11α proteins in a ligand-and-tissue dependent manner (Childers et al., 1993; Lauckner et al., 2005). Cannabinoids are also known to activate other extracellular targets such as the transient receptor potential cation channel (TRPV1) channel (Begg et al., 2005) and

other G protein-coupled receptors such as GPCR55 (Sawzdargo et al., 1999), GPCR119 (Overton et al., 2006), and GPCR18 (Kohno et al., 2006).

Endocannabinoids are short-range lipid-based signaling molecules synthesized from arachidonic acid. AEA is biosynthesized from membrane lips by *N*-acyltransferase and phospholipase D (Kuwae et al., 1999). 2-AG can be synthesized from arachidonic acid-containing diacylglycerol derived from increased inositol phospholipid metabolism by the action of diacylglycerol lipase (DAGL) (Prescott and Majerus, 1983). Fatty acid amide hydrolase and monoacylglycerol lipase (MAGL) are intracellular enzymes responsible for the degradation of endocannabinoids (Figure 1.2) (Cravatt et al., 2001; Deutsch and Chin, 1993).

In the central nervous system, endocannabinoids are involved in neuromodulatory signaling, which is predominately regulated by (1) the CB1 receptor, (2) a putative membrane cannabinoid transporter, and (3) hydrolytic enzymes involved in both synthesis, and degradation of cannabinoids. Endocannabinoids can act as retrograde signaling molecules, whereby postsynaptic depolarization leads to “on demand” endocannabinoid synthesis, release, and subsequent diffusion in a retrograde fashion across the synapse to activate CB receptors on the presynaptic terminal of both excitatory and inhibitory neurons. Termination of endocannabinoid signaling may require cellular reuptake via a transport process and are enzymatically degraded within the cell membrane (Fegley et al., 2004; Piomelli et al., 1999).

Cannabinoids and olfaction

Several peripheral sensory systems are modulated by the endocannabinoid system. In the retina, cannabinoids accelerate phototransduction deactivation cascade

in the outer segment of the cones (Straiker et al., 1999; Struik et al., 2006). Additionally, CB1 receptors are expressed on type II taste cells, and endocannabinoid signaling enhances the sweet taste (Yoshida et al., 2010). Finally, CB1 receptors are expressed on dorsal root ganglion cells (Bridges et al., 2003; Hohmann et al., 1999) and play a role in the spinal nociceptive system (Agarwal et al., 2007; Morisset et al., 2001).

In the central olfactory system, CB receptors have been identified in the rodent OB glomeruli, a spherical structure where afferents from OSNs make their primary synapses (Wang et al., 2012). Neurons in the glomerular layer are immunoreactive to endocannabinoids synthesis enzymes (Okamoto et al., 2007; Piomelli, 2003; Wang et al., 2012). Electrophysiological studies have shown the endocannabinoid system plays a functional role in regulating neuronal activity and signaling in the glomeruli through retrograde signaling and depolarization-induced suppression of inhibition (Wang et al., 2012). CB1 receptor protein is also found in high density in the granule cell layer of the OB. The granule cell layer is the deepest layer in the OB and is comprised of γ-aminobutyric acid (GABAergic) interneurons that provide lateral inhibition through dendro-dendritic synapses onto mitral glomeruli cells (Soria-Gomez et al., 2014). Endocannabinoids, 2-AG and AEA are also present in the main OB (Soria-Gomez et al., 2014).

Although CB1 receptors were localized in the OB several decades ago, (Cesa et al., 2001; Egertova et al., 2003; Egertova and Elphick, 2000; Herkenham et al., 1991) the first identification of cannabinoids in the peripheral olfactory system was only recently, when CB1 receptor mRNA was detected in the OE of *Xenopus laevis* tadpoles (Migliarini et al., 2006). Tadpole CB1 receptors are expressed on dendrites of OSNs

and 2-AG is synthesized in both OSNs and glial-like sustentacular cells (Breunig et al., 2010a; Czesnik et al., 2007). Thus, 2-AG acts through CB1 receptors located on OSN dendrites in a paracrine and autocrine manner. Odor-evoked increases in calcium in the tadpole OE are modulated by endocannabinoid signaling (Breunig et al., 2010a; Czesnik et al., 2007).

Olfactory epithelium

The OE is a pseudostratified neuroepithelium that contains several cell types (Figure 1.3) that will be identified through immunoreactivity in this chapter. Bipolar OSNs have dendritic knobs that lie above the surface of the epithelium with cilia that extend from the knobs to the external environment where volatile chemical molecules come into contact with olfactory receptors on the cilia. Mature OSNs have a cell soma located in the middle third of the epithelium and an unmyelinated axon that projects to the OB. Non-neuronal cells, such as glial-like sustentacular cells and microvillous cells, span the height of the OE. Sustentacular cells have a large cell soma located in the upper third of the epithelium and a thin cytoplasmic extension that terminates in an endfoot process, occasionally around basal cells. Basal cells are proliferative multipotent progenitor cells that lie near the basal lamina, and are the stem cells of the OE. There are two types of basal cells: globose basal cells that proliferate at a high rate and give rise to both OSNs and sustentacular cells (Chen et al., 2004a; Huard et al., 1998), and flat horizontal basal cells that lie adjacent to the basal lamina and have a lower mitotic index (Holbrook et al., 1995).

The present chapter seeks to determine if a functional cannabinoid system is present in the mouse OE. To identify a mouse OE cannabinoid system, the presence of

CB receptors, endocannabinoid ligands, and metabolic enzymes will be investigated through RT-PCR, western blot, immunohistochemistry, liquid chromatography-mass spectrometry, and confocal calcium imaging.

Methods

Animals

Adult male (6-8 weeks old) Swiss Webster (CFW) and C57BL/6 control (wildtype; WT) mice were obtained from Charles River, Portage, MI. Cannabinoid receptor 1 and cannabinoid receptor 2 deficient (CB1/CB2 knockout) mice were kindly provided by Dr. Norbert Kaminski (Michigan State University, MI) who obtained them from Dr. Andreas Zimmer (University of Bonn, Germany). The CB1/CB2 receptor KO mice were developed through replacement of the CB receptor coding region with non-CB receptor DNA using homologous recombination. Subsequently, heterozygote mice were mated to obtain the $CB1^{-/-}/CB2^{-/-}$ mice (Jarai et al., 1999). Inositol Triphosphate Receptor 3 (IP3R3)-tauGFP mice (kindly provided by Dr. Diego Restrepo, University of Colorado Denver, Aurora, CO), have the first exon of the Itpr3 gene replaced by the coding region for a fusion protein of tau and green fluorescent protein (Hegg et al., 2010). Only homozygous $IP3R3^{-/-}tauGFP^{+/+}$mice (denoted as $IP3R3^{-/-}$) in which the biallelic expression of the IP3R3-tauGFP transgene eliminates IP3R3 expression were used. Mice were given food and water *ad libitum*. Animal rooms were kept at 21–24°C and 40–60% relative humidity with a 12-h light/dark cycle. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of

Laboratory Animals as approved by Michigan State University Institutional Animal Care and Use Committee.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

All reagents used for RT-PCR were of molecular biology grade and were purchased from Promega (Madison, WI), unless otherwise noted. Anesthetized (65mg/kg ketamine with 5 mg/kg xylazine, i.p.) C57BL/6 and Swiss Webster (CFW) adult male animals were decapitated. The olfactory epithelia were immediately dissected and stored at -80 °C. Total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH). All isolated RNA samples were confirmed to be free of DNA contamination, as determined by the absence of product following PCR amplification in the absence of RT (Gibco-BRL/Invitrogen). Briefly, known amounts of total RNA and internal standard mRNA were reverse-transcribed simultaneously in the same reaction tube into cDNA using oligo $(dT)_{15}$ as primers. A PCR master mixture consisting of PCR buffer, 4 mM $MgCl_2$, 6 pmol each of the forward and reverse primers, and 1.25 U Taq DNA polymerase was added to the cDNA samples, which were then heated to 94°C for 4 minutes and cycled 25 times at 94°C for 15 s, 59°C for 30 s, and 72°C for 30 s, after which, an additional extension step at 72°C for 5 minutes was included. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME) and visualized using ethidium bromide staining. The number of transcripts was calculated from a standard curve, generated by using the density ratio between the gene of interest and the different IS concentrations used. The primer sequences from 5' to 3' for CB1 and CB2 are forward primer, ACCTGATGTTCTGGATCGGA; reverse primer, TGTTTATCTAGAGGCTGCGCA; and

forward primer, TTCTTACCTGCCGCTCATG; reverse primer, CGGATCTCTCCACTCCGTAG, respectively. The primers for IS were as follows: IS forward, 5'-T7 promoter (TAATACGACTCACTATAGG) + IFN-γ forward (as above) + "spacer" rat ß-globin forward (AAGCCTGATGCTGTAGAGCC); IS reverse, 5'-oligo dT_{18} + IFN-γ reverse (as above) + spacer rat ß-globin reverse (AACCTGGATACCAACCTGCC).

Western blot

Anesthetized adult male (6-8 weeks old) C57BL/6 mice were decapitated. The olfactory epithelia were immediately dissected and stored at -80 °C. The olfactory tissue was processed following the protocol described previously (Jia et al., 2009). Briefly, tissues were homogenized by sonication in Tris buffer. Homogenates (30µg/lane) were resolved on 12.5% gels and transferred to nitrocellulose membranes. Membranes were left overnight at 4 °C after incubation with primary antibodies (Table 1); rabbit anti-CB1 antibody ([1:250], a kind gift from Dr. Ken Mackie, Indiana University, Bloomington), rabbit anti-CB2 antibody ([1:200], Cayman Chemical, Ann Arbor, MI, USA), goat anti-DAGLα ([1:1000], Abcam,Cambridge, MA, USA), or rabbit anti-MAGL ([1:500], Abcam,Cambridge, MA, USA) made in blocking buffer (0.2% g/LI-Block, Millipore, Bedford, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (Jackson Laboratory, West Grove, PA, USA). Immunoreactive proteins were detected with a chemiluminescence reagent (ECL, Amersham Biosciences, Piscataway, NJ, USA) and then exposed to Kodak X-ray film.

Immunohistochemistry

Untreated adult male (6-8 weeks old) C57BL/6 mice were deeply anesthetized (65–80 mg/kg ketamine with 5–10 mg/kg xylazine), transcardially perfused with saline, and fixed with 4% g/L paraformaldehyde. Heads were decalcified in EDTA (0.5 M, pH = 8) for 5 days, cryoprotected with 30% g/L sucrose and embedded in Tissue Tek OCT (Sakura Finetek, Torrance, CA) as previously described (Jia et al., 2010). Frozen coronal sections of OE (20 μm) from level 3 of the mouse nasal cavity taken at the level of the second palatal ridge were obtained were collected using a cryostat and mounted onto Superfrost Plus slides (Electron Microscopy Sciences, Hatfield, PA, USA). Tissue sections were rehydrated with phosphate buffered saline (PBS), permeabilized with 0.2% ml/l triton x-100 and blocked with 1% blocking reagent. Tissue sections were incubated with (Table 1) rabbit anti-CB1 antibody [1:500],, or rabbit anti-MAGL ([1:50], Abcam, Cambridge, MA, USA). Double-labeled immunohistochemistry sections were processed as described above for CB1 immunoreactivity followed by incubation with goat anti-olfactory marker protein (OMP, 1:1000, Wako Chemical, Plano, TX, USA), mouse anti–mammalian achaete-schute homolog 1 (MASH 1 [1:20], BD Pharmingen, Franklin Lakes, NJ,USA), rabbit anti-cytokeratin 18 (CK18, 1:100, Abcam, Cambridge, MA), rabbit anti-P75 (P75, [1:200] Abcam, Cambridge, MA), or in IP3R3 GFP mice followed by TRITC-conjugated donkey anti-goat or anti-rabbit immunoglobin (1:50 or 1:200, Jackson Immunoresearch Lab, West Grove, PA, USA). Vectashield mounting medium for fluorescence (Vector, Burlingame, CA) was applied and immunoreactivity was visualized on an Olympus FV1000 Confocal laser scanning microscope (Olympus America Inc., Center Valley, PA, USA). FITC and TRITC dyes were excited at 488 and

543 nm and low pass filtered at 505–525 and 560–620 nm, respectively. For detection of MAGL and MASH1, antigen retrieval was performed before permeabilization by heating sections in a citrate buffer (pH = 6) in a microwave oven (700 W; 2×6 minutes low power). After antigen retrieval, MAGL primary antibody was left overnight on a shaker in a 4°C cold room. Antibody specificity was examined by omitting the primary antibody, secondary antibody or using a blocking peptide. No specific immunoreactivity was observed in any of the controls.

Measurement of endocannabinoids

Anesthetized adult male (6-8 weeks old) wildtype C57BL/6 and CB1/CB2 receptor KO mice were decapitated. The olfactory epithelia were immediately dissected and stored at -80 °C. OE sections were subjected to a lipid extraction process as described previously (Patel et al., 2003). Tissue samples were weighed and placed into borosilicate glass culture tubes containing two ml of acetonitrile with 84 pmol of [2H8] anandamide and 186 pmol of [2H8]2-AG. Tissue was homogenized with a glass rod and sonicated for 30 minutes. Samples were incubated overnight at -20°C to precipitate proteins, and then centrifuged at 1,500 x *g* to removed particulates. The supernatants were removed to a new glass tube and evaporated to dryness under N2 gas. The samples were resuspended in 300 µl of methanol to recapture any lipids adhering to the glass tube, and dried again under N2 gas. Final lipid extracts were suspended in 20 µl of methanol, and stored at -80°C until analysis. The contents of the two primary endocannabinoids AEA and 2-AG, as well as three other fatty acid lipid mediators, palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), and 2-oxogluteric acid (2-OG) within lipid extracts in methanol from brain tissue were determined using isotope-

dilution, liquid chromatography-mass spectrometry as described previously (Patel et al., 2005). PEA and OEA are non-cannabinoid fatty acid ethanolamides which share biosynthetic and metabolic pathways with AEA, but do not activate CB receptors.

Solutions

Ringer's solution contained (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose; pH 7.4, 290–320 mOsms. Probenecid (500 μM), an inhibitor of the organic anion transporter, was included to aid in the loading and retention of fluo-4 AM (Di et al., 1990; Manzini et al., 2008)(Di et al., 1990; Manzini et al., 2008). Odorant mixture included odorants n-amyl acetate, R-carvone, hetanol, cineole, and octanol (50 μM each) added directly to Ringer's solution. Concentrated stocks of ATP (10 μM) and odorant mixes were made in Ringer's solution and stored at −20 °C and reconstituted on the day of the experiment. Concentrated stocks of CB receptor agonist WIN 55212-2 (5 μM), enatomer WIN 55212-3 (5 μM), and CB1 receptor antagonist AM251 (5 μM) stocks were made in DMSO (1% final concentration DMSO) and stored at −20 °C and reconstituted and diluted in Ringer's solution on the day of the experiment.

Preparation of olfactory epithelium slices

To prepare OE slices, neonatal (P0-4) Swiss Webster (CFW) mice (Charles River, Portage, MI) were quickly decapitated, and the skin and lower jaw were removed. Tissue was embedded in a carrot, mounted on a vibratome-cutting block in cold Ringer's solution and 400 μm slices were made. Slices were loaded with 18 μM fluo-4 AM (Molecular Probes, Eugene, OR) for 90 minutes at room temperature. A stock solution of fluo-4 AM was prepared weekly in dimethyl sulfoxide (DMSO) containing 20% ml/l pluronic F-127 (Invitrogen, Carlsbad, CA). Ringer's solution was added to this

stock solution for a final concentration of 18 μM fluo-4 AM, 0.04% pluronic F-127 and 0.4% DMSO.

Confocal calcium imaging

Slices were placed in a laminar flow chamber (Warner Instruments, Hamden, CT) and perfused continuously with Ringer's solution at a flow rate of 1.5–2.0 ml/minutes. Test solutions were applied using bath exchange and a small volume loop injector (200 μl). Imaging occurred 50–100 μm below the surface of the slice to avoid damaged cells. An Olympus Fluoview 1000 laser scanning confocal microscope (Olympus, Center Valley, PA) was used for data collection and analysis. An argon ion laser was used for fluorescence excitation at 488 nm. Fluorescence emissions were filtered at 510 nm. Time series experiments were performed collecting 640× 256 pixel images at 0.2 – 1 Hz. The fluorometric signals obtained are expressed as relative fluorescence (F) change, $\Delta F/F = (F-F0)/F0$, in which F0 is the basal fluorescence level (mean F of first 5 frames). At the end of an experiment, fluorescein, an inert fluorescent compound, was used to monitor perfusion rates.

Imaging data analysis

Experiments were performed by sequentially obtaining (1) initial control agonist-evoked calcium transients, (2) calcium transients evoked by co-application of agonist in the presence of antagonists and (3) recovery agonist-evoked calcium transients. Regions of interest (ROIs) were drawn around areas, presumably individual cells that exhibited increases in calcium using Fluoview software (FV10-ASW Version 3.0). To be included in the data set, the peak amplitude of the recovery calcium transient had to be at least 75% of the initial transient amplitude. All data were normalized to the initial

calcium transient. Paired Student's t-tests were used to determine significant differences ($p < 0.05$) between the antagonist (AM251) peak amplitude and the recovery of agonist peak amplitude. Odorant mixture (50 μM of each heptanol, cineole, carvone, octanol, and amyl acetate) and ATP were superfused onto slices at the end of an experiment to distinguish between odorant-insensitive purinergic sustentacular cells, and OSNs. In addition, the locations of sustentacular cell somas in apical OE, versus OSN somas, in middle OE, were used to identify cell types.

Table 1. Description of antibodies against cell specific markers and dyes.

Antisera	Target	Source	Dilution	MW
Goat α - OMP	Mature Olfactory Neurons	Waco Chemicals	1:2000	
Rabbit α - CB1	Cannabinoid Receptor 1	Dr. Ken Mackie	1:500(IHC) 1:200(WB)	60
Rabbit α - CB2	Cannabinoid Receptor 2	Cayman Chemical	1:200	39-45
Mouse α - Mash1	Transit Amplifying Progenitor	BD Pharmingen	1:20	
Goat α - DAGLα	2-AG synthesis enzyme	Abcam	1:50 (IHC) 1:200(WB)	115
Rabbit α - MAGL	2-AG degradation enzyme	Abcam	1:200	33
Mouse α - P75 NGF	Sustentacular cell	Abcam	1:100	
Rabbit α - CK18	Sustentacular cell	Abcam	1:200	
Mouse α - Actin	Housekeeping gene			42

Results

CB receptor expression in the mouse OE

An endocannabinoid system has been identified peripherally in the tadpole OE (Czesnik et al., 2007) and centrally in the rodent OB (Soria-Gomez et al., 2014; Wang et al., 2012); however, it is unknown if the rodent OE contains an endocannabinoid system. The present study sought to confirm the presence of CB1 receptors in the mouse OE. CB receptor mRNA and protein was measured in OE tissue from C57BL/6 and CFW adult mice. CB1 and CB2 receptor mRNA was present in both species of mice (Figure 2.1A). Data are expressed in cycle threshold levels (i.e., the cycle number in which the florescence generated within a reaction crosses a set threshold), which is a relative measure of the concentration of target mRNA. Cycle threshold levels of both CB receptors were at moderate levels in both mouse species. Delta cycle threshold levels of CB1 receptor (CFW, 15.8; C57, 15.3) represents the cycle threshold value (CFW, 28.7; C57, 27.0) minus the internal control cycle threshold levels (18S: CFW, 12.9; C57, 11.6). CB2 receptors showed a similar cycle threshold value profile (CFW, 33.0, C57; 33.0) (Figure 2.1A). Using Western blot analysis, CB1 receptor protein was detected at 60 kD and CB2 receptor protein at around 40 kD, further verifying the presence of both CB receptors in the mouse OE (Figure 2.1B).

The distribution of only CB1 receptor expression in the mouse OE was further examined using immunohistochemistry. CB2 receptors were not further characterized as data in later chapters suggest CB1 receptors are primarily responsible for cannabinoid actions in the mouse OE. Images for CB1 receptor immunoreactivity (IR) were chosen from ectoturbinate 2 in the mouse OE (Figure 2.2A).

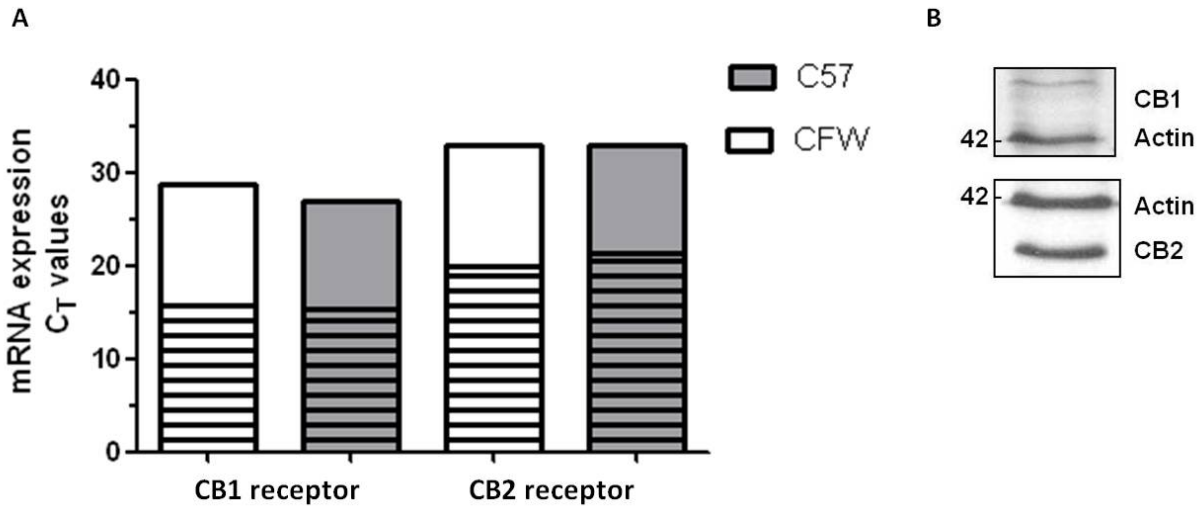

Figure 2.1 mRNA and protein expression of cannabinoid receptors. **(A)** RT-PCR cycle threshold (CT; solid and striped portion of bars) and ΔCT (striped portion of bars only) values for both the CB1 and CB2 receptors in Swiss Webster (CFW; white bars) and C57BL/6 (C57; grey bars) mice. **(B)** Representative immunoblots for both CB1 and CB2 receptors and actin in C57 mouse OE.

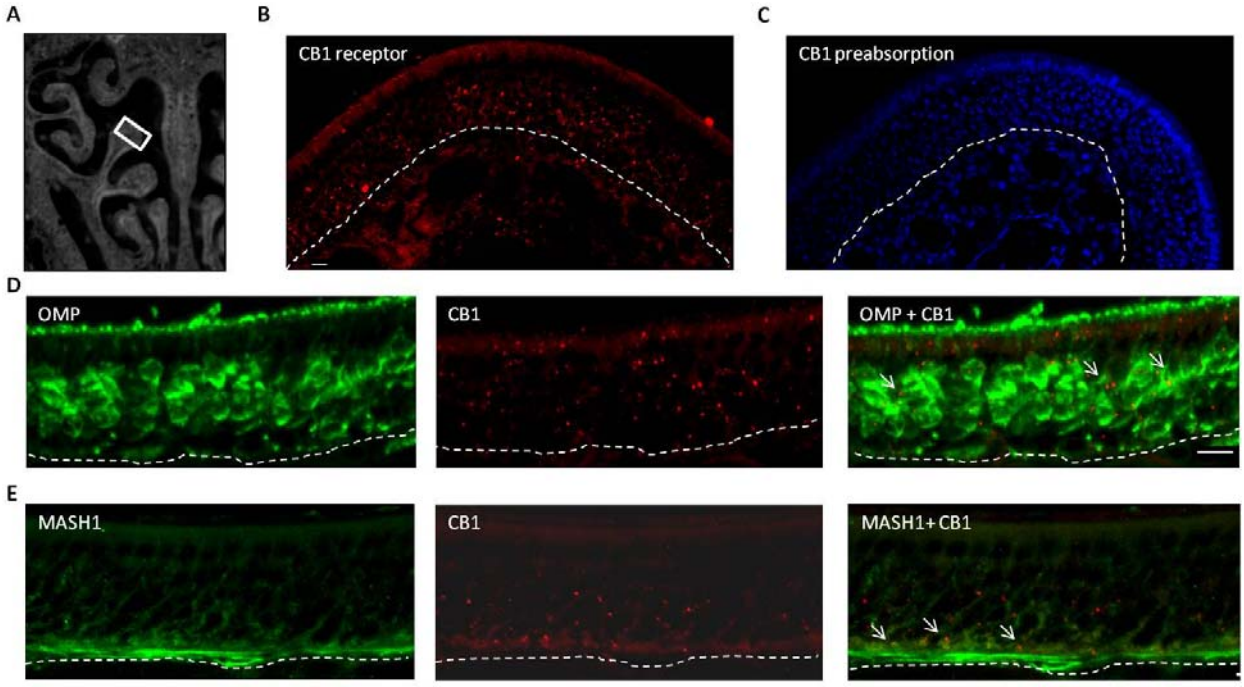

Figure 2.2 CB1 receptor expression is diffuse and co-localizes with multiple OE cell types. (A) Representative coronal section through the mouse OE at level 3 showing one side of the nasal cavity. The ectoturbinate 2, indicated by a white box, is the region shown in panels B-E. **(B**) CB1 receptor-IR (red) is diffuse throughout the OE. **(C)** Preabsorption of the CB1 receptor antibody with the antigen eliminates immunoreactivity. DAPI marks the nuclei (blue). **(D-H)** CB1 receptor-IR (red) co-localizes with **(D)** olfactory sensory neurons (OMP, green), **(E)** globose basal cells (Mash1, green), and non-neuronal **(F-G)** sustentacular cells (P75 and CK18, green) and **(H)** microvillous cells (GFP, green). Arrows indicate location of CB1 receptor co-localization. Scale bar = 10 μm. Dashed white lines indicate basement membrane.

Figure 2.2 (cont'd).

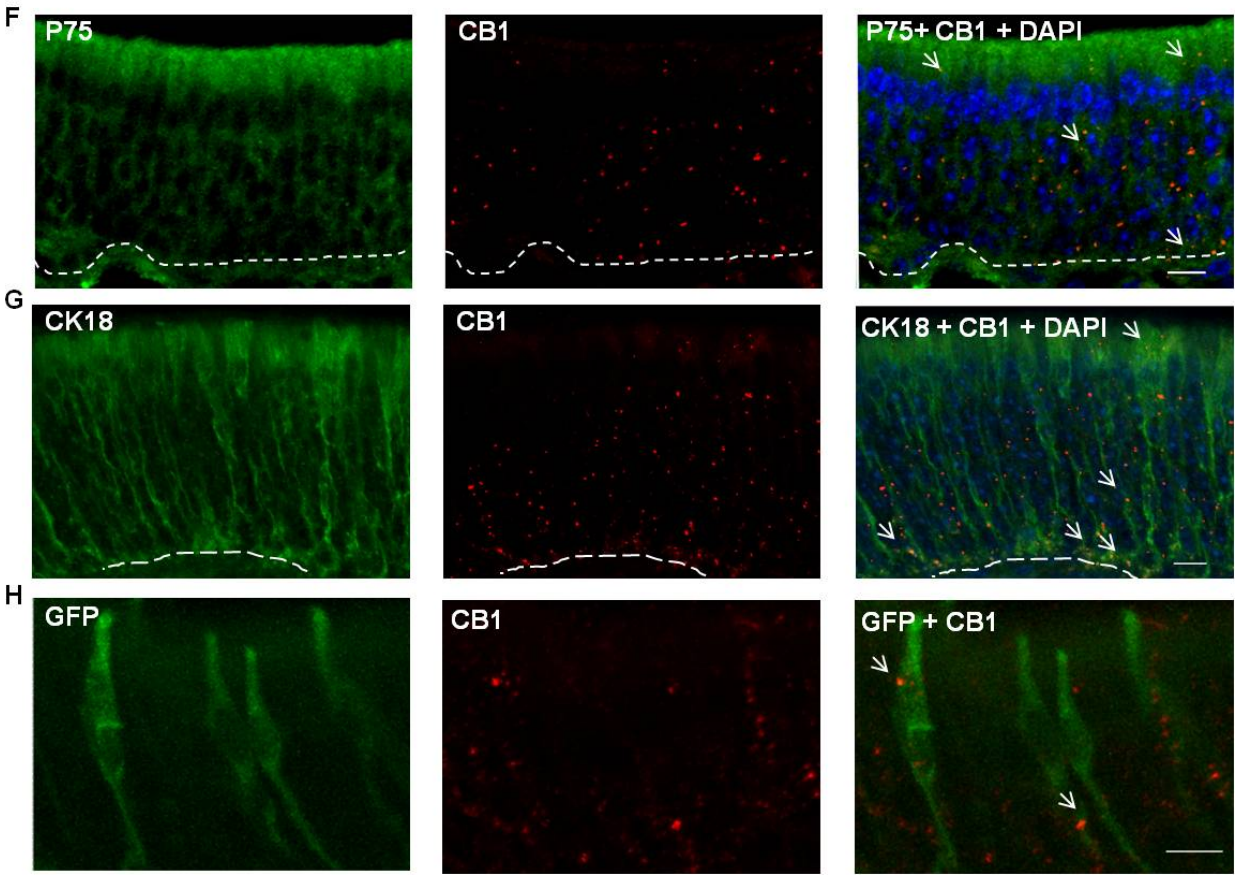

Figure 2.2 (cont'd). CB1 receptor expression is diffuse and co-localizes with multiple OE cell types. CB1 receptor-IR (red) co-localizes with non-neuronal **(F-G)** sustentacular cells (P75 and CK18, green), and **(H)** microvillous cells (GFP, green). Arrows indicate location of CB1 receptor co-localization. Scale bar = 10 μm. Dashed white lines indicate basement membrane.

Diffuse expression of CB1 receptor-IR was noted throughout the epithelium (Figure 2.2B), suggesting CB1 receptor protein localization on multiple cell types. Preabsorption of the primary antibody with its immunizing peptide eliminated the CB1 immunoreactivity, indicating specificity of the CB1 antibody (Figure 2.2C). CB1 receptor expression colocalized with OSN specific antibody olfactory marker protein (OMP) in the neuronal layer of the OE (Figure 2.2D). CB1 immunoreactivity was also present in MASH-1 positive GBCs (Figure 2.2E), suggesting that CB1 receptor action could influence basal cell proliferation in the mouse OE. CB1 receptor protein was also expressed in non-neuronal cell populations in the apical layer; including sustentacular cells marked by P75 and CK18 antibodies (Figure 2.2F-G) and the IP3R3–expressing subpopulation of microvillous cells (Figure 2.2H). Notably, the distribution of CB1 receptors was not uniform through the layers of OE tissue. CB1 receptor-IR is found more frequently on the processes of OSNs and on the cytoplasmic extensions of sustentacular cells, rather than on the cell bodies. These are the first data to indicate the presence of CB receptors in the mouse OE.

Endocannabinoid 2-AG is present in the mouse OE

Two main endocannabinoid ligands have been previously described in the central nervous system and olfactory systems. AEA, is present in pmol levels in the rodent brain, while 2-AG, is detected in nmol levels in the rodent brain, and pmol levels within the murine OB and *Xenopus laevis* larval OE (Breunig et al., 2010b; Buczynski and Parsons, 2010; Soria-Gomez et al., 2014). Lipid extracts of endocannabinoids 2-AG and AEA were quantified in the mouse OE (Table 2).

Table 2. Levels of endocannabinoids and select analogs in mouse olfactory wildtype and CB1/CB2 receptor KO turbinate tissue.

(nmol/g tissue)	**2-AG**	**2-OG**	**OEA**	**PEA**
Wildtype C57	26.5	9.0	0.026	0.076
CB1/CB2 KO	22.3	8.1	0.032	0.211

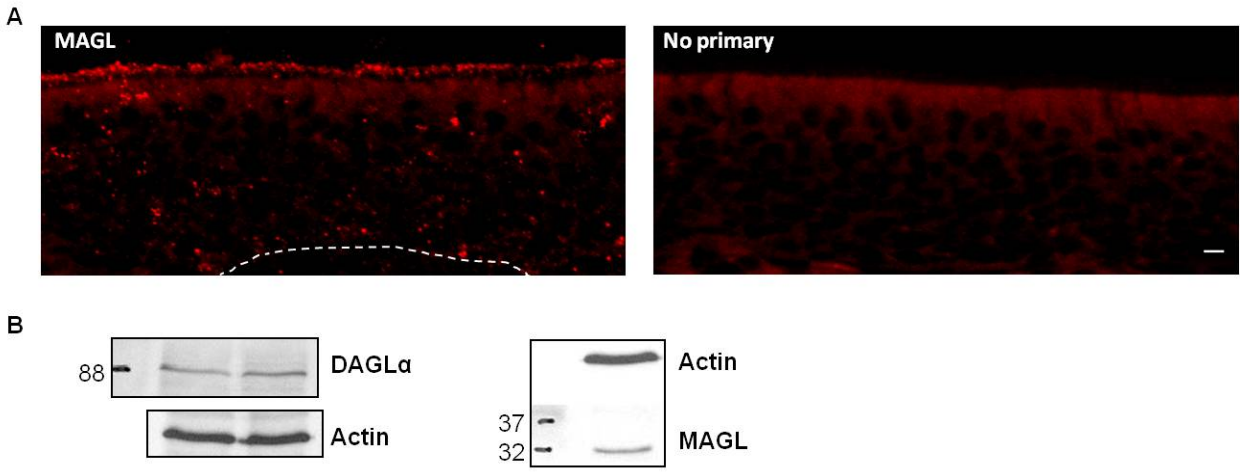

Figure 2.3 2-AG synthetic and degradative enzyme expression in the adult mouse OE. (A) Hydrolytic endocannabinoid enzyme monoacylglycerol lipase (MAGL) is distributed throughout the OE. Scale bar = 10μm. Dashed white lines indicate basement membrane. **(B)** Western blot images from C57 olfactory turbinate tissue showing 2-AG synthetic enzyme DAGLα and 2-AG degradative enzyme MAGL and constitutively.

High physiological levels of 2-AG were present in both C57 wildtype mice and CB1/CB2 receptor KO mice, although no detectable levels of AEA were present in either tissue. Mouse OE 2-AG levels were measured in the nmol range, while tadpole OE levels were measured at pmol levels (Breunig et al., 2010b). Additionally, 2-AG congener 2-oleoylglycerol (2-OG), and AEA congeners N-Palmitoyl ethanolamide (PEA) and N-Oleoyl ethanolamide (OEA), endogenous lipid signaling molecules proposed as members of the endocannabinoid family, were detected. The metabolic enzymes involved in the synthesis and degradation of 2-AG were examined next. 2-AG synthesis enzyme DAGLα protein was present in the mouse OE (Figure 2.3B). Similarly, the 2-AG metabolic enzyme MAGL was present and distributed throughout the mouse OE with notable immunoreactivity apically, possible in the knobs of OSNs (Figure 2.3A). These data conclusively show that 2-AG is synthesized, present at physiological condition, and degraded within the mouse OE.

CB receptors respond to WIN55212-2 stimulation

The above data suggest the presence of an endocannabinoid system in the mouse. Calcium imaging was performed to further investigate if the receptors are functional. The synthetic aminoalkylindole, WIN55,212-2 (5 μM, WIN), was applied to a neonatal mouse OE slice preparation loaded with the fluorescent calcium indicator dye Fluo-4 AM. Multiple successive applications of 5 μM WIN produced reproducible increases in intracellular calcium (Fig 2.4), however, only 17% of collective WIN-responsive cells (161/929 cells from 36 slices) responded to 3 successive WIN applications. Cells that only responded once or twice to WIN application were not included in the data set.

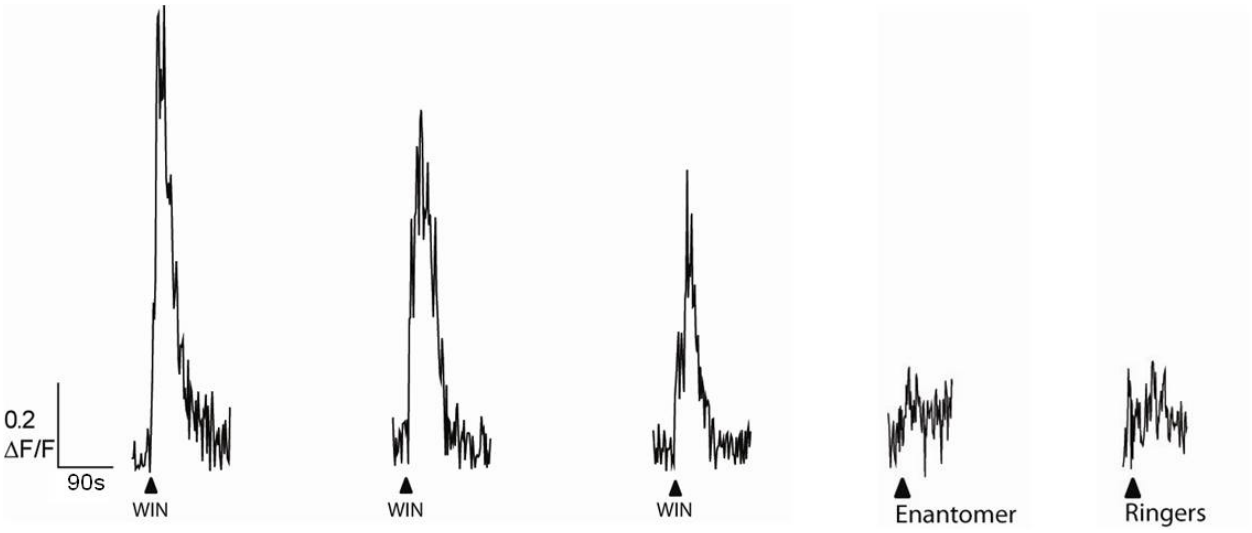

Figure 2.4 WIN-evoked calcium transients in the mouse OE. Changes in intracellular calcium from a single cell following three applications of 5 μM WIN 55212-2 (WIN), 5 μM WIN enantiomer, or control Ringer's application. Note the reproducible responses to WIN and absence of a response from the enantiomer and control Ringers solution. ▲indicates time of drug superfusion. In this figure and all subsequent figures, breaks in traces correspond to 5 minutes period when images were not collected.

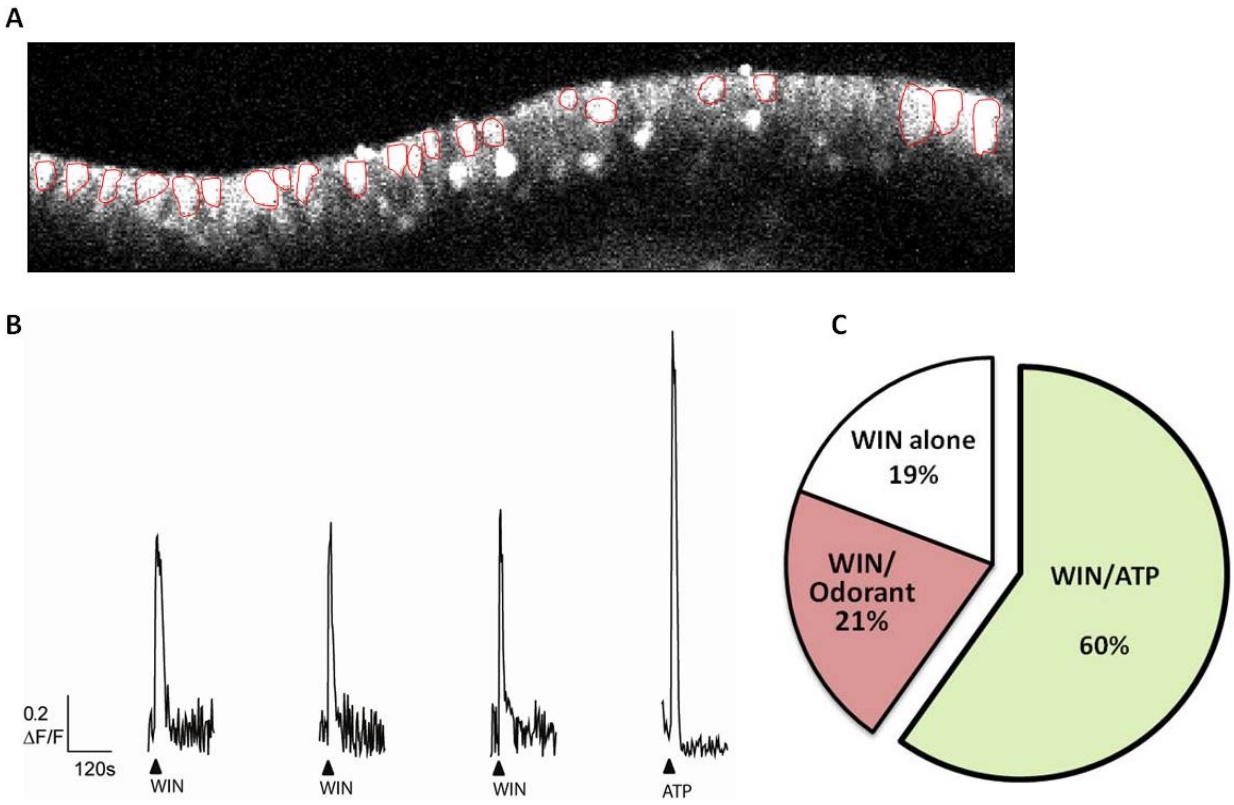

Figure 2.5 WIN induces calcium transients in olfactory sensory neurons. (A) Representative image of fluo-4AM loaded neonatal slice with ATP-responsive cells highlighted in red. **(B)** Representative calcium transient traces from one cell superfused with synthetic non-specific agonist 5 μM WIN followed by 10μM ATP. ▲indicates time of superfusion. **(C)** Pie chart depicting response profile of WIN responsive cells that also respond to ATP, odorants, or WIN alone.

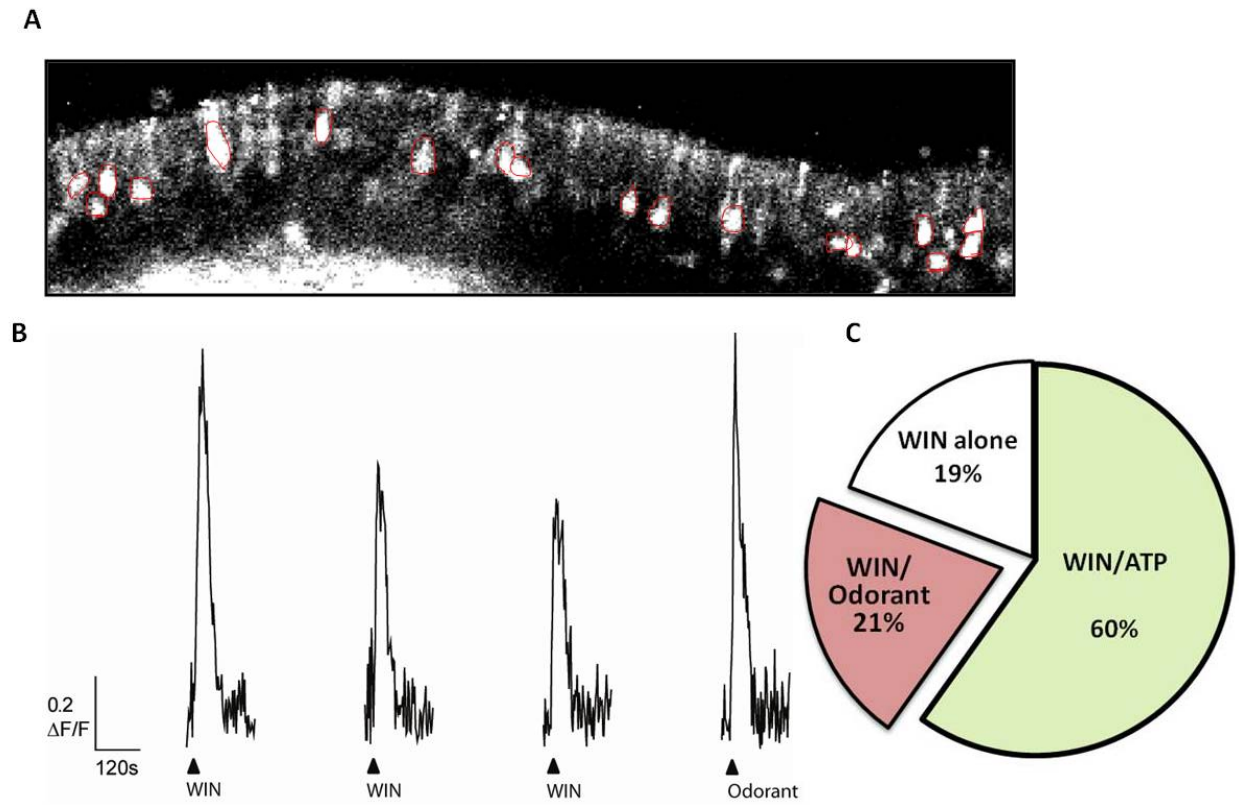

Figure 2.6 WIN induces calcium transients in non-neuronal cells. (A) Representative image of fluo-4AM loaded neonatal slice with odorant-responsive cells highlighted in red. **(B)** Representative calcium transient traces from two cells superfused with synthetic non-specific agonist WIN (5 μM) followed by a cocktail of odorants. ▲indicates time of superfusion. **(C)** Pie chart depicting response profile of WIN responsive cells that also respond to ATP, odorants, or WIN alone.

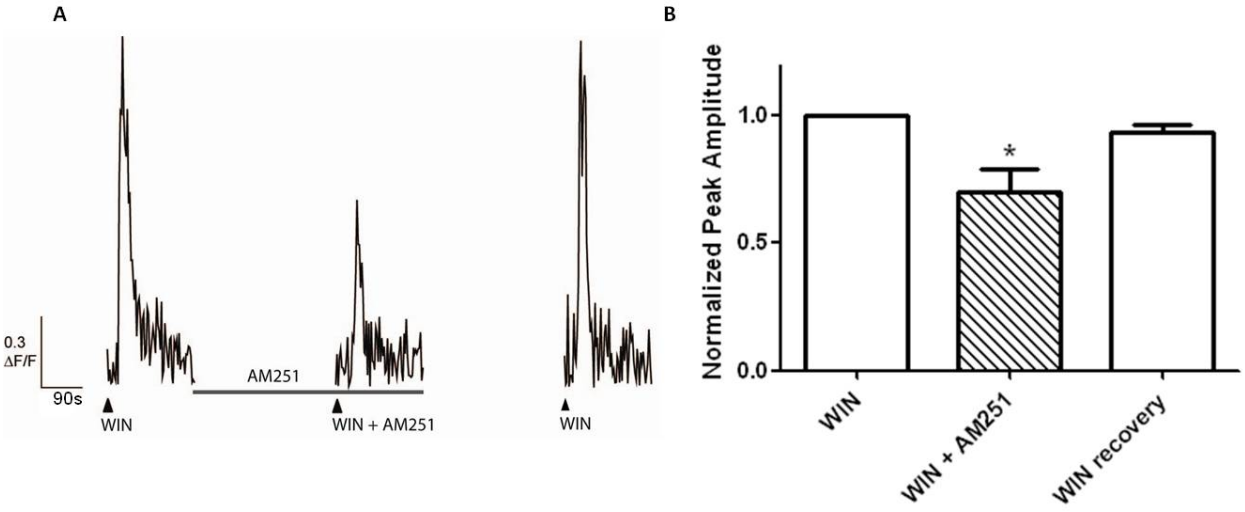

Figure 2.7 CB1 receptor activation induces calcium transients in mouse OE. Fluo-4AM loaded neonatal slices were superfused with WIN, then the bath solution was switch to contain (5μM) AM251 for 5 minutes before a second WIN application in the presence of AM251 was performed. Recovery involved 5 minute washout with Ringers alone before a third application of WIN. **(A)** Representative WIN-evoked calcium transients inhibited by superfusion of CB1 receptor specific antagonist AM251. ▲indicates time of WIN superfusion. **(B)** Bar graph depicting the normalized peak amplitudes of WIN-induced calcium transients in the presence or absence of AM251. AM251 significantly and reversibly reduced the WIN-evoked calcium transients (* $p<0.05$ vs. recovery; Student's t-test; n=27 regions of interest, 8 slices from 5 litters).

The majority of WIN positive cells did not respond to the inactive WIN enantiomer, WIN 55212-3 (10μM; Figure 2.4). This lack of response was not due to linear run-down or slice preparation deteriorization since no response was seen with WIN enantiomer when given before CB agonist application. Application of the enantiomer WIN 55212-3 led to calcium changes in a small percentage of cells (7.5%; 28/374 cells from13 slices) that were also responsive to WIN, suggesting some cells respond to either stereoisomer, however, the percentage of cells that responded to the enantiomer was less than the percentage of WIN-responsive cells that also responded to Ringer's (control) application (68/781cells from 34 slices). This observation likely reflects a small number of cells that respond to mechanical stimulation or exhibit spontaneous changes in calcium (Furuya et al., 1993). Next, to determine which cell types were responsive to WIN application, cell morphology, localization, and responsiveness to other stimuli were used as indicators. OSNs were responsive to a mixture of odorants and had a cell soma in the middle of the epithelium, while non-neuronal cells (sustentacular cells or microvillous cells) had cell somas located in the upper 1/3 of the epithelium and were responsive to ATP, but not odorants (Figure 2.5A). Using these parameters, 60% of WIN-responsive cells also responded to ATP (Figure 2.5C; 732/1223 cells from 46 slices), while 21% responded to the odorant mixture (Figure 2.6C; 256/1223 cells from 46 slices). Previous data suggest that WIN-responsive cells signal through CB1 receptors; therefore, the CB1 receptor specific antagonist AM251 (5 μM) was used to determine the CB receptor identity. AM251 was superfused over OE slices for 5 minutes prior to and during WIN application. AM251 significantly reduced the WIN-induced response by 30% (0.7±0.09 vs. 0.9 ± 0.03; $p<0.05$) of the initial WIN calcium transient

peak (Figure 2.7, 27 cells from 4 slices; $p < 0.05$). AM251 did not induce calcium transients when applied to OE tissue alone (data not shown). These data indicate that CB1 receptors are functional in the mouse OE.

Discussion

This chapter identifies a functional endocannabinoid system in the mouse OE for the first time. Previous studies have found an endocannabinoid system in the rodent OB (Wang et al., 2012) and the tadpole OE (Czesnik et al., 2007). This chapter extends that knowledge to the mouse OE, with the identification of both CB1 and CB2 receptor mRNA and protein. Furthermore, previous studies have only focused on a neuronal population of CB1 receptors, whereas here, the distribution of CB1 receptors has been identified in both neuronal and non-neuronal cells in the mouse OE. An in house prepared CB1 receptor antibody in the Mackie laboratory at Indiana University was used for all CB1 receptor IHC studies due to its superior specificity compared to commercially available antibodies. The current primary CB1 receptor antibody is a rabbit polyclonal antibody raised against the last 15 amino acids of the C-terminus of the CB1 receptor (Carey et al., 2010). Previous antibody controls have been conducted (Nyiri et al., 2005) demonstrating the specificity by showing the lack of immunostaining in the hippocampus and neocortex (Bodor et al., 2005; Hajos et al., 2000) of CB1 receptor KO mice (Ledent et al., 1999). CB1 receptors identified on mouse OSNs could be exhibiting neuromodulatory effects upon odor stimulation. As seen in the tadpole, CB1 receptor antagonists decrease the amplitude of odor-evoked calcium traces and increase the latency to activation (Czesnik et al., 2007). CB1 receptors located on mouse GBCs

could be involved in proliferation signaling, since the endocannabinoid system influences proliferation and neurogenesis in adult neurogenic regions of the CNS (Aguado et al., 2006; Compagnucci et al., 2013). Unfortunately, HBC co-localization with the CB1 receptor was unable to be performed despite attempting several IHC techniques, because the antibodies were raised in the same species for both CB1 and horizontal basal cell antibodies. CB1 receptor activation on non-neuronal cells could be involved in homeostatic regulation since sustentacular cells are capable of complex signaling pathways and function as glial cells that provide physiological and metabolic support to the OE (Hegg et al., 2009; Rodriguez et al., 2008).

Endogenous cannabinoid signaling in the mouse OE is possible through 2-AG given its synthetic enzyme DAGLα and degradative enzyme MAGL are both present. In the tadpole, 2-AG is synthesized by DAGLα in sustentacular cells and DAGLβ isoform in OSNs (Breunig et al., 2010a). Additionally, 2-AG is detected at high physiological levels in the mouse OE, over, 1,000 times the amount reported in the rodent OB and tadpole OE. 2-AG levels were similar between C57 wildtype and CB1/CB2 receptor KO mice, suggesting CB receptor availability does not influence endocannabinoid production. Few other rodent brain areas express such robust physiological endocannabinoid levels. Some regions that do express a high quantity of endocannabinoid include the cerebellum (Bisogno et al., 1999; Bortolato et al., 2007), hypothalamus (Gonzalez et al., 2004; Rubio et al., 2007), frontal cortex (Richardson et al., 2007), and hippocampus (Bisogno et al., 1999; Di Marzo and Petrosino, 2007; Richardson et al., 2007). Such high levels of physiological 2-AG could suggest a role for homeostatic signaling or tonic release of endocannabinoids in addition to activity

dependent synthesis. Calcium imaging studies done in the tadpole OE suggest a tonic release of cannabinergic substances as well (Czesnik et al., 2006), however, increases in 2-AG could result from experimental conditions, as phospholipid metabolites are known to rise within 15 seconds postmortem in rat brain tissue (Sugiura et al., 2001).This rapid increase in endocannabinoids, likely caused by postischemic rises in the concentrations of free calcium (Kempe et al., 1996), may confuse quantitative analysis unless appropriate precautions are taken to prevent it. OE tissue was frozen within 30 seconds of decapitation, therefore it is possible postmortem ischemic lipid breakdown was detected (Bazan, 1970), however, no detectable levels of AEA were present in mouse OE tissue, which is also subject to postmortem increases in synthesis even though small quantities of AEA are found in both rodent OB and tadpole OE, Therefore, 2-AG levels measured in the mouse OE are within a reasonably accurate range.

Endocannabinoids have low stability in solution, are easily oxidized when exposed to air, and hydrolyze rapidly *in vivo* (Mechoulam et al., 1998). Therefore the synthetic cannabinoid WIN 55212-2 (WIN) was used during experiments in this dissertation. WIN is an aminoalkylindole derivative, which produces effects similar to THC and endocannabinoids, but has a different chemical structure (Compton et al., 1992). WIN is a full agonist for the CB1 receptor with a higher affinity than THC (Kuster et al., 1993). WIN does not activate cannabinoid-like GPCRs, GPR55 or GPR18 (McHugh et al., 2010; Ryberg et al., 2007), but can bind to PPARs (Fakhfouri et al., 2012). The concentration of WIN used in the following experiments is within the range whereby WIN can inhibit TRPV1 channels, which are present in the mouse OE (Wang

et al., 2012). The concentration of WIN was based on an effective concentration seen to increase intracellular calcium in hippocampal neuron cultures (Lauckner et al., 2005). Usable WIN concentrations were limited by its solubility in Ringer's solution, since increasing DMSO concentrations to approximately 5% are not viable for calcium imaging.

To determine if CB receptors present in the mouse OE were functional, calcium imaging experiments were performed. In an acute slice preparation of the mouse OE, WIN agonist was able to reproducibly evoke calcium transients that were not seen with the stereoisomer WIN 55212-3. Furthermore, WIN-evoked calcium transients were seen in a population of OSNs that also responded to odorants, as previously found in the tadpole OE (Czesnik et al., 2006), however, in the mouse OE, WIN-evoked calcium transients were also seen in a population of non-neuronal cells. Unfortunately, calcium imaging of basal cells is not possible with our current preparation, perhaps due to insufficient fluo-4 loading. Therefore, functional CB receptors were identified in both sustentacular and OSNs supporting previous identification by immunohistochemistry.

CB1 receptors are partially responsible for WIN-induced calcium transient seen in the mouse OE. CB1 receptor specific antagonist AM251 reduced WIN-evoked calcium transients by 30% in a population of cells. Individual ROIs showed a range of calcium inhibition after WIN stimulation, similar to that seen in odor-evoked calcium transients in the tadpole OE (Czesnik et al., 2006). However, calcium transients were not completely blocked, suggesting possible off target effects of WIN. CB2 receptor mRNA and protein is present in the mouse OE. Therefore WIN, a CB1 CB2 receptor agonist, could also activate CB2 receptors. Additionally, although WIN does not act on

TRPV1 receptors directly (Price et al., 2004), TRPV1 receptors can be dephosphorylated via WIN-mediated TRPA1 activation in trigeminal sensory neurons (Jeske et al., 2006), all of which are present in the mouse OE (data not shown). Pharmacological agents could be used to further investigate the signaling pathway of WIN-induced calcium transients. For example, pertussis toxin treatment will identify if cannabinoid signaling is through Gi/o GPCRs, and CB2 receptor antagonist SR 144528 will identify involvement of CB2 receptors. Only one concentration of AM251 was used in the above studies. It was based on the effective concentration used to modulate odor-evoked calcium transients in the tadpole OE (Czesnik et al., 2006). Again, the concentration of AM251 was limited by its solubility in physiological Ringer's solution, as increasing DMSO concentrations damage the live slice to an extent that calcium imaging cannot be performed. Additionally, the duration of AM251 in the bath solution may not have been sufficient to inhibit all available CB1 receptors in the mouse OE. Although wash-out of AM251 can cause complete recovery of the WIN-evoked calcium transient, not all cells recovered from AM251 inhibition. Recovery was only seen in about 30% of the cells that responded to the first application of WIN (32/108 cells). Increasing the wash-out duration might increase the number of recovered cells; however, previous experiments that increased the washout periods did not help calcium transient recovery (Hegg et al., 2009). A CB receptor agonist was applied in the bath solution to improve odorant-induced recovery after cannabinoid antagonism in the tadpoles OE (Czesnik et al., 2006); however, this is also unlikely to improve recovery in our model since we are not measuring cannabinoid effects on odorant responses. It is

possible that the observed recovery rate is a reflection of the inconsistent reproducibility of responses to multiple WIN applications within the same region of interest.

CB receptors are GPCRs that primarily recruit Gi/o proteins that do not directly lead to calcium mobilization, but rather an inhibition of adenylyl cyclase and a decrease in cAMP. Data in this chapter demonstrating increases in intracellular calcium, thus, are interesting. However, other groups have reported instances in which CB1 receptor activation increases intracellular calcium (Filipeanu et al., 1997; Lograno and Romano, 2004; Netzeband et al., 1999; Sugiura et al., 1997). Generally, these studies reported a modest increase in calcium mediated by pertussis toxin sensitive Gi/o and βγ acting via PLC to release calcium from intracellular stores. The involvement of the phospholipase C pathway is typically associated with Gq proteins (Exton, 1996). However, the βγ subunit of Gi/o proteins do activate phospholipase C (Exton, 1996) in some cases, providing a pathway by which Gi/o-coupled receptors such as the cannabinoid receptors could enhance intracellular calcium levels. In addition to the possibility of non-CB receptor activation, WIN-evoked calcium transients could also be through CB1 receptor recruitment of alternative G proteins coupled to different effector systems. The coupling of CB1 receptors to other alpha subunits has been observed previously and is agonist- tissue- and brain region-specific. Indeed, WIN can stimulate Gq G proteins and consequently induce calcium efflux from intracellular stores in hippocampal cultures and in CB1 receptor transfected cells (Lauckner et al., 2005).One possibility is that CB1 receptors could exist in several active states and the relative ratio of each is a function of the ligand. Therefore, WIN may stabilize a conformation of CB1 that couples more readily to Gq/11 (Lauckner et al., 2005). CB1 receptors also couple to Gs proteins under

conditions in which Gi/o proteins are saturated (Glass and Felder, 1997) and dependent on the agonist used (Bonhaus et al., 1998). For example, WIN specifically induces an alternative conformation change in the CB1 receptor than do other agonists which allows recognition of different G proteins (Glass and Northup, 1999). For example, at high concentrations WIN can stimulate cAMP accumulation in a slice preparation of rat globus pallidus, which can be inhibited with a CB1 receptor antagonist (Maneuf and Brotchie, 1997). However, the concentration of WIN used to induce cAMP production is 20 fold higher than that used in my experiments. No conclusions can be made as to which signaling pathway is activated during WIN-induced calcium transient in the mouse OE. Further experiments are needed to identify which pathways are involved, starting with testing pertussis toxin sensitivity of CB1 receptors to determine G-protein specific coupling. Coupling to alternative GPCRs could account for the limited number of cells that responded to 3 consecutive applications of WIN. Collectively, these data indicate that CB1 receptor-mediated effects can greatly differ, depending on the type of agonist used length of stimulation used and tissue.

Conclusion

Previous studies have demonstrated the presence of a cannabinoid system in the tadpole OE (Czesnik et al., 2007), and the rodent OB (Soria-Gomez et al., 2014). The above studies provide the first identification of a functional cannabinoid system in the mouse OE. Based on the distribution of CB1 receptors on multiple cell types and the high unstimulated levels of 2-AG, it is most likely that functional consequences of endocannabinoid signaling result from 2-AG activation at CB1 receptors in the mouse

OE. Due to the differential G-protein coupling of CB receptors, future studies involving the signaling pathways of CB receptor activation are warranted. Additionally, future studies investigating the endocannabinoid synthesizing capabilities of individual cell types are needed to pinpoint a stimulus-based mechanism for endocannabinoid production in the mouse OE.

Chapter 3: Examination of OE morphology and olfactory-mediated behaviors in CB1/CB2 receptor KO mice.

Introduction

Cannabinoid receptors

Endocannabinoid ligands, together with their molecular targets and metabolic enzymes, form the endocannabinoid system (Figure 1.2). The main receptor targets for both exogenous and endogenous cannabinoids are CB1 and CB2 G protein-coupled receptors (Di Marzo and De Petrocellis, 2012; Pertwee, 2009, 2012). CB1 receptors are widely expressed in the nervous system and are localized on axon terminals of central and peripheral neurons. CB1 receptors are involved in retrograde signaling, inhibiting both excitatory and inhibitory neurotransmission and modulation of cognitive, memory, and motor functions (Bari et al., 2005; Maccarrone, 2008b; Yu et al., 2010). Centrally, long and short term CB1-mediated neurotransmitter inhibition relies on Gβγ subunit inhibition of voltage sensitive calcium channels (Wilson et al., 2001). CB2 receptors are predominately expressed in immune cells and are commonly associated with the regulation of various immune functions (Cencioni et al., 2010; Pandey et al., 2009). Recent studies have localized CB2 receptors in the brainstem (Izzo et al., 2009; Van Sickle et al., 2005), activated microglia and astrocytes (Ehrhart et al., 2005; Ramirez et al., 2005; Shoemaker et al., 2007), and in certain neuronal populations after injury. These findings expand the presence of CB2 receptors centrally with functional implications during chronic inflammation of the nervous system (Guindon and Hohmann, 2008; Gutierrez-Martin et al., 2011). CB1 and CB2 receptors usually couple

to Gi/o proteins and trigger the canonical signaling pathway of inhibition of adenylyl cyclase activity and reduction of cAMP levels (Castillo et al., 2012). Under certain conditions (GPCR protein availability, dimerization with other GPCRs, or particular cell environments) CB1 receptors may recruit Gq/11 or Gs proteins leading to alternative signaling cascades (Pertwee et al., 2010). Both CB receptors regulate various protein kinase cascades involved in cell proliferation and survival, thus functionally affecting progenitor cell proliferation and cell fate decisions (Galve-Roperh et al., 2013).

Cannabinoid receptor-deficient mice

Within this chapter, I utilize a genetic mouse model in which both CB1 and CB2 receptors are omitted. In the CB1 receptor KO mouse, most of the CB1 coding sequence was replaced with a PGK-neo, in which a hybrid gene consisting of the phosphoglycerate kinase 1 promoter drives the neomycin phosphotransferase gene' through homologous recombination in the embryonic stem cells between amino acids 32 and 448 (Zimmer et al., 1999). The *Cnr1* gene cytoplasmic domain is from amino acid 401- 473 leaving the CB1 receptor KO mouse with a truncated c-terminus domain. Unfortunately, the CB1 receptor antibody used in this thesis targets the last 15 amino acids of the *Cnr1* C-terminus, therefore overlapping with a non-deleted sequence in the CB1/CB2 receptor KO mouse. Therefore, CB1 receptor antibody specificity is unable to be confirmed using the CB1/CB2 receptor KO mouse. CB1 receptor KO mice appeared healthy, fertile, and maintained normal body weight, yet exhibit a higher number of spontaneous deaths occurring over the first 2 years of life (Zimmer et al., 1999). However, few if any spontaneous deaths occurred after 6-8 weeks of age, which is the chosen age of mice for the experiments performed in this dissertation. Four behavioral

tests commonly used to assess cannabinoid effects in rodents were tested in CB1 receptor KO mice including hypothermia, spontaneous activity in the open field, catalepsy in the ring test, and nociceptive responses (Martin et al., 1991). CB1 receptor KO mice exhibited no change in body temperature, but increased immobility in the catalepsy test, hypoactivity in the open field test, and hyoalgesia (Ledent et al., 1999; Zimmer et al., 1999). Additionally, in the CB2 receptor KO mouse model no changes in body temperature or immobility were detected between C57 wildtype and CB2 receptor KO mice (Buckley et al., 2000). Additional functional consequences of CB1 receptor KO mice include an increased sensitivity to the harmful effects of ischemia, excitotoxic, and oxidative injury (Kim et al., 2006). CB1 receptor KO mice also exhibit a resistance towards obesity when given an obesity-prone diet, suggesting that CB1 receptors are implicated in feeding control and peripheral metabolic regulations (Ravinet Trillou et al., 2004).

Endocannabinoid signaling regulates progenitor cell fate during developmental and in adulthood

Endocannabinoid signaling is present and helps to regulate progenitor cell fate in the nervous system and in the periphery both developmentally and during adulthood. Endocannabinoids regulate hematopoietic and mesenchymal stem cells that play a key role in determining the formation of several cell types in peripheral tissues, including blood cells, adipocytes, osteoblasts, and epithelial cells. In the early stages of development, a functional endocannabinoid system has been identified in both embryonic stem cells and multipotent progenitor cells (Bari et al., 2011; Maccarrone, 2008a; Paria and Dey, 2000). CB1 receptors are expressed in neuroepithelial progenitor

cells from early embryonic stages, and their levels increase through neural differentiation. Endocannabinoid signaling, likely through CB1 receptors, is involved in cell fate determination of embryonic stem cells (Jiang et al., 2007; Nones et al., 2010). CB2 receptors, normally absent in neurons, are functionally active in undifferentiated neural cells and may participate, along with CB1 receptors, in the regulation of neural progenitor cell fate decisions including cell proliferation, cell cycle maintenance (Goncalves et al., 2008; Palazuelos et al., 2006). Overall, at early embryonic states CB receptors contribute to normal embryo growth and control of stem cell populations (Wang et al., 2004).

Loss of CB1 receptor signaling leads to a decrease in progenitor cell numbers and/or a loss of progenitor cell function. In the developing mammalian cortex, neural progenitor cells are distributed in the ventricular and SVZ, and are identified by their selective expression of the transcription factors Pax6 and Tbr2 (Guillemot et al., 2006; Osumi et al., 2008). Pax6, a paired domain-containing transcription factor, regulates the network of neural stem cell decision-making genes (Englund et al., 2005; Sansom et al., 2009), while Tbr2 is essential for neuronal amplification and tightly controls the balance between neural stem cell renewal and neurogenesis (Guillemot et al., 2006). In CB1 receptor-deficient mouse embryos, there is a decrease in progenitor cell numbers in both Pax6 and Tbr2 cell populations *in vivo* (Diaz-Alonso et al., 2014). Pax6 and Tbr2 mRNA levels were also reduced in the absence of CB1 receptors, suggesting that CB1 receptor signaling regulates embryonic cortical progenitor cell survival in the ventricular and SVZ (Diaz-Alonso et al., 2014). In the same animal model, mRNA levels of Sox2, a transcription factor essential for neural stem cell proliferation and self-renewal

(Guillemot et al., 2006) are also reduced, suggesting that a decrease in progenitor cell numbers leads to a decrease in neuronal cell populations in CB1-deficient cells (Diaz-Alonso et al., 2014).

Neurodevelopmental contributions to the regulation of progenitor cell fate can induce altered functions of the adult nervous system. For example, unbalanced excitatory or inhibitory neurogenesis during development could underlie the higher susceptibility to seizures and increased severity seen in CB1 receptor-deficient mice (Katona and Freund, 2008). Additionally, embryonic loss of CB1 receptor signaling has been shown to exert alterations in adult neuronal differentiation that influence motor activity (Diaz-Alonso et al., 2012). Deficits in adult cerebral neuronal numbers in CB1-receptor deficient mice may be caused by alterations of neural progenitor cell proliferation and cell cycle control (Aguado et al., 2005; Mulder et al., 2008). Genetic depletion of CB1 receptors leads to an early onset of age-related memory decline, affecting both reward and aversion-driven learning (Albayram et al., 2012). In the adult nervous system, the endocannabinoid system regulates progenitor stem cells in restricted neurogenic areas including the SVZ and dentate gyrus. Progenitor cell function is also inhibited in CB receptor-deficient mice. Loss of CB1 receptor signaling reduces hippocampal and SVZ proliferation, as well as reduces the self-renewal ability of neural progenitor cells in the adult brain (Aguado et al., 2005; Jin et al., 2004). CB receptor-mediated regulation of progenitor/stem cell number and fate could have functional and behavioral consequence for mice lacking these receptors.

Cannabinoids and olfaction

The endocannabinoid system has been implicated in several neurological driven behaviors including food intake (Di Marzo and Matias, 2005), reinforcing characteristic of drugs of abuse (De Vries and Schoffelmeer, 2005), and cognitive processing including executive (Marsicano et al., 2002a; Varvel et al., 2005) and memory functions (Marsicano et al., 2002b; Takahashi et al., 2005). Olfaction can be defined by olfactory threshold, identification of odorants, and discrimination between odorants. Olfactory threshold is likely to be influenced by the peripheral OE, while odorant identification and discrimination are influenced by the central olfactory structures, the OB and cortex (Enwere et al., 2004; Kovacs, 2004). Olfactory threshold can be measured as an index of odorant sensitivity (Witt et al., 2009). Functional decline in olfaction could be related to changes in the cellular structure of the olfactory system. CB1 receptor activity influences neuronal activation, odorant detection, and food intake in fasted mice (Breunig et al., 2010b; Soria-Gomez et al., 2014). Neuronal activity in the granule cell layer of the OB is regulated by CB1 receptors. A decrease in c-Fos positive cells, a marker of neuronal activity, is seen in C57 wildtype but not CB1 receptor-deficient mice 24 hours after fasting, suggesting that CB1 receptors contribute to the activation of OB neurons (Soria-Gomez et al., 2014). CB1 receptor signaling in the main OB alters olfactory processing in fasted mice. Using an olfactory behavioral assay, exogenous cannabinoids decreased olfactory habituation index, suggesting that CB receptor signaling leads to an increase in odorant exploration even after the scent is no longer novel (Soria-Gomez et al., 2014). Exogenous cannabinoid stimulation after fasting decreases odorant threshold, thereby enhancing odorant detection (Breunig et al.,

2010b; Soria-Gomez et al., 2014). A behavioral assay scoring exploration time of increasing concentrations of an odorant (almond or banana) was used to show cannabinoid treatment, either intra-olfactory bulb THC injection or increasing endogenous AEA production, increases the exploration time of odorants at concentrations 100 fold lower compared to vehicle treated mice (Soria-Gomez et al., 2014). Additionally, OB CB1-receptor deficient mice displayed a physiological decrease in odorant detection threshold under vehicle conditions, suggesting that increased odorant detection in a fasted state is CB1 receptor-specific (Soria-Gomez et al., 2014). Similarly in the tadpole OE, calcium imaging studies have shown CB receptor signaling can increase the odorant sensitivity of OSNs (Breunig et al., 2010b). Odorant threshold sensitivity is increased when 2-AG synthetic enzyme DAGL is inhibited pharmacologically, suggesting that endogenous OSN odorant sensitivity is mediated by the endocannabinoid 2-AG (Breunig et al., 2010b). Mice with a selective CB1-receptor deficiency in the granule cell layer of the OB ate less food 24 hours after fasting than fasted control mice (Soria-Gomez et al., 2014). After pharmacological manipulation of the cannabinoid system, food intake could be increased with exogenous cannabinoids and blocked with the CB1 receptor antagonist AM251 in wildtype mice. Therefore, CB1 receptors influence feeding increases after fasting. Collectively, these data suggest that endocannabinoid signaling via CB1 receptors helps link the physiological state of hunger to odorant detection thresholds and food intake.

Previous research in our lab utilizing a transgenic mice lacking the inositol trisphosphate receptor 3 (IP3R3), which regulates the release of NPY from non-neuronal microvillous cells (Jia et al., 2013), have identified changes in cellular

composition, proliferation, and olfactory-mediated behaviors. IP3R3-null mice exhibit a decrease in basal cell and immature neuron cell populations starting from 2 months of age and lasting throughout adulthood (Jia et al., 2013). The decrease in basal cell numbers corresponded with a compromised ability in neuronal differentiation in IP3R3-null mice. Furthermore, the IP3R3-null mouse showed a limited capacity to proliferate after injury, possibly due to a decrease in NPY signaling in the IP3R3-null mice (Jia et al., 2013). These data indicate that changes in trophic factor signaling can alter OE cellular morphology and the ability to proliferate and recover after injury. Using the same animal model, a decrease in mature OSN numbers and proliferation levels was seen in aged IP3R3-null mice (unpublished results.) Olfactory-mediated behaviors were also compromised in the aged IP3R3-null mice, possibly as a consequence of impaired proliferative ability and decreased OSN numbers (unpublished results). Collectively, our studies using the IP3R3-null mice have associated a decrease in the proliferative factor NPY with altered OE morphology, compromised proliferative ability, and deficits in olfaction.

The present chapter describes experiments that examine if CB1/CB2 receptor KO mice have gross morphological or olfactory-mediated behavioral deficits. Given the central hypothesis that the cannabinoid system promotes neurogenesis, CB receptor knockout mice may therefore have altered OE cell populations or a decline in olfactory-mediated behaviors.

Methods

Animals

Adult male (6-8 weeks old) C57BL/6 control wildtype mice were obtained from Charles River, Portage, MI. CB1/CB2 receptor KO mice were kindly provided by Dr. Norbert Kaminski (Michigan State University, MI) who obtained them from Dr. Andreas Zimmer (University of Bonn, Germany). The CB1/CB2 receptor KO mice were developed through replacement of the CB receptor coding region with non-CB receptor DNA using homologous recombination. Subsequently, heterozygote mice were mated to obtain the CB1/CB2 receptor KO mice (Jarai et al., 1999). Mice were given food and water *ad libitum*. Animal rooms were kept at 21–24°C and 40–60% relative humidity with a 12-h light/dark cycle. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by Michigan State University Institutional Animal Care and Use Committee.

Immunohistochemistry

Frozen coronal OE tissue sections (20 μm) were rehydrated with PBS, permeabilized with 0.01-0.3% triton x-100 and incubated with 10% (v/v) normal donkey serum. Tissue sections were incubated with goat anti-OMP (1:1000, Waco Chemicals, Richmond, VA), rabbit anti-cytokeratin 5 (CK5; 1:100, Abcam, Cambridge, MA), mouse anti-mammalian achaete-scute complex homolog-1 (MASH1; 1:20, BD Pharmingen, San Diego, CA), over night at 4 °C. Immunoreactivity was detected by Cy3-conjugated donkey anti-goat, mouse or rabbit immunoglobin (1:50 or 1:200, Jackson ImmunoResearch Lab, West Grove, PA). The nuclei were counterstained with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratory,

Burlingame, CA). For detection of CK5 and MASH1, antigen retrieval was performed before permeabilization by placing sections in a citrate buffer (pH=6) or HCl (2 M) and heating in a microwave oven (700 W) at low power for 2 x 6 minutes and cooling for 20 minutes. Antibody immunoreactivity was visualized using an Olympus FV1000 confocal laser scanning microscope (Pleasant Valley, PA). Antibody specificity was examined by omitting the primary antibody or secondary antibody. No immunoreactivity was observed in any of the controls.

The number of CK5 positive or MASH1 positive in the ectoturbinate 2 and endoturbinate II on three consecutive coronal sections of OE was counted by an experimenter blinded to the treatments and genotypes (n = 3-6 mice/group). Data were normalized to the length of OE on which the immunopositive cells were scored and expressed as number per linear millimeter OE.

A stereological approach was used to estimate the quantity of OMP positive neurons given their large numbers. The percent volume density of OMP positive cells was calculated in one coronal section of OE between levels 3 and 4 in each animal using STEPanizer® software at www.stepanizer.com (Tschanz et al., 2011). At six regions in the ectoturbinate 2, four locations in the endo-turbinate II and one location in the septum, a 250x250 μm 144-point overlay was randomly placed (total area analyzed = 62500 μm^2/location). The volume density of OMP positive cells was determined by manual point counting and expressed as the percentage of the ratio of the number of test points hitting OMP-immunoreactive OSNs, divided by the total number of points hitting the OE.

Olfactory behavioral test - Buried food test

A trial was administered every other day for 7 days (4 trials total). Wildtype and CB1/CB2 receptor KO 6-8 week old male mice (n = 12 mice/group) were fasted 16-18 hours prior to each trial day. For the first 3 trials, a mouse was first acclimated in a cage filled only with fresh wood chip bedding for 5 minutes, transferred to a second cage for 5 minutes and then to a third cage that contained a piece of sugary cereal that was buried beneath the bedding in a randomly selected location. On the 4th trial, the sugary cereal was placed on the surface of bedding. The latency to uncovering and eating the buried food was measured. Trial 1 measures naïve olfactory-mediated finding, trial 2 and 3 examine improvement based on positive reinforcement received in the previous trials and indicate olfactory-mediated learning and memory, while trial 4 with visible cereal is used to assess locomotor activity. Only mice that could find the buried food within 5 minutes and eat the food were included in the data analysis. The average improvement factor was calculated as $\sum(T1/T3)/n$ (Le Pichon et al., 2009).

Olfactory behavioral test - Habituation/dishabituation

The habituation/dishabituation test was performed and analyzed as described previously (Le Pichon et al., 2009). Briefly, wildtype and CB1/CB2 receptor KO male mice (n = 11-16 mice/group) were acclimated in the test cage for 30 minutes with a clean dry cotton applicator inserted through the hole on the cage lid prior to the beginning of trials to reduce novelty-induced exploratory activity during the subsequent trials. Distilled water (100 µl), peppermint extract or almond extract (100 µl of 1:100 with distilled water, McCormick & Co., Hunt Valley, MD) was applied to a cotton applicator and inserted through the hole on the cage lid. For each trial an odorant was delivered

for 2 minutes with a 30 second delay before the next trial began. The testing consisted of 3 trials of distilled water, 3 trials of peppermint, then 3 trials of almond. Investigation was defined as active sniffing within a 1 cm radius of the cotton applicator with the snout oriented towards the applicator. The cumulative investigation time during the 2 minutes odorant presentation was recorded by a single observer blind to genotypes. Investigation time for trial 1 was calculated for each animal as ∑[(Trial 1distilled water + Trial 1peppermint+Trial 1almond)]/3. Odorant habituation was assessed by analyzing the investigation times of repeated exposure to the same odorant. The habituation index for each animal was calculated as ∑[(Trial 3 distilled water)+(Trial 3 peppermint)+(Trial 3 almond)]/3. The cross-habituation index for each animal was calculated as ∑[Trial 1 peppermint-Trial 3 distilled water)+(Trial 1 almond-Trial 3 peppermint)]/2.

Statistical analysis

Student's t-test, and one-way ANOVA followed by the Bonferroni multiple comparison test was performed using Prism 5 (Graphpad Software, San Diego, CA). Two-way ANOVA or repeated-measures two-way ANOVA was performed followed by the Newman-Keul post hoc test using GB-Stat v9.0 (Dynamic Microsystems, Inc., Silver Spring, MD).

Results

OE tissue composition in CB1/CB2 receptor KO mice

A change in endocannabinoid signaling could alter the microenvironment and ultimately affect the behavior and function of basal progenitor cells. First, to determine if

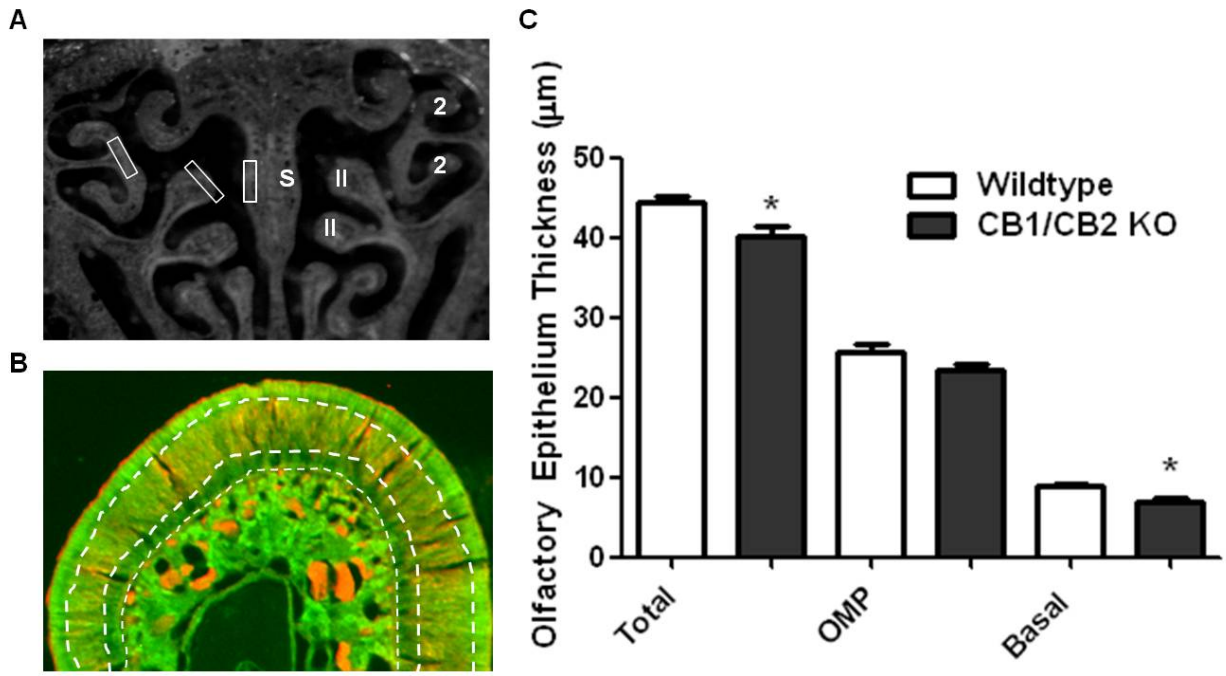

Figure 3.1 Decreased OE thickness in CB1/CB2 receptor KO adult mice. (A) Representative sagittal cross section of mouse OE tissue indicating the locations of endoturbinates II (II), ectoturbinates 2 (2), and septum (s). White boxes indicate regions where thickness was measured. **(B)** Olfactory marker protein immunoreactivity (OMP; red) depicts the thickness of OE cell layers. Background auto fluorescence (green) is used to help visualize OE boarders. Thin dashed lines delineate basal lamina and thick dashed lines boarder the OMP cell layer. Scale bars = 10 µm. **(C)** Total OE and basal layer thickness is decreased in CB1/CB2 receptor KO mice. (* $p<0.05$ vs. respective control in wildtype; student's t-test for layer; n = 12 sections from 4 mice).

CB1/CB2 receptor KO mice exhibit gross OE morphological changes, the thickness of OE cell layers were measured. OSNs were labeled with olfactory marker protein (OMP) to distinguish between the pseudostratified layers of the OE. The height above the neuronal layer is classified as the apical layer, the OMP positive layer is the middle or neuronal layer, and the width below OMP positive cells to the basement membrane is deemed the basal layer (Figure 3.1B). The thickness of several OE regions was measured including one region from the septum, and ten regions from the turbinates (ectoturbinate 2 and endoturbinate II) (Figure 3.1A). CB1/CB2 receptor KO mice showed a decrease in basal cell thickness, which was reflected in a decrease in total OE thickness (Figure 3.1C; $p < 0.05$).

To further investigate any morphological changes in the CB1/CB2 receptor KO mice, the complement of cells in the OE of adult C57 wildtype and CB1/CB2 receptor KO mice was examined. Compared to C57 wildtype mice, the number of cells expressing cytokeratin 5 (CK5), located in horizontal basal cells and MASH1, a proneural transcription factor expressed in a subpopulation of global basal cells, was significantly reduced in the OE of CB1/CB2 receptor KO mice compared to wildtype ($p<0.05$, Figure 3.2, Table 3). This indicates that in the CB1/CB2 receptor KO mice, there are fewer progenitor cells, although horizontal basal cell numbers were only decreased 23% and GBC numbers were decreased 26%. However, this decrease in basal cell numbers could be sufficient to induce changes in differentiated cell populations in the OE of CB1/CB2 receptor KO mice. Indeed, the number of OMP positive mature OSNs in CB1/CB2 receptor KO mice was significantly decreased ($p < 0.05$, Figure 3.3, Table 3). This suggests that the pool of progenitor cells in the

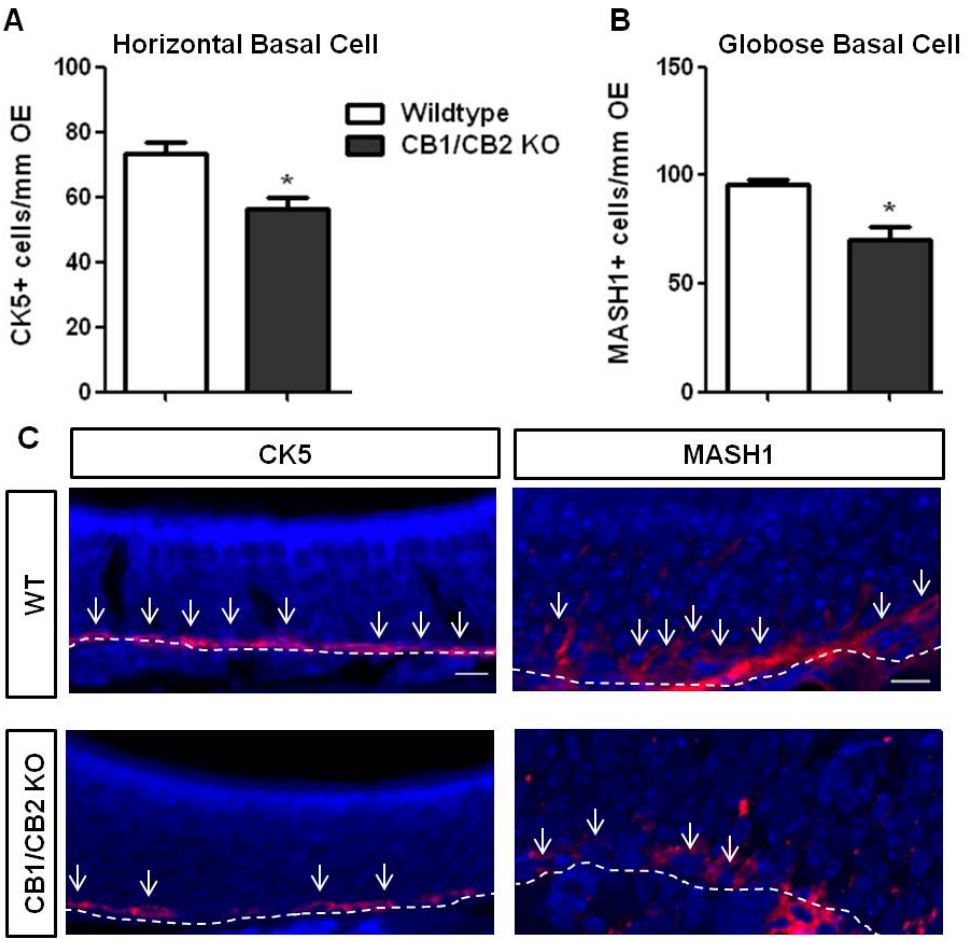

Figure 3.2 CB1/CB2 receptor KO mice have fewer basal cells in the OE. (A) The number of cytokeratin 5 (CK5) positive horizontal basal cells is reduced in CB1/CB2 receptor KO OE. **(B)** The number of proneural transcription factor MASH1 positive globose basal cells is reduced in CB1/CB2 receptor KO OE. **(C)** Representative immunoreactivity to cellular markers in adult C57BL/6 wildtype (WT) and CB1/CB1 receptor KO mice. (* $p<0.05$ vs. respective C57 wildtype control; Student's t-test for each cell marker; n = 12 sections from 4 mice). DAPI (blue) demarcates the nuclei. Dashed white lines indicate basement membrane. Arrows indicate positive immunoreactivity. Scale bar = 10 μm.

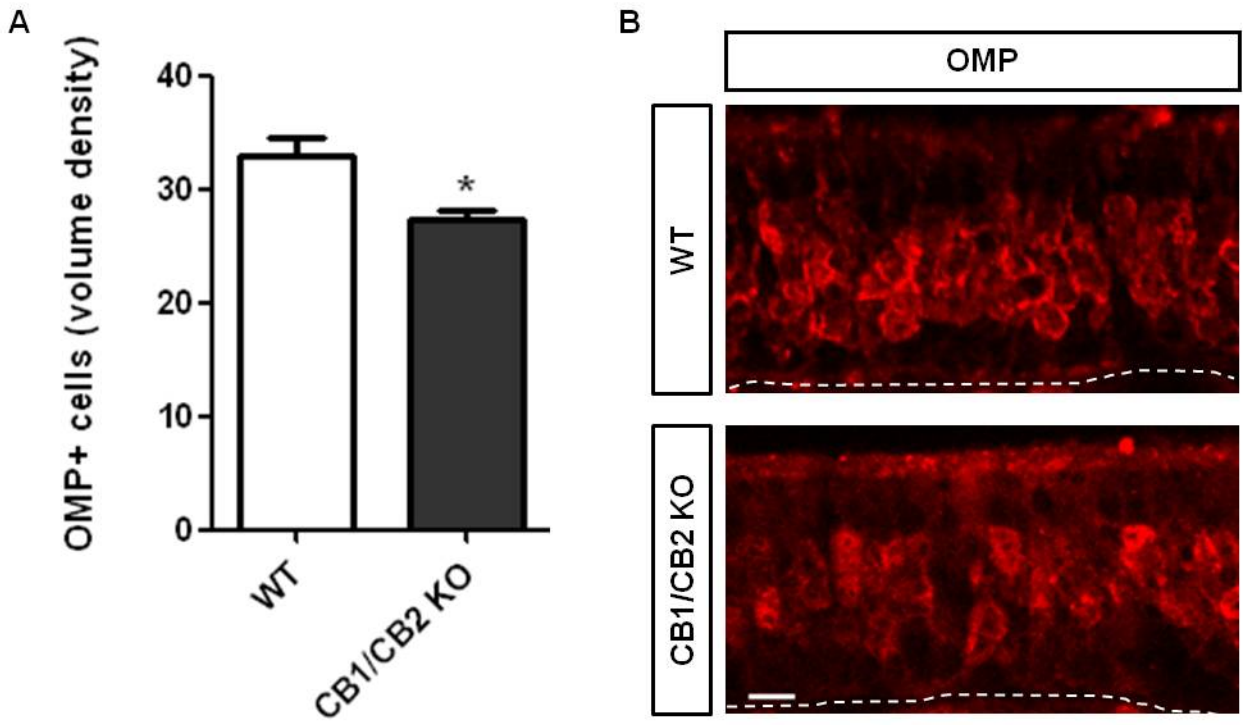

Figure 3.3 CB1/CB2 receptor KO adult mice have fewer mature olfactory sensory neurons in the OE. (A) The number of olfactory marker protein (OMP) positive neurons in the OE of CB1/CB2 receptor KO mice is less than C57BL/6 wildtype (WT) adult mice ($p < 0.05$; Student's t-test; $n = 12$ sections from 4 mice.) **(B)** Representative immunoreactivity to mature neuronal marker, OMP. Dashed white lines indicate basement membrane. Arrows indicate positive immunoreactivity. Scale bar = 10 μm.

Table 3. Basal cell counts and OMP density numbers in wildtype and CB1/CB2 receptor KO mice

Antibody (cell type)	WT	CB1/CB2 KO
Mash1 (Globose Basal Cells)	95.59 ± 2.5	70.18 ± 6.2 *
CK5 (Horizontal Basal Cells)	73.23 ± 3.4	56.25 ± 3.8 *
OMP (Mature Olfactory Neurons)	32.88 ± 1.7	27.25 ± .80 *

Data are expressed as the average number of cells ± S.E.M. * $p < 0.01$; Student's t-test

CB1/CB2 receptor KO mouse is not sufficient to maintain the number of mature neurons. Similar to the significant albeit slight decrease in basal cell numbers, CB1/CB2 receptor KO mice only exhibited a 16% decrease in OMP-positive cells compared to wildtype. Collectively, these data indicate that a significant decrease in progenitor cell numbers leads to impaired differentiation and subsequent decrease in mature OSNs in the OE of CB1/CB2 receptor KO mice.

Olfactory function in CB1/CB2 receptor KO mice

To determine if the decreases in basal cell and mature OSN numbers seen in CB1/CB2 receptor KO mice lead to impaired olfaction, two olfactory-mediated behavioral tests were performed. In the buried food test, the latency to find a piece of buried food was measured over 3 trials in fasted untreated C57 wildtype and CB1/CB2 receptor KO mice (Figure 3.4). The first trial measures the ability to smell via naïve olfactory-mediated detection, while the subsequent trials are a measure of olfactory-mediated learning and memory by assessing the ability to improve based on positive reinforcement from previous trials (Le Pichon et al., 2009).

CB1/CB2 receptor KO mice exhibited a significant increase in the latency of trial 1, used to measure naïve olfactory-mediated investigation, compared to C57BL/6 wildtype mice ($p<0.05$; Figure 3.4A). However, CB1/CB2 receptor KO mice exhibited a significant increase in the latency to approach a piece of visible food ($p<0.05$; Figure 3.4B), indicating that the increased latency in trial 1 times in CB1/CB2 receptor KO mice could either be due to a deficit in olfaction or an overall decrease in mobility. Indeed, a hallmark of CB1 receptor KO mice is decreased locomotion resulting from the high

density of CB receptors in the basal ganglia and cerebellar cortex (Elphick and Egertova, 2001; Zimmer et al., 1999). Due to the global knockout of CB1/CB2 receptors in our genetic mouse model, it is possible that higher processing centers of olfactory information are also compromised. The average improvement factor was calculated as the ratio of trial 1 to trial 3 latencies, an indicator of olfactory-mediated learning and memory. No change in improvement factor is seen in CB1/CB2 receptor KO mice, indicating that CB receptor deficiency does not impair olfactory learning and memory (Figure 3.4C). Overall, the analysis of the latency to find buried food using a repeated measures two-way ANOVA showed that there were significant overall effects of repeated measure trials ($F_{(2,22)} = 7.52$, $p < 0.0016$) as well as a genotype effect ($F_{(1,23)} = 8.50$, $p = 0.008$), but no interaction between these measures. The significant overall effect on repeated measures suggests that the ability to find buried food in both C57 wildtype and CB1/CB2 receptor KO mice was improved due to the positive reinforcement of repetition as all mice have a reduced latency in trials 2 and 3.

To further investigate if CB receptor signaling alters olfactory-mediated behaviors, a more sensitive behavioral assay was performed. The olfactory habituation/dishabituation test relies on the natural tendency to investigate novel smells and is used to examine the ability to detect and differentiate between different odorants (Sundberg et al., 1982; Yang and Crawley, 2009). Generally, mice spend more time on a novel odorant and less time on a previously investigated odorant. This test eliminates the factors associated with traditional operant odorant discrimination tests, such as cognitive or learning-dependent training, nutritional deprivation, and sensorimotor control, and is therefore used as a test of spontaneous odorant discrimination (Linster et

al., 2002; Wesson et al., 2008). The habituation/dishabituation test includes the measurement of novel odorant investigation in the first trial, odorant habituation by exposure to the same odorant, and odorant dishabituation by exposure to a new odorant. Novel odorant investigation was measured by combining the investigation times of the first trial for each odorant (water1, peppermint1, almond1) in CB1/CB2 receptor KO and C57 wildtype mice (Figure 3.5B). The pooled investigation times of trial 1 were similar between C57 wildtype and CB1/CB2 receptor KO mice, indicating no deficiency in novel odorant investigation (Figure 3.5B). We next assessed odorant habituation over repeated odorant exposures. All mice showed decreases in investigation times in trials 2 and 3 (Figure 3.5A; habituation) and an increase in investigation times with novel odorant presentation, trial 1 (Figure 3.5A; dishabituation). There were no significant differences in the habituation index among ages (Figure 3.5C), indicating that all mice have a similar ability to habituate to repeated odorant exposure. The ability to discriminate odorants was assessed using the cross-habituation index, and no genotype differences were observed indicating the ability of CB1/CB2 receptor KO mice to discriminate between different odorants (Figure 3.5D). Two-way ANOVA revealed no significant effect of genotype ($F_{(1,36)} = 3.68$, $p < 0.0632$), but a significant main effect of repeated measures ($F(1,29) = 9.75$, $p < .0001$). Therefore, the sensitivity to novel odorant detection or odorant discrimination is not affected in CB1/CB2 receptor KO mice despite a significant loss in OSN population compared to C57 wildtype.

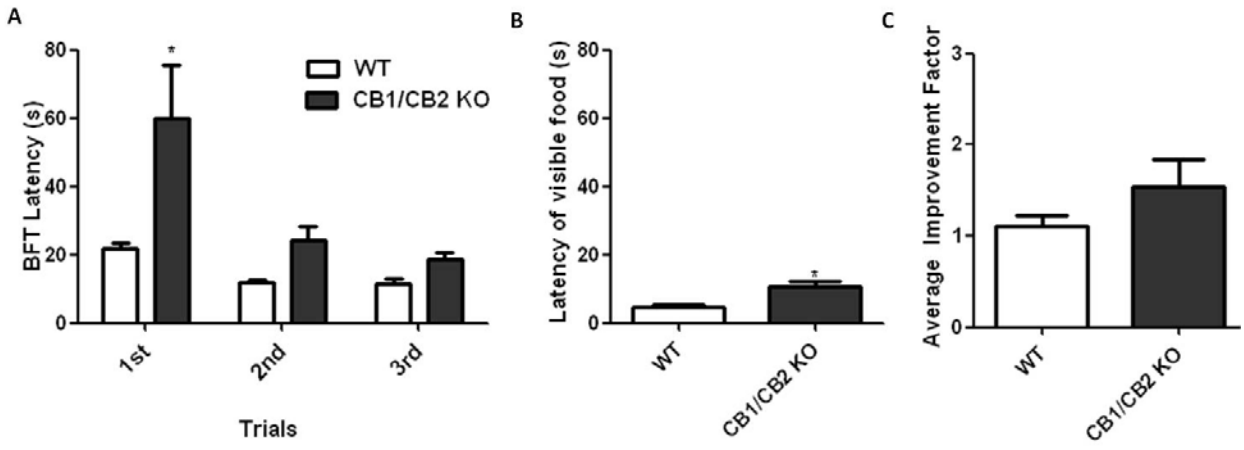

Figure 3.4 Olfactory-mediated food detection is impaired in CB1/CB2 receptor KO mice. Buried food behavioral tests were performed every other day in fasted CB1/CB2 receptor KO mice. **(A)** Histogram showing the average (± SEM) latency of trials 1-3 for CB1/CB2 receptor KO mice (dark bars) and C57 wildtype (WT) mice (white bars) mice. (*, $p<0.05$ for trial 1 compared to C57 wildtype control; repeated measures two-way ANOVA followed by Tukey/Kramer Procedure post-hoc test; n = 11-16 mice per group). **(B)** Histogram showing the latency of trial 4 with visible food, an assessment of locomotor activity in C57 wildtype and CB1/CB2 receptor KO mice. ($p<0.05$; Student's t-test; n = 10-12 mice per group). **(C)** Average improvement factor calculated as the ratio of trial 1 vs. trial 3 latencies, an indicator of olfactory-mediated learning and memory.

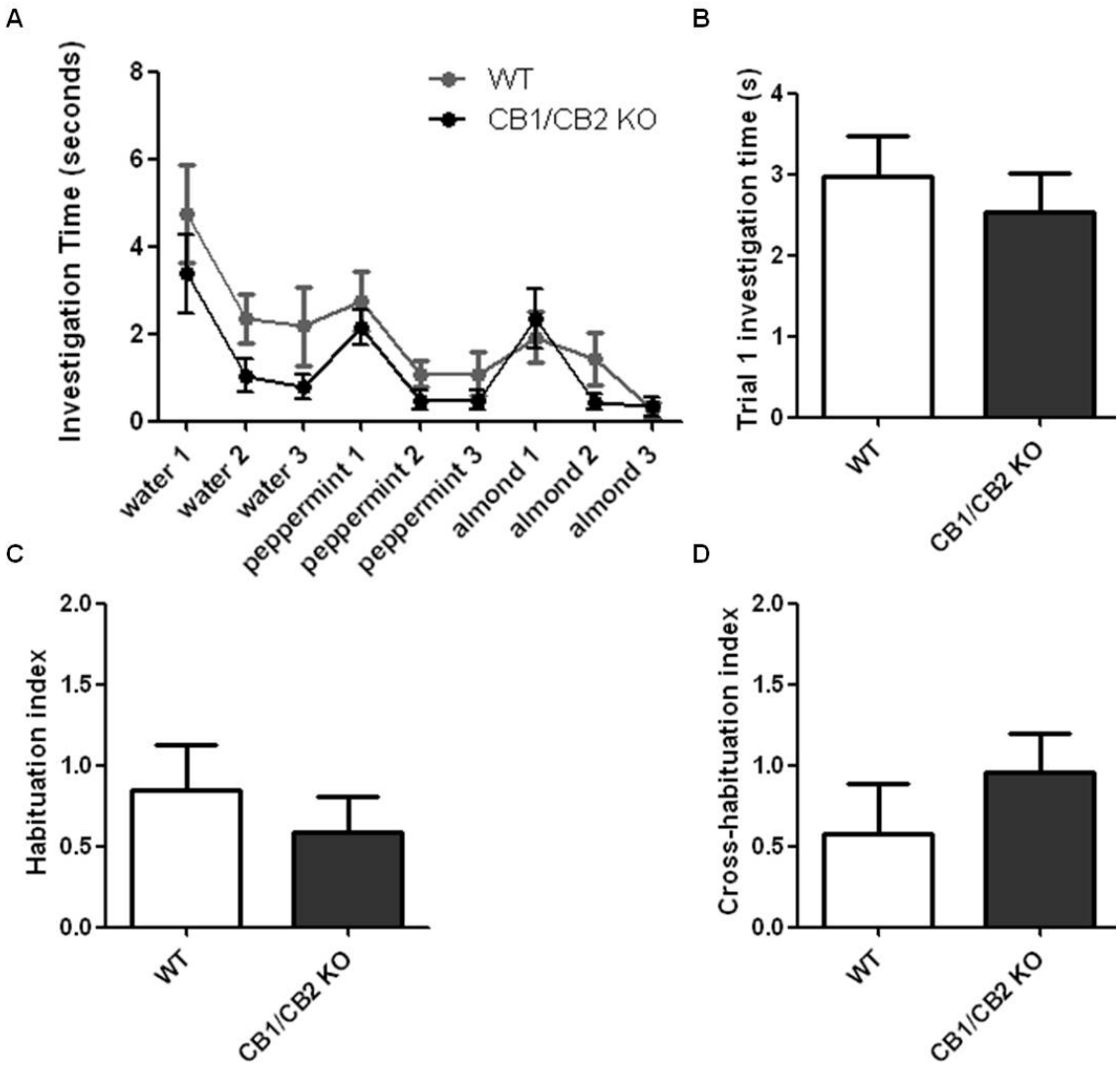

Figure 3.5 CB1/CB2 receptor KO mice show no deficit in novel odorant investigation time, discrimination, or habituation. The olfactory habituation/dishabituation behavioral test was performed in C57 wildtype (WT) and CB1/CB2 receptor KO mice. **(A)** Investigation times in seconds (+SEM) for trials 1-3 for three odorants are shown. All mice showed a decrease in investigation time during trial 2 and 3 (habituation) and no change in investigation time to a novel odorant presentation (dishabituation). **(B)** The investigation time of the trial 1 odorants (the

average for all three first odorants) is comparable between CB1/CB2 receptor KO and C57 wildtype mice ($p<0.05$, Student's t-test; n = 11-16 mice per group). **(C)** The habituation index was comparable across genotypes ($p<0.05$, Student's t-test; n = 11-16 mice per group). **(D)** The ability to discriminate between odorants, measured by the cross-dishabituation index, was comparable across genotypes. ($p<0.05$, Student's t-test; n = 11-16 mice per group).

Discussion

The endocannabinoid system helps regulate the survival and function of progenitor cells in the developing mammal as well as in adulthood. Neurosphere cultures of embryonic neural progenitor cells derived from CB1 and CB2 receptor-null mice have reduced cell proliferation and impaired self-renewal (Aguado et al., 2005; Palazuelos et al., 2006). Accordingly, pharmacological regulation with selective CB1 and CB2 receptor agonists or antagonists exerts a positive or negative action, respectively, on neural progenitor cell division (Aguado et al., 2005; Goncalves et al., 2008; Jiang et al., 2005; Palazuelos et al., 2006). *In vivo*, CB1 receptor deficiency causes altered cortical and hippocampal development (Aguado et al., 2005; Berghuis et al., 2005) and reduced progenitor cell proliferation (Mulder et al., 2008; Trazzi et al., 2010).

In the OE, progenitor cells that contribute to ongoing neurogenesis lie near the basal lamina. This basal germinal zone is comprised of GBCs and HBCs. GBC function as the major proliferative population of the OE (Caggiano et al., 1994), and give rise to neurons and sustentacular cells (Chen et al., 2004b; Huard et al., 1998). During differentiation into OSNs, GBC progenitors sequentially express the proneural genes *Mash1* and *Neurog 1* (Cau et al., 2002). Mash1-positive GBCs are the earliest OSN precursors (Calof et al., 2002).The second basal cell population are HBCs, which are defined by their: (1) presence as a single-cell layer below the GBCs, (2) specific expression of cytokeratin 5 and 14, and (3) direct contact with the basal lamina (Holbrook et al., 1995). HBCs rarely self-renew *in vivo* (Caggiano et al., 1994; Mackay-Sim and Kittel, 1991) but undergo a regulated, transient proliferative burst to repopulate

the OE generating proneural intermediates under conditions of extensive OE cellular depletion (Leung et al., 2007a).

The number of HBCs and GBCs is decreased in the CB1/CB2 receptor KO mouse. Chapter 2 identified CB1 receptor immunoreactivity on GBCs. As in the developing brain, endocannabinoid signaling via CB1 receptors on basal cells could help maintain basal cell population and self-renewal. The number of OSNs was also decreased in CB1/CB2 receptor KO mice, either reflecting the reduction in survival of the basal cell population or possible alterations in differentiation. Although the thickness of the apical non-neuronal layer was measured, specific non-neuronal cell numbers were not counted due to limitations in antibody detection of sustentacular cells. Additionally, numbers of immature neurons were not measured, which could provide a clue as to when along the neuronal differentiation lineage CB1/CB2 receptor KO mice begin to show alterations in neuronal populations. The ability of CB1/CB2 receptor KO mice to undergo proliferation will be examined in Chapter 4, in which I will further investigate the neurotrophic effects of cannabinoids on basal cells. Similar cell population profiles as in the CB1/CB2 receptor KO mouse are seen in genetic mouse models in which trophic factor receptors have been ablated. For example, in NPY receptor Y1-null mice there is a significant decrease in mature OSNs and MASH1 positive basal progenitor cells (Doyle et al., 2008; Hansel et al., 2001). In a mouse lacking the IP3R3, a receptor that contributes to the release of neurotrophic factor NPY through non-neuronal microvillous cells, a decrease in both GBCs and HBCs is seen (Jia et al., 2013). These similarities suggest that endocannabinoids play a role in neural progenitor cell homeostasis. The decrease in OMP positive cells in the CB1/CB2

receptor KO mice suggests that endocannabinoid signaling also influences neuronal differentiation under physiological conditions.

The function of the endocannabinoid system in the mouse OE is unknown, but previous work in the rat OB and tadpole OE suggests the cannabinoid system helps connect the physiological state of hunger to alterations in olfaction resulting in increases in food intake (Breunig et al., 2010b; Soria-Gomez et al., 2014). Olfaction is defined by olfactory threshold, odorant identification, and odorant discrimination. Olfactory threshold is likely to be influenced by the peripheral OE, while odorant identification and discrimination are influenced by the central olfactory structures, the OB and olfactory cortex (Enwere et al., 2004; Kovacs, 2004). Declines in olfactory function could be related to changes in the structure of the olfactory system. Deficits in olfaction may be primarily due to changes in the OE tissue composition; however, changes in OB and cortex cannot be excluded. This chapter examines CB receptor-dependent changes in mouse olfactory function using olfactory-mediated behavioral assays, the buried food test and olfactory habituation/dishabituation test. The buried food test, which relies on the natural tendency to use olfactory cues for foraging, is used to examine the ability to smell volatile odorants (Yang and Crawley, 2009). The latency to find a piece of buried cereal was measured in food deprived mice. The first trial measures the ability to smell via naïve olfactory-mediated finding and the subsequent trials indicate olfactory-mediated learning and memory by measuring the ability to improve based on the positive reinforcements from previous trials (Le Pichon et al., 2009). CB1/CB2 receptor KO mice exhibited prolonged latencies to detect a buried odorant (i.e., food), which reflects impairment in olfaction. However, CB1/CB2 receptor KO mice also showed

decreased locomotion when visible food was presented. This could reflect the previously characterized decreases in mobility and increases in freezing behavior seen in CB1 receptor KO mice (Zimmer et al., 1999). Given the physiological 16% loss of OSNs in CB1/CB2 receptor KO mice it is possible that these mice exhibit natural impairments in olfaction and sporadic decreases in locomotion. During trials 2 and 3, CB1/CB2 receptor KO mice did not exhibit increased latencies to detected buried food, suggesting impairments in mobility were not consistent throughout each trial compared to C57 wildtype controls. Therefore it is likely trial 1 increases in latency are olfactory-mediated. However, these data need to be replicated to confirm if CB1/CB2 receptor KO mice exhibit any deficits in the buried food test.

Due to the inconclusive results from the buried food test, the olfactory habituation/dishabituation test was used to examine novel odorant investigation, odorant discrimination and odorant habituation. A single sniff of an odorant is sufficient for odorant detection and discrimination (Wesson et al., 2008), while prolonged sniffing response is an indicator of arousal and motivation (Linster et al., 2002; Wachowiak et al., 2009). The present study demonstrates no differences between C57 wildtype and CB1/CB2 receptor KO mice in novel odorant investigation, suggesting no changes in odorant sensitivity despite significant decreases in OSNs. There are also no significant alterations in odorant habituation or dishabituation between CB1/CB2 receptor KO and C57 wildtype mice. Odorant habituation is associated with adaptation at OSN in the OE (Reisert and Matthews, 2001; Verhagen et al., 2007), mitral cells in the OB (Kadohisa and Wilson, 2006a), and in the prefrontal cortex (Kadohisa and Wilson, 2006b).

Previous studies using olfactory behavior tests detected an increase in exploration after THC treatment, reflecting habituation inability, suggesting that CB receptor signaling participates in olfactory-mediated behaviors. Moreover, OB CB1 receptor-deficient mice did not show any evident spontaneous phenotype in olfactory habituation experiments (Soria-Gomez et al., 2014).These observations support my data in that no naïve impairment in habituation is seen in either global CB1/CB2 receptor KO mice or specific OB CB1 receptor-null mice. Future studies should investigate odorant-mediated behaviors after exogenous cannabinoid administration to compare activated CB receptors in C57 wildtype mice to CB1/CB2 receptor KO mice. However, OB CB1 receptor-null mice did exhibit a naïve increase in threshold odorant detection compared to wildtype mice 24 hours after fasting (Soria-Gomez et al., 2014). Similarly, CB1-receptor mediated odorant threshold was decreased after 24 hours of fasting in the tadpole OE (Breunig et al., 2010b). In the current experiments, mice were fasted for roughly 16 hours to insure proper motivation to perform olfactory-mediated tasks. Different signaling mechanisms may come into play in 24 hour fasted mice that are not seen in mice fasted overnight. Although a decrease in basal cell and OSN numbers was observed in CB1/CB2 receptor KO mice, this did not translate to impairment in olfactory-mediated behaviors. Similarly, IP3R3-null mice exhibit a robust 50% decrease in basal cells numbers (Jia et al., 2013), but no deficits in olfactory-mediated buried food test or habituation/dishabituation tests (unpublished results). However, a significant deficit was detected when comparing aged IP3R3 KO mice to aged wildtype mice in both behavior paradigms (unpublished result), suggesting that the presumed neurotrophic compensatory mechanisms functioning normally in the adult

animals are compromised with aging, resulting in decreased olfaction. Therefore, decreases in cannabinoid signaling and OSN number may show deficits in olfactory-mediated behavior in the aged animal whereas no deficits were seen in 6-8 week old mice.

Conclusion

These data indicate that CB receptor signaling contribute to the survival and proper regulation of the basal cell population, given the decrease in both HBC and GBC numbers in the CB1/CB2 receptor KO mouse. A decrease was also seen in OSN numbers in the CB1/CB2 receptor KO mouse, although the remaining population of neurons was sufficient to adequately perform olfactory-mediated behaviors. This suggests olfactory signaling can adapt within the constraints of a limited OSN population. Cannabinoid signaling in olfaction could further be investigated under altered physiological conditions, such as hunger state, or under pathophysiological conditions seen in advanced aging or with neurological disease. Because the endocannabinoid system contributes to OE tissue homeostasis, reduced cannabinoid signaling together with an increase in cellular stress seen with aging or diseased states (i.e., limited availability of trophic factors, increased inflammation, or a reduced capacity to regenerate) could further accelerate the rate of OE tissue damage and increase likelihood of anosmia.

Chapter 4: Cannabinoid contribution to neurogenesis in the mouse olfactory epithelium

Introduction

Stem cells during development

Stem cell biology has rapidly expanded since the identification of hematopoietic stem cells and adult neurogenesis in the 1960's (Altman, 1969; Till and Mc, 1963). Stem cells are undifferentiated cells that have the potential to develop into many differentiated cell types. In mammals, there are two main types of stem cells: embryonic stem cells, which are isolated from blastocytes, and adult stem cells, which are found in various CNS regions (Hipp and Atala, 2008). Stem cells are able to go through numerous cycles of cell division while maintaining an undifferentiated state. During embryogenesis the zygote is considered totipotent since it has the ability to divide and produce an entire organism, while embryonic stem cells are pluripotent, and have the ability to differentiate into any of the three germ layers. These layers then produce multipotent progenitor cells having the potential to give rise to cells from multiple, albeit limited, lineages (Figure 1.3) (Hipp and Atala, 2008). Multipotent progenitor cells are the variety found in the OE. Until recently, it was thought only certain tissue areas had the ability to renew, but it is becoming more evident that many adult tissues have a small percentage of progenitor or stem cells acting to replace damaged cells (Sartipy, 2013).

Adult proliferation

Adult neurogenesis plays an active and important role in maintaining normal homeostatic processes and contributes to the plasticity of the central nervous system (Lledo and Gheusi, 2006; Ming and Song, 2011). Postnatal neurogenesis involves the proliferation, migration, differentiation, integration, and survival of newly generated cells in both humans and non-human primates (Eriksson et al., 1998; Gould et al., 1999; Reynolds and Weiss, 1992). Adult pluripotent neural stem cells are defined by their ability to self-replicate and differentiate into multiple cell types found in the CNS, including neurons, astrocytes, and oligodendrocytes (Gage, 2000), and neural progenitor cells (Potten and Loeffler, 1990). However, compared to embryonic stem cells, adult neural stem cells have restricted neurogenic potential which could be due to a change in the microenvironment of the stem cell. Neurogenic regions consist of a resident population of neural stem cells and a microenvironment that not only physically supports stem cells, but also functionally controls their development (Li and Xie, 2005). Adult neurogenesis in humans and rodents has been most studied in two specialized neurogenic regions: (1) the subventricular zone of the lateral ventricles (SVZ) where new cells are generated and migrate into the OB, and (2) the subgranular zone of the dentate gyrus where new hippocampal cells are generated (Gage et al., 1995; Reynolds and Weiss, 1992). Neural progenitor cells are not limited to these neurogenic regions of the brain, and proliferation can be observed in many brain regions, especially after injury. However, in non-neurogenic regions it appears that neurogenesis is actively repressed by the local environment. Like the rodent olfactory system, the human olfactory system also remains pliable into adulthood. Adult neurogenesis is considered

important for regulation of cognition and mood (Zhao et al., 2008), contributes to hippocampal and olfactory learning and memory (Deng et al., 2010; Kitamura et al., 2009), and enhances perceptual and neural olfactory discrimination (Li et al., 2008; Li et al., 2006).

Olfactory epithelium regeneration

Along with the DG, the olfactory system is the only other area in the mammalian brain that exhibits continual adult neurogenesis (Ming and Song, 2005b). Central olfactory neurogenesis occurs in the SVZ where newborn cells migrate along a well defined pathway, the rostral migratory stream, into the OB where they differentiate into several types of olfactory interneurons (Alvarez and Eichenbaum, 2002; Lledo and Lagier, 2006). Similarly, the OE is dynamic, and undergoes continuous replacement of OSNs derived from local basal progenitor cell population. Natural turnover of OSNs is required to maintain the sense of smell. Even after a virtually complete loss of OSNs, the population recovers in terms of neuronal number and topography of odorant receptor protein expression (Iwema et al., 2004; Schwob et al., 1999). These newly-generated OSNs must reestablish axonal connections with their appropriate targets in the OB; however, this process is subject to errors unseen during development (Blanco-Hernandez et al., 2012; Costanzo, 2000; John and Key, 2003) that could have functional implications. For example, errors in the reinnervation of glomeruli may underlie olfactory dysfunction in humans recovering from olfactory loss due to trauma or infection (Meisami et al., 1998).

Progenitor cells residing in the basal layer continually turnover, differentiate, and move apically towards the neuronal and apical layers (Graziadei and Graziadei, 1979).

Multipotent horizontal and GBCs give rise to mature neurons, marked by expression of OMP. During integration, OSNs extend axons that must find the proper synaptic targets in the OB after approximately two week (Figure 1.3) (Beites et al., 2005; Caggiano et al., 1994; Leung et al., 2007b; Schwob et al., 1994). Four stages of OE neuronal lineage have been identified. (1) *Sox2*-expressing stem cells, which reside in the basal compartment of the epithelium, are thought to commit to the OSN lineage via expression of the proneural gene, *Mash1. (*2) *Mash1*-expressing early progenitor cells, which divide and may act as transit-amplifying cells (Gordon et al., 1995), in turn give rise to (3) late-stage transit-amplifying cells, also known as immediate neuronal precursors, which express a second proneural gene, *Ngn1* (Wu et al., 2003). Immediate neuronal precursors give rise to daughter cells that undergo terminal differentiation into (4) postmitotic neural cell adhesion molecule -expressing OSNs (Figure 1.3). Non-neuronal sustentacular cells can be self-derived or renewed from Bowman's gland cells or basal cells (Huard et al., 1998). The natural turn-over rate of cells in the OE is not saturated, since the rate of proliferation increases after injury. The rate of basal cell proliferation is markedly increased with OSN injury induced through surgical bulbectomy (Kastner et al., 2000), axonomy (Suzuki and Takeda, 1991), or intranasal exposure to chemical toxicants such as zinc sulfate and methyl bromide gas (Leung et al., 2007b; Margolis et al., 1974; McBride et al., 2003; Schwob et al., 1995). Cell proliferation and neuronal differentiation in the OE is tightly regulated by multiple signals produced in the stem cell microenvironment, which is complex and remains to be fully defined (Kanekar et al., 2009; Kawauchi et al., 2004; Mackay-Sim and Chuah, 2000). Our lab has investigated ATP, Neuropeptide Y, transforming growth factor, and fibroblast growth

factor 2 as OE proliferation signaling molecules (Jia et al., 2011a; Jia and Hegg, 2010; Kanekar et al., 2009); yet more work needs to be done to elucidate fully the mechanisms underlying proliferation.

The peripheral OE is an ideal system with which to investigate mechanisms of neuroproliferative factors as neurogenesis continues to occur throughout the life under both physiological and injury conditions. Several features of the OE make it an ideal system in which to study proliferation. (1) The OE is the only system in which sensory neurons are in direct contact with the environment and therefore can be easily damaged by environmental toxicants.(2) The sense of smell is essential for survival (through foraging) and reproduction in many mammals (and is fundamental to health and nutrition in humans). (3) OSNs provide a route for viruses and bacteria to enter the brain, and exhibit (4) lifelong capacity to regenerate neuronal and non neuronal cell types. (5) Newly born neurons retain capacity to reinnervate OB, thereby restoring sensory function. (6) Its peripheral location makes the OE easy to access and isolate.

Cannabinoid system and proliferation

It is well established that the cannabinoid system modulates progenitor cell survival and function throughout development (Diaz-Alonso et al., 2012). CB1 receptors are detected in rodent embryonic neuronal progenitors (Rueda et al., 2002b), suggesting a developmental role for the endocannabinoid system starting at the earliest stages of brain development. The endocannabinoid system has a cell specific and spatio-temporal distribution from very early stages of embryonic development, even before the appearance of the neural tube (Sun and Dey, 2009). Mammalian CB1 receptor expression during neural development is predominately present in white matter

during neurogenesis and axonal migration prior to synaptic maturation and signaling. Not until postnatal stages and into adulthood are CB1 receptors expressed in grey matter (Berrendero et al., 1998), suggesting neurodevelopmental CB1 receptor function is likely to be independent of their neuromodulatory role at the mature neuronal synapse.

Studies examining cannabinoid-effects on the survival of developmental neuronal progenitors have been performed with various pharmacological agents. AEA analog (ACEA) and THC analog (HU-210) enhance progenitor survival via CB1 receptors (Molina-Holgado et al., 2002; Wolf et al., 2010), while FAAH inhibition and THC seemed to have no effect (Aguado et al., 2006; Molina-Holgado et al., 2002; Wolf et al., 2010). *In vitro*, pharmacological CB1 receptor activation enhances progenitor proliferation and neurosphere generation via activation of Gi/o proteins and ERK phosphorylation (Aguado et al., 2006). CB1 receptor signaling activates SVZ progenitor cells to generate pyramidal cell precursors that subsequently migrate to the developing neocortex (Wonders and Anderson, 2006). This suggests endocannabinoid signaling in local environments could provide extracellular cues that modulate the fate of neural progenitor cells. The presence and function of the cannabinoid system in embryonic progenitor cells is preserved in the adult brain in neurogenic regions.

In the mature central nervous system, CB1 and CB2 receptors can modulate neural stem cell proliferation *in vitro* (Molina-Holgado et al., 2007; Rueda et al., 2002a) and *in vivo* (Aguado et al., 2005; Aguado et al., 2006; Jiang et al., 2005; Jin et al., 2004). Neurospheres are non-adherent *in vitro* cultures of neuronal stem cells that grow as free-floating clusters. Neurospheres from postnatal rodents express CB receptors,

metabolic endocannabinoid enzymes, and produce endocannabinoids (Aguado et al., 2005; Molina-Holgado et al., 2007; Palazuelos et al., 2006). CB1 receptor activation promotes progenitor cell proliferation and neurosphere generation (Aguado et al., 2006), which can be prevented in CB1 receptor-deficient cells and increased in neurospheres with robustly elevated AEA levels (Aguado et al., 2005). Neurosphere proliferation can be regulated by both CB1 and CB2 receptor stimulation through the PI3K/AKT signaling pathway (Molina-Holgado et al., 2007). CB2 receptor activation *in vitro* promotes neural progenitor cell proliferation and neurosphere generation which can be decreased with CB2 receptor specific antagonists (Molina-Holgado et al., 2007).

Similar to neurosphere generation, CB receptor signaling regulates adult progenitor cell proliferation *in vivo*. Neural progenitor cells reside in the SVZ and the DG of the hippocampus, and in the OB. Adult neural progenitor cells express a functional endocannabinoid system (Aguado et al., 2006), including CB1 and CB2 receptors, biosynthetic and endocannabinoid degradation enzymes, and produce endocannabinoid ligands in response to increases in intracellular calcium concentrations (Jiang et al., 2005; Palazuelos et al., 2006). Experiments involving pharmacological manipulation and genetic mouse models have shown endocannabinoids stimulate hippocampal and SVZ neural progenitor cell proliferation via CB1 and CB2 receptors and modulate the self-renewal ability of neural progenitor cells (Aguado et al., 2007; Rubio-Araiz et al., 2008). Furthermore, CB1 receptors are present in dividing cells identified by BrdU labeling and the expression of endogenous cell cycle marker Ki-67 (Aguado et al., 2005; Mulder et al., 2008). CB1 receptor knockout mice show nearly a 50% reduction in dividing BrdU positive cells in the SVZ and DG (Jin et al., 2004). Progenitor proliferation was observed

in CB2 receptor deficient mice in both physiological conditions as well as after excitotoxicity (Palazuelos et al., 2006). Indirectly increasing the endocannabinoid AEA, by inhibiting its degradation enzyme FAAH, induces progenitor cell proliferation in the DG (Aguado et al., 2005; Aguado et al., 2006). 2-AG synthesis enzymes, DAGLα and β both contribute to endocannabinoid regulation of adult neurogenesis in the SVZ and DG (Gao et al., 2010b; Goncalves et al., 2008). Young adult mice show an almost 50% reduction in SVZ proliferation in mice lacking the biosynthesis enzyme DAGLα (Gao et al., 2010b). Extracellular signaling cues can mobilize endocannabinoids in progenitor cells. Fibroblast growth factor in coordination with neural cell adhesion molecule, increases 2-AG levels via DAGL coupled with PLC activation (Williams et al., 2003). Chronic treatment with the cannabinoid agonist HU-210 also leads to an increase in progenitor proliferation (Jiang et al., 2005), yet mice treated with THC or cannabidiol during 6 weeks have a lower number of proliferating cells in the DG in comparison with the controls (Wolf et al., 2010). These data suggest that CB receptor-induced proliferation can be tissue-and drug-dependent.

In young mice, CB2 receptor and DAGL antagonists inhibit cell proliferation in the SVZ, which is associated with a reduction in the appearance of new neurons in the OB (Goncalves et al., 2008). CB2 receptor agonists can stimulate cell proliferation in the SVZ of older animals and increase the appearance of new neurons in the OB (Goncalves et al., 2008), suggesting that CB2 receptor signaling may counteract the natural decline in adult neurogenesis that is associated with aging (Goncalves et al., 2008; Marchalant et al., 2009b; Marchalant et al., 2008). Similarly, CB1 receptor-deficient mice show an early onset of age-related memory deficits (Albayram et al.,

2012). Additionally, a low dose of the synthetic cannabinoid agonist WIN 55212-2 (WIN) can partially restore proliferation and neurogenesis in the hippocampus of aged rats (Marchalant et al., 2009b; Marchalant et al., 2008). Overall, these data indicate a clear role of CB receptor signaling in progenitor cell proliferation throughout the lifespan of the animal; with contributions to normal mammalian development and functional consequences during aging.

Endocannabinoid signaling regulates post-proliferative mechanisms

The endocannabinoid system plays a major role in regulating glial or neuronal lineage fate of multipotent progenitor cells. However, cannabinoid influence on cell lineage is not consistent and is dependent on location, age, pharmacology, pathophysiology, and ligand duration and bioavailability (i.e., locally generated endocannabinoid versus acute injections of endocannabinoid synthetic agonists). CB1 receptor activation promotes the differentiation of neuronal progenitors into astroglial cells *in vivo*, confirmed by decreased numbers of differentiated astroglial cells in CB1 receptor-null mice (Aguado et al., 2005; Aguado et al., 2006). Data from neuroblastoma cells indicate that AEA, but not 2-AG or WIN, could also inhibit neuronal differentiation in a CB1 receptor-independent manner (Rueda et al., 2002b). Synthetic CB receptor agonist WIN, did not influence the survival of neuronal progenitors from cortices (Aguado et al., 2006), but enhanced oligodendrocyte cell numbers via survival mechanisms involving PI3K/AKT signaling (Molina-Holgado et al., 2002; Molina-Holgado et al., 2007). Interestingly, a four-day treatment of AEA promotes glial differentiation, whereas a seven-day treatment promotes neuronal differentiation *in vivo*

(Soltys et al., 2010), suggesting that signaling duration may also influence neural progenitor cell lineage.

In contrast to gliogenesis, the impact of CB1 receptor activation on neurogenesis seems to be variable, even though the effect of the cannabinoid system to regulate neuronal progenitor cell differentiation has been extensively evaluated. Studies show AEA decreases differentiation of cortical neuron progenitor cells through CB1 receptor-dependent ERK activation (Rueda et al., 2002b). Methanandamide, a non-hydrolyzable AEA analog, also significantly decreases neurogenesis in the adult DG of rats (Rueda et al., 2002b). In contrast, endocannabinoids can increase expression of selective markers of immature and mature neurons, such as β-III-tubulin and neuron-specific nuclear protein (NeuN) that colocalize with proliferation marker BrdU in SVZ cultures (Xapelli et al., 2013). Endocannabinoid signaling also promotes cortical and hippocampal differentiation of adult progenitors through high concentrations of AEA in FAAH-deficient mice (Aguado et al., 2006). HU210, a synthetic CB1 receptor agonist, increases hippocampal neurogenesis in rats (Jiang et al., 2005). In SVZ cell cultures, both AEA and CB1 activation enhance neural progenitor cell differentiation into neurons, without affecting astrocyte or oligodendrocyte differentiation, while also upregulating the expression of pro-neural genes (Compagnucci et al., 2013; Xapelli et al., 2013). In addition, 2-AG is present in neurogenic niches and plays an active role in neural progenitor cell regulation through tuning of 2-AG levels by DAGL and MAGL activity (Reisenberg et al., 2012). Genetic ablation of DAGLα, but not the β isoform, interferes with hippocampal and SVZ-derived neurogenesis (Gao et al., 2010a). In neuroblastoma cells retinoic acid-induced neuronal differentiation also leads to an increase in

expression of DAGLα and DAGLβ (Jung et al., 2011). In both adult spinal cord primary progenitor cell cultures treated with the CB1 receptor antagonist AM251 and *in vitro* CB1 receptor-null mice slice preparation resulted in enhanced neuronal differentiation (Sideris et al., 2012). CB1 receptor antagonist, SR141716, increases the rates of neuronal differentiation of neural progenitors (Jin et al., 2004; Rueda et al., 2002b). Endocannabinoids may also have indirect actions that promote neurogenesis via crosstalk with growth factor signaling pathways essential for progenitor cell proliferation (Williams et al., 2003). Additionally, cannabinoids oppose the anti-neurogenic effect of neuronal nitric oxide (Kim et al., 2006). Collectively, these studies point to the existence of an endogenous cannabinoid tone that actively modulates neural progenitor differentiation through the CB1 receptors. These findings also indicate that characteristics of a given CB1 receptor agonist, together with the particular CB receptor signaling context, could determine the fate of progenitor cell lineage that is modulated by the CB receptors.

The cannabinoid system can also influence post-differentiation processes such as migration, maturation, and cell survival. Mouse migratory neuroblasts express CB receptors and 2-AG synthesis and degradation enzymes. Activation of CB1 receptor signaling can influence neurite outgrowth and synaptogenesis, processes required to generate functionally mature neurons. CB1 receptor activation can enhance the maturation of neural stem cells through long term inhibition of ERK1/2 pathway (Compagnucci et al., 2013). Additionally, pharmacological activation of CB1 receptors increases migration of interneurons both *in vitro* and *in vivo* through a mechanism involving the activation of TrkB receptor (BDNF/NT-3 growth factor receptor) (Williams

et al., 2003), whereas inhibition of CB1 receptors decreases neuroblast migration in rostral migratory stream explants (Oudin et al., 2011). Depletion of the AEA tone in the developing cortex interferes with radial migration of neural progenitors (Mulder et al., 2008), a process that is required for appropriate neuronal differentiation and maturation. Recent evidence indicates that AEA and WIN together with BDNF, the principal pro-differentiating neurotrophin, induce long-distance migration of interneurons to populate the embryonic cortex (Berghuis et al., 2005). In neuroblastoma cells, AEA and HU210 promote neurite outgrowth through Gi/o mediated activation of GTPase Rap1 (Jordan et al., 2005). Inhibition of DAGL activity or CB receptors substantially decreases migration along the rostral migratory stream (Oudin et al., 2011). 2-AG can stimulate neurite outgrowth of cannabinoid neurons which is depend on intrinsic DAGL activity within axonal growth cones, whereas CB1 receptor antagonists abolish neurite extension (Williams et al., 2003), indicating that autocrine endocannabinoid signaling can regulate aspects of differentiation and axon guidance (Bisogno et al., 2003). Similarly, THC increases the density of interneurons in the rat hippocampus *in vivo* (Berghuis et al., 2005); suggesting that endocannabinoid signaling is permissive for neuronal migration. Overall, it appears CB receptor activation promotes the proliferation, survival, and migration of progenitor cells but interferes with the transition from the multipotent proliferating progenitor cell towards a differentiated mature cell. Thus, further investigation is needed to clarify the discrepancy of endocannabinoid influence on postnatal progenitor cells. Specific intracellular signaling mechanisms have also not been fully elucidated. Since not all proliferating cells in regions of adult neurogenesis give rise to neurons (Kriegstein and Alvarez-Buylla, 2009), it is important to distinguish

between the effects of CB receptor signaling on progenitor proliferation from neurogenesis.

Cannabinoids take part in the control of neural cell fate, thereby modulating the balance between cell death and survival (Guzman, 2003; Mechoulam et al., 2002). A fine balance between progenitor cell proliferation and programmed death is necessary to provide appropriate cell populations and insure tissue homeostasis. Both CB1 and CB2 receptors regulate neural progenitor commitment, survival, and cell-cycle maintenance (Aguado et al., 2006; Guzman, 2003). During development, an inverse relation between expression of CB1 receptors and the stage of cell differentiation is seen in neuronal and glial cells (Palazuelos et al., 2006), indicating that CB1 receptors might function to prevent a differentiated state in favor of a non-differentiated, proliferative state. Similarly, CB2 receptors increase with de-differentiation of glia (Sanchez et al., 2001) and some tumors (Caffarel et al., 2006). Conversely, studies in glioma or astrocytoma cells (Sanchez et al., 2001) and in various non-neuronal cancers (Caffarel et al., 2006) show that activation of CB2 receptors induces apoptosis and inhibits tumor growth in host mice. Substantial *in vivo* and *in vitro* results show that cannabinoids can also protect neurons from death (Mechoulam et al., 2002; Romero et al., 2002). This neuroprotection might be relevant for the treatment of both acute brain injury (e.g. cerebral ischemia and trauma) and chronic neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease or multiple sclerosis. Studies have implicated CB receptors in the neuroprotective activity thorough glia-dependent anti-inflammatory actions (Fernandez-Ruiz et al., 2007). Conversely, administration of cannabinoids can inhibit the survival of various tumor cells in culture

and curb the growth of different models of tumor xenografts in rats and mice (Guzman, 2003). This antitumor effect has been mainly studied in tumors of the CNS, specifically gliomas (Velasco et al., 2004). The varying actions of CB receptors can provide cell protection (Romero et al., 2002) or elicit apoptosis (Guzman, 2003). However, these data demonstrate that CB receptors clearly influence cell homeostasis involved in cell survival and death.

Measuring cell homeostasis

Common markers for neural stem cells and neural progenitor cells such as nestin, BrdU, and DCX can also detect reactive astrocytes or cancer cells (Kaneko et al., 2000; Komitova and Eriksson, 2004; Lendahl et al., 1990). BrdU labeling in conjunction with cell specific markers is a common method of studying adult neurogenesis. For this dissertation, I used BrdU instead of other proliferative markers such as Ki-67 or PCNA because they are not specific to the phase of the cell cycle, exhibit nonspecific immunoreactivity outside of proliferation, are subject to variability depending on fixation, and cannot be used for cell lineage analysis (Ohta and Ichimura, 2000; Taupin, 2007). BrdU incorporation occurs primarily in cells undergoing DNA synthesis. For example, in the OE following OB ablation in young adult rodents, apoptotic cells were positive for TUNEL, a cell death marker, but rarely incorporated BrdU (Bauer and Patterson, 2005). This suggests that dying neurons do not reenter the cell cycle following bulbectomy lesion and that BrdU incorporation is an appropriate experimental approach to monitor proliferation in the olfactory system (Brann and Firestein, 2010).

The cannabinoid contributions to neurogenesis in the mouse OE were studied in this chapter. Proliferation, differentiation, and cell death were measured after manipulation of the cannabinoid system in the following ways: (1) addition of exogenous cannabinoid WIN, (2) inhibition of cannabinoid degradation enzyme to indirectly increase endogenous cannabinoids, and (3) inhibition of CB1 receptors. All pharmacological agents were also employed on CB1/CB2 receptor KO mice as a further control. Adult neurogenesis was measured by: (1) basal cell proliferation via BrdU incorporation, (2) differentiation via BrdU colocalization with OSNs, and (3) survival using a marker of apoptotic endpoint.

Methods

Animals

Adult male (6-8 weeks old) Swiss Webster (CFW), neonatal (P0-4), and C57BL/6 control wildtype mice were obtained from Charles River, Portage, MI. CB1/CB2 receptor KO mice were kindly provided by Dr. Norbert Kaminski (Michigan State University, MI) who obtained them from Dr. Andreas Zimmer (University of Bonn, Germany). The CB1/CB2 receptor KO mice were developed through replacement of the CB1/CB2 receptor coding region with non-CB receptor DNA using homologous recombination. Subsequently, heterozygote mice were mated to obtain CB1/CB2 receptor KO mice (Jarai et al., 1999). Mice were given food and water *ad libitum*. Animal rooms were kept at 21–24°C and 40–60% relative humidity with a 12-h light/dark cycle. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care

and Use of Laboratory Animals as approved by Michigan State University Institutional Animal Care and Use Committee.

In vivo apoptosis, proliferation, and lineage studies

Intranasal instillation was performed via a pipette tip placed immediately above the nares while the mouse was in a supine position. Test compounds were intranasally delivered drop wise into the nares, and were subsequently aspirated. Neonatal (P4) Swiss Webster (CFW; n=3-4 mice/group) mice aspirated a small amount (10 µl) of vehicle (1:1 ethanol: saline or 1% DMSO in saline), WIN 55212,2 (WIN, 10µM), or 2-AG ether (1µM, 5µM, 10µM in 1:1 ethanol:saline) (Figure 4.1A). Anesthetized (4% isoflurane) adult (6-9 weeks) male CFW, C57BL/6 or CB1/CB1 receptor KO mice (n = 3–4 mice/group) aspirated a bolus of WIN (10µM), AM251 (10µM), a cocktail of JZL184 (10µM) with URB597 (100µM) or an equivalent volume (50 µl) of vehicle (1% DMSO in saline) placed on the nares. Some mice intranasally aspirated CB1 receptor antagonist AM251 (10µM) or vehicle (1% DMSO in saline) 30 minutes prior, to examine CB1-receptor specific effect of cannabinoid manipulation (Figure 4.1B).

In order to detect proliferation through BrdU-incorporation, animals received two BrdU injections (i.p., 18 mg/kg) at either 2 and 4 hours (neonates) or 6 and 3 hours (adults) prior to tissue collection. In the neurogenesis lineage study, BrdU injections were given 42 hours and 45 hours after intranasal instillation. OE tissue was collected at 8 hours, 24 hours, 48 hours, 72 hours, or 16 days post-instillation (Figure 4.1C,D), and processed as described previously (Jia et al., 2009; Jia and Hegg, 2010).

Neonates were quickly decapitated and heads were post-fixed for 2 hours in 4% paraformaldehyde. Adult mice were anesthetized (65mg/kg ketamine + 5mg/kg

xylazine, ip), transcardially perfused with ice-cold 0.1 M PBS followed by 4% paraformaldehyde (PFA) and decapitated. The lower jaw and skin was removed and tissue was postfixed overnight in 4% and decalcified with EDTA (0.5 M, pH = 8) for 4–5 days. After decalcification both neonatal and adult tissue was cryoprotected with 20% sucrose and embedded in Tissue Tek OCT (Sakura Finetek, Torrance, CA). Frozen coronal sections of OE (20 μm) from level 3 of the mouse nasal cavity taken at the level of the second palatal ridge (Young, 1981) were collected using a cryostat and mounted onto Superfrost Plus slides (Electron Microscopy Sciences, Hatfield, PA, USA). Tissue was always compared from equivalent levels between treatment groups (n = 3–4 animals per group).

Immunohistochemistry

BrdU immunoreactivity was assessed as described previously (Jia and Hegg, 2010). Briefly, cryostat sections (20 μm) of the OE were rehydrated with 0.1 M PBS for 20 minutes and permeabilized with 0.3% Triton X-100 for 20 minutes. Non-specific binding was blocked with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in 0.1 M PBS for 1 hr. Tissue sections were incubated in 2 M HCl for 30 minutes at 65°C to denature DNA, and then incubated with rat anti-BrdU IgG (1:100; Abcam Inc., Cambridge, MA) in 10% normal donkey serum overnight at 4° C. Coronal sections of OE (20 μm) were collected to detect the levels of apoptosis by Terminal dUTP Nick End Labeling (TUNEL). TUNEL was performed with an *In Situ* Cell Death Detection Kit (TMR Red; Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions. The number of BrdU positive or TUNEL positive cells in

ectoturbinate 2 and endoturbinate II from three tissue sections of each mouse (n = 3-4 mice per group) were manually counted by an experimenter blinded to the treatments. The linear length of OE was measured using Metamorph® software (Molecular Devices, Sunnyvale, CA). Data are expressed as a ratio of BrdU or TUNEL-positive cells to the linear length of OE scored. For cell linage studies, goat anti-olfactory marker protein (OMP, 1:1000, Waco Chemicals, Richmond, VA) antibody was included in the BrdU incubation. Immunoreactivity was detected using fluorescein isothiocyanate (FITC)-conjugated donkey anti-rat IgG ± Cy3-conjugated donkey anti-goat immunoglobin (Jackson ImmunoResearch; 1:200 in 0.3% triton X 100 + 10% donkey serum in PBS for 2 hours at 37° C). Sections were mounted with Vectashield mounting media for fluorescence (Vector Laboratories, Burlingame, CA). The nuclei were counterstained with Vectashield mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratory, Burlingame, CA). Antibody specificity was examined by omitting the primary antibody; no immunoreactivity was ever observed. Immunoreactivity was visualized using a Nikon Eclipse 2000-U microscope equipped with an Excite 120 Fluorescence Illumination system or an Olympus FV1000 confocal laser scanning microscope (Pleasant Valley, PA). Fluorescein isothiocyanate (FITC) and tetramethylrhodamine (TRITC) dyes were excited at 488 and 543 nm and low pass filtered at 505–525 and 560–620 nm, respectively. In some instances, the brightness and contrast of the fluorescent images were altered *post-hoc*. In all cases, the same changes were applied to all images collected on that given day, and it was verified that immunoreactivity was not observed in the antibody specificity controls under the new settings. No immunoreactivity was observed in any of the controls.

Data analysis

An experimenter blinded to the treatments manually counted the number of BrdU positive cells from 3 tissue sections from each mouse (3-4 mice/group). It was previously determined that the intranasal instillation was effectively reaching the OE via detection of trypan blue (0.4%) staining following instillation into the nares (Jia et al., 2009). The septal OE (septum) and ectoturbinate 2 and endoturbinate II (turbinates) were regions that were heavily stained with trypan blue (data not shown), indicating that compounds instilled into the nares permeate into the OE at these regions, although it is not known how long compounds remain in the nasal cavity before they are cleared. Thus, BrdU positive cells were tabulated in the turbinates. Data are expressed as a ratio of BrdU positive cells to linear length of OE, and is presented as entire OE unless specified otherwise. Neonatal data were normalized to the length of OE on which the immunoreactive cells were scored and expressed as number per linear millimeter OE. The length of the ectoturbinate 2 and endoturbinate II were measured using Metamorph® software (Molecular Devices, Sunnyvale, CA). In some experiments, BrdU positive cells were counted in 3 different layers of the OE, (1) the apical sustentacular cell layer, defined as the region where the apical-most cell nuclei reside and above OMP immunoreactivity, (2) the middle neuronal layer, defined as the region where OMP immunoreactivity can be observed, and (3) the basal cell layer, defined as the region below OMP immunoreactivity (Figure 3.1B). The entire breadth of all three layers is designated “Total OE”. This was performed to distinguish between basal cell, neuronal, and sustentacular cell proliferation, and to examine differentiation qualitatively. Student’s t-test, and one-way ANOVA followed by the Bonferroni multiple comparison

test were performed using Prism 5 (Graphpad Software, San Diego, CA). Two-way ANOVA or repeated-measures two ANOVA were performed followed by the Newman-Keul post hoc test using GB-Stat v9.0 (Dynamic Microsystems, Inc., Silver Spring, MD).

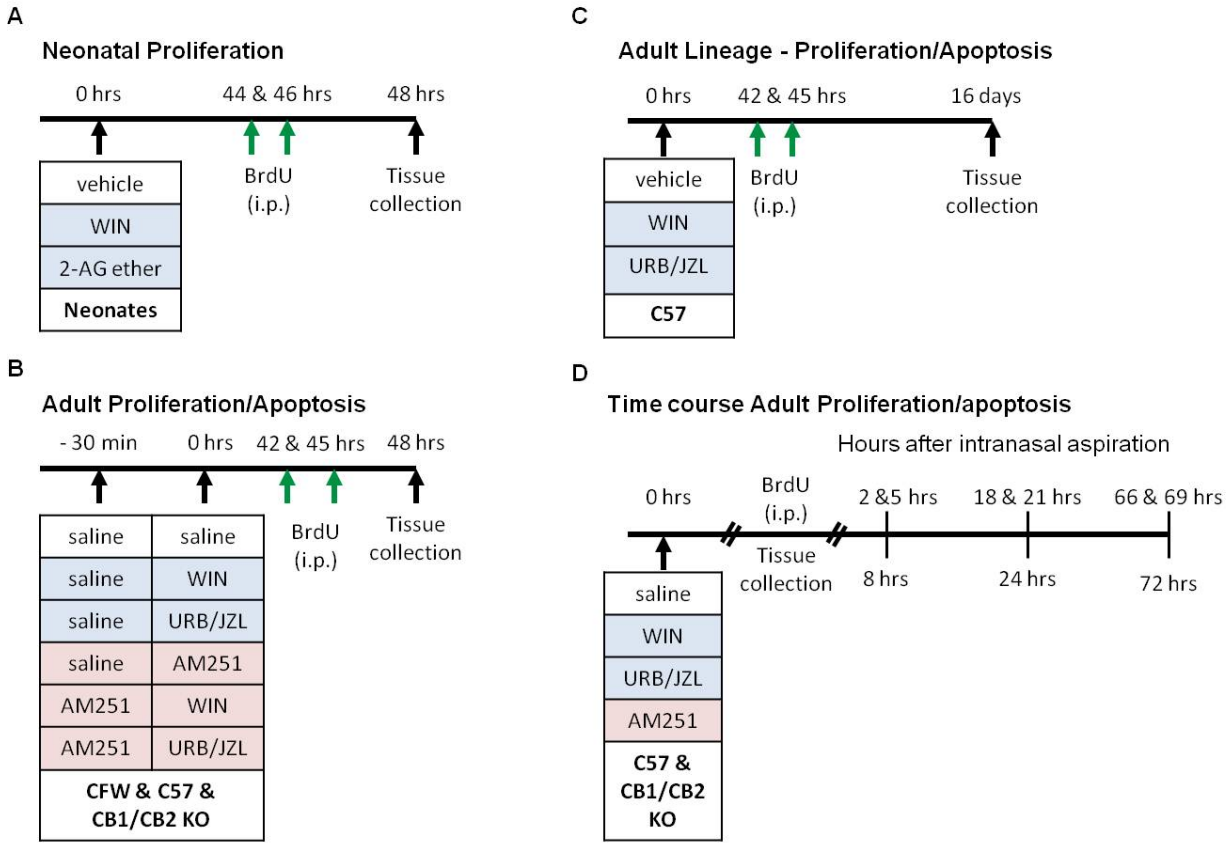

Figure 4.1 Experimental protocol for *in vivo* studies. (A) Swiss Webster (CFW) neonatal mice were instilled with vehicle (1% DMSO or 1:1 ethanol in saline), WIN (10μM), or 2-AG ether (1μM, 5μM, 10μM). BrdU was injected (i.p., 18 mg/kg) at 2 and 4 hours prior to tissue collection 48 hours after treatment. **(B)** Adult male 6-8 week old C57 wildtype, CFW, and CB1/CB2 receptor KO mice intranasally aspirated vehicle (1% DMSO in saline), CB1 receptor antagonist AM251 (10μM), CB1/CB2 receptor agonist WIN (10μM), or a cocktail of JZL184/URB597 (10μM, 100μM, respectively), or AM251 30 minutes prior to WIN. BrdU was injected (i.p., 18 mg/kg) 3 and 6 hours before tissue collection 48 hours post-instillation. **(C)** Adult C57 mice intranasally aspirated vehicle (1% DMSO in saline), CB1/CB2 receptor agonist WIN (10μM), or a cocktail of

JZL184/URB597 (10μM, 100μM, respectively). BrdU was injected (i.p., 18 mg/kg) 42 and 45 hours after administration and tissue was collected 16 days later. **(D)** Adult male 6-8 week old C57 and CB1/CB2 receptor KO mice (8hr time point only) intranasally aspirated vehicle (1%DMSO in saline), CB1 receptor antagonist AM251 (10μM), CB1/CB2 receptor agonist WIN (10μM), or a cocktail of JZL184/URB597 (10μM, 100μM, respectively), and BrdU was injected (i.p.,18 mg/kg) 3 and 6 hours before tissue collection 8, 24, 48, and 72 hours post-instillation.

Results

Exogenous and endogenous cannabinoids increase proliferation in neonatal OE

To determine if cannabinoids act as a proliferative factor throughout development, BrdU incorporation was first assessed in neonatal mouse OE after intranasal instillation of both exogenous and endogenous cannabinoids. Endogenous cannabinoid analog, 2-AG ether was used because of its increased chemically stability over 2-AG, with an endogenous half-life of hours rather than minutes (Laine et al., 2002). However, 2-AG ether can be at least 10-fold less potent than 2-AG in eliciting typical CB1-mediated responses (Sugiura et al., 1999), therefore a range of concentrations were used. Initial proliferation experiments were done in neonates because they exhibit a high level of proliferation (Jia et al., 2009; Weiler and Farbman, 1997a) and endocannabinoid signaling strongly influences proliferation during development and early postnatal periods (Berrendero et al., 1999; Galve-Roperh et al., 2013). Neonatal mice (P4) intranasally aspirated the synthetic CB1/CB2 receptor agonist WIN 55212-2 (WIN; 10μM) or 2-AG ether (1μM, 5μM, 10μM) and levels of BrdU incorporation were measured 48 hours after cannabinoid manipulation. Intranasal instillation of WIN significantly increased BrdU positive cells in the OE by 35% above vehicle (Figure 4.2, $p<0.05$, n = 3-4 animals/group). Intranasal treatment with increasing concentrations of 2-AG ether all showed similar significant increased levels (68%, 81%, 86%, respectively) of BrdU positive cells compared to vehicle (Figure 4.2, $p<0.01$, n=3-4 animals/group). These data indicate that both exogenous and endogenous cannabinoids can promote proliferation in the neonatal mouse OE.

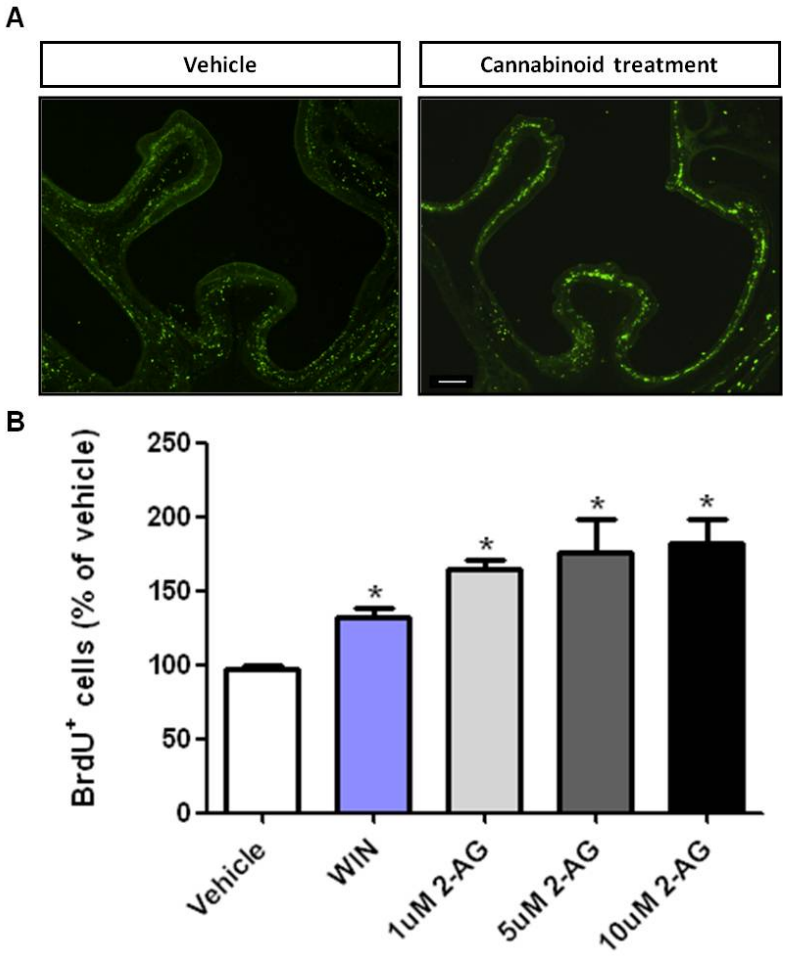

Figure 4.2 Exogenous and endogenous cannabinoids increase proliferation in neonatal OE. (A) Representative images from vehicle control groups (left) or cannabinoid treatment groups (right). **(B)** CFW neonatal mice were instilled with vehicle (1%DMSO or 50% ethanol; white), WIN (5 μM; blue), or 2-AG ether (light grey, 1μM; dark grey, 5μM; or black, 10μM). BrdU incorporation was measured 48 hours after intranasal instillation. Synthetic WIN showed an increase in neonatal BrdU incorporation, and 2-AG ether increased BrdU positive cells dose-dependently. Data were normalized to respective vehicle groups (n=3-4 mice/group). (*, $p<0.05$ vs. vehicle; one-way ANOVA followed by the Bonferroni multiple comparison test).

CB1 receptor signaling promote proliferation in the adult CFW mice

Given the increase in neonatal proliferation after cannabinoid administration and the vast body of data showing cannabinoid effects on central adult proliferation (Galve-Roperh et al., 2013), CB1 receptor-mediated proliferation the adult OE was examined next. First, adult proliferation levels were examined in Swiss Webster (CFW) strain of mice. CFW mice were used in Chapter 1 for the calcium imaging studies. Adult male mice (6-8 weeks) intranasally aspirated vehicle (1% DMSO in saline), CB1 receptor specific antagonist AM251 (10μM), or WIN (10μM) and BrdU-incorporation was quantified in the OE 48 hours post-administration. WIN significantly increased the number of BrdU positive cells compared to vehicle control in the OE of CFW mice (Figure 4.3, 20.1 ± 1.1 vs. 26.9 ± 2.2 cell/mm OE, *$p<0.01$). Pre-treatment with CB1 receptor-specific antagonist AM251 did not alter the number of BrdU positive cells in the OE of saline vehicle-instilled control animals but significantly blocked the WIN-induced increase in BrdU incorporation (Figure 4.3, 26.9 ± 2.2 vs. 15.7 ± 0.80 cells/mm OE, # $p<0.001$), indicating that CB1 receptors are involved in WIN-induced increase of cell proliferation in the OE. Additionally, AM251 alone did not alter BrdU levels, as they were not different from vehicle control (Figure 4.3, 20.1 ± 1.1 vs. 15.3 ± 0.78 cells/mm OE).

Endogenous cannabinoids promotes proliferation in adult C57 mice

Next, a transgenic animal model in which the functional portions of both CB1 and CB2 receptor are omitted was used in addition to pharmacological techniques. CB1/CB2 receptor KO mice were back bred on a C57BL/6 (C57) strain of mice; therefore C57 mice are used in the following experiments as control wildtype mice.

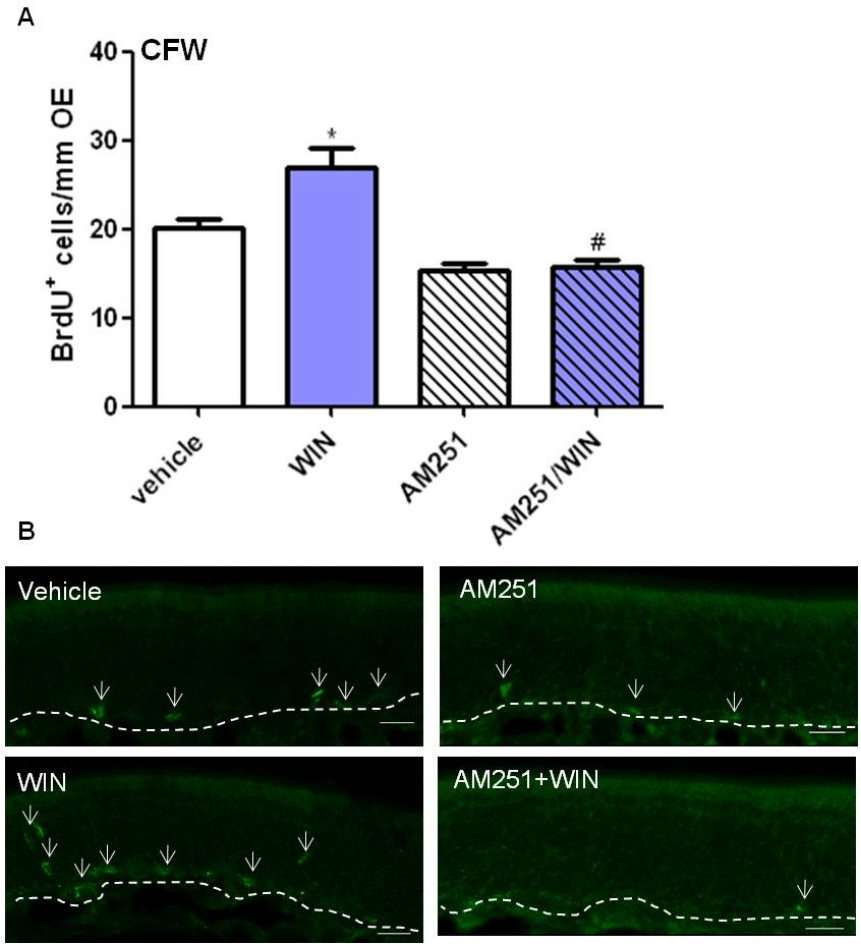

Figure 4.3 WIN promotes proliferation in the adult mouse OE via CB1-receptors. **(A)** CB1 receptors mediate WIN-induced increase in cell proliferation. CFW mice intranasally aspirated vehicle (1% DMSO in saline) or the CB1 receptor antagonist AM251 (10μM) 30 minutes prior to vehicle, CB receptor synthetic agonist WIN (10μM), or AM251. BrdU (i.p., 18 mg/kg) was injected 3 and 6 hours before tissue collection 48 hours post-instillation. * $p<0.05$ vs. vehicle; # $p<0.05$ vs. WIN. (One way ANOVA followed by Bonferroni multiple comparison test; n=6 animals). **(B)** Representative images from each treatment group. Arrow heads indicate BrdU positive cells (green). Dashed white lines delineate basement membrane. Scale bar, 10μm.

Male 6-8 week old wildtype and CB1/CB2 receptor KO mice intranasally aspirated vehicle (1% DMSO in saline), WIN (10μM), AM251 (10μM), AM251 30 minutes prior to WIN, or a cocktail of JZL184/URB597 (10μM and 100μM, respectively), which inhibits degradative enzymes of endocannabinoids 2-AG and AEA. BrdU-incorporation was quantified in the OE 48 hours post-administration and basal cell proliferation was quantified by measuring BrdU-incorporation in the ectoturbinate 2 and endoturbinate II OE (Figure 4.1B). Similar to CFW mice, WIN-treated C57 mice showed a significant 57% increase in BrdU incorporation over vehicle treated control mice (Figure 4.4A; 49.6 ± 3.5 vs. 31.6 ± 1.1 cell/mm OE; $p<0.01$). The WIN-induced increase in proliferation was also blocked by CB1 receptor specific antagonist AM251 (Figure 4.4A; 49.6 ± 3.5 vs. 34.4 ± 3.7 $p<0.05$). Additionally, 2-AG and AEA degradation enzyme (MAGL/FAAH) inhibitors JZL/URB greatly increased the number of BrdU positive cells over vehicle control by 109% (Figure 4.1A; 31.6 ± 1.1 vs. 65.9 ± 3.6 cell/mm OE; $p<0.001$), suggesting that endogenous cannabinoids increase progenitor cell proliferation in the mouse OE. Furthermore, JZL/URB treatment further increased BrdU incorporation compared to the WIN-induced BrdU proliferative effect (Figure 4.1A; 49.6 ± 3.5 vs. 65.9 ± 3.6 cell/mm OE; $p<0.05$), indicating that endocannabinoids are equally if not more effective at increasing basal cell proliferation than exogenous synthetic cannabinoids at the current concentrations. A slight but insignificant decrease was noticed in AM251 treated animals compared to vehicle control (Figure 4.4A; AM251, 24.8 ± 1.8 vs. vehicle, 31.6 ± 1.1 cell/mm OE). This suggests that tonic release of endocannabinoids through CB1 receptor signaling is not primarily responsible for baseline levels of proliferation in the mouse OE.

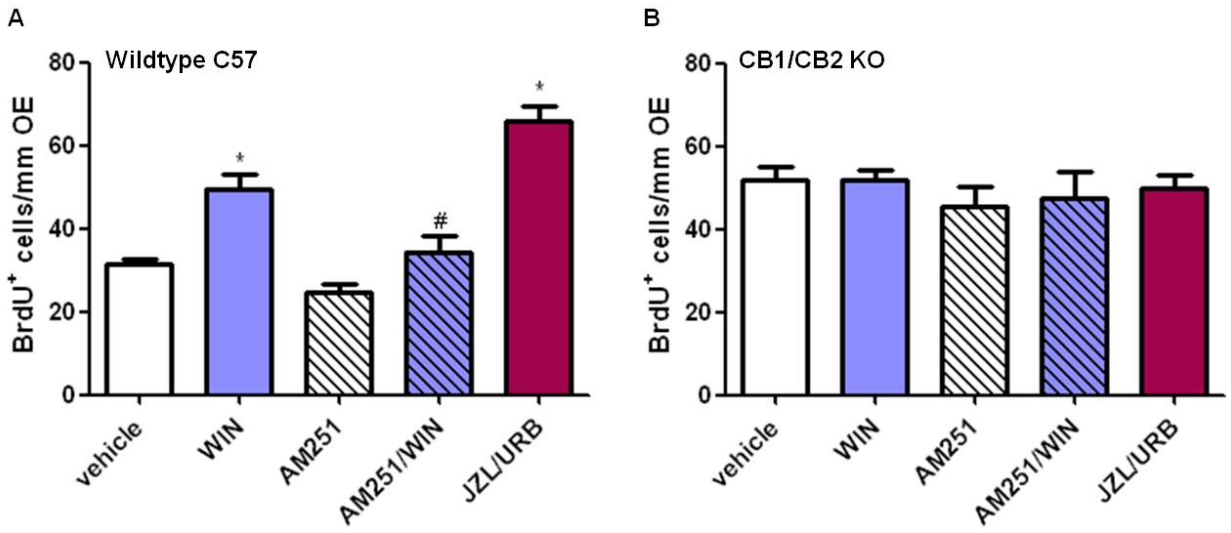

Figure 4.4 Endogenous cannabinoids promote proliferation in wildtype but not CB1/CB2 receptor KO mice. Adult male 6-8 week old C57 (wildtype) and CB1/CB2 receptor KO mice intranasally aspirated vehicle (1%DMSO in saline), CB1 receptor antagonist AM251 (10μM), CB1/CB2 receptor agonist WIN (10μM), or a cocktail of JZL184/URB597 (10μM, 100μM, respectively), or AM251 30 minutes prior to WIN. BrdU was injected (i.p., 18 mg/kg) 3 and 6 hours before tissue collection 48 hours post-instillation. **(A)** Quantification of BrdU positive cells in ectoturbinate 2 and endoturbinate II (One way ANOVA followed by Bonferroni multiple comparison test; n=3-4 mice.). * $p<0.05$ vs. vehicle; # $p<0.05$ vs. WIN. **(B)** Quantification of BrdU positive cells in CB1/CB2 receptor KO mice show no effect of cannabinoid treatment.

cannabinoids at the current concentrations. A slight but insignificant decrease was noticed in AM251 treated animals compared to vehicle control (Figure 4.4A; AM251, 24.8 ± 1.8 vs. vehicle, 31.6 ± 1.1 cell/mm OE). This suggests that tonic release of endocannabinoids through CB1 receptor signaling is not primarily responsible for baseline levels of proliferation in the mouse OE. As expected, CB1/CB2 receptor KO mice showed no change in BrdU incorporation in response to any cannabinoid pharmacological manipulation (Figure 4.4B).

Cannabinoids increase proliferation up to 3 days after administration

Previous work in our lab has targeted 48 hours after intranasal instillation as an ideal time point to detect proliferation after ATP-induced NPY release (Jia et al., 2009; Jia and Hegg, 2010). Similarly, 48 hours after cannabinoid manipulation yielded positive changes in proliferation (Figure 4.4A). However, to better characterize the time course of cannabinoid-induced proliferation in the mouse OE, BrdU positive cells were counted 8, 24, and 72 hours after intranasal aspiration (Figure 4.1D). Male 6-8 week old wildtype and CB1/CB2 receptor KO mice intranasally aspirated vehicle (1% DMSO in saline), WIN (10μM), AM251 (10μM), or a cocktail of JZL184/URB597 (10μM and 100μM, respectively), and BrdU-incorporation was quantified in the OE 8 hours post-administration. No changes in BrdU positive cells were detected in wildtype mice (Figure 4.5A) or CB1/CB2 receptor KO mice (Figure 4.5B) across treatments at 8 hours. However, variability is seen among vehicle-treated animals. An increase in the vehicle-treated group in wildtype mice was detected at 8 hours compared to 24 and 72 hours post-instillation (data not graphically represented; 46.9 ± 2.4 vs. 38.9 ± 2.2, and 39.8 ± 2.7 respectively; $p<0.05$). 24 hours after intranasal aspiration, a 53% increase in BrdU+

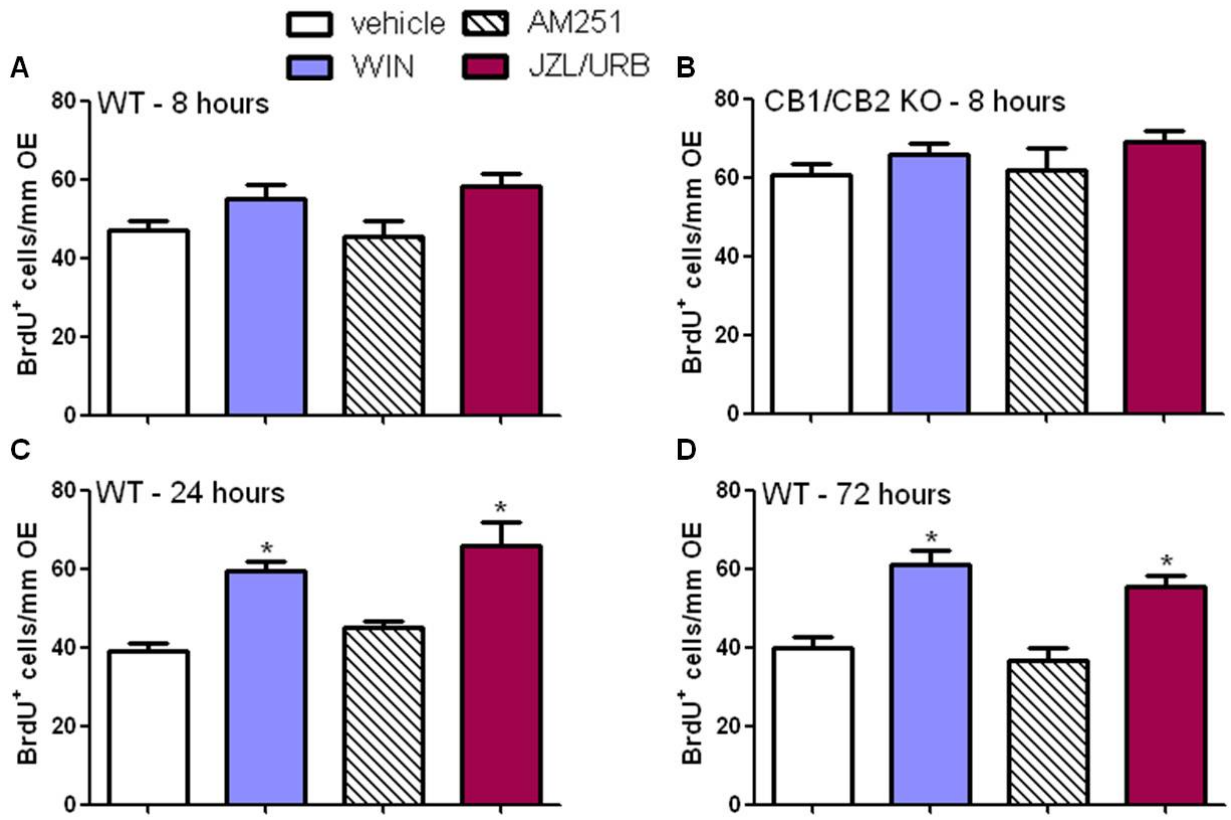

Figure 4.5 Cannabinoid-induced proliferation is detected up to 72 hours after administration. Adult male 6-8 week old C57 wildtype (WT) and CB1/CB2 receptor KO mice intranasally aspirated vehicle (1%DMSO in saline), CB1 receptor antagonist AM251 (10μm), CB1/CB2 receptor agonist WIN (10μm), or a cocktail of JZL184/URB597 (10μm, 100μm, respectively), and BrdU (i.p.,18 mg/kg) was injected 3 and 6 hours before tissue collection 8, 24, and 72 hours post-instillation. Quantification of BrdU positive cells in **(A)** C57 wildtype mice **(B)** and CB1/CB2 receptor KO mice 8 hours after instillation. Quantification of BrdU positive cells in wildtype mice **(C)** 24 hours post-instillation and **(D)** hours post-instillation show increases in WIN and JZL/URB groups (* $p<0.05$ from vehicle; one way ANOVA followed by Bonferroni multiple comparison test; n=2-6 mice).

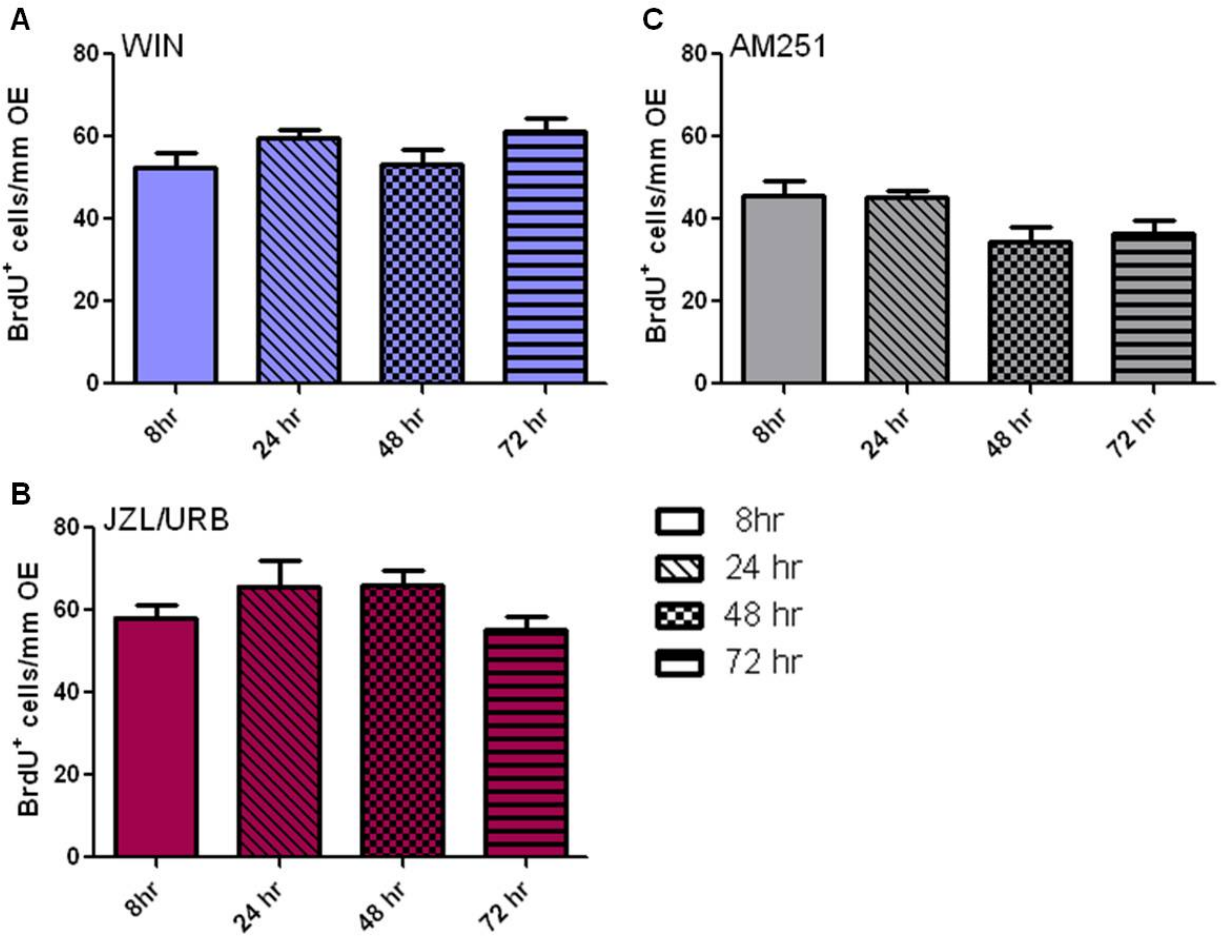

Figure 4.6 Cannabinoid treatment sustains progenitor cell proliferation up to 72 hours after administration. Adult male 6-8 week old C57 wildtype mice intranasally aspirated vehicle (1%DMSO in saline), CB1 receptor antagonist AM251 (10μm), CB1/CB2 receptor agonist WIN (10μm), or a cocktail of JZL184/URB597 (10μm, 100μm, respectively), and BrdU was injected (i.p., 18 mg/kg) 3 and 6 hours before tissue collection 8, 24, 48, and 72 hours post-instillation. Quantification of BrdU positive cells in C57 wildtype mice 8, 24, 48, and 72 hours after **(A)** WIN **(B)** AM251 and **(C)** JZL/URB administration. (One way ANOVA followed by Bonferroni multiple comparison test; n=3-4 mice).

cells was detected in WIN-treated mice compared to vehicle (Figure 4.5C; 59.5 ± 2.3 vs. 38.9 ± 2.2; $p<0.001$), and a 70% increase in JZL/URB treated mice (Figure 4.5C; 65.9 ± 6.1 vs. 38.9 ± 2.2; $p<0.001$). This robust increase in proliferation was still detectable 72 hours after intranasal administration with a 54% increase after cannabinoid WIN treatment compared to control (Figure 4.5D; 39.79 ± 2.7 vs. 61.2 ± 3.3, $p<0.001$) and a 39% increase after inhibiting endocannabinoid degradative enzymes compared to control (Figure 4.5D; 38.9 ± 2.2 vs. 55.3 ± 3.2; $p<0.001$). No change in BrdU-incorporation was seen at 24 or 72 hours after AM251 treatment vs. vehicle control levels (Figure 4.5C-D; vehicle, 24 hours 38.9 ± 2.2 vs.45.2 ± 1.6, and 72 hours 39.79 ± 2.7 vs. 36.5 ± 3.3). Overall, these data indicate intranasal instillation of exogenous cannabinoids or increased endocannabinoids promotes progenitor cell proliferation 24 hours after administration, and is sustained for up to 72 hours.

To determine if the same cannabinoid treatment changes BrdU levels across time, the data displayed in Figure 4.5 were reorganized to reflect changes within each treatment from 8-72 hours after administration. No changes in WIN-induced (Figure 4.6A), AM251-induced (Figure 4.6B), or JZL/URB-induced (Figure 4.6C) proliferation were seen across time (8, 24, 48, or 72 hr post intranasal aspiration). Although no significant increases in proliferation were detected in cannabinoid-treated wildtype mice 8 hours post-instillation (Figure 4.5A), proliferation in the WIN and JZL/URB-treated groups were not significantly lower at 8 hours compared to 24, 48, or 72 hours (Figure 4.6A-C). This observation reflects the increased variability in vehicle control-treated mice at 8 hours post-instillation compared to vehicle control at all other time points measured (Figure 4.5). These data indicate that WIN or JZL/URB treatment is able to

increase basal cell proliferation 24 hours after treatment and sustain the same levels of proliferation for up to 72 hours.

Single administration of cannabinoids does not regulate differentiation

The cannabinoid system contributes to the regulation of central neural precursors committed to a neuronal or glial lineage (Aguado et al., 2006). Therefore, to determine if cannabinoids induce the proliferation of neuronal and sustentacular progenitor cells, a new BrdU incorporation assay was performed extending the end point to 16 days following WIN or JZL/URB application in C57BL/6 adult mice (Figure 4.1C). This extended period allows for the basal cells to proliferate and differentiate into mature OSNs, a process that takes a minimum of 10 days (Schwob et al., 1995). To analyze the fate of cannabinoid-induced cell proliferation, BrdU positive cells in the apical sustentacular cell layer, the middle OSN layer, and the basal progenitor cell layer were counted. Two days after intranasal administration, cannabinoid-induced BrdU positive cells were increased in the basal layer (Figure 4.7B; 73% and 99% of the total vehicle control; $p<0.01$), confirming the previous results (Figure 4.4A). No increase in BrdU positive cell in the total OE was seen after intranasal instillation of either WIN (37.2 ± 1.0 vs. 34.6 ± 3.2 cell/mm OE) or JZL/URB (33.7 ± 2.8 vs. 34.6 ± 3.2 cell/mm OE) compared to vehicle control 16 day post treatment (Figure 4.7A). The number of WIN (2 day, 53.5 ± 1.7 16 day 37.2 ± 1.0 cell/mm OE; $p<0.05$) and JZL/URB-(2 day, 68.1 ± 5.0; 16day 33.7 ± 2.8 cell/mm OE; $p<0.001$) induced BrdU positive cells was significantly lower 16 days after treatment compared to 2 days, suggesting that newly generated cells at 2 days did not survive to 16 days (Figure 4.7A).

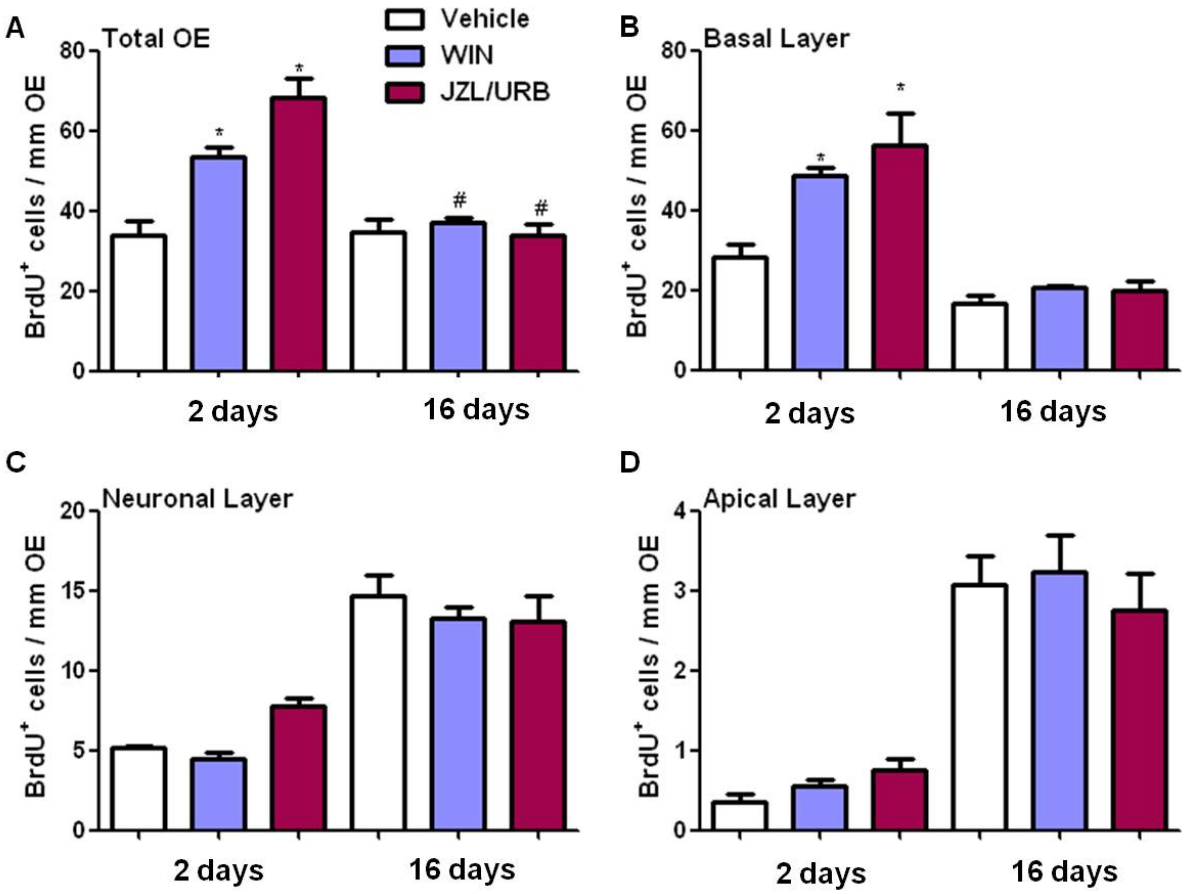

Figure 4.7 Cannabinoid administration does not induce neurogenesis. Adult C57 wildtype mice intranasally aspirated vehicle (1% DMSO in saline), CB1/CB2 receptor agonist WIN (10μm), or a cocktail of JZL184/URB597 (10μm, 100μm, respectively). BrdU was injected (i.p.,18 mg/kg) 42 and 45 hours after administration and tissue was collected 2 or 16 days later. Quantitative analysis of BrdU incorporation in the **(A)** entire OE, **(B)** basal layer, **(C)** neuronal layer, or **(D)** apical layer 2 and 16 days following cannabinoid instillation. Data are expressed as mean (±SEM) number of BrdU positive cells per linear length of the entire OE. (* $p<0.01$ vs. respective 2 day vehicle, # $p<0.05$ vs. same treatment as 2day; one way ANOVA followed by Bonferroni multiple comparison test; n=4 mice).

No change in BrdU-incorporation was seen in vehicle treated mice between 2 and 16 days (Figure 4.7A; 34.0 ± 3.5 vs. 34.6 ± 3.2 cell/mm OE). Additionally, no significant change in the neuronal layer was seen 16 days post-instillation (Figure 4.7C), indicating additional neurogenesis does not occur after WIN or JZL/URB treatment compared to vehicle. Similarly, no change was detected in the apical layer 16 days after cannabinoid manipulation (Figure 4.7D), suggesting gliogenesis is not affected by WIN or JZL/URB administration. Collectively, these data suggest that cannabinoid-induced proliferation does not lead to an increase in differentiated cells above control levels in the mature OE. Although, the newly proliferated cells do not incorporate into the OE as mature neurons and glia, tightly regulated homeostatic mechanisms in the OE imply that that cell death is occurring before maturation to maintain homeostasis.

Inhibiting endogenous cannabinoid signaling does not increase cell death levels

Cannabinoid signaling is neuroprotective in the CNS (Kim et al., 2006), promoting the survival of neural progenitor cells during development and in adulthood (Galve-Roperh et al., 2013). Therefore, we assessed whether pharmacological or genetic inhibition of CB receptors has a protective function under physiological conditions by examining apoptosis by counting the number of TUNEL-positive cells in ectoturbinate 2 and endoturbinate II in the mouse OE. Inhibition of CB1 receptors with AM251 did not significantly increase TUNEL positive cells over vehicle in C57 control or CFW mice (Figure 4.8A, C). However, there was a significant main effect of genotype after two-way ANOVA comparison of wildtype C57 and CB1/CB2 receptor KO mice ($F_{(1,80)} = 38.18$, $p < 0.0001$), but no significant interaction between treatment and

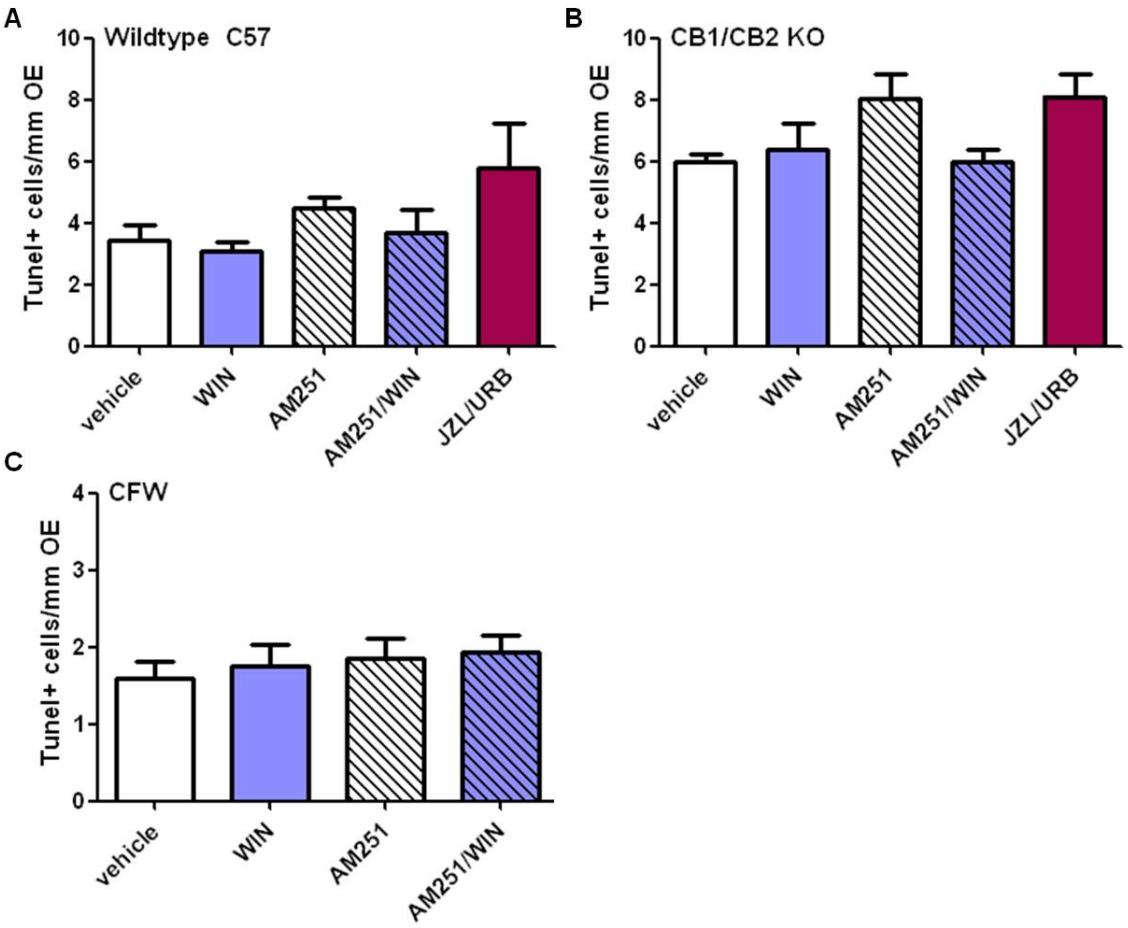

Figure 4.8 Inhibiting endocannabinoid signaling does not influence incidence of cell death. Adult male 6-8 week old C57 wildtype and CB1/CB2 receptor KO mice intranasally aspirated vehicle (1% DMSO in saline), CB1 receptor antagonist AM251 (10μm), CB1/CB2 receptor agonist WIN (10μm), or a cocktail of JZL184/URB597 (10μm, 100μm, respectively), or AM251 30 min prior to WIN. The levels of apoptosis were measured by TUNEL, 8 hours, 72 hours, or 16 days after cannabinoid administration. Quantification of TUNEL positive cells from ectoturbinate 2 and endoturbinate II in **(A)** C57 wildtype mice, **(B)** CB1/CB2 receptor KO mice, and **(C)** CFW mice. Two-way ANOVA followed by Tukey/Kramer Procedure post-hoc test or one way ANOVA followed by Bonferroni multiple comparison test; n=3-4 mice).

genotype (Figure 4.8; $F_{(4,76)} = .31$, $p < 0.87$). Collectively, these data show that pharmacological inhibition of CB1 receptors or within individual treatment groups of genetic CB1/CB2 receptor KO mice do not protect against cell death under physiological conditions.

Incidence of cell death increases 72 hours after cannabinoid administration

Given the cannabinoid-induced increase in proliferation but not maturation, cell death was assessed at multiple time points after cannabinoid administration. As at the 48 hour time point, no interaction of treatment and genotype was seen in TUNEL positive cells 8 hours after cannabinoid instillation in wildtype C57 or CB1/CB2 receptor KO mice (Figure 4.9A-B). WIN and JZL/URB treated mice showed a 48.8% and 50.7% increase in apoptosis 72 hours after intranasal aspiration (Figure 4.9C; vehicle 6.5 ± .98 vs. WIN 12.7 ± .63 and JZL/URB 13.2 ± 1.0 cell/mm OE; $p<0.01$; $n=3$). Accordingly, TUNE positive cells in both WIN (Figure 4.9E; 9.1 ± .4 vs. 12.7 ± .6 and 12.9 ±.9 cell/mm OE; $p<0.05$) and JZL/URB (Figure 4.9F; 9.5 ± .46 vs. 13.6 ± .88 and 15.6 ± 1.2 cell/mm OE; $p<0.05$) treated groups is significantly lower at 16 days-post treatment. These data indicate that the cannabinoid-induced increase in proliferation is matched with an increase in cell death starting at 72 hours after and expiring within 2 weeks after instillation.

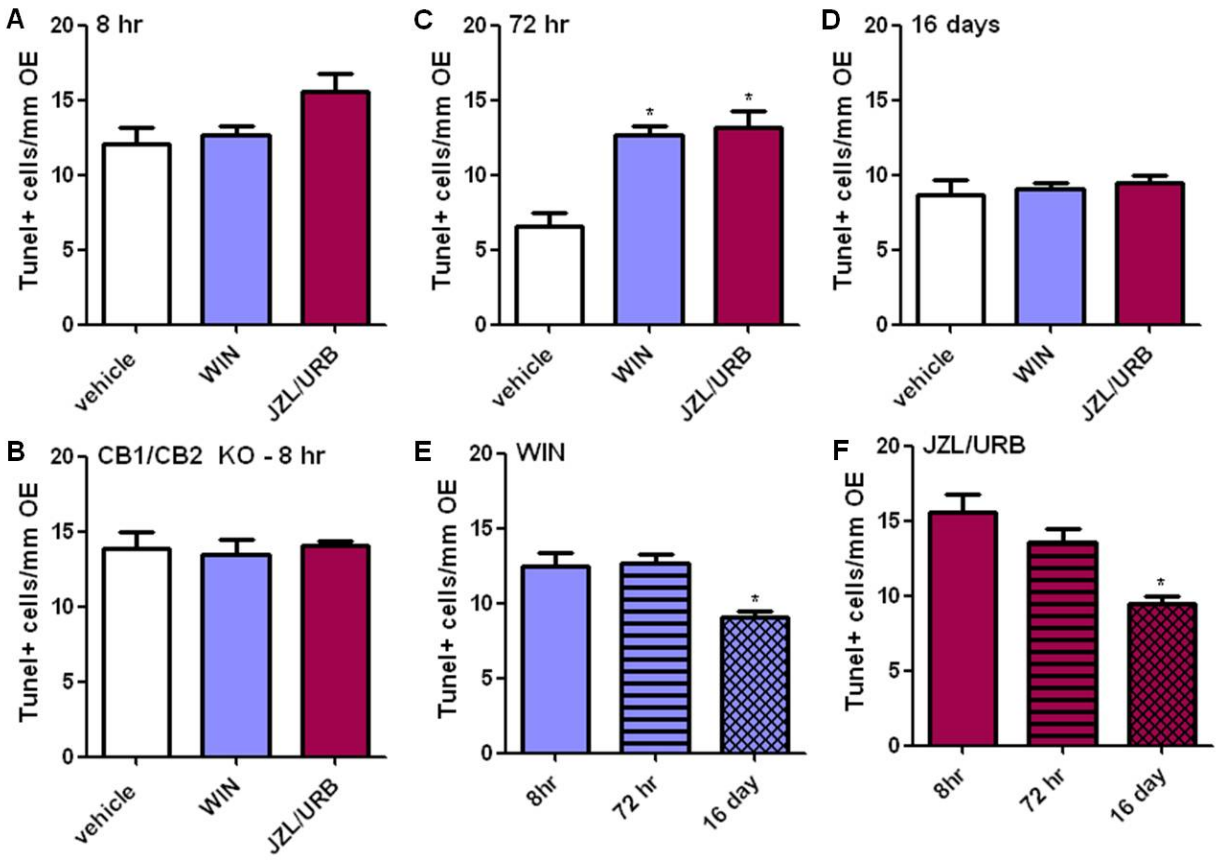

Figure 4.9 Cannabinoids increase apoptosis 72 hours after administration. Adult C57 wildtype mice intranasally aspirated vehicle (1% DMSO in saline), CB receptor agonist WIN (10μm), or a cocktail of JZL184/URB597 (10μm, 100μm, respectively).The levels of apoptosis were measured by TUNEL, 8 hours, 72 hours, or 16 days after cannabinoid administration. Quantification of TUNEL positive cells in **(A)** C57 wildtype and **(B)** CB1/CB2 receptor KO mice 8 hours after cannabinoid administration (n=3). **(C)** Quantification of TUNEL positive cells in C57 wildtype mice 72 hours and **(D)** 16 days after instillation (n=3 mice). (*p<0.05 vs. vehicle; one way ANOVA followed by Bonferroni's post hoc test). Quantification of TUNEL positive cells in C57 wildtype mice across time in (E) WIN and (F) JZL/URB treated groups (n=3 mice). (*p<0.05 vs. from

each time point within respective treatment; one-way ANOVA followed by Bonferroni's post hoc test).

Discussion

This chapter outlined experiments that investigated the effect of the cannabinoid system on adult neurogenesis in the mouse OE *in vivo*. Neurogenesis is a finely tuned process involving the proliferation of progenitor cells, successful differentiation, and functional integration into the surrounding tissue. First, proliferation was measured by BrdU-incorporation in two strains of mice, Swiss Webster (CFW) and C57BL/6, and at two ages, postnatal day 4 and adult 6-8 week old mice. These two ages were chosen because cannabinoids can influence proliferation during both late development stages and into adulthood. CFW neonatal mice were used in Chapter 2 to investigate CB receptor signaling by calcium imaging, therefore it is important to establish a similar functional role of cannabinoids between neonates and adults. Additionally, neonatal mice exhibit higher levels of proliferation compared to adult mice and endocannabinoids are well known to regulate development during early post-natal periods. Neonatal CFW mice showed an increase in BrdU positive cells 48 hours after intranasal aspiration of exogenous cannabinoid WIN, similar to that seen in the adult OE. Additionally, BrdU-incorporation was increased in neonates with intranasal aspiration of 2-AG ether at varying concentrations. Not surprisingly, neonates expressed an inherently greater amount of cells undergoing proliferation in the vehicle treatment groups. However, when neonatal data were normalized to compare results across different experiments and vehicle groups, a similar trend was observed, suggesting that similar mechanisms and chemical signals may mediate both post-natal and adult neurogenesis under normal conditions.

Two strains of mice were used for *in vivo* proliferation experiments. C57BL/6 (C57) mice were used as control background mice for the CB1/CB2 receptor KO mice, and CFW mice were used to verify proliferation in the mouse strain used for calcium imaging data and to recapitulate reproducibility of results. Accordingly, similar increases in WIN-induced proliferation were seen in both CFW and C57 adult mice. However, the inherent amount of proliferation that occurred in the vehicle-treated groups varied by about 12 BrdU positive cells between strains. The Swiss Webster strain is an outbred strain, and thus has substantial genetic variability between individual mice. The variability with outbred strains, coupled with significant results, suggests that the cannabinoid effect on proliferation is quite strong. In our experience, as well as in other laboratories, the C57BL/6 inbred strain has a significantly higher level of proliferation under control conditions than does the Swiss Webster, indicating that there may be both strain differences as well as individual differences in basal cell proliferation. These data suggest that cannabinoids may be a proliferative factor throughout development and into adulthood in the mouse OE. Additional differences in BrdU-incorporation were detected in vehicle treated groups 8 hours post-instillation compared to 24, 48, or 72 hours (Figure 4.5). No significant difference in vehicle group was seen between 24 hours, 72 hours, 48 hours, and 16 days. This could possibly reflect damaging effects of an acute application of 1% DMSO in the solution causing BrdU incorporation during DNA repair, natural fluctuations in proliferation levels, or an experimenter error in BrdU preparation or administration. Further investigation with alternative cell proliferative markers such as PCNA and Ki-67 are needed to verify results. Variability in vehicle-treated groups, although statistically significant may not be physiologically significant.

Therefore, individual control groups for each experiment are essential. Notably, all statistical analyses were performed on individual time controls since there was differences between vehicle groups at some time points. BrdU-incorporation was increased with both exogenous cannabinoid WIN and by increasing endocannabinoids by blocking their principal degradative enzymes (JZL/URB treatment) in control C57 mice. In the transgenic CB1/CB2 receptor KO mice, no difference in BrdU positive cells was seen in any treatment group. These data suggest cannabinoid-induced increases in proliferation are mediated by CB receptors and not at off target receptors. Additionally, the CB1 receptor specific antagonist, AM251 was sufficient to inhibit WIN-induced proliferation, further implicating CB1 receptors in this phenomenon. However, AM251 instillation alone did not significantly affect the proliferation rate, suggesting that tonic release of endocannabinoids does not act alone to regulate cell turn over and additional signals are involved. The effect of AM251 on cannabinoid-induced proliferation is similar to that seen with other trophic factor receptors in the OE, such as NPY Y1 (Jia and Hegg, 2010), fibroblast growth factor, and epidermal growth factor receptors (Jia et al., 2011a). Despite this observation, tonic release of 2-AG is still possible given the high steady-state levels of 2-AG found in the mouse OE (Chapter 2), presumably not from stimulus driven biosynthesis. High basal 2-AG levels are also detected in the rat brain (Stella et al., 1997), coupled with multiple roles in lipid metabolism (Piomelli et al., 2007), indicating that some “reserve” 2-AG is not immediately released, and could function as a messenger for intercellular signaling (Blankman et al., 2007; Di Marzo, 1999; Piomelli, 2003). Previous reports of 2-AG synthesis and release in the tadpole OE have suggested both autocrine and paracrine signaling routes (Breunig et al., 2010b),

therefore, similar mechanisms could be at work in the mouse OE. Future studies involving genetic depletion of 2-AG synthesis enzyme DAGL, could further investigate the contribution of tonic endocannabinoid stimulation on basal cell cycle regulation and proliferation. Cannabinoids may have a role in normal maintenance of basal cell proliferation state in the OE, but other factors may also contribute.

A natural increase in BrdU positive cells is seen in vehicle and all treated groups in CB1/CB2 receptor KO mice compared to the wildtype controls, although no effect of cannabinoid treatment was detected. Curiously, the increase in physiological BrdU incorporation persists despite decreased numbers of basal cells and OSNs in CB1/CB2 receptor KO mice. This observation conflicts with previous reports of a robust physiological decrease in BrdU positive cells in the both the DG and SVZ in CB1 receptor KO mice (Jin et al., 2004), and in the DG of CB2 receptor KO mice (Palazuelos et al., 2006). However, no studies have examined proliferation levels in the double CB1/CB2 receptor KO mice. It would be interesting to evaluate the natural proliferation levels of CB1 or CB2 receptor-null mice individually to compare to the results seen in the double KO mouse and to compare OE proliferation levels to those seen in the CNS. Perhaps a compensatory mechanism by the remaining basal cells is providing CB1/CB2 receptor KO mice with additional trophic factor support. For example, NPY release from microvillous cells supplies basal cells with an important physiological and injury-induced neurotrophic factor (Jia et al., 2013; Jia and Hegg, 2010, 2012). NPY Y1 receptor expression on basal cells may be upregulated in the CB1/CB2 receptor KO mouse, thereby increasing pro-proliferative signaling mechanisms. These data could also indicate that the decrease in basal cell numbers in CB receptor-deficient mice, albeit

statistical, may not be physiologically relevant. In addition to basal cell compensatory mechanisms, a decrease in anti-proliferative signaling from the decreased mature population of OSNs could contribute to increased proliferation in the CB1/CB2 receptor KO mouse. Following specific OSN damage, the rates of progenitor cell division decrease as an increasing number of neurons mature and integrate until OE homeostasis is achieved (Calof et al., 1996; Holcomb et al., 1995b; Schwob et al., 1992). These observations suggest that progenitor cells in the OE are able to detect appropriate levels of OSNs present in the OE and respond by altering proliferation and differentiation accordingly. Indeed, *in vitro* studies have shown that purified OE neuronal progenitor cells exhibit reduced levels of neurogenesis when grown in the presence of a large number of mature OSNs, indicating that OSNs produce an autocrine signal(s) that inhibits neurogenesis (Mumm et al., 1996). Additionally, the natural increase in BrdU is not mirrored with overall increases in cell death. Despite a major effect of genotype detected in TUNEL staining 48 hours after cannabinoid administration, no individual treatment increases were seen when comparing CB1/CB2 receptor KO mice to wildtype. To maintain homeostasis, a tonic increase in newly dividing cells should correspond to a consistent level of cell death; however, that relationship was not fully detected in my experiments. Further experiments using additional markers of proliferation such as Ki-67 and PCNA are needed to establish if the increased BrdU levels in CB receptor KO mice are valid. Cell lineage studies in CB1/CB2 receptor KO mice will also advance our understanding of newborn cell survival and differentiation. Additionally, measuring the number of immature neural precursors at physiological levels and 6 days after BrdU incorporation are needed to further investigate the

regulation of cell populations in CB receptor KO mice. Overall, CB1/CB2 receptor KO mice display an abnormal proliferation to apoptosis ratio, indicating cell cycle dysregulation and further implicate the cannabinoid system as integral for proper homeostatic regulation of basal cells in the mouse OE.

In the mature OE, basal stem cells proliferate into neuronal precursor cells and then differentiate into immature followed by mature OSNs to replace dying and dead OSNs (Graziadei and Graziadei, 1979; Graziadei and Monti-Graziadei, 1978). The rate of cell proliferation and neuronal differentiation in the OE is tightly regulated by multiple chemical signals produced by the different cell types in the OE (Kawauchi et al., 2004; Mackay-Sim and Chuah, 2000).Cell lineage experiments measuring BrdU location in either the basal layer, middle layer (indicative of neurogenesis), or apical layer (indicative of gliogenesis) of the OE was performed in wildtype C57 adult mice 16 days after cannabinoid administration. No increases in BrdU positive cells over control were seen in any layer 16 days after WIN or JZL/URB treatment, suggesting that increases in proliferation did not lead to additional neurogenesis or gliogenesis. Additionally, a significant decrease in total BrdU cell numbers was seen 16 days after WIN and JZL/URB instillation compared to 2 days, suggesting the cannabinoid-induced increases in proliferation at 2 days are not maintained and newly proliferated cells undergo apoptosis before 14 days after treatment. These data further indicate that the cannabinoid system regulates the proliferative state of basal cells but not differentiation. In this regard, cannabinoid signaling is unlike purinergic ATP signaling in the mouse OE, which is sufficient to increase both neuronal and glial cell differentiation (Jia et al., 2009). However, only one instillation of cannabinoid agonist was given while other

studies examining the role of cannabinoids on differentiation involve multiple treatments (Goncalves et al., 2008; Jiang et al., 2005). Additionally, the level of neurogenesis is tightly regulated by a multitude of chemical signals produced by OSNs or sustentacular cells that influence the proliferation and differentiation of endogenous neuronal progenitor basal cells. Negative autoregulators of neurogenesis such as GDF11 (Wu et al., 2003), are released from neurons and inhibit proliferation and generation of new neurons in the OE. Thus, destruction of neurons removes the source of the anti-proliferation signal, and promotes neurogenesis. Therefore, differentiation might not have been seen due to competing signaling pathways involved in cell population tissue homeostasis. During development, an inverse relation between expression of CB1 receptors and the stage of cell differentiation is seen in neuronal and glial cells (Palazuelos et al., 2006), indicating that CB1 receptors might function to prevent a differentiated state in favor of a non-differentiated, proliferative state. Similarly, CB2 receptors increase with de-differentiation of glial (Sanchez et al., 2001) and some tumors (Caffarel et al., 2006). Results obtained from this chapter suggest that cannabinoid signaling actively promotes basal cell proliferation while not influencing differentiation in wildtype mice. Additional studies outlining cell lineage progression in CB1/CB2 receptor KO mice would greatly contribute to the interpretation of wildtype data.

Cannabinoids take part in the control of tissue homeostasis, thereby modulating the balance between cell death and survival (Guzman, 2003; Mechoulam et al., 2002). Both CB1 and CB2 receptors regulate neural progenitor commitment, survival, and cell-cycle maintenance (Aguado et al., 2006; Guzman, 2003). The varying actions of CB

receptors can provide cell protection (Romero et al., 2002) or elicit apoptosis (Guzman, 2003). Studies in glioma or astrocytoma cells (Sanchez et al., 2001) and in various non-neuronal cancers (Caffarel et al., 2006), show that activation of CB receptors induces apoptosis and inhibits tumor growth in host mice. Administration of cannabinoids can inhibit the survival of various tumor cells in culture and curb the growth of different models of tumor xenografts in rats and mice (Guzman, 2003). Most antitumor effects of cannabinoid signaling have been studied in tumors of the CNS, specifically gliomas (Velasco et al., 2004). In the current chapter, a cannabinoid-induced increase in proliferation was matched by an increase in apoptosis 72 hours after WIN or JZL/URB administration. Levels of cell death were elevated by around 50% in both treatment groups. However, despite a significant increase in cell death, the number of cannabinoid-induced proliferating cells is 4 to 5 times higher than the increased numbers of TUNEL positive cells. This suggests that apoptosis of newly generated cells takes place over an extended period of time or that apoptosis peaks after the 3 day time point. Cannabinoid-induced increases in BrdU positive cells is still seen 3 days after administration, falling within an previously characterized length of basal cell proliferation, so it is unsurprising that cell death numbers at 72 hours are not sufficient to restore balance between proliferation and cell death numbers. Additionally, TUNEL positive cells significantly decrease 16 days post cannabinoid instillation compared to 8 and 72 hours, suggesting that the range of apoptosis falls between 3 and 16 days.

Cannabinoids are also neuroprotective in a variety of neuronal injury and degenerative models (Guzman et al., 2002). The literature on neuroprotective properties of endocannabinoids suggests tissue, concentration, and injury-dependent recovery.

WIN protects hippocampal neurons in culture from glutamate-induced excitotoxicity through a CB1 receptor-dependent mechanism (Shen and Thayer, 1998). Genetic deletion of CB1 receptors renders mice more sensitive to the harmful effects of ischemia, excitotoxic effects of glutamate, and oxidative injury (Kim et al., 2006). WIN exhibits neuroprotection on both hippocampal neurons in a cerebral ischemia paradigm (Nagayama et al., 1999) and on hippocampal astrocytes from toxic doses of ceramide (Gomez Del Pulgar et al., 2002), suggesting that neuroprotective effects of the cannabinoid system can also act through glia. However, in the present study no increase in TUNEL positive cells was detected 48 hours after administration of CB1 receptor antagonist AM251 in either CFW or C57 mice. This suggests that potential tonic release of endocannabinoids does not function to prevent cell death. However, in CB1/CB2 receptor KO mice a major effect of genotype was detected in TUNEL staining 48 hours after cannabinoid administration, suggesting that there is an increase in basal levels of apoptosis in the absence of CB receptor signaling. Taken together, these data demonstrate that CB receptors clearly influence cell homeostasis involved in cell survival and death.

Cannabinoids regulate the state of proliferation, differentiation, and cell death via several proposed pathways (Harkany et al., 2007). CB1 and CB2 receptors can activate the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway in cortical progenitor cells during development and in mature neurons (Crino, 2011; Diaz-Alonso et al., 2014; Puighermanal et al., 2012). mTORC1 is involved in the control of a plethora of cell functions by acting through the regulation of protein synthesis via phosphorylation of its downstream targets (Foster et al., 2010), which are essential elements in neuronal

responses to synaptic activity and plasticity (Hoeffer and Klann, 2010). In the developing brain, mTORC1 signaling regulates neural cell fate, and a fine turning of this signaling pathway is essential for appropriate development (Crino, 2011). Additionally, mTORC1 activation in intermediate progenitor cells in adult neurogenic brain areas reverts progenitor quiescence to active proliferation in the aged brain (Paliouras et al., 2012). Thus, mTORC1 activation by CB receptors may contribute to injury-induced neural progenitor activation (Aguado et al., 2007; Palazuelos et al., 2012) and alleviate aging-associated decline of neurogenesis (Goncalves et al., 2008; Marchalant et al., 2009a). mTORC1 is a major target of the PI3K/Akt pathway and thus also plays a central role in neural cell survival/death decision (Foster et al., 2010). For example, activated mTORC1 is responsible for the hippocampal alterations that contribute to the development of epilepsy, including mossy fiber sprouting, neuronal cell death, and neurogenesis (Zeng et al., 2009).CB1 receptor signaling is able to engage PI3K/Akt/mTORC1 signaling pathway in the developing cortex (Diaz-Alonso et al., 2014) leading to proliferation. In cerebellar progenitors, CB1 receptor-induced cell proliferation is mediated by the PI3K/Akt pathway that, as a consequence of GSL3β inhibition, increases β-catenin nuclear translocation and cyclin D1 expression (Diaz-Alonso et al., 2014; Trazzi et al., 2010). Likewise, the CB2 receptor, normally absent from mature neuronal cells, but expressed in undifferentiated neural progenitors, promotes cell proliferation via PI3K/Akt/mTORC1 signaling (Palazuelos et al., 2012). Furthermore, it was found that CB2 receptor-induced neural progenitor proliferation via mTORC1 signaling is relevant in pathophysiological conditions such as proliferation during development and excitotoxicity-induced adult hippocampal neurogenesis

(Palazuelos et al., 2012). In addition to the involvement of PI3K/Akt signaling, a potential contribution of other pathways, such as the ERK cascade is plausible.

Conclusions

Previous studies in the olfactory system have examined cannabinoid-mediated olfaction after fasting (Breunig et al., 2010b; Soria-Gomez et al., 2014), but no attention has been paid to the mechanism of proliferation. The experiments performed in this chapter advance our understanding of stem cell lineage and the trophic factor/signaling mediators responsible for progenitor cell proliferation, differentiation, and survival in the mouse OE. Trophic factor requirements of OE progenitor cells are similar to progenitor cells in the CNS (Mackay-Sim and Chuah, 2000) and cannabinoid signaling can now be added to this list. OE progenitor cells are easily obtained via a simple biopsy (Feron et al., 1998) and are able to generate neurons and partially restore function after transplanted into the brain (Murrell et al., 2008). Future research on cannabinoid-induced mechanisms of neurogenesis could have direct implications for regenerative strategies using olfactory-derived progenitor cells in mouse models and therapeutic strategies for the human central nervous system.

Chapter 5: Discussion

Results of this thesis demonstrate a clear influence of the cannabinoid system in the regulation of proliferation and tissue homeostasis in the mouse OE. Using both a genetic and pharmacological approach to manipulate cannabinoid signaling, we have observed (1) the first identification of a cannabinoid system in the mouse OE, (2) basal cell and neuronal population alterations in CB1/CB2 receptor KO mice, (3) dysregulation of progenitor cell balance in CB1/CB2 receptor KO mice, and (4) increases in basal cell proliferation due to exogenous and endogenous cannabinoids. The studies in this thesis cohesively imply the balance of progenitor cell survival and proliferation in adult mouse OE is regulated by CB receptor signaling. Additional hypotheses and future implications derived from this work include the topics of (1) autocrine and paracrine endocannabinoid signaling in the OE, (2) variability in CB receptor genetic models, (3) production and release of endocannabinoids through tonic and stimulated mechanisms, (4) signaling pathways of proliferation, and broader implications of progenitor cell regulation, including cannabinoid system involvement in (5) age-related declines in olfactory function and (6) pathological declines in olfactory function.

Additional hypotheses and future implications

Non-retrograde cannabinoid signaling in the OE

Cannabinoid signaling in OE progenitor cells is not the canonical retrograde signaling observed in the central nervous system, which has a modulatory function. Instead, CB signaling most resembles that seen in the progenitor cell niche in the CNS since (1) OE tissue morphology is similar to that seen in central adult and

developmental stem cell niches (2) OSNs are primary neurons making their first synapse in the OB, (3) autocrine and paracrine endocannabinoid signaling is seen in *Xenopus laevis* OE, and (4) trigeminal nerves innervating the mouse OE are likely not involved in CB receptor-mediated effects.

A progenitor cell niche is defined by the proximal extracellular matrix, local paracrine and autocrine signaling from neighboring cells, and cell specific properties, i.e., available receptors that will define the ability to respond to extracellular signals (Mackay-Sim and Kittel, 1991). An endocannabinoid system is present in embryonic neural stem cell niches, and its regulatory role in neuronal generation and maturation is preserved in neurogenic niches of the adult brain. Studies in the developing brain and in adult neurogenic niches have shown that CB1 and CB2 receptor activation can modulate neural stem cell proliferation *in vitro* and *in vivo.* These neural progenitors have the capacity to synthesize endocannabinoids, express CB receptors, and the metabolic enzymes for endocannabinoid degradation, resulting in a local signaling loop. This suggests that cannabinoid signaling on progenitor cells is contained within the neurogenic microenvironment, outside of the canonical signaling roles of cannabinoids in retrograde transmission. Additionally, non-retrograde endocannabinoid signaling in the CNS includes cannabinoid-mediated slow self-inhibition in neocortical interneurons and pyramidal neurons (Bacci et al., 2004; Marinelli et al., 2009). This form of autoregulation results from activity-dependent rises in intracellular calcium, mobilization of 2-AG, and activation of CB1 receptors (Bacci et al., 2004; Marinelli et al., 2008). CB2 receptors localized to intracellular compartments also self-inhibit neuronal activation in medial prefrontal cortical pyramidal neurons (den Boon et al., 2012). These data

suggest that cannabinoid signaling in development and in mature progenitor cell niches are through local paracrine or autocrine pathways.

The mouse olfactory mucosa is divided into epithelium and lamina propria by separation of the basement membrane. The OE contains only one type of neuron, OSNs, which receives no synaptic innervations within the epithelium. OSNs send a dendrite to the apical surface where their cilia extend into the mucus lining the lumen of the nasal cavity. OSN axons leave the epithelium and enter the lamina propria where they gather in bundles before entering the OB where they synapse with mitral/tufted cells and interneurons. The apical surface of the epithelium contains the cell bodies of sustentacular and microvillous cells that send processes to the basement membrane. Adjacent to the basement membrane are horizontal basal cells, the neural stem cell, and atop horizontal cells are the multipotent progenitor GBCs. OE tissue composition represents an isolated peripheral system that contains a progenitor cell niche. Non-synaptic signaling has also been identified in the tadpole OE. Tadpole OSNs appear to be involved in autocrine signaling, whereby neuronal 2-AG synthesis leads to CB1 receptor activation on OSNs, and paracrine signaling whereby 2-AG synthesis in sustentacular cells leads to CB1 receptor activation on OSNs (Breunig et al., 2010b). Therefore, tissue morphology of the mouse OE is more similar to that seen in neural progenitor cells during development, and in adult neurogenic niches. It is unlikely that cannabinoids are acting in a retrograde signaling mechanism as they do in brain regions in which synaptic transmission dominates cell to cell signaling modalities, such as the OB. Unlike the mouse OE, the OB contains several types of neurons with complex synapses that influence the perception of odors. Primary OSNs first synapse within

discrete anatomical areas called glomeruli. Each glomerulus contains (1) mitral and tufted relay neurons, which project axons to the olfactory cortex, (2) periglomerular interneurons that encircle the glomerulus, and (3) granule cell interneurons that provide negative feedback circuits and are located deeper within the bulb. Cannabinoid signaling can regulate OB perception of odors through cannabinergic synapses between granule cells in the OB and centrifugal fibers that project from the anterior olfactory nucleus and piriform cortex to the OB (Soria-Gomez et al., 2014). This is an example of cannabinoid mediated inhibition of synaptic signaling in the olfactory system, similar to that seen involved in short and long term potentiation in the hippocampus (Chevaleyre et al., 2006).

In addition to the previously mentioned cell types, the mammalian OE is innervated by trigeminal nerve branches (Bouvet et al., 1987). Trigeminal afferents mediate sensations of touch, pressure, temperature, and nociception (Finger et al., 1990; Sekizawa and Tsubone, 1994). Trigeminal activity can influence olfactory processing through central processing in the medio-dorsal thalamus (Inokuchi et al., 1993), in the OB (Stone et al., 1968), and through the release of Substance P from innervating trigeminal fibers in the OE (Finger et al., 1990). The interaction between the olfactory and trigeminal systems influences odor perception, but also monitors the physiological state of the OE environment. Furthermore, data have shown an interaction between the cannabinoid system and trigeminal nerves. CB receptors have been located on dorsal root ganglion primary afferent terminals, which can facilitate substance P release through disinhibiton (Zhang et al., 2010). WIN exerts both excitatory and inhibitory effects on trigeminal sensory neurons, however not through

CB1, CB2, or TRPV1 receptor signaling (Price et al., 2009). CB1 and CB2 receptor signaling was also not involved in WIN-induced inhibition of TRPV1 in primary sensory neurons (Wang et al., 2012). Despite an evident association between WIN signaling and trigeminal nerve effects, it is unlikely that trigeminal nerves are a source of endocannabinoids or participate in any conical cannabinoid signaling pathways in the mouse OE. However, it would be interesting to investigate the relationship between trigeminal fibers, cannabinoid signaling, and substance P release during inflammatory insult in the mouse OE.

Variability in CB receptor genetic models

Although the evidence described in this thesis supports a broad role for endocannabinoid signaling during OE progenitor cell proliferation, the CB1/CB2 receptor KO mouse doesn't exhibit a clear relationship between basal cell proliferation and olfactory-mediated functional consequence resulting from no CB receptor signaling. However, CB1/CB2 receptor KO mice exhibit a dysreguation in basal cell proliferation to cell death ratio and alterations in basal cell and OSN numbers. Additionally, proliferation levels in the CB1/CB2 receptor KO mouse model conflict with previous reports in single CB receptor KO mice (Jin et al., 2004; Palazuelos et al., 2006). The endocannabinoid signaling network has recently become more complicated with the identification of alternative synthetic and degradation pathways for 2-AG and AEA (Dinh et al., 2002; Liu et al., 2006; Simon and Cravatt, 2006), and the increasing spectrum of endocannabinoid ligands together with other lipid signaling mediators (Bradshaw and Walker, 2005; Harkany et al., 2007). The similarities and redundancy of the spatial and

temporal occurrence of lipid mediators in the brain, their receptor selectivity, and physiological actions, suggest compensatory mechanisms could occur if a key endocannabinoid system component is ablated. At the receptor level; the constitutive deletion of CB1 receptors might upregulate the expression of other cannabinergic receptors (e.g. GPR55) (Baker et al., 2006). Activation at these sites could substitute for a lack of CB receptor signaling in proliferation. Therefore, the genetic restriction of cannabinoid signaling using conditional rather than global transgenic alterations could offer more specific insight into cannabinoid signaling in the OE during neurogenesis. Controlling in which progenitor cell types a given cannabinoid-related gene is ablated would help localize functions related to cannabinoid signaling in the OE. For example, in the mouse OB neuronal specific deletion of CB1 receptors on interneurons resulted in specific mechanistic results not previously obtainable with global CB receptor KO mice (Soria-Gomez et al., 2014).

Tonic endocannabinoid synthesis/release mechanisms

Although many tissues have a resident pool of progenitor cells only certain adult neurogenic niches display neurogenesis under physiological conditions. The regulation of these progenitor cells are attributed, in part, to paracrine signaling from the microenvironment. Recently, it has been discovered that tonic paracrine signaling through GABAergic interneurons in the adult DG regulates the proliferative status (quiescent or active) of local progenitor cells (Song et al., 2012). This suggests that adult proliferation and differentiation are tonically influenced by the local signaling network reflecting the activity and number of neurons. Likewise, tonic and stimulated

release of ATP has been identified in the mouse OE, and implicated in normal progenitor cell turn-over with constitutive release, and neurogenesis with injury induced release of ATP (Hayoz et al., 2012).

Recent pharmacological and genetic studies suggest that synthesis and release of 2-AG are not always tightly coupled. Basal levels of 2-AG in DAGLα mice are much lower than in wildtype mice (Gao et al., 2010a; Tanimura et al., 2010), indicating a substantial amount of 2-AG is present in unstimulated brains. This result also suggests that DAGLα may be intrinsically active, or low levels of spontaneous neuronal activity may be a sufficient stimulus. Three different models of 2-AG signaling have been proposed (Alger and Kim, 2011). In a conventional on-demand synthesis model, 2-AG synthesis is tightly linked to demand triggered by neuronal activation. Stimulation induces intracellular calcium elevation and/or G protein (Gq/11) activation leading to 2-AG synthesis by DAGL. Second, in an on-demand release model 2-AG synthesis is uncoupled from release. An unstimulated neuron constitutively synthesizes, but does not immediately release 2-AG, suggesting 2-AG is able to be stored within the cell. Activation of DAGL in resting neurons may be sensitive to the unstimulated levels of intracellular calcium fluctuation. A third combined model involves both constitutive release from unstimulated cells and stimulus dependent 2-AG-mobilization. 2-AG is synthesized in unstimulated cells, but is also tonically released into the extracellular space. Neuronal stimulation can further increase synthesis and release of additional 2-AG. This third mode of endocannabinoid mobilization could be acting in the mouse OE. Evidence for tonic cannabinoid signaling gained from this thesis includes (1) high levels of unstimulated 2-AG concentrations in mouse OE, presumable from a preformed pool,

and not from stimulus-driven biosynthesis, (2) detectable changes in intracellular calcium in unstimulated cells (occasional increases in intracellular calcium are detected when physiological solution alone is applied to OE slices), and (3) CB1/CB2 receptor KO mice exhibit physiological changes in tissue composition and proliferation dysfunction compared to wildtype.

Stimulation-dependent endocannabinoid synthesis/release

Endocannabinoid production is triggered by both calcium mobilization and purinergic receptor signaling. Calcium mobilization causes endocannabinoid synthesis under both physiological and injury-induced conditions. Stimulus-based endocannabinoid synthesis from membrane phospholipids is in response to an increase in intracellular calcium alone, or combined with activation of Gq/11 proteins, such as group I metabotropic glutamate receptors (Bisogno et al., 2013; Pertwee et al., 2010; Simon and Cravatt, 2006). For example, ionotropic and metabotropic glutamate receptors can engage 2-AG generation via Gq/11-PLC activation and/or increased calcium levels (Castillo et al., 2012), which may also occur during spontaneous neuronal activity during adult hippocampal proliferation (Nochi et al., 2012). Endocannabinoid production can be further increased after CNS injury and serve in a neuroprotective role. Ischemic and traumatic brain injuries are CNS pathologies in which high intracellular calcium accumulation is among the earliest events. The synthesis and accumulation of endocannabinoids in response to injury, in addition to their role as anti-oxidants, anti-inflammatory agents, inhibitors of excitotoxicity, and stimulators of neurogenesis, suggest that cannabinoid signaling is involved in

neuroprotective and neuroregenerative responses (Shohami et al., 2011). Several *in vivo* studies have shown that CB1 receptor activation prevents neuronal death in response to ischemia (Hayakawa et al., 2007; Leker et al., 2003; Nagayama et al., 1999). Pretreatment with CB receptor agonist WIN prior to ischemia produced a significant increase in the number of surviving neurons in a CB1 receptor dependent manor (Fernandez-Lopez et al., 2010; Nagayama et al., 1999). 2-AG levels were also elevated in a closed head injury model lasting from 1-24 hours after injury, and this rise in 2-AG served to protect hippocampal neurons from ischemic injury (Panikashvili et al., 2001). CB1 receptor agonists reduce infarct volume following stroke in a mouse model (Nagayama et al., 1999). In the same stroke model, CB1 receptor-null mice exhibit increased mortality and increased severity at the injury site (Parmentier-Batteur et al., 2002).

Endocannabinoid synthesis can also be regulated by ATP signaling. The cytoplasm of neural cells contain millimolar amounts of ATP, which flood the extracellular space after injury, activating purinergic receptors expressed by glia and other cell types. Recently, it was shown that astrocyte and microglia can produce 2-AG, and that ATP signaling increases 2-AG microglial production threefold (Walter et al., 2004; Witting et al., 2004). Increased 2-AG production from astrocytes involves ATP activation of purinergic P2X receptors, sustained rise in intracellular calcium, and DAGL activity (Walter et al., 2004). Therefore, calcium and ATP signaling from stimulated or injured tissue can increase endocannabinoid synthesis and release.

A major difference between OSNs and other neurons is their proximity to the external environment, which increases their risk of death. Cilia containing sensory

receptors reside on apical OSN dendritic extensions into the overlaying mucus layer (Menco, 1980). Inhaled toxic particles can destroy the OE and the neurons within it. Additionally, OSNs provide a route for viruses and bacteria to enter the brain (Owen et al., 2009), and as a consequence may be vulnerable to apoptosis as a protective mechanism (Harris et al., 2009). Given the increased risk of apoptosis, the OE has developed a remarkable ability to regenerate both neuronal and non-neuronal cell types. Several injury models have been used to study neuronal regeneration in the OE. Olfactory bulbectomy is one such model that induces neuronal degeneration and subsequent neurogenesis, since the OB is the direct synaptic target of OSNs. Olfactory bulbectomy causes a selective degeneration of OSNs, followed by near-complete regeneration of OSNs within a specific time period (Costanzo and Graziadei, 1983; Holcomb et al., 1995b; Schwartz Levey et al., 1991; Schwob et al., 1992). Neuronal regeneration following bulbectomy involves the proliferation and differentiation of a defined sequence of cellular intermediates including Mash1-expressing progenitors and progeny (Figure 1.3). As new OSNs are generated, the rate of progenitor cell division decreases until steady-state is restored, around ten days after bulbectomy (Calof et al., 1996; Holcomb et al., 1995b; Schwob et al., 1992). These observations suggest that progenitor cells in the OE are able to detect appropriate levels of OSNs present in the OE and respond by altering proliferation and differentiation accordingly. Indeed, *in vitro* studies have shown that purified OE neuronal progenitor cells exhibit reduced levels of neurogenesis when grown in the presence of a large number of mature OSNs, indicating that OSNs produce an autocrine signal(s) that inhibits neurogenesis (Mumm et al., 1996; Simpson et al., 2003). This ability to detect changes in OSN number is

essential to respond to natural fluctuations in neuronal numbers that occur as the OE is subjected to infection and toxic insult during life (Hinds et al., 1984; Mackay-Sim and Kittel, 1991).

Although olfactory bulbectomy is a good model to study the process of neurogenesis, models involving environmental toxicants are more appropriate to understand natural injury process. Our lab has studied the impact of such toxic compounds including, diesel particles, satratoxin G (Islam et al., 2006; Jia et al., 2011b), nickel sulfate (Jia et al., 2010), and fine particulate matter. During such toxic insults, OSNs and other cell types are damaged or die, releasing their intracellular contents, into the extracellular space. Two important molecules, ATP and calcium, are involved in injury-induced cell regeneration in both the central and peripheral nervous systems. In the CNS, ATP, is released by injured neurons and astrocytes via activation of P2 purinergic receptors (Franke and Illes, 2006; Neary and Zimmermann, 2009) and can regulate cell proliferation and neuroregeneration by triggering localized neurotrophic factor release (Neary et al., 1996; Rathbone et al., 1992; Rathbone et al., 1999). Similarly, in the OE, toxic insult induces ATP release through P2 receptors resulting in basal cell proliferation (Hegg and Lucero, 2006; Jia et al., 2011a; Jia and Hegg, 2010). ATP activation of either P2X or P2Y receptors evokes (1) increases in intracellular calcium in OSNs, sustentacular cells, and microvillous cells (Hassenklöver et al., 2009; Hegg et al., 2003; Hegg et al., 2009), (2) release of trophic factors (Jia et al., 2011a; Kanekar et al., 2009), and (3) increases in basal cell proliferation, differentiation, and maturation of OSNs (Hassenklöver et al., 2009; Jia et al., 2009). Additionally, activation of purinergic receptors in the glial-like sustentacular cells or the microvillous cells could

induce the release of trophic factors to promote basal cell proliferation. Indeed, ATP increases the release of fibroblast growth factor 2 and NPY through a purinergic receptor-dependent mechanism (Jia et al., 2011a; Jia and Hegg, 2010). Therefore, ATP and calcium signaling activate a cascade of intracellular signals and the induction of calcium-dependent release of neurotrophic factors, possibly from sustentacular cell endfeet, microvillous cell processes, or neuronal axons.

Sustentacular cells and microvillous cells have the molecular machinery to signal via Gq/11 pathways (Hegg et al., 2009; Jia and Hegg, 2010, 2012). Calcium imaging has shown activation of purinergic P2Y receptors leads to phospholipase C accumulation and production of DAG and IP3, subsequently releasing calcium from intracellular stores (Hegg et al., 2009). The increase in cytosolic calcium induces calcium-dependent calcium release via the ryanodine receptor. This pathway may control secretion, proliferation, and development in sustentacular cells. Importantly, calcium-dependent exocytosis in non-neuronal cells may produce and release chemical signals, such as endocannabinoids, that act on basal cells, neuronal precursors, or OSNs. Endocannabinoid production through metabotropic glutamate receptors are not likely, since previous work in the mouse OE has shown no calcium responses to the application of glutamate (Hegg et al., 2009).

Although no direct evidence of injury-induced cannabinoid signaling has been collected in this thesis, the above literature overwhelmingly suggests that (1) the OE is exposed to multiple toxicants, therefore injury-induced neurogenesis/gliogenesis is a likely event, (2) calcium and ATP are known mediators of injury-induced recovery in the mouse OE, and (3) endocannabinoids are synthesized in a calcium-dependent manner.

Therefore, endocannabinoid-mediated neuronal and glial proliferation, differentiation, and integration after toxic insult are an extremely plausible mechanism for repair in the mouse OE. Furthermore, an extensive *in vivo* study was performed for this thesis that would have addressed this idea. However, the injury model used did not induce significant cell damage as previously seen. Therefore no results were obtained on the endocannabinoid effect of regeneration after injury.

Potential mechanisms of CB receptor-mediated progenitor cell signaling

The results from this dissertation will add to the body of work that implicates cannabinoid signaling in the modulation of a progenitor cell niche. What remains to be investigated is the mechanism by which CB receptor activation initiates progenitor cell proliferation. Although CB receptor signaling at neuronal synapses has been examined extensively (Porter and Felder, 2001), specific signaling mechanisms in progenitor cells remain to be fully characterized. However, some work has been done to elucidate signaling transduction mechanisms regulated by CB receptor in neural progenitors (Figure 5.1). The following mechanisms described below may be occurring during cannabinoid-induced proliferation and progenitor cell homeostasis in the mouse OE.

CB receptors belong to the Gi/o protein-coupled receptor superfamily (Pertwee, 1997). Cellular responses triggered with CB receptor activation include inhibition of adenylyl cyclase and voltage-gated calcium channels, and activation of potassium channels, mitogen-activated protein kinase (MAPK), and phosphoinositide-3 kinase (PI3K)/Akt signaling pathways (Bouaboula et al., 1995; Gomez del Pulgar et al., 2000). CB1 receptor-mediated proliferative and pro-survival actions have been attributed to the activation of the PI3K/Akt axis and extracellular signal-related protein kinase (ERK)

(Guillemot et al., 2006). Gi/o protein-coupled receptors can activate the PI3K/Akt and ERK pro-survival pathways through the βγ subunit either directly (via activation of targets such as PI3Ks) or indirectly (via transactivation of growth factor tyrosine kinase receptors and subsequent canonical PI3K/Akt and ERK pathways) (Dorsam and Gutkind, 2007). PI3K/Akt signaling also plays a role in self-renewal and protection of neural stem cells (Watanabe et al., 2006) and in oligodendrocyte progenitor cell survival (Ebner et al., 2000; Vemuri and McMorris, 1996).

The PI3K/Akt pathway promotes neuronal and glial survival by both enhancing the expression of anti-apoptotic proteins and inhibiting the activity of proapoptotic ones, such as direct intracellular target glycogen synthase kinase (GSK-3β) (Brunet et al., 2001). Several well known prosurvival factors for oligodendrocyte progenitors are also strong activators of PI3K/Akt, suggesting the ability of cannabinoids to activate the PI3K/Akt pathway may account for their protective role (Barres et al., 1993; Flores et al., 2000). For example, cannabinoid agonist-stimulated oligodendrocyte proliferation was reduced by an Akt translocation inhibitor (Molina-Holgado et al., 2007). Additionally, trophic deprivation-induced cell death was prevented by CB receptor activation and recovery of Akt activity (Molina-Holgado et al., 2002).

In neuronal precursors from both the cerebellum and SVZ, CB1 receptors promote proliferation through the PI3K/AKT/GSK-3β/β-catenin pathway, suggesting that this pathway plays a pivotal role in the CB1 receptor-dependent modulation of neuronal proliferation. CB receptor agonist HU-210 treatment increased phosphorylation of AKT and GSK-3β, and that inhibition of PI3K and AKT suppressed the CB1 receptor-mediated proliferation increase of granuale cell progenitors (Trazzi et al., 2010). GSK-

3β is inactivated through phosphorylation, leading to β-catenin stabilization and accumulation in the cytosol and can be available for translocation to the nucleus where it functions as a transcriptional regulator (Dominguez and Green, 2001; Grimes and Jope, 2001). Therefore, CB1 receptor activation can increase β-catenin nuclear localization which can then activate transcription factors that induce proliferation, thereby modulating cell cycle regulatory genes such as cyclin D1 (Trazzi et al., 2010). Recent evidence shows that β-catenin plays an important role in regulating proliferation of neural stem cells by decreasing cell cycle exit (Chenn and Walsh, 2003). Furthermore, inhibition of GSK-3β activity blocks apoptosis of neurons, whereas overexpression of active GSK-3β or transfection with a GSK-3β mutant that cannot be phosphorylated (inhibited) induces apoptosis (Culbert et al., 2001; Hetman et al., 2000). Collectively, these results indicate that CB1 receptor activation is able to activate Akt via pertussis-sensitive Gi/o-proteins through a PI3K-dependent signaling pathway (Molina-Holgado et al., 2007).

Similarly, homeostatic factors can signal through the PI3K/Akt pathway to help control levels of proliferation and differentiation in the mouse OE. Neurotrophin (NT)-3 , a member of the neurotrophin family including nerve growth factor and brain-derived neurotrophic factor, has been localized in a subpopulation of OSNs within the OE (Vigers et al., 2003), and was able to either directly or indirectly activate both the MAPK and PI3K/Akt pathways in vitro (Simpson et al., 2003). Activation of the Akt pathway preceded activation of the MAPK pathway, and inhibition of the PI3K/Akt pathway resulted in decreased survival of mature neurons (Simpson et al., 2003). Therefore, cannabinoid regulation of OE homeostasis could be acting though the PI3K/Akt pathway

Additionally, CB1 signaling in neural cells may involve the activation of mammalian target of rapamycin complex 1 (mTORC1), a protein kinase that regulates cell growth, proliferation, and survival (Hoeffer and Klann, 2010). CB1 receptor stimulation in hippocampal neurons activates mTORC1 and phosphorylation of downstream targets that result in protein synthesis (Puighermanal et al., 2012; Puighermanal et al., 2009), which can explain some long-term cannabinoid actions on neuronal plasticity and cognition (Hoeffer and Klann, 2010). The CB2 receptor has also been implicated in mTORC1 signaling in neural progenitor cells both in the developing cortex and subgranular zone of the adult hippocampus (Palazuelos et al., 2012), which play a role in the balance of neural progenitor cell proliferation versus differentiation (Qiu and Knopfel, 2009) and cell survival (Puighermanal et al., 2009).

CB1 receptor signaling through canonical Gi/o mediated inhibition of adenylyl cyclase and cAMP concentration, can lead to ERK activation via de-inhibiting the ERK pathway by PKA (Davis et al., 2003; Derkinderen et al., 2003). Additionally, G protein βγ subunit mobilization can activate the ERK pathway in a PI3K-dependent manner (Galve-Roperh et al., 2002). Cannabinoid administration leads to CB1 receptor-mediated ERK pathway activation in the hippocampus (Derkinderen et al., 2003), striatum (Valjent et al., 2001), frontal cortex (Moranta et al., 2007), and cerebellum (Tonini et al., 2003). Accordingly, a decrease in endocannabinoid-induced neuronal differentiation is also accompanied by reduced activation of ERK (Rueda et al., 2002b). Direct endocannabinoid effects on neural progenitor differentiation may occur through ERK signaling through a mechanism that involves the upstream inhibition of small GTPase Rap1 and proto-oncogene B-Raf (Rueda et al., 2002b). This differential control

of ERK activity could also contribute to the divergent effects of endocannabinoids on neural and glial lineage because glia do not express significant amounts of B-Raf, which is an essential component of this signaling cascade (Galve-Roperh et al., 2006). However, ERK activation can be promiscuous and CB1 receptors can couple to both Gs and Gi proteins that activate the Raf-MEK-ERK pathway (Chen et al., 2010). Finally, MEK-ERK signaling is seen in the mouse OE in response to neurogenic signaling factor NPY (Jia and Hegg, 2012). This indicates that CB receptor activation could also lead to proliferation via the MEK/ERK pathway, and that convergence of trophic factor signaling could occur between NPY and endocannabinoids in the mouse OE.

Given the results from the genetic and pharmacological studies in this thesis along with the literature previously cited, I hypothesize that the endocannabinoid system plays a role in OE tissue homeostasis under both physiological and injury-induced conditions. I hypothesize that during normal cell turnover, 2-AG is continually synthesized in the mouse OE and small quantities are tonically released and activate CB1 receptors on basal cells which helps promote survival and balance the proliferative state of basal cells, by potentially suppressing neurogenesis. Supporting evidence for this hypothesis from my thesis include: (1) presence of CB1 receptors on basal cells, (2) presence of 2-AG synthesis and degradation enzymes, (3) resting high levels of unstimulated 2-AG synthesis, (4) a dysregulation in proliferation and a decrease in horizontal and globose basal cell numbers in the CB1/CB2 receptor KO mouse despite similar levels of 2-AG production (suggesting there is no feedback loop from CB receptor activation/presence to physiological production of 2-AG), and (5) an overall effect of increased apoptosis in CB1/CB2 receptor KO mice.

A further hypothesis is that after acute injury, brief increases in intracellular and free ATP lead to on-demand production and release of endocannabinoids from sustentacular, microvillous, or OSNs in the mouse OE. The injury-induced surge of endocannabinoid activates CB1 receptors on basal cells sufficient to promote proliferation, but not differentiation or survival. Evidence for this hypothesis is based on the rationale that a single intranasal instillation of exogenous cannabinoids or pharmacologically increasing endogenous cannabinoid is likened to the brief production of endocannabinoids after acute injury. A single administration of CB receptor synthetic agonist WIN is sufficient to increase basal cell proliferation marked by BrdU-incorporation well above control levels. Likewise, increase endocannabinoid bioavailability through inhibiting their degradative enzymes, results in an even greater increase in BrdU positive cells over vehicle. This phenomenon is not seen in CB1/CB2 receptor KO mice, suggesting CB receptor specificity and eliminating off target signaling. Additionally, the CB1 receptor-specific antagonist AM251 significantly decreased WIN-induced proliferation in the mouse OE, further implicating CB1 receptors. Cannabinoid-induced proliferation was noticed 24 hours after administration and lasted up to 3 days. Due to the lack of continued CB receptor activation, other trophic support, or inhibiting signals from mature OSNs, newly generated cells begin to undergo apoptosis 72 hours post CB receptor activation. This is supported by an increase in TUNEL positive cells in WT mice after WIN or JZL/URB instillation.

A final hypothesis is that multiple surges of cannabinoids over time, elicited through increased injury intensity, are sufficient for survival and differentiation of proliferating cells through a synergistic effect of cannabinoid signaling and increased

circulation of other trophic factors such as NPY. Additionally, endocannabinoids could have a protective effect on basal cells from toxic insult. This hypothesis is supported by the previously cited literature, including (1) increased calcium mobilization and ATP-induced calcium signaling in the mouse OE after injury, (2) on demand endocannabinoid synthesis from increases in intracellular calcium, (3) endocannabinoid signaling is increased after brain injury, and (4) provides protection and quicken recovery after injury.

However, it is also possible CB1 receptor activation can recruit Gq/11 proteins based on the calcium imaging data collected in Chapter 2, and evidence of WIN-induced Gq signaling in cultured hippocampal neurons (Lauckner et al., 2005). Gq protein activation, via the βγ subunit, has been shown to induced proliferation through PI3K/Akt/ GSK-3β signaling in osteoblasts (Katz et al., 2011), myocyte (Gosens et al., 2007), and neurons (Fournier et al., 2012). Similarly, *in vivo* and *in vitro* experiments indicate proliferative signal, vascular endothelial growth factor, can activate ERK and Akt signaling pathways in the adult rodent hippocampus and in cultures of hippocampal neuronal progenitor cells (Fournier et al., 2012). Additionally, proliferative actions of vascular endothelial growth factor require activation of both ERK and Akt signaling cascades, which are stimulated almost exclusively in BrdU positive proliferating neuronal progenitor cells (Fournier et al., 2012). Furthermore, Akt activation preceded ERK stimulation, suggesting that PI3K/Akt signaling might initiate proliferation, and subsequent ERK activation may help to stabilize and maintain cell cycle progression. Indeed, there is evidence that the cell survival PI3K/Akt pathway and mitogenic Ras/Ref/MEK/ERK cascades rarely act independently, and that there is cross-talk

between these signals (Aksamitiene et al., 2010). Therefore, alternative G-protein coupling to CB1 receptor action can converge on similar molecular pathways to include proliferation.

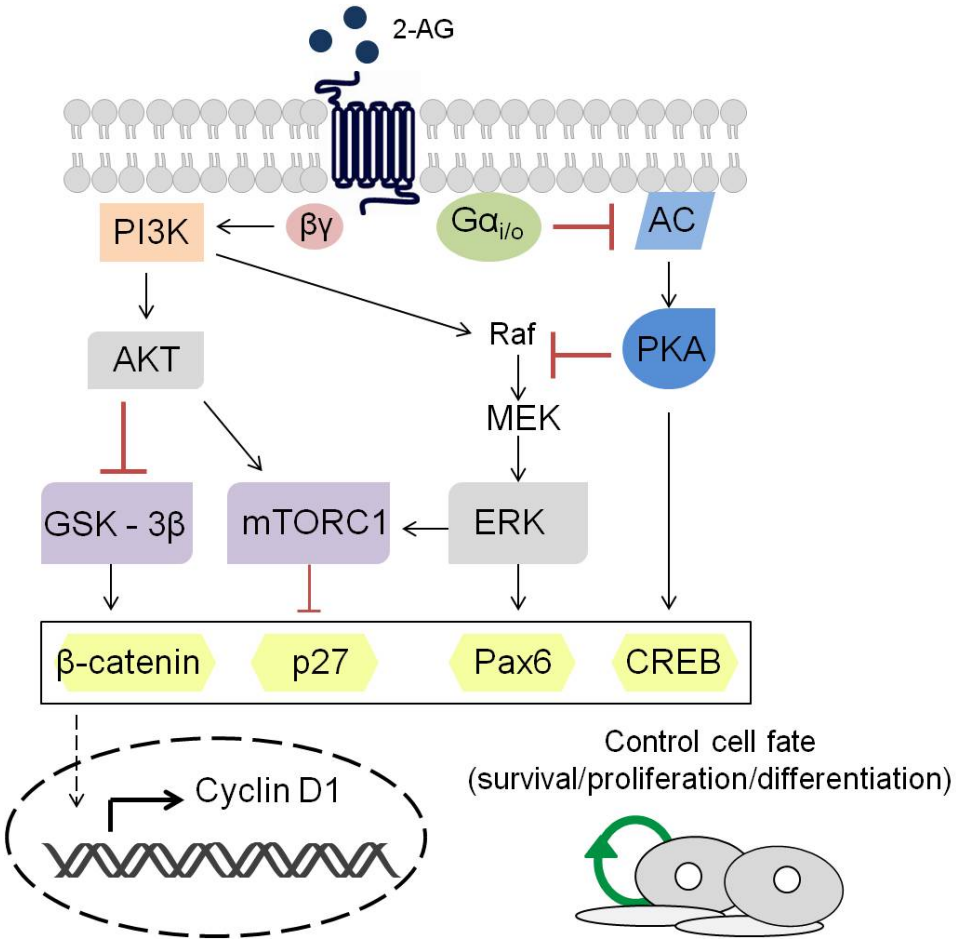

Figure 5.1 Schematic drawing of possible CB1 receptor signaling pathways in the regulation of progenitor cell fate. CB receptors are coupled to Gi proteins, thereby mediating the inhibition of adenylyl cyclase (AC) and PKA. CB receptor signaling is also associated with activation of the extracellular signal-regulated kinase (ERK) pathways through PKA disinhibition of Raf leading to MEK/ERK activation. CB1 receptor activation increases PI3K/Akt activity followed by phosphorylation of GSK-3β and β-catenin stabilization and translocation to the nucleus where it functions as a transcriptional regulator, modulating the expression of genes, such as *cyclin D1*, involved in the regulation of cell proliferation. PI3K/Akt can also activate mammalian target of

rapamycin complex 1 (mTOC1) pathway which, by inhibiting its downstream target p27, induces NP proliferation. Activation of CB receptors can ultimately control different transcriptional regulators, including CREB, p21,PAX-6, and β-catenin.

Broader impact of OE progenitor cell research

OE progenitor cell research has important implications for human health and disease states. Two major strategies currently used in stem cell-based therapy include transplantation of exogenous neural stem/progenitor cells into damaged brain tissue and stimulation of existing endogenous neural stem/progenitor cells (Taupin, 2006). Human OE progenitor cells are more readily available and easier to biopsy than in central stem cell niches. OE derived tissue and cells can be safely and easily collected from live human subjects and may provide a window into neural processes involved in disorders while avoiding the limitations of using postmortem brain samples or neuronal tissue. For example, OE-derived cell cultures from schizophrenic patients showed increased levels of a particular miRNA leading to increased gene expression through the fibroblast growth factor signaling pathway not found in non-schizophrenic patient OE cultured biopsies (Mor et al., 2013). This indicates that OE derived samples might serve to strengthen current studies when trying to identify biomarkers in disease states. An additional study involving Parkinson's disease patients showed that cultured OE neural progenitors from Parkinson's disease patients yielded dopaminergic cells in cultures as well as after implantation into the rodent brain (Murrell et al., 2008). Additionally, after transplantation, there was a reduction in behavioral deficits induced by ablation of site specific dopaminergic neurons in a rat model of Parkinson's disease (Murrell et al., 2008). Therefore, cannabinoid action on OE progenitor cells could contribute to the cultivation and transplantation of biopsied OE tissue to treat human neurological disease.

The research in this thesis could also have implications for intranasally delivered cannabinoid-based therapies in human health. Intranasal drug deliver is an important delivery option for brain targeting given that large and many small molecule drugs do not cross the blood-brain barrier, making the intranasal delivery route particularly attractive for drugs targeting neurodegenerative diseases. Drugs can reach the brain via trigeminal fibers or along neural bundle pathways (Hanson and Frey, 2008; Thorne et al., 2004). Previous studies have demonstrated that intranasal administration offers a simple, practical, non-invasive, cost-effective route for rapid drug delivery to the brain (Dorman et al., 2002; Liu et al., 2001; Vyas et al., 2005). Additionally, intranasal drug delivery not only circumvents the BBB, but also avoids systemic and other mechanisms of drug dilution, therefore lower effective doses are needed leading to reduced toxicity risks. One oralmucosal delivery route of a cannabinoid drug is patented and approved in Europe to treat symptoms in adult patients with moderate to severe spasticity due to multiple sclerosis. Therefore, all *in vivo* research performed in this thesis could contribute to an intranasal drug delivery route for cannabinoid based therapies. Cannabinoid-mediated proliferation in the mouse OE could monitor other physiological and pathophysiological conditions including olfaction during aging and under various neurodegenerative diseases.

OE and the cannabinoid system during aging

OE homeostasis is maintained through the proliferation and differentiation of basal progenitor cells throughout adulthood. Homeostatic changes associated with physiological aging of the OE include decreases in (1) olfactory progenitor cell proliferation, (2) numbers of neurons and sustentacular cells, and (3) rate of cell death

(Kondo et al., 2010; Loo et al., 1996; Weiler and Farbman, 1997a, 1998). Similar changes are seen in the OE from aged humans exhibiting reduced epithelium thickness and reduced number of sensory neurons (Naessen, 1971; Nakashima et al., 1984). Despite a remarkable ability to regenerate, OE impairment with age can be caused by both extrinsic mechanisms, such as cumulative damage from environmental pollutants, and intrinsic mechanisms, such as a reduction in trophic factor signaling. Age-related loss of olfactory function has also been observed in rodents (Enwere et al., 2004) but olfactory deficits are more pronounced in the human population in which half of the population of 65 years old suffers from olfaction deficits (Murphy et al., 2002; Stevens and Cain, 1987). Functional decline in olfaction could be related to changes in the structure of the olfactory system leading to functional decreases in proliferative and neurogenic capacity (Loo et al., 1996). For instance, neural progenitors grown from human olfactory biopsy are able to generate dopamine neurons in vitro, and after transplantation into the rat brain. These newly generated dopamine neurons were able to reduce symptoms of neurodegenerative disease in a rat model of Parkinson's disease demonstrating the ability to restore function (Murrell et al., 2008). The olfactory mucosa is accessible in living adult humans and is therefore a source of tissue useful for studying the biology of adult neurogenesis in health and disease (Mackay-Sim et al., 2008). It is also a source of cells for transplantation (regenerative medicine) of the nervous system (Feron et al., 2005; Mackay-Sim et al., 2008). Understanding the biology of olfactory neural stem cells and their niche will be very important for optimizing clinical applications.

Similarly, in the CNS, the ability and frequency of adult neural stem cells to self renew is compromised with age (Ahn and Joyner, 2005; Kuhn et al., 1996), with a concurrent decline in transcription factors (Nishino et al., 2008). Progenitor cell proliferation in the SVZ declines dramatically with age (Luo et al., 2006) leading to decreases in migration and interneuron integration in the OB. In 6 month old mice proliferation has decreased approximately 85% compared to younger (6 week old) animals. CB2 receptor agonist or WIN application was able to increase proliferation in the SVZ and migration and integration into the OB in aged mice (Goncalves et al., 2008). Additionally, 20 month old mice showed nearly no proliferation in the SVZ, but ten days of WIN treatment restored neurogenesis to the level normally found in the 6 month old mouse. This suggests. CB receptors are still present in older animals and are functionally coupled to cell proliferation and neurogenesis, yet endocannabinoid synthesis and release may still be compromised (Goncalves et al., 2008). Additionally, a low dose of WIN administration for 3 weeks can partially restore neurogenesis in the hippocampus of aged (23 months) rats (Marchalant et al., 2009a). The importance of an endocannabinoid regulatory tone on neurogenesis is also highlighted by the observation that CB1 receptor KO mice suffer from early onset age-related cognitive impairment (Bilkei-Gorzo et al., 2005), a potential consequence of aging-associated decrease in cortical neurogenesis in the absence of CB1 receptors (Lie et al., 2004). CB1 receptor KO mice also exhibit early onset age-related memory decline in tasks investigating reward and aversion-driven learning (Albayram et al., 2012). Cannabinoid-mediated beneficial effect in the aged brain may signal through mTORC1 pathways. For example, mTORC1 activation in progenitor cells in adult neurogenic brain areas reverts progenitor

quiescence to active proliferative states in the aged brain (Paliouras et al., 2012). Thus, mTORC1 activation by CB receptors may contribute to injury-induced neural progenitor activation (Aguado et al., 2007; Palazuelos et al., 2012) and alleviate aging-associated decline of neurogenesis (Goncalves et al., 2008; Marchalant et al., 2009a). Collectively, these data suggest that the cannabinoid system, through regulation of proliferation, migration, and integration of new neurons, can help restore cell numbers and potentially function of the CNS during aging. However, no work has been done to investigate the effect of the cannabinoid system in physiological aging in the OE, but the above data suggest a possible role for the cannabinoid system in olfactory restoration during aging.

OE and the cannabinoid system in neurodegenerative diseases

In addition to physiological aging, the cannabinoid system could contribute to the pathological aged state of the OE. The role of adult neurogenesis in neurological disorders and disease is still poorly characterized and may help to repair the CNS in response to brain injury or disease. Olfactory dysfunction is a common and early symptom of many neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Albers et al., 2006; Haehner et al., 2011). The neuropathological changes in olfactory dysfunction in neurodegenerative diseases may involve the OE, OB tract, primary olfactory corticies, and their secondary targets. Olfactory dysfunction presents in approximately 90% of early cases of PD and can precede the onset of motor symptoms by years (Doty, 1989). Recent studies of olfactory dysfunction in neurodegenerative disorders target alterations in olfaction as an early biomarker for the diagnosis and disease progression (Attems et al., 2014). Many environmental risk factors for PD, including older age, head trauma, and exposure to

metal ions, viruses, and pesticides are also risk factors for anosmia, independent of PD. Air pollution and toxic nanoparticles can enter the brain via the OE and induce inflammatory responses and PD like neuropathology in the OB and other brain structures. Toxicants found in diesel exhaust and air pollution initiate PD and AD like neuropathology in human OB (Calderon-Garciduenas et al., 2004; Levesque et al., 2011). Furthermore, postmortem studies have identified the accumulation of ultrafine particulate matter and in the OB and OE along with abnormal immunoreactivity to indicators of PD (Calderon-Garciduenas et al., 2011; Calderon-Garciduenas et al., 2010; Calderon-Garciduenas et al., 2012). In a meta-analysis of the effect of AD and PD on olfaction, results indicate that AD and PD patients are more impaired in odorant identification and recognition tasks than in odorant detection threshold tasks. In addition, PD patients are more impaired on detection threshold tasks than AD patients (Rahayel et al., 2012). These results suggest that PD patients have increased dysfunction during low-level perceptual olfactory tasks, whereas AD patients are more limited on higher-order olfactory tasks involving specific cognitive process.

Additionally, deregulation of the endocannabinoid system is seen in neurological disorders associated with executive dysfunctions such as schizophrenia (Ashton and Moore, 2011), AD (Koppel et al., 2009), and PD (Benarroch, 2007; Fernandez-Ruiz, 2009). CB receptors are known to be neuroprotective, further implicating their effect on acute and chronic neurodegenerative disorders. In AD, CB1 and CB2 receptors are localized to senile plaques along with microglia activation markers (Ramirez et al., 2005). Additionally, cortical CB1 positive neurons are lost and CB1 receptor expression and function are markedly decreased in AD (Ramirez et al.,

2005). Furthermore, in AD models, *in vivo* cannabinoids partially prevent cognitive impairment, while reducing the loss of neuronal and glial cells (Ramirez et al., 2005; van der Stelt et al., 2006). CB2 receptors are also implicated in AD. CB2 receptors are unregulated both in reactive microglia on senile plaques in individuals with AD (Benito et al., 2003), and in lesioned striatum in a rat model replicating human AD pathology (Fernandez-Ruiz and Gonzales, 2005). In PD patients endocannabinoid levels in the cerebrospinal fluid are increased (Pisani et al., 2005) and basal ganglia CB1 receptor mRNA is upregulated in a rodent model of PD. Despite this upregulation of the CB system that is observed at intermediate and late stages of the disease process, in the pre-symptomatic phase of PD, CB1 receptors are down regulated (Garcia-Arencibia et al., 2009) which may render the basal ganglia more vulnerable to excitotoxic injury due to the loss of CB1 receptor -mediated presynaptic inhibition of glutamate release (van der Stelt et al., 2005). Collectively, these data suggest that impairment in olfaction can be used as a viable clinical early indication of neuronal dysfunction, and that targeting the cannabinoid system for therapeutic actions could prevent the progression of the disease state. Therefore, olfactory-mediated endpoints of therapeutic cannabinoid intervention are possible. However, no work has been done to investigate the effect of the cannabinoid system in physiological aging in the OE, but the above data suggest a possible role for the cannabinoid system in olfactory restoration during aging.

Conclusions

For the first time in the mouse OE, a cannabinoid system has been identified and found to contribute to tissue homeostasis through the regulation of cell survival, proliferation, and differentiation. The studies in this thesis cohesively imply the balance of progenitor cell survival and proliferation in adult mouse OE is regulated by CB receptor signaling. Identification of signaling molecules that influence progenitor cell proliferation in physiological conditions will lead to investigation of the mechanisms responsible for initiating enhanced neuroregeneration under aging and pathological conditions. Most importantly, biopsied olfactory tissue for neuronal degenerative diseases research will contain the necessary molecular components to establish a functional cannabinoid system as a therapeutic target. Additionally, as new cannabinoid-based therapeutic become available, research using an intranasal drug deliver approach will advance pre-clinical safety and drug efficacy studies.

LITERATURE CITED

LITERATURE CITED

Agarwal N, Pacher P, Tegeder I, Amaya F, Constantin CE, Brenner GJ, Rubino T, Michalski CW, Marsicano G, Monory K, Mackie K, Marian C, Batkai S, Parolaro D, Fischer MJ, Reeh P, Kunos G, Kress M, Lutz B, Woolf CJ, Kuner R (2007) Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. Nat Neurosci 10:870-879

Aguado T, Monory K, Palazuelos J, Stella N, Cravatt B, Lutz B, Marsicano G, Kokaia Z, Guzman M, Galve-Roperh I (2005) The endocannabinoid system drives neural progenitor proliferation. FASEB J 19:1704-1706

Aguado T, Palazuelos J, Monory K, Stella N, Cravatt B, Lutz B, Marsicano G, Kokaia Z, Guzman M, Galve-Roperh I (2006) The endocannabinoid system promotes astroglial differentiation by acting on neural progenitor cells. J Neurosci 26:1551-1561

Aguado T, Romero E, Monory K, Palazuelos J, Sendtner M, Marsicano G, Lutz B, Guzman M, Galve-Roperh I (2007) The CB1 cannabinoid receptor mediates excitotoxicity-induced neural progenitor proliferation and neurogenesis. J Biol Chem 282:23892-23898

Ahluwalia J, Urban L, Bevan S, Nagy I (2003) Anandamide regulates neuropeptide release from capsaicin-sensitive primary sensory neurons by activating both the cannabinoid 1 receptor and the vanilloid receptor 1 in vitro. Eur J Neurosci 17:2611-2618

Ahn S, Joyner AL (2005) In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 437:894-897

Akerman S, Kaube H, Goadsby PJ (2004) Anandamide is able to inhibit trigeminal neurons using an in vivo model of trigeminovascular-mediated nociception. J Pharmacol Exp Ther 309:56-63

Aksamitiene E, Kholodenko BN, Kolch W, Hoek JB, Kiyatkin A (2010) PI3K/Akt-sensitive MEK-independent compensatory circuit of ERK activation in ER-positive PI3K-mutant T47D breast cancer cells. Cell Signal 22:1369-1378

Albayram O, Bilkei-Gorzo A, Zimmer A (2012) Loss of CB1 receptors leads to differential age-related changes in reward-driven learning and memory. Front Aging Neurosci 4:34

Albers MW, Tabert MH, Devanand DP (2006) Olfactory dysfunction as a predictor of neurodegenerative disease. Curr Neurol Neurosci Rep 6:379-386

Alger BE, Kim J (2011) Supply and demand for endocannabinoids. Trends Neurosci 34:304-315

Alger BE, Tang AH (2010) Do cannabinoids reduce brain power? Nat Neurosci 15:499-501

Ali AB, Todorova M (2010) Asynchronous release of GABA via tonic cannabinoid receptor activation at identified interneuron synapses in rat CA1. Eur J Neurosci 31:1196-1207

Altman J (1969) Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol 137:433-457

Alvarez-Buylla A (1997) Mechanism of migration of olfactory bulb interneurons. Semin Cell Dev Biol 8:207-213

Alvarez P, Eichenbaum H (2002) Representations of odors in the rat orbitofrontal cortex change during and after learning. Behav Neurosci 116:421-433

Amstrup J, Novak I (2003) P2X7 receptor activates extracellular signal-regulated kinases ERK1 and ERK2 independently of Ca2+ influx. Biochem J 374:51-61

Anand U, Otto WR, Sanchez-Herrera D, Facer P, Yiangou Y, Korchev Y, Birch R, Benham C, Bountra C, Chessell IP, Anand P (2008) Cannabinoid receptor CB2 localisation and agonist-mediated inhibition of capsaicin responses in human sensory neurons. Pain 138:667-680

Arevalo-Martin A, Garcia-Ovejero D, Rubio-Araiz A, Gomez O, Molina-Holgado F, Molina-Holgado E (2007) Cannabinoids modulate Olig2 and polysialylated neural cell adhesion molecule expression in the subventricular zone of post-natal rats through cannabinoid receptor 1 and cannabinoid receptor 2. Eur J Neurosci 26:1548-1559

Aroniadou-Anderjaska V, Zhou FM, Priest CA, Ennis M, Shipley MT (2000) Tonic and synaptically evoked presynaptic inhibition of sensory input to the rat olfactory bulb via GABA(B) heteroreceptors. J Neurophysiol 84:1194-1203

Ashton CH, Moore PB (2011) Endocannabinoid system dysfunction in mood and related disorders. Acta Psychiatr Scand 124:250-261

Ashton CH, Moore PB, Gallagher P, Young AH (2005) Cannabinoids in bipolar affective disorder: a review and discussion of their therapeutic potential. J Psychopharmacol 19:293-300

Aso E, Ozaita A, Valdizan EM, Ledent C, Pazos A, Maldonado R, Valverde O (2008) BDNF impairment in the hippocampus is related to enhanced despair behavior in CB1 knockout mice. J Neurochem 105:565-572

Attems J, Walker L, Jellinger KA (2014) Olfactory bulb involvement in neurodegenerative diseases. Acta Neuropathol 127:459-475

Bacci A, Huguenard JR, Prince DA (2004) Long-lasting self-inhibition of neocortical interneurons mediated by endocannabinoids. Nature 431:312-316

Baker D, Pryce G, Davies WL, Hiley CR (2006) In silico patent searching reveals a new cannabinoid receptor. Trends Pharmacol Sci 27:1-4

Bari M, Battista N, Fezza F, Finazzi-Agro A, Maccarrone M (2005) Lipid rafts control signaling of type-1 cannabinoid receptors in neuronal cells. Implications for anandamide-induced apoptosis. J Biol Chem 280:12212-12220

Bari M, Tedesco M, Battista N, Pasquariello N, Pucci M, Gasperi V, Scaldaferri ML, Farini D, De Felici M, Maccarrone M (2011) Characterization of the endocannabinoid system in mouse embryonic stem cells. Stem Cells Dev 20:139-147

Barres BA, Schmid R, Sendnter M, Raff MC (1993) Multiple extracellular signals are required for long-term oligodendrocyte survival. Development 118:283-295

Bash R, Rubovitch V, Gafni M, Sarne Y (2003) The stimulatory effect of cannabinoids on calcium uptake is mediated by Gs GTP-binding proteins and cAMP formation. Neurosignals 12:39-44

Bauer S, Patterson PH (2005) The cell cycle-apoptosis connection revisited in the adult brain. J Cell Biol 171:641-650

Bayer SA (1982) Changes in the total number of dentate granule cells in juvenile and adult rats: a correlated volumetric and 3H-thymidine autoradiographic study. Exp Brain Res 46:315-323

Bazan NG, Jr. (1970) Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. Biochim Biophys Acta 218:1-10

Becker AJ, Mc CE, Till JE (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 197:452-454

Begg M, Pacher P, Batkai S, Osei-Hyiaman D, Offertaler L, Mo FM, Liu J, Kunos G (2005) Evidence for novel cannabinoid receptors. Pharmacol Ther 106:133-145

Beites CL, Kawauchi S, Crocker CE, Calof AL (2005) Identification and molecular regulation of neural stem cells in the olfactory epithelium. Exp Cell Res 306:309-316

Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, Piomelli D (1997) Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science 277:1094-1097

Benarroch E (2007) Endocannabinoids in basal ganglia circuits: implications for Parkinson disease. Neurology 69:306-309

Benito C, Nunez E, Tolon RM, Carrier EJ, Rabano A, Hillard CJ, Romero J (2003) Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. J Neurosci 23:11136-11141

Berghuis P, Dobszay MB, Wang X, Spano S, Ledda F, Sousa KM, Schulte G, Ernfors P, Mackie K, Paratcha G, Hurd YL, Harkany T (2005) Endocannabinoids regulate interneuron migration and morphogenesis by transactivating the TrkB receptor. Proc Natl Acad Sci U S A 102:19115-19120

Berrendero F, Romero J, Garcia-Gil L, Suarez I, De la Cruz P, Ramos JA, Fernandez-Ruiz JJ (1998) Changes in cannabinoid receptor binding and mRNA levels in several brain regions of aged rats. Biochim Biophys Acta 1407:205-214

Berrendero F, Sepe N, Ramos JA, Di Marzo V, Fernandez-Ruiz JJ (1999) Analysis of cannabinoid receptor binding and mRNA expression and endogenous cannabinoid contents in the developing rat brain during late gestation and early postnatal period. Synapse 33:181-191

Bilkei-Gorzo A, Racz I, Valverde O, Otto M, Michel K, Sastre M, Zimmer A (2005) Early age-related cognitive impairment in mice lacking cannabinoid CB1 receptors. Proc Natl Acad Sci U S A 102:15670-15675

Bisogno T, Howell F, Williams G, Minassi A, Cascio MG, Ligresti A, Matias I, Schiano-Moriello A, Paul P, Williams EJ, Gangadharan U, Hobbs C, Di Marzo V, Doherty P (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. J Cell Biol 163:463-468

Bisogno T, Mahadevan A, Coccurello R, Chang JW, Allara M, Chen Y, Giacovazzo G, Lichtman A, Cravatt B, Moles A, Di Marzo V (2013) A novel fluorophosphonate inhibitor of the biosynthesis of the endocannabinoid 2-arachidonoylglycerol with potential anti-obesity effects. Br J Pharmacol 169:784-793

Bisogno T, Melck D, De Petrocellis L, Di Marzo V (1999) Phosphatidic acid as the biosynthetic precursor of the endocannabinoid 2-arachidonoylglycerol in intact mouse neuroblastoma cells stimulated with ionomycin. J Neurochem 72:2113-2119

Blanco-Hernandez E, Valle-Leija P, Zomosa-Signoret V, Drucker-Colin R, Vidaltamayo R (2012) Odor memory stability after reinnervation of the olfactory bulb. PLoS One 7:e46338

Blankman JL, Simon GM, Cravatt BF (2007) A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. Chem Biol 14:1347-1356

Bodor AL, Katona I, Nyiri G, Mackie K, Ledent C, Hajos N, Freund TF (2005) Endocannabinoid signaling in rat somatosensory cortex: laminar differences and involvement of specific interneuron types. J Neurosci 25:6845-6856

Bonfanti L, Theodosis DT (1994) Expression of polysialylated neural cell adhesion molecule by proliferating cells in the subependymal layer of the adult rat, in its rostral extension and in the olfactory bulb. Neuroscience 62:291-305

Bonhaus DW, Chang LK, Kwan J, Martin GR (1998) Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. J Pharmacol Exp Ther 287:884-888

Bortolato M, Mangieri RA, Fu J, Kim JH, Arguello O, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D (2007) Antidepressant-like activity of the fatty acid amide hydrolase inhibitor URB597 in a rat model of chronic mild stress. Biol Psychiatry 62:1103-1110

Bouaboula M, Hilairet S, Marchand J, Fajas L, Le Fur G, Casellas P (2005) Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation. Eur J Pharmacol 517:174-181

Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G, Casellas P (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. Biochem J 312 (Pt 2):637-641

Bouvet JF, Delaleu JC, Holley A (1987) Olfactory receptor cell function is affected by trigeminal nerve activity. Neurosci Lett 77:181-186

Bovetti S, Veyrac A, Peretto P, Fasolo A, De Marchis S (2009) Olfactory enrichment influences adult neurogenesis modulating GAD67 and plasticity-related molecules expression in newborn cells of the olfactory bulb. PLoS One 4:e6359

Bradshaw HB, Walker JM (2005) The expanding field of cannabimimetic and related lipid mediators. Br J Pharmacol 144:459-465

Brann JH, Firestein S (2010) Regeneration of new neurons is preserved in aged vomeronasal epithelia. J Neurosci 30:15686-15694

Breunig E, Czesnik D, Piscitelli F, Di Marzo V, Manzini I, Schild D (2010a) Endocannabinoid modulation in the olfactory epithelium. Results Probl Cell Differ 52:139-145

Breunig E, Manzini I, Piscitelli F, Gutermann B, Di Marzo V, Schild D, Czesnik D (2010b) The endocannabinoid 2-arachidonoyl-glycerol controls odor sensitivity in larvae of Xenopus laevis. J Neurosci 30:8965-8973

Bridges D, Rice AS, Egertova M, Elphick MR, Winter J, Michael GJ (2003) Localisation of cannabinoid receptor 1 in rat dorsal root ganglion using in situ hybridisation and immunohistochemistry. Neuroscience 119:803-812

Bromberg KD, Ma'ayan A, Neves SR, Iyengar R (2008) Design logic of a cannabinoid receptor signaling network that triggers neurite outgrowth. Science 320:903-909

Bruel-Jungerman E, Davis S, Rampon C, Laroche S (2006) Long-term potentiation enhances neurogenesis in the adult dentate gyrus. J Neurosci 26:5888-5893

Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11:297-305

Buckland ME, Cunningham AM (1999) Alterations in expression of the neurotrophic factors glial cell line-derived neurotrophic factor, ciliary neurotrophic factor and brain-derived neurotrophic factor, in the target-deprived olfactory neuroepithelium. Neuroscience 90:333-347

Buckley NE, McCoy KL, Mezey E, Bonner T, Zimmer A, Felder CC, Glass M (2000) Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. Eur J Pharmacol 396:141-149

Buczynski MW, Parsons LH (2010) Quantification of brain endocannabinoid levels: methods, interpretations and pitfalls. Br J Pharmacol 160:423-442

Cadas H, Gaillet S, Beltramo M, Venance L, Piomelli D (1996a) Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. J Neurosci 16:3934-3942

Cadas H, Schinelli S, Piomelli D (1996b) Membrane localization of N-acylphosphatidylethanolamine in central neurons: studies with exogenous phospholipases. J Lipid Mediat Cell Signal 14:63-70

Caffarel MM, Sarrio D, Palacios J, Guzman M, Sanchez C (2006) Delta9-tetrahydrocannabinol inhibits cell cycle progression in human breast cancer cells through Cdc2 regulation. Cancer Res 66:6615-6621

Caggiano M, Kauer JS, Hunter DD (1994) Globose basal cells are neuronal progenitors in the olfactory epithelium: a lineage analysis using a replication-incompetent retrovirus. Neuron 13:339-352

Calderon-Garciduenas L, Engle R, Mora-Tiscareno A, Styner M, Gomez-Garza G, Zhu H, Jewells V, Torres-Jardon R, Romero L, Monroy-Acosta ME, Bryant C, Gonzalez-Gonzalez LO, Medina-Cortina H, D'Angiulli A (2011) Exposure to severe urban air pollution influences cognitive outcomes, brain volume and systemic inflammation in clinically healthy children. Brain Cogn 77:345-355

Calderon-Garciduenas L, Franco-Lira M, Henriquez-Roldan C, Osnaya N, Gonzalez-Maciel A, Reynoso-Robles R, Villarreal-Calderon R, Herritt L, Brooks D, Keefe S, Palacios-Moreno J, Torres-Jardon R, Medina-Cortina H, Delgado-Chavez R, Aiello-Mora M, Maronpot RR, Doty RL (2010) Urban air pollution: influences on olfactory function and pathology in exposed children and young adults. Exp Toxicol Pathol 62:91-102

Calderon-Garciduenas L, Reed W, Maronpot RR, Henriquez-Roldan C, Delgado-Chavez R, Calderon-Garciduenas A, Dragustinovis I, Franco-Lira M, Aragon-Flores M, Solt AC, Altenburg M, Torres-Jardon R, Swenberg JA (2004) Brain inflammation and Alzheimer's-like pathology in individuals exposed to severe air pollution. Toxicol Pathol 32:650-658

Calderon-Garciduenas L, Serrano-Sierra A, Torres-Jardon R, Zhu H, Yuan Y, Smith D, Delgado-Chavez R, Cross JV, Medina-Cortina H, Kavanaugh M, Guilarte TR (2012) The impact of environmental metals in young urbanites' brains. Exp Toxicol Pathol 65:503-511

Calignano A, La Rana G, Giuffrida A, Piomelli D (1998) Control of pain initiation by endogenous cannabinoids. Nature 394:277-281

Calof AL, Bonnin A, Crocker C, Kawauchi S, Murray RC, Shou J, Wu HH (2002) Progenitor cells of the olfactory receptor neuron lineage. Microsc Res Tech 58:176-188

Calof AL, Hagiwara N, Holcomb JD, Mumm JS, Shou J (1996) Neurogenesis and cell death in olfactory epithelium. J Neurobiol 30:67-81

Cameron HA, Woolley CS, McEwen BS, Gould E (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56:337-344

Carey MR, Myoga MH, McDaniels KR, Marsicano G, Lutz B, Mackie K, Regehr WG (2010) Presynaptic CB1 receptors regulate synaptic plasticity at cerebellar parallel fiber synapses. J Neurophysiol 105:958-963

Carrier EJ, Kearn CS, Barkmeier AJ, Breese NM, Yang W, Nithipatikom K, Pfister SL, Campbell WB, Hillard CJ (2004) Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism. Mol Pharmacol 65:999-1007

Carter LA, MacDonald JL, Roskams AJ (2004) Olfactory horizontal basal cells demonstrate a conserved multipotent progenitor phenotype. J Neurosci 24:5670-5683

Castillo PE, Younts TJ, Chavez AE, Hashimotodani Y (2012) Endocannabinoid signaling and synaptic function. Neuron 76:70-81

Cau E, Casarosa S, Guillemot F (2002) Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development 129:1871-1880

Cencioni MT, Chiurchiu V, Catanzaro G, Borsellino G, Bernardi G, Battistini L, Maccarrone M (2010) Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors. PLoS One 5:e8688

Cesa R, Mackie K, Beltramo M, Franzoni MF (2001) Cannabinoid receptor CB1-like and glutamic acid decarboxylase-like immunoreactivities in the brain of Xenopus laevis. Cell Tissue Res 306:391-398

Chanda PK, Gao Y, Mark L, Btesh J, Strassle BW, Lu P, Piesla MJ, Zhang MY, Bingham B, Uveges A, Kowal D, Garbe D, Kouranova EV, Ring RH, Bates B, Pangalos MN, Kennedy JD, Whiteside GT, Samad TA (2010) Monoacylglycerol lipase activity is a critical modulator of the tone and integrity of the endocannabinoid system. Mol Pharmacol 78:996-1003

Chen X, Fang H, Schwob JE (2004a) Multipotency of purified, transplanted globose basal cells in olfactory epithelium. J Comp Neurol 469:457-474

Chen X, Fang H, Schwob JE (2004b) Multipotency of purified, transplanted globose basal cells in olfactory epithelium. J Comp Neurol 469:457-474

Chen XP, Yang W, Fan Y, Luo JS, Hong K, Wang Z, Yan JF, Chen X, Lu JX, Benovic JL, Zhou NM (2010) Structural determinants in the second intracellular loop of the human cannabinoid CB1 receptor mediate selective coupling to G(s) and G(i). Br J Pharmacol 161:1817-1834

Chenn A, Walsh CA (2003) Increased neuronal production, enlarged forebrains and cytoarchitectural distortions in beta-catenin overexpressing transgenic mice. Cereb Cortex 13:599-606

Cheung MC, Jang W, Schwob JE, Wachowiak M (2014) Functional recovery of odor representations in regenerated sensory inputs to the olfactory bulb. Front Neural Circuits 7:207

Chevaleyre V, Takahashi KA, Castillo PE (2006) Endocannabinoid-mediated synaptic plasticity in the CNS. Annu Rev Neurosci 29:37-76

Childers SR, Pacheco MA, Bennett BA, Edwards TA, Hampson RE, Mu J, Deadwyler SA (1993) Cannabinoid receptors: G-protein-mediated signal transduction mechanisms. Biochem Soc Symp 59:27-50

Collinson JM, Quinn JC, Hill RE, West JD (2003) The roles of Pax6 in the cornea, retina, and olfactory epithelium of the developing mouse embryo. Dev Biol 255:303-312

Compagnucci C, Di Siena S, Bustamante MB, Di Giacomo D, Di Tommaso M, Maccarrone M, Grimaldi P, Sette C (2013) Type-1 (CB1) cannabinoid receptor promotes neuronal differentiation and maturation of neural stem cells. PLoS One 8:e54271

Compton DR, Gold LH, Ward SJ, Balster RL, Martin BR (1992) Aminoalkylindole analogs: cannabimimetic activity of a class of compounds structurally distinct from delta 9-tetrahydrocannabinol. J Pharmacol Exp Ther 263:1118-1126

Cooper-Kuhn CM, Kuhn HG (2002) Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res 134:13-21

Costanzo RM (2000) Rewiring the olfactory bulb: changes in odor maps following recovery from nerve transection. Chem Senses 25:199-205

Costanzo RM, Graziadei PP (1983) A quantitative analysis of changes in the olfactory epithelium following bulbectomy in hamster. J Comp Neurol 215:370-381

Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc Natl Acad Sci U S A 98:9371-9376

Cravatt BF, Lichtman AH (2004) The endogenous cannabinoid system and its role in nociceptive behavior. J Neurobiol 61:149-160

Crawley JN, Corwin RL, Robinson JK, Felder CC, Devane WA, Axelrod J (1993) Anandamide, an endogenous ligand of the cannabinoid receptor, induces hypomotility and hypothermia in vivo in rodents. Pharmacol Biochem Behav 46:967-972

Cremer H, Lange R, Christoph A, Plomann M, Vopper G, Roes J, Brown R, Baldwin S, Kraemer P, Scheff S, et al. (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 367:455-459

Crino PB (2011) mTOR: A pathogenic signaling pathway in developmental brain malformations. Trends Mol Med 17:734-742

Cristino L, de Petrocellis L, Pryce G, Baker D, Guglielmotti V, Di Marzo V (2006) Immunohistochemical localization of cannabinoid type 1 and vanilloid transient receptor potential vanilloid type 1 receptors in the mouse brain. Neuroscience 139:1405-1415

Culbert AA, Brown MJ, Frame S, Hagen T, Cross DA, Bax B, Reith AD (2001) GSK-3 inhibition by adenoviral FRAT1 overexpression is neuroprotective and induces Tau dephosphorylation and beta-catenin stabilisation without elevation of glycogen synthase activity. FEBS Lett 507:288-294

Curtis MA, Eriksson PS, Faull RL (2007) Progenitor cells and adult neurogenesis in neurodegenerative diseases and injuries of the basal ganglia. Clin Exp Pharmacol Physiol 34:528-532

Czesnik D, Kuduz J, Schild D, Manzini I (2006) ATP activates both receptor and sustentacular supporting cells in the olfactory epithelium of Xenopus laevis tadpoles. Eur J Neurosci 23:119-128

Czesnik D, Schild D, Kuduz J, Manzini I (2007) Cannabinoid action in the olfactory epithelium. Proc Natl Acad Sci U S A 104:2967-2972

Dalton GD, Howlett AC (2012) Cannabinoid CB1 receptors transactivate multiple receptor tyrosine kinases and regulate serine/threonine kinases to activate ERK in neuronal cells. Br J Pharmacol 165:2497-2511

Davis JA, Reed RR (1996) Role of Olf-1 and Pax-6 transcription factors in neurodevelopment. J Neurosci 16:5082-5094

Davis MI, Ronesi J, Lovinger DM (2003) A predominant role for inhibition of the adenylate cyclase/protein kinase A pathway in ERK activation by cannabinoid receptor 1 in N1E-115 neuroblastoma cells. J Biol Chem 278:48973-48980

De March Z, Zuccato C, Giampa C, Patassini S, Bari M, Gasperi V, De Ceballos ML, Bernardi G, Maccarrone M, Cattaneo E, Fusco FR (2008) Cortical expression of brain derived neurotrophic factor and type-1 cannabinoid receptor after striatal excitotoxic lesions. Neuroscience 152:734-740

De Vries TJ, Schoffelmeer AN (2005) Cannabinoid CB1 receptors control conditioned drug seeking. Trends Pharmacol Sci 26:420-426

den Boon FS, Chameau P, Schaafsma-Zhao Q, van Aken W, Bari M, Oddi S, Kruse CG, Maccarrone M, Wadman WJ, Werkman TR (2012) Excitability of prefrontal cortical pyramidal neurons is modulated by activation of intracellular type-2 cannabinoid receptors. Proc Natl Acad Sci U S A 109:3534-3539

Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci 11:339-350

Derkinderen P, Valjent E, Toutant M, Corvol JC, Enslen H, Ledent C, Trzaskos J, Caboche J, Girault JA (2003) Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. J Neurosci 23:2371-2382

Deutsch DG, Chin SA (1993) Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. Biochem Pharmacol 46:791-796

Devane WA, Dysarz FA, 3rd, Johnson MR, Melvin LS, Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 34:605-613

Di Marzo V (1999) Biosynthesis and inactivation of endocannabinoids: relevance to their proposed role as neuromodulators. Life Sci 65:645-655

Di Marzo V (2011) Endocannabinoid signaling in the brain: biosynthetic mechanisms in the limelight. Nat Neurosci 14:9-15

Di Marzo V, Breivogel CS, Tao Q, Bridgen DT, Razdan RK, Zimmer AM, Zimmer A, Martin BR (2000) Levels, metabolism, and pharmacological activity of anandamide in CB(1) cannabinoid receptor knockout mice: evidence for non-CB(1), non-CB(2) receptor-mediated actions of anandamide in mouse brain. J Neurochem 75:2434-2444

Di Marzo V, Cristino L (2008) Why endocannabinoids are not all alike. Nat Neurosci 11:124-126

Di Marzo V, De Petrocellis L (2012) Why do cannabinoid receptors have more than one endogenous ligand? Philos Trans R Soc Lond B Biol Sci 367:3216-3228

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372:686-691

Di Marzo V, Matias I (2005) Endocannabinoid control of food intake and energy balance. Nat Neurosci 8:585-589

Di Marzo V, Petrosino S (2007) Endocannabinoids and the regulation of their levels in health and disease. Curr Opin Lipidol 18:129-140

Di VF, Steinberg TH, Silverstein SC (1990) Inhibition of Fura-2 sequestration and secretion with organic anion transport blockers. Cell Calcium 11:57-62

Diaz-Alonso J, Aguado T, de Salas-Quiroga A, Ortega Z, Guzman M, Galve-Roperh I (2014) CB1 Cannabinoid Receptor-Dependent Activation of mTORC1/Pax6 Signaling Drives Tbr2 Expression and Basal Progenitor Expansion in the Developing Mouse Cortex. Cereb Cortex

Diaz-Alonso J, Guzman M, Galve-Roperh I (2012) Endocannabinoids via CB(1) receptors act as neurogenic niche cues during cortical development. Philos Trans R Soc Lond B Biol Sci 367:3229-3241

Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D (2002) Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci U S A 99:10819-10824

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Regeneration of a germinal layer in the adult mammalian brain. Proc Natl Acad Sci U S A 96:11619-11624

Dominguez I, Green JB (2001) Missing links in GSK3 regulation. Dev Biol 235:303-313

Dorman DC, Brenneman KA, McElveen AM, Lynch SE, Roberts KC, Wong BA (2002) Olfactory transport: a direct route of delivery of inhaled manganese phosphate to the rat brain. J Toxicol Environ Health A 65:1493-1511

Dorsam RT, Gutkind JS (2007) G-protein-coupled receptors and cancer. Nat Rev Cancer 7:79-94

Doty RL (1989) Influence of age and age-related diseases on olfactory function. Ann NY Acad Sci 561:76-86

Doyle KL, Karl T, Hort Y, Duffy L, Shine J, Herzog H (2008) Y1 receptors are critical for the proliferation of adult mouse precursor cells in the olfactory neuroepithelium. J Neurochem 105:641-652

Dupret D, Fabre A, Dobrossy MD, Panatier A, Rodriguez JJ, Lamarque S, Lemaire V, Oliet SH, Piazza PV, Abrous DN (2007) Spatial learning depends on both the addition and removal of new hippocampal neurons. PLoS Biol 5:e214

Ebner S, Dunbar M, McKinnon RD (2000) Distinct roles for PI3K in proliferation and survival of oligodendrocyte progenitor cells. J Neurosci Res 62:336-345

Egertova M, Cravatt BF, Elphick MR (2003) Comparative analysis of fatty acid amide hydrolase and cb(1) cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. Neuroscience 119:481-496

Egertova M, Elphick MR (2000) Localisation of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB. J Comp Neurol 422:159-171

Ehrhart J, Obregon D, Mori T, Hou H, Sun N, Bai Y, Klein T, Fernandez F, Tan J, Shytle RD (2005) Stimulation of cannabinoid receptor 2 (CB2) suppresses microglial activation. J Neuroinflammation 2:29

Elphick MR, Egertova M (2001) The neurobiology and evolution of cannabinoid signalling. Philos Trans R Soc Lond B Biol Sci 356:381-408

Englund C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci 25:247-251

Enwere E, Shingo T, Gregg C, Fujikawa H, Ohta S, Weiss S (2004) Aging results in reduced epidermal growth factor receptor signaling, diminished olfactory neurogenesis, and deficits in fine olfactory discrimination. J Neurosci 24:8354-8365

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313-1317

Exton JH (1996) Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. Annu Rev Pharmacol Toxicol 36:481-509

Fabel K, Kempermann G (2008) Physical activity and the regulation of neurogenesis in the adult and aging brain. Neuromolecular Med 10:59-66

Fagundo AB, de la Torre R, Jimenez-Murcia S, Aguera Z, Pastor A, Casanueva FF, Granero R, Banos R, Botella C, Del Pino-Gutierrez A, Fernandez-Real JM, Fernandez-Garcia JC, Fruhbeck G, Gomez-Ambrosi J, Menchon JM, Moragrega I, Rodriguez R, Tarrega S, Tinahones FJ, Fernandez-Aranda F (2013) Modulation of the Endocannabinoids N-Arachidonoylethanolamine (AEA) and 2-Arachidonoylglycerol (2-AG) on Executive Functions in Humans. PLoS One 8:e66387

Fakhfouri G, Ahmadiani A, Rahimian R, Grolla AA, Moradi F, Haeri A (2012) WIN55212-2 attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR-gamma pathway. Neuropharmacology 63:653-666

Fang KM, Yang CS, Sun SH, Tzeng SF (2009) Microglial phagocytosis attenuated by short-term exposure to exogenous ATP through P2X receptor action. J Neurochem 111:1225-1237

Favaro R, Valotta M, Ferri AL, Latorre E, Mariani J, Giachino C, Lancini C, Tosetti V, Ottolenghi S, Taylor V, Nicolis SK (2009) Hippocampal development and neural stem cell maintenance require Sox2-dependent regulation of Shh. Nat Neurosci 12:1248-1256

Fegley D, Kathuria S, Mercier R, Li C, Goutopoulos A, Makriyannis A, Piomelli D (2004) Anandamide transport is independent of fatty-acid amide hydrolase activity and is blocked by the hydrolysis-resistant inhibitor AM1172. Proc Natl Acad Sci U S A 101:8756-8761

Felder CC, Joyce KE, Briley EM, Glass M, Mackie KP, Fahey KJ, Cullinan GJ, Hunden DC, Johnson DW, Chaney MO, Koppel GA, Brownstein M (1998) LY320135, a novel cannabinoid CB1 receptor antagonist, unmasks coupling of the CB1 receptor to stimulation of cAMP accumulation. J Pharmacol Exp Ther 284:291-297

Fernandez-Lopez D, Pradillo JM, Garcia-Yebenes I, Martinez-Orgado JA, Moro MA, Lizasoain I (2010) The cannabinoid WIN55212-2 promotes neural repair after neonatal hypoxia-ischemia. Stroke 41:2956-2964

Fernandez-Ruiz J (2009) The endocannabinoid system as a target for the treatment of motor dysfunction. Br J Pharmacol 156:1029-1040

Fernandez-Ruiz J, Gonzales S (2005) Cannabinoid control of motor function at the basal ganglia. Handb Exp Pharmacol:479-507

Fernandez-Ruiz J, Romero J, Velasco G, Tolon RM, Ramos JA, Guzman M (2007) Cannabinoid CB2 receptor: a new target for controlling neural cell survival? Trends Pharmacol Sci 28:39-45

Feron F, Perry C, Cochrane J, Licina P, Nowitzke A, Urquhart S, Geraghty T, Mackay-Sim A (2005) Autologous olfactory ensheathing cell transplantation in human spinal cord injury. Brain 128:2951-2960

Feron F, Perry C, McGrath JJ, Mackay-Sim A (1998) New techniques for biopsy and culture of human olfactory epithelial neurons. Arch Otolaryngol Head Neck Surg 124:861-866

Ferrer B, Asbrock N, Kathuria S, Piomelli D, Giuffrida A (2003) Effects of levodopa on endocannabinoid levels in rat basal ganglia: implications for the treatment of levodopa-induced dyskinesias. Eur J Neurosci 18:1607-1614

Filipeanu CM, de Zeeuw D, Nelemans SA (1997) Delta9-tetrahydrocannabinol activates [Ca2+]i increases partly sensitive to capacitative store refilling. Eur J Pharmacol 336:R1-3

Finger TE, Getchell ML, Getchell TV, Kinnamon JC, Green BG, Mason JR, Kare MR (1990) Affector and effector functions of peptidergic innervation of the nasal cavity. Chem Senses: Irritation. Dekker, New York, pp 1-20

Fioravanti B, De Felice M, Stucky CL, Medler KA, Luo MC, Gardell LR, Ibrahim M, Malan TP, Jr., Yamamura HI, Ossipov MH, King T, Lai J, Porreca F, Vanderah TW (2008) Constitutive activity at the cannabinoid CB1 receptor is required for behavioral response to noxious chemical stimulation of TRPV1: antinociceptive actions of CB1 inverse agonists. J Neurosci 28:11593-11602

Flores AI, Mallon BS, Matsui T, Ogawa W, Rosenzweig A, Okamoto T, Macklin WB (2000) Akt-mediated survival of oligodendrocytes induced by neuregulins. J Neurosci 20:7622-7630

Foster KG, Acosta-Jaquez HA, Romeo Y, Ekim B, Soliman GA, Carriere A, Roux PP, Ballif BA, Fingar DC (2010) Regulation of mTOR complex 1 (mTORC1) by raptor Ser863 and multisite phosphorylation. J Biol Chem 285:80-94

Fournier NM, Lee B, Banasr M, Elsayed M, Duman RS (2012) Vascular endothelial growth factor regulates adult hippocampal cell proliferation through MEK/ERK- and PI3K/Akt-dependent signaling. Neuropharmacology 63:642-652

Franke H, Illes P (2006) Involvement of P2 receptors in the growth and survival of neurons in the CNS. Pharmacol Ther 109:297-324

Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, Piomelli D (2003) Oleylethanolamide

regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature 425:90-93

Furuya K, Enomoto K, Yamagishi S (1993) Spontaneous calcium oscillations and mechanically and chemically induced calcium responses in mammary epithelial cells. Pflugers Arch 422:295-304

Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438

Gage FH, Ray J, Fisher LJ (1995) Isolation, characterization, and use of stem cells from the CNS. Annu Rev Neurosci 18:159-192

Galve-Roperh I, Aguado T, Palazuelos J, Guzman M (2007) The endocannabinoid system and neurogenesis in health and disease. Neuroscientist 13:109-114

Galve-Roperh I, Aguado T, Rueda D, Velasco G, Guzman M (2006) Endocannabinoids: a new family of lipid mediators involved in the regulation of neural cell development. Curr Pharm Des 12:2319-2325

Galve-Roperh I, Chiurchiu V, Diaz-Alonso J, Bari M, Guzman M, Maccarrone M (2013) Cannabinoid receptor signaling in progenitor/stem cell proliferation and differentiation. Prog Lipid Res 52:633-650

Galve-Roperh I, Rueda D, Gomez del Pulgar T, Velasco G, Guzman M (2002) Mechanism of extracellular signal-regulated kinase activation by the CB(1) cannabinoid receptor. Mol Pharmacol 62:1385-1392

Gandelman M, Peluffo H, Beckman JS, Cassina P, Barbeito L (2010) Extracellular ATP and the P2X7 receptor in astrocyte-mediated motor neuron death: implications for amyotrophic lateral sclerosis. J Neuroinflammation 7:33

Gao L, Cao L, Qiu Y, Su Z, Burnstock G, Xiang Z, He C (2010a) Blocking P2X receptors can inhibit the injury-induced proliferation of olfactory epithelium progenitor cells in adult mouse. Int J Pediatr Otorhinolaryngol

Gao Y, Vasilyev DV, Goncalves MB, Howell FV, Hobbs C, Reisenberg M, Shen R, Zhang MY, Strassle BW, Lu P, Mark L, Piesla MJ, Deng K, Kouranova EV, Ring RH, Whiteside GT, Bates B, Walsh FS, Williams G, Pangalos MN, Samad TA, Doherty P (2010b) Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. J Neurosci 30:2017-2024

Garcia-Arencibia M, Garcia C, Fernandez-Ruiz J (2009) Cannabinoids and Parkinson's disease. CNS Neurol Disord Drug Targets 8:432-439

Ge S, Sailor KA, Ming GL, Song H (2008) Synaptic integration and plasticity of new neurons in the adult hippocampus. J Physiol 586:3759-3765

Gerdeman GL, Lovinger DM (2003) Emerging roles for endocannabinoids in long-term synaptic plasticity. Br J Pharmacol 140:781-789

Gheusi G, Lledo PM (2007) Control of early events in olfactory processing by adult neurogenesis. Chem Senses 32:397-409

Giuffrida A, Parsons LH, Kerr TM, Rodriguez de Fonseca F, Navarro M, Piomelli D (1999) Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. Nat Neurosci 2:358-363

Glass M, Felder CC (1997) Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. J Neurosci 17:5327-5333

Glass M, Northup JK (1999) Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. Mol Pharmacol 56:1362-1369

Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, Cassano T, Morgese MG, Debonnel G, Duranti A, Tontini A, Tarzia G, Mor M, Trezza V, Goldberg SR, Cuomo V, Piomelli D (2005) Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. Proc Natl Acad Sci U S A 102:18620-18625

Gomez Del Pulgar T, De Ceballos ML, Guzman M, Velasco G (2002) Cannabinoids protect astrocytes from ceramide-induced apoptosis through the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem 277:36527-36533

Gomez del Pulgar T, Velasco G, Guzman M (2000) The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. Biochem J 347:369-373

Gomez O, Sanchez-Rodriguez A, Le M, Sanchez-Caro C, Molina-Holgado F, Molina-Holgado E (2011) Cannabinoid receptor agonists modulate oligodendrocyte differentiation by activating PI3K/Akt and the mammalian target of rapamycin (mTOR) pathways. Br J Pharmacol 163:1520-1532

Goncalves MB, Suetterlin P, Yip P, Molina-Holgado F, Walker DJ, Oudin MJ, Zentar MP, Pollard S, Yanez-Munoz RJ, Williams G, Walsh FS, Pangalos MN, Doherty P (2008) A diacylglycerol lipase-CB2 cannabinoid pathway regulates adult subventricular zone neurogenesis in an age-dependent manner. Mol Cell Neurosci 38:526-536

Gong JP, Onaivi ES, Ishiguro H, Liu QR, Tagliaferro PA, Brusco A, Uhl GR (2006) Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. Brain Res 1071:10-23

Gonzalez S, Valenti M, de Miguel R, Fezza F, Fernandez-Ruiz J, Di Marzo V, Ramos JA (2004) Changes in endocannabinoid contents in reward-related brain regions of

alcohol-exposed rats, and their possible relevance to alcohol relapse. Br J Pharmacol 143:455-464

Gordon MK, Mumm JS, Davis RA, Holcomb JD, Calof AL (1995) Dynamics of MASH1 expression in vitro and in vivo suggest a non-stem cell site of MASH1 action in the olfactory receptor neuron lineage. Mol Cell Neurosci 6:363-379

Gosens R, Dueck G, Rector E, Nunes RO, Gerthoffer WT, Unruh H, Zaagsma J, Meurs H, Halayko AJ (2007) Cooperative regulation of GSK-3 by muscarinic and PDGF receptors is associated with airway myocyte proliferation. Am J Physiol Lung Cell Mol Physiol 293:L1348-1358

Gotz M, Stoykova A, Gruss P (1998) Pax6 controls radial glia differentiation in the cerebral cortex. Neuron 21:1031-1044

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999) Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2:260-265

Graziadei GA, Graziadei PP (1979) Neurogenesis and neuron regeneration in the olfactory system of mammals. II. Degeneration and reconstitution of the olfactory sensory neurons after axotomy. J Neurocytol 8:197-213

Graziadei PPC, Monti-Graziadei GA (1978) Continuous nerve cell renewal in the olfactory system. In: Jacobson M (ed) Handbook of Sensory Physiology. Springer, New York, pp 55-83

Grimes CA, Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65:391-426

Guillemot F, Molnar Z, Tarabykin V, Stoykova A (2006) Molecular mechanisms of cortical differentiation. Eur J Neurosci 23:857-868

Guindon J, Hohmann AG (2008) Cannabinoid CB2 receptors: a therapeutic target for the treatment of inflammatory and neuropathic pain. Br J Pharmacol 153:319-334

Gulyas AI, Cravatt BF, Bracey MH, Dinh TP, Piomelli D, Boscia F, Freund TF (2004) Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. Eur J Neurosci 20:441-458

Guo Z, Packard A, Krolewski RC, Harris MT, Manglapus GL, Schwob JE (2010) Expression of Pax6 and Sox2 in adult olfactory epithelium. J Comp Neurol 518:4395-4418

Gutierrez-Martin Y, Bustillo D, Gomez-Villafuertes R, Sanchez-Nogueiro J, Torregrosa-Hetland C, Binz T, Gutierrez LM, Miras-Portugal MT, Artalejo AR (2011) P2X7 receptors

trigger ATP exocytosis and modify secretory vesicle dynamics in neuroblastoma cells. J Biol Chem 286:11370-11381

Guzman M (2003) Neurons on cannabinoids: dead or alive? Br J Pharmacol 140:439-440

Guzman M, Sanchez C, Galve-Roperh I (2001) Control of the cell survival/death decision by cannabinoids. J Mol Med (Berl) 78:613-625

Guzman M, Sanchez C, Galve-Roperh I (2002) Cannabinoids and cell fate. Pharmacol Ther 95:175-184

Haehner A, Hummel T, Reichmann H (2011) Olfactory loss in Parkinson's disease. Parkinsons Dis 2011:450939

Hajos N, Katona I, Naiem SS, MacKie K, Ledent C, Mody I, Freund TF (2000) Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. Eur J Neurosci 12:3239-3249

Hansel DE, Eipper BA, Ronnett GV (2001) Neuropeptide Y functions as a neuroproliferative factor. Nature 410:940-944

Hanson LR, Frey WH, 2nd (2008) Intranasal delivery bypasses the blood-brain barrier to target therapeutic agents to the central nervous system and treat neurodegenerative disease. BMC Neurosci 9 Suppl 3:S5

Harkany T, Guzman M, Galve-Roperh I, Berghuis P, Devi LA, Mackie K (2007) The emerging functions of endocannabinoid signaling during CNS development. Trends Pharmacol Sci 28:83-92

Harris JA, West AK, Chuah MI (2009) Olfactory ensheathing cells: nitric oxide production and innate immunity. Glia 57:1848-1857

Hart S, Fischer OM, Ullrich A (2004) Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. Cancer Res 64:1943-1950

Hashimotodani Y, Ohno-Shosaku T, Kano M (2007) Presynaptic monoacylglycerol lipase activity determines basal endocannabinoid tone and terminates retrograde endocannabinoid signaling in the hippocampus. J Neurosci 27:1211-1219

Hassenklöver T, Schwartz P, Schild D, Manzini I (2009) Purinergic signaling regulates cell proliferation of olfactory epithelium progenitors. Stem Cells 27:2022-2031

Hayakawa K, Mishima K, Nozako M, Hazekawa M, Ogata A, Fujioka M, Harada K, Mishima S, Orito K, Egashira N, Iwasaki K, Fujiwara M (2007) Delta9-

tetrahydrocannabinol (Delta9-THC) prevents cerebral infarction via hypothalamic-independent hypothermia. Life Sci 80:1466-1471

Hayar A, Ennis M (2007) Endogenous GABA and glutamate finely tune the bursting of olfactory bulb external tufted cells. J Neurophysiol 98:1052-1056

Hayar A, Karnup S, Shipley MT, Ennis M (2004) Olfactory bulb glomeruli: external tufted cells intrinsically burst at theta frequency and are entrained by patterned olfactory input. J Neurosci 24:1190-1199

Hayoz S, Jia C, Hegg CC (2012) Mechanisms of constitutive and ATP-evoked ATP release in neonatal mouse olfactory epithelium. BMC Neurosci 13:53

Hegg CC, Au E, Roskams AJ, Lucero MT (2003) PACAP is Present in the Olfactory System and Evokes Calcium Transients in Olfactory Receptor Neurons. J Neurophysiol 90 2711-2719

Hegg CC, Irwin M, Lucero MT (2009) Calcium store-mediated signaling in sustentacular cells of the mouse olfactory epithelium. Glia 57:634-644

Hegg CC, Jia C, Chick WS, Restrepo D, Hansen A (2010) Microvillous cells expressing IP3R3 in the olfactory epithelium of mice. Eur J Neurosci 32:1632-1645

Hegg CC, Lucero MT (2006) Purinergic receptor antagonists inhibit odorant-induced heat shock protein 25 induction in mouse olfactory epithelium. Glia 53:182-190

Heifets BD, Castillo PE (2009) Endocannabinoid signaling and long-term synaptic plasticity. Annu Rev Physiol 71:283-306

Hentges ST, Low MJ, Williams JT (2005) Differential regulation of synaptic inputs by constitutively released endocannabinoids and exogenous cannabinoids. J Neurosci 25:9746-9751

Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC (1991) Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. J Neurosci 11:563-583

Hermann H, De Petrocellis L, Bisogno T, Schiano Moriello A, Lutz B, Di Marzo V (2003) Dual effect of cannabinoid CB1 receptor stimulation on a vanilloid VR1 receptor-mediated response. Cell Mol Life Sci 60:607-616

Hetman M, Cavanaugh JE, Kimelman D, Xia Z (2000) Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. J Neurosci 20:2567-2574

Hillard CJ, Manna S, Greenberg MJ, DiCamelli R, Ross RA, Stevenson LA, Murphy V, Pertwee RG, Campbell WB (1999) Synthesis and characterization of potent and

selective agonists of the neuronal cannabinoid receptor (CB1). J Pharmacol Exp Ther 289:1427-1433

Hinds JW, Hinds PL, McNelly NA (1984) An autoradiographic study of the mouse olfactory epithelium: evidence for long-lived receptors. Anat Rec 210:375-383

Hipp J, Atala A (2008) Sources of stem cells for regenerative medicine. Stem Cell Rev 4:3-11

Hoeffer CA, Klann E (2010) mTOR signaling: at the crossroads of plasticity, memory and disease. Trends Neurosci 33:67-75

Hoffman AF, Lupica CR (2000) Mechanisms of cannabinoid inhibition of GABA(A) synaptic transmission in the hippocampus. J Neurosci 20:2470-2479

Hohmann AG, Tsou K, Walker JM (1999) Intrathecal cannabinoid administration suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in rat spinal cord: comparison with morphine. Zhongguo Yao Li Xue Bao 20:1132-1136

Holbrook EH, Szumowski KE, Schwob JE (1995) An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium. J Comp Neurol 363:129-146

Holcomb JD, Mumm JS, Calof AL (1995a) Apoptosis in the neuronal lineage of the mouse olfactory epithelium: regulation in vivo and in vitro. Dev Biol 172:307-323

Holcomb JD, Mumm JS, Calof AL (1995b) Apoptosis in the neuronal lineage of the mouse olfactory epithelium: regulation in vivo and in vitro. Dev Biol 172:307-323

Howlett AC (1995) Pharmacology of cannabinoid receptors. Annu Rev Pharmacol Toxicol 35:607-634

Howlett AC (1998) The CB1 cannabinoid receptor in the brain. Neurobiol Dis 5:405-416

Huard JM, Youngentob SL, Goldstein BJ, Luskin MB, Schwob JE (1998) Adult olfactory epithelium contains multipotent progenitors that give rise to neurons and non-neural cells. J Comp Neurol 400:469-486

Hurst DP, Grossfield A, Lynch DL, Feller S, Romo TD, Gawrisch K, Pitman MC, Reggio PH (2010) A lipid pathway for ligand binding is necessary for a cannabinoid G protein-coupled receptor. J Biol Chem 285:17954-17964

Inokuchi A, Kimmelman CP, Snow JB, Jr. (1993) Convergence of olfactory and nasotrigeminal inputs and possible trigeminal contributions to olfactory responses in the rat thalamus. Eur Arch Otorhinolaryngol 249:473-477

Islam Z, Harkema JR, Pestka JJ (2006) Satratoxin G from the black mold Stachybotrys chartarum evokes olfactory sensory neuron loss and inflammation in the murine nose and brain. Environ Health Perspect 114:1099-1107

Iwema CL, Fang H, Kurtz DB, Youngentob SL, Schwob JE (2004) Odorant receptor expression patterns are restored in lesion-recovered rat olfactory epithelium. J Neurosci 24:356-369

Izzo AA, Piscitelli F, Capasso R, Aviello G, Romano B, Borrelli F, Petrosino S, Di Marzo V (2009) Peripheral endocannabinoid dysregulation in obesity: relation to intestinal motility and energy processing induced by food deprivation and re-feeding. Br J Pharmacol 158:451-461

Jang W, Chen X, Flis D, Harris M, Schwob JE (2014) Label-retaining, quiescent globose basal cells are found in the olfactory epithelium. J Comp Neurol 522:731-749

Jarai Z, Wagner JA, Varga K, Lake KD, Compton DR, Martin BR, Zimmer AM, Bonner TI, Buckley NE, Mezey E, Razdan RK, Zimmer A, Kunos G (1999) Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. Proc Natl Acad Sci U S A 96:14136-14141

Jeske NA, Patwardhan AM, Gamper N, Price TJ, Akopian AN, Hargreaves KM (2006) Cannabinoid WIN 55,212-2 regulates TRPV1 phosphorylation in sensory neurons. J Biol Chem 281:32879-32890

Jia C, Cussen AR, Hegg CC (2011a) ATP differentially upregulates fibroblast growth factor 2 and transforming growth factor alpha in neonatal and adult mice: effect on neuroproliferation. Neuroscience 177:335–346

Jia C, Doherty JD, Crudgington S, Hegg CC (2009) Activation of purinergic receptors induces proliferation and neuronal differentiation in Swiss Webster mouse olfactory epithelium. Neuroscience 163:120-128

Jia C, Hayoz S, Hutch CR, Iqbal TR, Pooley AE, Hegg CC (2013) An IP3R3- and NPY-expressing microvillous cell mediates tissue homeostasis and regeneration in the mouse olfactory epithelium. PLoS One 8:e58668

Jia C, Hegg CC NPY mediates ATP-induced neuroproliferation in adult mouse olfactory epithelium. Neurobiol Dis 38:405-413

Jia C, Hegg CC (2010) NPY mediates ATP-induced neuroproliferation in adult mouse olfactory epithelium. Neurobiol Dis 38:405-413

Jia C, Hegg CC (2012) Neuropeptide Y and extracellular signal-regulated kinase mediate injury-induced neuroregeneration in mouse olfactory epithelium. Mol Cell Neurosci 49:158-170

Jia C, Roman C, Hegg CC (2010) Nickel sulfate induces location-dependent atrophy of mouse olfactory epithelium: protective and proliferative role of purinergic receptor activation. Toxicol Sci 115:547-556

Jia C, Sangsiri S, Belock B, Iqbal T, Pestka JJ, Hegg CC (2011b) ATP mediates neuroprotective and neuroproliferative effects in mouse olfactory epithelium following exposure to satratoxin G in vitro and in vivo. Toxicol Sci 124:169-178

Jiang S, Fu Y, Williams J, Wood J, Pandarinathan L, Avraham S, Makriyannis A, Avraham HK (2007) Expression and function of cannabinoid receptors CB1 and CB2 and their cognate cannabinoid ligands in murine embryonic stem cells. PLoS One 2:e641

Jiang W, Zhang Y, Xiao L, Van Cleemput J, Ji SP, Bai G, Zhang X (2005) Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. J Clin Invest 115:3104-3116

Jin K, Xie L, Kim SH, Parmentier-Batteur S, Sun Y, Mao XO, Childs J, Greenberg DA (2004) Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. Mol Pharmacol 66:204-208

John JA, Key B (2003) Axon mis-targeting in the olfactory bulb during regeneration of olfactory neuroepithelium. Chem Senses 28:773-779

Jordan JD, He JC, Eungdamrong NJ, Gomes I, Ali W, Nguyen T, Bivona TG, Philips MR, Devi LA, Iyengar R (2005) Cannabinoid receptor-induced neurite outgrowth is mediated by Rap1 activation through G(alpha)o/i-triggered proteasomal degradation of Rap1GAPII. J Biol Chem 280:11413-11421

Jordt SE, Julius D (2002) Molecular basis for species-specific sensitivity to "hot" chili peppers. Cell 108:421-430

Jung KM, Astarita G, Thongkham D, Piomelli D (2011) Diacylglycerol lipase-alpha and -beta control neurite outgrowth in neuro-2a cells through distinct molecular mechanisms. Mol Pharmacol 80:60-67

Kadohisa M, Wilson DA (2006a) Olfactory cortical adaptation facilitates detection of odors against background. J Neurophysiol 95:1888-1896

Kadohisa M, Wilson DA (2006b) Separate encoding of identity and similarity of complex familiar odors in piriform cortex. Proc Natl Acad Sci U S A 103:15206-15211

Kanekar S, Jia C, Hegg CC (2009) Purinergic receptor activation evokes neurotrophic factor neuropeptide Y release from neonatal mouse olfactory epithelial slices. J Neurosci Res 87:1424-1434

Kaneko Y, Sakakibara S, Imai T, Suzuki A, Nakamura Y, Sawamoto K, Ogawa Y, Toyama Y, Miyata T, Okano H (2000) Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev Neurosci 22:139-153

Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092-1094

Kastner A, Moyse E, Bauer S, Jourdan F, Brun G (2000) Unusual regulation of cyclin D1 and cyclin-dependent kinases cdk2 and cdk4 during in vivo mitotic stimulation of olfactory neuron progenitors in adult mouse. J Neurochem 74:2343-2349

Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 9:76-81

Katona I, Freund TF (2008) Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. Nat Med 14:923-930

Katona I, Sperlagh B, Magloczky Z, Santha E, Kofalvi A, Czirjak S, Mackie K, Vizi ES, Freund TF (2000) GABAergic interneurons are the targets of cannabinoid actions in the human hippocampus. Neuroscience 100:797-804

Katona I, Sperlagh B, Sik A, Kafalvi A, Vizi ES, Mackie K, Freund TF (1999) Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. J Neurosci 19:4544-4558

Katona I, Urban GM, Wallace M, Ledent C, Jung KM, Piomelli D, Mackie K, Freund TF (2006) Molecular composition of the endocannabinoid system at glutamatergic synapses. J Neurosci 26:5628-5637

Katz S, Ayala V, Santillan G, Boland R (2011) Activation of the PI3K/Akt signaling pathway through P2Y(2) receptors by extracellular ATP is involved in osteoblastic cell proliferation. Arch Biochem Biophys 513:144-152

Kawauchi S, Beites CL, Crocker CE, Wu HH, Bonnin A, Murray R, Calof AL (2004) Molecular signals regulating proliferation of stem and progenitor cells in mouse olfactory epithelium. Dev Neurosci 26:166-180

Kempe K, Hsu FF, Bohrer A, Turk J (1996) Isotope dilution mass spectrometric measurements indicate that arachidonylethanolamide, the proposed endogenous ligand of the cannabinoid receptor, accumulates in rat brain tissue post mortem but is contained at low levels in or is absent from fresh tissue. J Biol Chem 271:17287-17295

Kim J, Alger BE (2010) Reduction in endocannabinoid tone is a homeostatic mechanism for specific inhibitory synapses. Nat Neurosci 13:592-600

Kim SH, Won SJ, Mao XO, Jin K, Greenberg DA (2006) Molecular mechanisms of cannabinoid protection from neuronal excitotoxicity. Mol Pharmacol 69:691-696

Kitamura T, Saitoh Y, Takashima N, Murayama A, Niibori Y, Ageta H, Sekiguchi M, Sugiyama H, Inokuchi K (2009) Adult neurogenesis modulates the hippocampus-dependent period of associative fear memory. Cell 139:814-827

Kiyokage E, Pan YZ, Shao Z, Kobayashi K, Szabo G, Yanagawa Y, Obata K, Okano H, Toida K, Puche AC, Shipley MT (2010) Molecular identity of periglomerular and short axon cells. J Neurosci 30:1185-1196

Kohno M, Hasegawa H, Inoue A, Muraoka M, Miyazaki T, Oka K, Yasukawa M (2006) Identification of N-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. Biochem Biophys Res Commun 347:827-832

Komitova M, Eriksson PS (2004) Sox-2 is expressed by neural progenitors and astroglia in the adult rat brain. Neurosci Lett 369:24-27

Kondo K, Suzukawa K, Sakamoto T, Watanabe K, Kanaya K, Ushio M, Yamaguchi T, Nibu K, Kaga K, Yamasoba T (2010) Age-related changes in cell dynamics of the postnatal mouse olfactory neuroepithelium: cell proliferation, neuronal differentiation, and cell death. J Comp Neurol 518:1962-1975

Koppel J, Bradshaw H, Goldberg TE, Khalili H, Marambaud P, Walker MJ, Pazos M, Gordon ML, Christen E, Davies P (2009) Endocannabinoids in Alzheimer's disease and their impact on normative cognitive performance: a case-control and cohort study. Lipids Health Dis 8:2

Kovacs T (2004) Mechanisms of olfactory dysfunction in aging and neurodegenerative disorders. Ageing Res Rev 3:215-232

Kriegstein A, Alvarez-Buylla A (2009) The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32:149-184

Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027-2033

Kuster JE, Stevenson JI, Ward SJ, D'Ambra TE, Haycock DA (1993) Aminoalkylindole binding in rat cerebellum: selective displacement by natural and synthetic cannabinoids. J Pharmacol Exp Ther 264:1352-1363

Kuwae T, Shiota Y, Schmid PC, Krebsbach R, Schmid HH (1999) Biosynthesis and turnover of anandamide and other N-acylethanolamines in peritoneal macrophages. FEBS Lett 459:123-127

Lafon-Cazal M, Adjali O, Galeotti N, Poncet J, Jouin P, Homburger V, Bockaert J, Marin P (2003) Proteomic analysis of astrocytic secretion in the mouse. Comparison with the cerebrospinal fluid proteome. J Biol Chem 278:24438-24448

Lafourcade M, Elezgarai I, Mato S, Bakiri Y, Grandes P, Manzoni OJ (2007) Molecular components and functions of the endocannabinoid system in mouse prefrontal cortex. PLoS One 2:e709

Laine K, Jarvinen K, Mechoulam R, Breuer A, Jarvinen T (2002) Comparison of the enzymatic stability and intraocular pressure effects of 2-arachidonylglycerol and noladin ether, a novel putative endocannabinoid. Invest Ophthalmol Vis Sci 43:3216-3222

Lander AD, Gokoffski KK, Wan FY, Nie Q, Calof AL (2009) Cell lineages and the logic of proliferative control. PLoS Biol 7:e15

Lauckner JE, Hille B, Mackie K (2005) The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. Proc Natl Acad Sci U S A 102:19144-19149

Laywell ED, Rakic P, Kukekov VG, Holland EC, Steindler DA (2000) Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. Proc Natl Acad Sci U S A 97:13883-13888

Lazarini F, Mouthon MA, Gheusi G, de Chaumont F, Olivo-Marin JC, Lamarque S, Abrous DN, Boussin FD, Lledo PM (2009) Cellular and behavioral effects of cranial irradiation of the subventricular zone in adult mice. PLoS One 4:e7017

Le Pichon CE, Valley MT, Polymenidou M, Chesler AT, Sagdullaev BT, Aguzzi A, Firestein S (2009) Olfactory behavior and physiology are disrupted in prion protein knockout mice. Nat Neurosci 12:60-69

Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F, Bohme GA, Imperato A, Pedrazzini T, Roques BP, Vassart G, Fratta W, Parmentier M (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. Science 283:401-404

Leker RR, Gai N, Mechoulam R, Ovadia H (2003) Drug-induced hypothermia reduces ischemic damage: effects of the cannabinoid HU-210. Stroke 34:2000-2006

Lendahl U, Zimmerman LB, McKay RD (1990) CNS stem cells express a new class of intermediate filament protein. Cell 60:585-595

Leuner B, Mendolia-Loffredo S, Kozorovitskiy Y, Samburg D, Gould E, Shors TJ (2004) Learning enhances the survival of new neurons beyond the time when the hippocampus is required for memory. J Neurosci 24:7477-7481

Leung CT, Coulombe PA, Reed RR (2007a) Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat Neurosci 10:720-726

Leung CT, Coulombe PA, Reed RR (2007b) Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat Neurosci 10:720-726

Levenes C, Daniel H, Soubrie P, Crepel F (1998) Cannabinoids decrease excitatory synaptic transmission and impair long-term depression in rat cerebellar Purkinje cells. J Physiol 510 (Pt 3):867-879

Levesque S, Surace MJ, McDonald J, Block ML (2011) Air pollution & the brain: Subchronic diesel exhaust exposure causes neuroinflammation and elevates early markers of neurodegenerative disease. J Neuroinflammation 8:105

Li L, Xie T (2005) Stem cell niche: structure and function. Annu Rev Cell Dev Biol 21:605-631

Li W, Howard JD, Parrish TB, Gottfried JA (2008) Aversive learning enhances perceptual and cortical discrimination of indiscriminable odor cues. Science 319:1842-1845

Li W, Luxenberg E, Parrish T, Gottfried JA (2006) Learning to smell the roses: experience-dependent neural plasticity in human piriform and orbitofrontal cortices. Neuron 52:1097-1108

Lie DC, Song H, Colamarino SA, Ming GL, Gage FH (2004) Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annu Rev Pharmacol Toxicol 44:399-421

Linster C, Johnson BA, Morse A, Yue E, Leon M (2002) Spontaneous versus reinforced olfactory discriminations. J Neurosci 22:6842-6845

Liu H, Iacono RP, Szalay AA (2001) Detection of GDNF secretion in glial cell culture and from transformed cell implants in the brains of live animals. Mol Genet Genomics 266:614-623

Liu J, Wang L, Harvey-White J, Osei-Hyiaman D, Razdan R, Gong Q, Chan AC, Zhou Z, Huang BX, Kim HY, Kunos G (2006) A biosynthetic pathway for anandamide. Proc Natl Acad Sci U S A 103:13345-13350

Lledo PM, Gheusi G (2006) [Adult neurogenesis: from basic research to clinical applications]. Bull Acad Natl Med 190:385-400; discussion 400-382

Lledo PM, Lagier S (2006) Adjusting neurophysiological computations in the adult olfactory bulb. Semin Cell Dev Biol 17:443-453

Lledo PM, Merkle FT, Alvarez-Buylla A (2008) Origin and function of olfactory bulb interneuron diversity. Trends Neurosci 31:392-400

Lograno MD, Romano MR (2004) Cannabinoid agonists induce contractile responses through Gi/o-dependent activation of phospholipase C in the bovine ciliary muscle. Eur J Pharmacol 494:55-62

Long JZ, Nomura DK, Cravatt BF (2009) Characterization of monoacylglycerol lipase inhibition reveals differences in central and peripheral endocannabinoid metabolism. Chem Biol 16:744-753

Loo AT, Youngentob SL, Kent PF, Schwob JE (1996) The aging olfactory epithelium: neurogenesis, response to damage, and odorant-induced activity. Int J Dev Neurosci 14:881-900

LoVerme J, La Rana G, Russo R, Calignano A, Piomelli D (2005) The search for the palmitoylethanolamide receptor. Life Sci 77:1685-1698

Ludanyi A, Eross L, Czirjak S, Vajda J, Halasz P, Watanabe M, Palkovits M, Magloczky Z, Freund TF, Katona I (2008) Downregulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus. J Neurosci 28:2976-2990

Luo J, Daniels SB, Lennington JB, Notti RQ, Conover JC (2006) The aging neurogenic subventricular zone. Aging Cell 5:139-152

Maccarrone M (2008a) CB2 receptors in reproduction. Br J Pharmacol 153:189-198

Maccarrone M (2008b) Good news for CB1 receptors: endogenous agonists are in the right place. Br J Pharmacol 153:179-181

Maccarrone M, Rossi S, Bari M, De Chiara V, Fezza F, Musella A, Gasperi V, Prosperetti C, Bernardi G, Finazzi-Agro A, Cravatt BF, Centonze D (2008) Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum. Nat Neurosci 11:152-159

Mackay-Sim A, Chuah MI (2000) Neurotrophic factors in the primary olfactory pathway. Prog Neurobiol 62:527-559

Mackay-Sim A, Feron F, Cochrane J, Bassingthwaighte L, Bayliss C, Davies W, Fronek P, Gray C, Kerr G, Licina P, Nowitzke A, Perry C, Silburn PA, Urquhart S, Geraghty T (2008) Autologous olfactory ensheathing cell transplantation in human paraplegia: a 3-year clinical trial. Brain 131:2376-2386

Mackay-Sim A, Kittel PW (1991) On the Life Span of Olfactory Receptor Neurons. Eur J Neurosci 3:209-215

Mackie K, Hille B (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proc Natl Acad Sci U S A 89:3825-3829

Mackie K, Lai Y, Westenbroek R, Mitchell R (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. J Neurosci 15:6552-6561

Mackie K, Stella N (2006) Cannabinoid receptors and endocannabinoids: evidence for new players. AAPS J 8:E298-306

Maejima T, Ohno-Shosaku T, Kano M (2001) Endogenous cannabinoid as a retrograde messenger from depolarized postsynaptic neurons to presynaptic terminals. Neurosci Res 40:205-210

Maneuf YP, Brotchie JM (1997) Paradoxical action of the cannabinoid WIN 55,212-2 in stimulated and basal cyclic AMP accumulation in rat globus pallidus slices. Br J Pharmacol 120:1397-1398

Manzini I, Schweer TS, Schild D (2008) Improved fluorescent (calcium indicator) dye uptake in brain slices by blocking multidrug resistance transporters. J Neurosci Methods 167:140-147

Marchalant Y, Brothers HM, Norman GJ, Karelina K, DeVries AC, Wenk GL (2009a) Cannabinoids attenuate the effects of aging upon neuroinflammation and neurogenesis. Neurobiol Dis 34:300-307

Marchalant Y, Brothers HM, Wenk GL (2009b) Cannabinoid agonist WIN-55,212-2 partially restores neurogenesis in the aged rat brain. Mol Psychiatry 14:1068-1069

Marchalant Y, Cerbai F, Brothers HM, Wenk GL (2008) Cannabinoid receptor stimulation is anti-inflammatory and improves memory in old rats. Neurobiol Aging 29:1894-1901

Margolis FL, Roberts N, Ferriero D, Feldman J (1974) Denervation in the primary olfactory pathway of mice: biochemical and morphological effects. Brain Res 81:469-483

Marinelli S, Pacioni S, Bisogno T, Di Marzo V, Prince DA, Huguenard JR, Bacci A (2008) The endocannabinoid 2-arachidonoylglycerol is responsible for the slow self-inhibition in neocortical interneurons. J Neurosci 28:13532-13541

Marinelli S, Pacioni S, Cannich A, Marsicano G, Bacci A (2009) Self-modulation of neocortical pyramidal neurons by endocannabinoids. Nat Neurosci 12:1488-1490

Marrs WR, Blankman JL, Horne EA, Thomazeau A, Lin YH, Coy J, Bodor AL, Muccioli GG, Hu SS, Woodruff G, Fung S, Lafourcade M, Alexander JP, Long JZ, Li W, Xu C, Moller T, Mackie K, Manzoni OJ, Cravatt BF, Stella N (2010) The serine hydrolase

ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors. Nat Neurosci 13:951-957

Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A, Azad SC, Cascio MG, Gutierrez SO, van der Stelt M, Lopez-Rodriguez ML, Casanova E, Schutz G, Zieglgansberger W, Di Marzo V, Behl C, Lutz B (2003) CB1 cannabinoid receptors and on-demand defense against excitotoxicity. Science 302:84-88

Marsicano G, Moosmann B, Hermann H, Lutz B, Behl C (2002a) Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1. J Neurochem 80:448-456

Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglgansberger W, Di Marzo V, Lutz B (2002b) The endogenous cannabinoid system controls extinction of aversive memories. Nature 418:530-534

Martin BR, Compton DR, Thomas BF, Prescott WR, Little PJ, Razdan RK, Johnson MR, Melvin LS, Mechoulam R, Ward SJ (1991) Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs. Pharmacol Biochem Behav 40:471-478

McBride K, Slotnick B, Margolis FL (2003) Does intranasal application of zinc sulfate produce anosmia in the mouse? An olfactometric and anatomical study. Chem Senses 28:659-670

McHugh D, Hu SS, Rimmerman N, Juknat A, Vogel Z, Walker JM, Bradshaw HB (2010) N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor. BMC Neurosci 11:44

McPartland JM, Glass M (2003) Functional mapping of cannabinoid receptor homologs in mammals, other vertebrates, and invertebrates. Gene 312:297-303

Mechoulam R (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol 50:83-90

Mechoulam R, Fride E, Di Marzo V (1998) Endocannabinoids. Eur J Pharmacol 359:1-18

Mechoulam R, Spatz M, Shohami E (2002) Endocannabinoids and neuroprotection. Sci STKE 2002:re5

Meisami E, Mikhail L, Baim D, Bhatnagar KP (1998) Human olfactory bulb: aging of glomeruli and mitral cells and a search for the accessory olfactory bulb. Ann NY Acad Sci 855:708-715

Menco BP (1980) Qualitative and quantitative freeze-fracture studies on olfactory and nasal respiratory epithelial surfaces of frog, ox, rat, and dog. III. Tight-junctions. Cell Tissue Res 211:361-373

Migliarini B, Marucci G, Ghelfi F, Carnevali O (2006) Endocannabinoid system in Xenopus laevis development: CB1 receptor dynamics. FEBS Lett 580:1941-1945

Ming GL, Song H (2005a) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28:223-50.:223-250

Ming GL, Song H (2005b) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28:223-250

Ming GL, Song H (2011) Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70:687-702

Moldrich G, Wenger T (2000) Localization of the CB1 cannabinoid receptor in the rat brain. An immunohistochemical study. Peptides 21:1735-1742

Molina-Holgado E, Vela JM, Arevalo-Martin A, Almazan G, Molina-Holgado F, Borrell J, Guaza C (2002) Cannabinoids promote oligodendrocyte progenitor survival: involvement of cannabinoid receptors and phosphatidylinositol-3 kinase/Akt signaling. J Neurosci 22:9742-9753

Molina-Holgado F, Rubio-Araiz A, Garcia-Ovejero D, Williams RJ, Moore JD, Arevalo-Martin A, Gomez-Torres O, Molina-Holgado E (2007) CB2 cannabinoid receptors promote mouse neural stem cell proliferation. Eur J Neurosci 25:629-634

Mor E, Kano S, Colantuoni C, Sawa A, Navon R, Shomron N (2013) MicroRNA-382 expression is elevated in the olfactory neuroepithelium of schizophrenia patients. Neurobiol Dis 55:1-10

Moranta D, Esteban S, Garcia-Sevilla JA (2007) Acute, chronic and withdrawal effects of the cannabinoid receptor agonist WIN55212-2 on the sequential activation of MAPK/Raf-MEK-ERK signaling in the rat cerebral frontal cortex: short-term regulation by intrinsic and extrinsic pathways. J Neurosci Res 85:656-667

Moreno MM, Linster C, Escanilla O, Sacquet J, Didier A, Mandairon N (2009) Olfactory perceptual learning requires adult neurogenesis. Proc Natl Acad Sci U S A 106:17980-17985

Morisset V, Ahluwalia J, Nagy I, Urban L (2001) Possible mechanisms of cannabinoid-induced antinociception in the spinal cord. Eur J Pharmacol 429:93-100

Mu Y, Lee SW, Gage FH (2010) Signaling in adult neurogenesis. Curr Opin Neurobiol 20:416-423

Mukhopadhyay S, McIntosh HH, Houston DB, Howlett AC (2000) The CB(1) cannabinoid receptor juxtamembrane C-terminal peptide confers activation to specific G proteins in brain. Mol Pharmacol 57:162-170

Mulder J, Aguado T, Keimpema E, Barabas K, Ballester Rosado CJ, Nguyen L, Monory K, Marsicano G, Di Marzo V, Hurd YL, Guillemot F, Mackie K, Lutz B, Guzman M, Lu HC, Galve-Roperh I, Harkany T (2008) Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning. Proc Natl Acad Sci U S A 105:8760-8765

Mumm JS, Shou J, Calof AL (1996) Colony-forming progenitors from mouse olfactory epithelium: evidence for feedback regulation of neuron production. Proc Natl Acad Sci U S A 93:11167-11172

Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365:61-65

Murphy C, Schubert CR, Cruickshanks KJ, Klein BE, Klein R, Nondahl DM (2002) Prevalence of olfactory impairment in older adults. JAMA 288:2307-2312

Murphy GJ, Darcy DP, Isaacson JS (2005) Intraglomerular inhibition: signaling mechanisms of an olfactory microcircuit. Nat Neurosci 8:354-364

Murrell W, Feron F, Wetzig A, Cameron N, Splatt K, Bellette B, Bianco J, Perry C, Lee G, Mackay-Sim A (2005) Multipotent stem cells from adult olfactory mucosa. Dev Dyn 233:496-515

Murrell W, Wetzig A, Donnellan M, Feron F, Burne T, Meedeniya A, Kesby J, Bianco J, Perry C, Silburn P, Mackay-Sim A (2008) Olfactory mucosa is a potential source for autologous stem cell therapy for Parkinson's disease. Stem Cells 26:2183-2192

Naessen R (1971) An enquiry on the morphological characteristics and possible changes with age in the olfactory region of man. Acta Otolaryngol 71:49-62

Nagayama T, Sinor AD, Simon RP, Chen J, Graham SH, Jin K, Greenberg DA (1999) Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. J Neurosci 19:2987-2995

Nakashima T, Kimmelman CP, Snow JB, Jr. (1984) Structure of human fetal and adult olfactory neuroepithelium. Arch Otolaryngol 110:641-646

Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP, Burnstock G (1996) Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci 19:13-18

Neary JT, Zimmermann H (2009) Trophic functions of nucleotides in the central nervous system. Trends Neurosci 32:189-198

Netzeband JG, Conroy SM, Parsons KL, Gruol DL (1999) Cannabinoids enhance NMDA-elicited Ca2+ signals in cerebellar granule neurons in culture. J Neurosci 19:8765-8777

Newman MP, Feron F, Mackay-Sim A (2000) Growth factor regulation of neurogenesis in adult olfactory epithelium. Neuroscience 99:343-350

Nie J, Lewis DL (2001) The proximal and distal C-terminal tail domains of the CB1 cannabinoid receptor mediate G protein coupling. Neuroscience 107:161-167

Nishino J, Kim I, Chada K, Morrison SJ (2008) Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression. Cell 135:227-239

Nochi R, Kato T, Kaneko J, Itou Y, Kuribayashi H, Fukuda S, Terazono Y, Matani A, Kanatani S, Nakajima K, Hisatsune T (2012) Involvement of metabotropic glutamate receptor 5 signaling in activity-related proliferation of adult hippocampal neural stem cells. Eur J Neurosci 36:2273-2283

Nones J, Spohr TC, Furtado DR, Sartore RC, Paulsen BS, Guimaraes MZ, Rehen SK (2010) Cannabinoids modulate cell survival in embryoid bodies. Cell Biol Int 34:399-408

Nyiri G, Szabadits E, Cserep C, Mackie K, Shigemoto R, Freund TF (2005) GABAB and CB1 cannabinoid receptor expression identifies two types of septal cholinergic neurons. Eur J Neurosci 21:3034-3042

O'Sullivan SE (2007) Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. Br J Pharmacol 152:576-582

Oddi S, Fezza F, Pasquariello N, De Simone C, Rapino C, Dainese E, Finazzi-Agro A, Maccarrone M (2008) Evidence for the intracellular accumulation of anandamide in adiposomes. Cell Mol Life Sci 65:840-850

Ohta Y, Ichimura K (2000) Proliferation markers, proliferating cell nuclear antigen, Ki67, 5-bromo-2'-deoxyuridine, and cyclin D1 in mouse olfactory epithelium. Ann Otol Rhinol Laryngol 109:1046-1048

Okamoto Y, Wang J, Morishita J, Ueda N (2007) Biosynthetic pathways of the endocannabinoid anandamide. Chem Biodivers 4:1842-1857

Oliet SH, Baimoukhametova DV, Piet R, Bains JS (2007) Retrograde regulation of GABA transmission by the tonic release of oxytocin and endocannabinoids governs postsynaptic firing. J Neurosci 27:1325-1333

Osumi N, Shinohara H, Numayama-Tsuruta K, Maekawa M (2008) Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. Stem Cells 26:1663-1672

Oudin MJ, Gajendra S, Williams G, Hobbs C, Lalli G, Doherty P (2011) Endocannabinoids regulate the migration of subventricular zone-derived neuroblasts in the postnatal brain. J Neurosci 31:4000-4011

Overstreet-Wadiche LS, Westbrook GL (2006) Functional maturation of adult-generated granule cells. Hippocampus 16:208-215

Overton HA, Babbs AJ, Doel SM, Fyfe MC, Gardner LS, Griffin G, Jackson HC, Procter MJ, Rasamison CM, Tang-Christensen M, Widdowson PS, Williams GM, Reynet C (2006) Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. Cell Metab 3:167-175

Owen SJ, Batzloff M, Chehrehasa F, Meedeniya A, Casart Y, Logue CA, Hirst RG, Peak IR, Mackay-Sim A, Beacham IR (2009) Nasal-associated lymphoid tissue and olfactory epithelium as portals of entry for Burkholderia pseudomallei in murine melioidosis. J Infect Dis 199:1761-1770

Palazuelos J, Aguado T, Egia A, Mechoulam R, Guzman M, Galve-Roperh I (2006) Non-psychoactive CB2 cannabinoid agonists stimulate neural progenitor proliferation. FASEB J 20:2405-2407

Palazuelos J, Ortega Z, Diaz-Alonso J, Guzman M, Galve-Roperh I (2012) CB2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling. J Biol Chem 287:1198-1209

Paliouras GN, Hamilton LK, Aumont A, Joppe SE, Barnabe-Heider F, Fernandes KJ (2012) Mammalian target of rapamycin signaling is a key regulator of the transit-amplifying progenitor pool in the adult and aging forebrain. J Neurosci 32:15012-15026

Palmer TD, Takahashi J, Gage FH (1997) The adult rat hippocampus contains primordial neural stem cells. Mol Cell Neurosci 8:389-404

Pan B, Wang W, Zhong P, Blankman JL, Cravatt BF, Liu QS (2011) Alterations of endocannabinoid signaling, synaptic plasticity, learning, and memory in monoacylglycerol lipase knock-out mice. J Neurosci 31:13420-13430

Pandey R, Hegde VL, Singh NP, Hofseth L, Singh U, Ray S, Nagarkatti M, Nagarkatti PS (2009) Use of cannabinoids as a novel therapeutic modality against autoimmune hepatitis. Vitam Horm 81:487-504

Panikashvili D, Simeonidou C, Ben-Shabat S, Hanus L, Breuer A, Mechoulam R, Shohami E (2001) An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. Nature 413:527-531

Paquette JJ, Wang HY, Bakshi K, Olmstead MC (2007) Cannabinoid-induced tolerance is associated with a CB1 receptor G protein coupling switch that is prevented by ultra-low dose rimonabant. Behav Pharmacol 18:767-776

Parent JM (2007) Adult neurogenesis in the intact and epileptic dentate gyrus. Prog Brain Res 163:529-540

Paria BC, Dey SK (2000) Ligand-receptor signaling with endocannabinoids in preimplantation embryo development and implantation. Chem Phys Lipids 108:211-220

Parmentier-Batteur S, Jin K, Mao XO, Xie L, Greenberg DA (2002) Increased severity of stroke in CB1 cannabinoid receptor knock-out mice. J Neurosci 22:9771-9775

Patel S, Carrier EJ, Ho WS, Rademacher DJ, Cunningham S, Reddy DS, Falck JR, Cravatt BF, Hillard CJ (2005) The postmortal accumulation of brain N-arachidonylethanolamine (anandamide) is dependent upon fatty acid amide hydrolase activity. J Lipid Res 46:342-349

Patel S, Rademacher DJ, Hillard CJ (2003) Differential regulation of the endocannabinoids anandamide and 2-arachidonylglycerol within the limbic forebrain by dopamine receptor activity. J Pharmacol Exp Ther 306:880-888

Pazos MR, Sagredo O, Fernandez-Ruiz J (2008) The endocannabinoid system in Huntington's disease. Curr Pharm Des 14:2317-2325

Pertwee RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol Ther 74:129-180

Pertwee RG (2009) Emerging strategies for exploiting cannabinoid receptor agonists as medicines. Br J Pharmacol 156:397-411

Pertwee RG (2012) Targeting the endocannabinoid system with cannabinoid receptor agonists: pharmacological strategies and therapeutic possibilities. Philos Trans R Soc Lond B Biol Sci 367:3353-3363

Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, Elphick MR, Greasley PJ, Hansen HS, Kunos G, Mackie K, Mechoulam R, Ross RA (2010) International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2). Pharmacol Rev 62:588-631

Petreanu L, Alvarez-Buylla A (2002) Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. J Neurosci 22:6106-6113

Pevny L, Placzek M (2005) SOX genes and neural progenitor identity. Curr Opin Neurobiol 15:7-13

Pevny LH, Lovell-Badge R (1997) Sox genes find their feet. Curr Opin Genet Dev 7:338-344

Piomelli D (2003) The molecular logic of endocannabinoid signalling. Nat Rev Neurosci 4:873-884

Piomelli D, Astarita G, Rapaka R (2007) A neuroscientist's guide to lipidomics. Nat Rev Neurosci 8:743-754

Piomelli D, Beltramo M, Glasnapp S, Lin SY, Goutopoulos A, Xie XQ, Makriyannis A (1999) Structural determinants for recognition and translocation by the anandamide transporter. Proc Natl Acad Sci U S A 96:5802-5807

Piomelli D, Giuffrida A, Calignano A, Rodriguez de Fonseca F (2000) The endocannabinoid system as a target for therapeutic drugs. Trends Pharmacol Sci 21:218-224

Pisani A, Fezza F, Galati S, Battista N, Napolitano S, Finazzi-Agro A, Bernardi G, Brusa L, Pierantozzi M, Stanzione P, Maccarrone M (2005) High endogenous cannabinoid levels in the cerebrospinal fluid of untreated Parkinson's disease patients. Ann Neurol 57:777-779

Porter AC, Felder CC (2001) The endocannabinoid nervous system: unique opportunities for therapeutic intervention. Pharmacol Ther 90:45-60

Potten CS, Loeffler M (1990) Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. Development 110:1001-1020

Prescott SM, Majerus PW (1983) Characterization of 1,2-diacylglycerol hydrolysis in human platelets. Demonstration of an arachidonoyl-monoacylglycerol intermediate. J Biol Chem 258:764-769

Price DA, Martinez AA, Seillier A, Koek W, Acosta Y, Fernandez E, Strong R, Lutz B, Marsicano G, Roberts JL, Giuffrida A (2009) WIN55,212-2, a cannabinoid receptor agonist, protects against nigrostriatal cell loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. Eur J Neurosci 29:2177-2186

Price TJ, Patwardhan A, Akopian AN, Hargreaves KM, Flores CM (2004) Cannabinoid receptor-independent actions of the aminoalkylindole WIN 55,212-2 on trigeminal sensory neurons. Br J Pharmacol 142:257-266

Puighermanal E, Busquets-Garcia A, Maldonado R, Ozaita A (2012) Cellular and intracellular mechanisms involved in the cognitive impairment of cannabinoids. Philos Trans R Soc Lond B Biol Sci 367:3254-3263

Puighermanal E, Marsicano G, Busquets-Garcia A, Lutz B, Maldonado R, Ozaita A (2009) Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. Nat Neurosci 12:1152-1158

Qiu DL, Knopfel T (2009) Presynaptically expressed long-term depression at cerebellar parallel fiber synapses. Pflugers Arch 457:865-875

Rahayel S, Frasnelli J, Joubert S (2012) The effect of Alzheimer's disease and Parkinson's disease on olfaction: a meta-analysis. Behav Brain Res 231:60-74

Rakic P (2004) Neuroscience: immigration denied. Nature 427:685-686

Ramirez BG, Blazquez C, Gomez del Pulgar T, Guzman M, de Ceballos ML (2005) Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. J Neurosci 25:1904-1913

Rathbone MP, Christjanson L, Deforge S, Deluca B, Gysbers JW, Hindley S, Jovetich M, Middlemiss P, Takhal S (1992) Extracellular purine nucleosides stimulate cell division and morphogenesis: pathological and physiological implications. Med Hypotheses 37:232-240

Rathbone MP, Middlemiss PJ, Gysbers JW, Andrew C, Herman MA, Reed JK, Ciccarelli R, Di Iorio P, Caciagli F (1999) Trophic effects of purines in neurons and glial cells. Prog Neurobiol 59:663-690

Ravinet Trillou C, Delgorge C, Menet C, Arnone M, Soubrie P (2004) CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. Int J Obes Relat Metab Disord 28:640-648

Reisenberg M, Singh PK, Williams G, Doherty P (2012) The diacylglycerol lipases: structure, regulation and roles in and beyond endocannabinoid signalling. Philos Trans R Soc Lond B Biol Sci 367:3264-3275

Reisert J, Matthews HR (2001) Response properties of isolated mouse olfactory receptor cells. J Physiol 530:113-122

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707-1710

Richardson D, Ortori CA, Chapman V, Kendall DA, Barrett DA (2007) Quantitative profiling of endocannabinoids and related compounds in rat brain using liquid chromatography-tandem electrospray ionization mass spectrometry. Anal Biochem 360:216-226

Robbe D, Kopf M, Remaury A, Bockaert J, Manzoni OJ (2002) Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. Proc Natl Acad Sci U S A 99:8384-8388

Rochefort C, Gheusi G, Vincent JD, Lledo PM (2002) Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. J Neurosci 22:2679-2689

Rochefort C, Lledo PM (2005) Short-term survival of newborn neurons in the adult olfactory bulb after exposure to a complex odor environment. Eur J Neurosci 22:2863-2870

Rodriguez S, Sickles HM, Deleonardis C, Alcaraz A, Gridley T, Lin DM (2008) Notch2 is required for maintaining sustentacular cell function in the adult mouse main olfactory epithelium. Dev Biol 314:40-58

Romero J, Lastres-Becker I, de Miguel R, Berrendero F, Ramos JA, Fernandez-Ruiz J (2002) The endogenous cannabinoid system and the basal ganglia. biochemical, pharmacological, and therapeutic aspects. Pharmacol Ther 95:137-152

Ross HH, Levkoff LH, Marshall GP, 2nd, Caldeira M, Steindler DA, Reynolds BA, Laywell ED (2008) Bromodeoxyuridine induces senescence in neural stem and progenitor cells. Stem Cells 26:3218-3227

Rouzer CA, Marnett LJ (2011) Endocannabinoid oxygenation by cyclooxygenases, lipoxygenases, and cytochromes P450: cross-talk between the eicosanoid and endocannabinoid signaling pathways. Chem Rev 111:5899-5921

Rubio-Araiz A, Arevalo-Martin A, Gomez-Torres O, Navarro-Galve B, Garcia-Ovejero D, Suetterlin P, Sanchez-Heras E, Molina-Holgado E, Molina-Holgado F (2008) The endocannabinoid system modulates a transient TNF pathway that induces neural stem cell proliferation. Mol Cell Neurosci 38:374-380

Rubio M, McHugh D, Fernandez-Ruiz J, Bradshaw H, Walker JM (2007) Short-term exposure to alcohol in rats affects brain levels of anandamide, other N-acylethanolamines and 2-arachidonoyl-glycerol. Neurosci Lett 421:270-274

Rueda D, Galve-Roperh I, Haro A, Guzman M (2000) The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. Mol Pharmacol 58:814-820

Rueda D, Navarro B, Martinez-Serrano A, Guzman M, Galve-Roperh I (2002a) The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway. J Biol Chem 277:46645-46650

Rueda D, Navarro B, Martinez-Serrano A, Guzman M, Galve-Roperh I (2002b) The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway. J Biol Chem 277:46645-46650

Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol 152:1092-1101

Saario SM, Salo OM, Nevalainen T, Poso A, Laitinen JT, Jarvinen T, Niemi R (2005) Characterization of the sulfhydryl-sensitive site in the enzyme responsible for hydrolysis of 2-arachidonoyl-glycerol in rat cerebellar membranes. Chem Biol 12:649-656

Sahay A, Hen R (2007) Adult hippocampal neurogenesis in depression. Nat Neurosci 10:1110-1115

Sanai N, Tramontin AD, Quinones-Hinojosa A, Barbaro NM, Gupta N, Kunwar S, Lawton MT, McDermott MW, Parsa AT, Manuel-Garcia Verdugo J, Berger MS, Alvarez-Buylla A (2004) Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature 427:740-744

Sanchez C, de Ceballos ML, Gomez del Pulgar T, Rueda D, Corbacho C, Velasco G, Galve-Roperh I, Huffman JW, Ramon y Cajal S, Guzman M (2001) Inhibition of glioma growth in vivo by selective activation of the CB(2) cannabinoid receptor. Cancer Res 61:5784-5789

Sanchez C, Galve-Roperh I, Canova C, Brachet P, Guzman M (1998) Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells. FEBS Lett 436:6-10

Sansom SN, Griffiths DS, Faedo A, Kleinjan DJ, Ruan Y, Smith J, van Heyningen V, Rubenstein JL, Livesey FJ (2009) The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet 5:e1000511

Sartipy P (2013) Advancing pluripotent stem cell culture: it is a matter of setting the standard. Stem Cells Dev 22:1159-1161

Sawzdargo M, Nguyen T, Lee DK, Lynch KR, Cheng R, Heng HH, George SR, O'Dowd BF (1999) Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain. Brain Res Mol Brain Res 64:193-198

Schlosburg JE, Blankman JL, Long JZ, Nomura DK, Pan B, Kinsey SG, Nguyen PT, Ramesh D, Booker L, Burston JJ, Thomas EA, Selley DE, Sim-Selley LJ, Liu QS, Lichtman AH, Cravatt BF (2010) Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. Nat Neurosci 13:1113-1119

Schwartz Levey M, Chikaraishi DM, Kauer JS (1991) Characterization of potential precursor populations in the mouse olfactory epithelium using immunocytochemistry and autoradiography. J Neurosci 11:3556-3564

Schwob JE, Huard JM, Luskin MB, Youngentob SL (1994) Retroviral lineage studies of the rat olfactory epithelium. Chem Senses 19:671-682

Schwob JE, Szumowski KE, Stasky AA (1992) Olfactory sensory neurons are trophically dependent on the olfactory bulb for their prolonged survival. J Neurosci 12:3896-3919

Schwob JE, Youngentob SL, Mezza RC (1995) Reconstitution of the rat olfactory epithelium after methyl bromide-induced lesion. J Comp Neurol 359:15-37

Schwob JE, Youngentob SL, Ring G, Iwema CL, Mezza RC (1999) Reinnervation of the rat olfactory bulb after methyl bromide-induced lesion: timing and extent of reinnervation. J Comp Neurol 412:439-457

Sekizawa SI, Tsubone H (1994) Nasal receptors responding to noxious chemical irritants. Respir Physiol 96:37-48

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153-7160

Shen M, Thayer SA (1998) Cannabinoid receptor agonists protect cultured rat hippocampal neurons from excitotoxicity. Mol Pharmacol 54:459-462

Shoemaker JL, Seely KA, Reed RL, Crow JP, Prather PL (2007) The CB2 cannabinoid agonist AM-1241 prolongs survival in a transgenic mouse model of amyotrophic lateral sclerosis when initiated at symptom onset. J Neurochem 101:87-98

Shohami E, Cohen-Yeshurun A, Magid L, Algali M, Mechoulam R (2011) Endocannabinoids and traumatic brain injury. Br J Pharmacol 163:1402-1410

Sideris A, Bekker T, Chan WS, Montoya-Gacharna JV, Blanck TJ, Recio-Pinto E (2012) A Role for the Cannabinoid 1 Receptor in Neuronal Differentiation of Adult Spinal Cord Progenitors in vitro is Revealed through Pharmacological Inhibition and Genetic Deletion. Front Neurosci 6:4

Simon GM, Cravatt BF (2006) Endocannabinoid biosynthesis proceeding through glycerophospho-N-acyl ethanolamine and a role for alpha/beta-hydrolase 4 in this pathway. J Biol Chem 281:26465-26472

Simpson PJ, Wang E, Moon C, Matarazzo V, Cohen DR, Liebl DJ, Ronnett GV (2003) Neurotrophin-3 signaling maintains maturational homeostasis between neuronal populations in the olfactory epithelium. Mol Cell Neurosci 24:858-874

Soltys J, Yushak M, Mao-Draayer Y (2010) Regulation of neural progenitor cell fate by anandamide. Biochem Biophys Res Commun 400:21-26

Song J, Zhong C, Bonaguidi MA, Sun GJ, Hsu D, Gu Y, Meletis K, Huang ZJ, Ge S, Enikolopov G, Deisseroth K, Luscher B, Christian KM, Ming GL, Song H (2012) Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. Nature 489:150-154

Soria-Gomez E, Bellocchio L, Reguero L, Lepousez G, Martin C, Bendahmane M, Ruehle S, Remmers F, Desprez T, Matias I, Wiesner T, Cannich A, Nissant A, Wadleigh A, Pape HC, Chiarlone AP, Quarta C, Verrier D, Vincent P, Massa F, Lutz B, Guzman M, Gurden H, Ferreira G, Lledo PM, Grandes P, Marsicano G (2014) The endocannabinoid system controls food intake via olfactory processes. Nat Neurosci 17:407-415

Spalding KL, Bhardwaj RD, Buchholz BA, Druid H, Frisen J (2005) Retrospective birth dating of cells in humans. Cell 122:133-143

Starowicz K, Nigam S, Di Marzo V (2007) Biochemistry and pharmacology of endovanilloids. Pharmacol Ther 114:13-33

Stella N, Schweitzer P, Piomelli D (1997) A second endogenous cannabinoid that modulates long-term potentiation. Nature 388:773-778

Stevens JC, Cain WS (1987) Old-age deficits in the sense of smell as gauged by thresholds, magnitude matching, and odor identification. Psychol Aging 2:36-42

Stone H, Williams B, Carregal EJ (1968) The role of the trigeminal nerve in olfaction. Exp Neurol 21:11-19

Straiker AJ, Maguire G, Mackie K, Lindsey J (1999) Localization of cannabinoid CB1 receptors in the human anterior eye and retina. Invest Ophthalmol Vis Sci 40:2442-2448

Struik ML, Yazulla S, Kamermans M (2006) Cannabinoid agonist WIN 55212-2 speeds up the cone response to light offset in goldfish retina. Vis Neurosci 23:285-293

Sugiura T, Kodaka T, Kondo S, Nakane S, Kondo H, Waku K, Ishima Y, Watanabe K, Yamamoto I (1997) Is the cannabinoid CB1 receptor a 2-arachidonoylglycerol receptor? Structural requirements for triggering a Ca2+ transient in NG108-15 cells. J Biochem 122:890-895

Sugiura T, Kodaka T, Nakane S, Miyashita T, Kondo S, Suhara Y, Takayama H, Waku K, Seki C, Baba N, Ishima Y (1999) Evidence that the cannabinoid CB1 receptor is a 2-arachidonoylglycerol receptor. Structure-activity relationship of 2-arachidonoylglycerol, ether-linked analogues, and related compounds. J Biol Chem 274:2794-2801

Sugiura T, Waku K (2002) Cannabinoid receptors and their endogenous ligands. J Biochem 132:7-12

Sugiura T, Yoshinaga N, Waku K (2001) Rapid generation of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand, in rat brain after decapitation. Neurosci Lett 297:175-178

Suh H, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH (2007) In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. Cell Stem Cell 1:515-528

Sullivan JM (2000) Cellular and molecular mechanisms underlying learning and memory impairments produced by cannabinoids. Learn Mem 7:132-139

Sultan S, Mandairon N, Kermen F, Garcia S, Sacquet J, Didier A (2010) Learning-dependent neurogenesis in the olfactory bulb determines long-term olfactory memory. FASEB J 24:2355-2363

Sun X, Dey SK (2009) Cannabinoid/Endocannabinoid signaling impact on early pregnancy events. Curr Top Behav Neurosci 1:255-273

Sundberg H, Doving K, Novikov S, Ursin H (1982) A method for studying responses and habituation to odors in rats. Behav Neural Biol 34:113-119

Sutterlin P, Williams EJ, Chambers D, Saraf K, von Schack D, Reisenberg M, Doherty P, Williams G (2013) The molecular basis of the cooperation between EGF, FGF and eCB receptors in the regulation of neural stem cell function. Mol Cell Neurosci 52:20-30

Suzuki Y, Takeda M (1991) Basal cells in the mouse olfactory epithelium after axotomy: immunohistochemical and electron-microscopic studies. Cell Tissue Res 266:239-245

Syed SK, Bui HH, Beavers LS, Farb TB, Ficorilli J, Chesterfield AK, Kuo MS, Bokvist K, Barrett DG, Efanov AM (2012) Regulation of GPR119 receptor activity with endocannabinoid-like lipids. Am J Physiol Endocrinol Metab 303:E1469-1478

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-676

Takahashi RN, Pamplona FA, Fernandes MS (2005) The cannabinoid antagonist SR141716A facilitates memory acquisition and consolidation in the mouse elevated T-maze. Neurosci Lett 380:270-275

Tanimura A, Uchigashima M, Yamazaki M, Uesaka N, Mikuni T, Abe M, Hashimoto K, Watanabe M, Sakimura K, Kano M (2010) Synapse type-independent degradation of the endocannabinoid 2-arachidonoylglycerol after retrograde synaptic suppression. Proc Natl Acad Sci U S A 109:12195-12200

Taupin P (2006) Autologous transplantation in the central nervous system. Indian J Med Res 124:613-618

Taupin P (2007) BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev 53:198-214

Temple S (2001) Stem cell plasticity--building the brain of our dreams. Nat Rev Neurosci 2:513-520

Thorne RG, Pronk GJ, Padmanabhan V, Frey WH, 2nd (2004) Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. Neuroscience 127:481-496

Till JE, Mc CE (1963) Early repair processes in marrow cells irradiated and proliferating in vivo. Radiat Res 18:96-105

Tomasiewicz H, Ono K, Yee D, Thompson C, Goridis C, Rutishauser U, Magnuson T (1993) Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system. Neuron 11:1163-1174

Tonini T, Rossi F, Claudio PP (2003) Molecular basis of angiogenesis and cancer. Oncogene 22:6549-6556

Trazzi S, Steger M, Mitrugno VM, Bartesaghi R, Ciani E (2010) CB1 cannabinoid receptors increase neuronal precursor proliferation through AKT/glycogen synthase kinase-3beta/beta-catenin signaling. J Biol Chem 285:10098-10109

Tschanz SA, Burri PH, Weibel ER (2011) A simple tool for stereological assessment of digital images: the STEPanizer. J Microsc 243:47-59

Tsou K, Brown S, Sanudo-Pena MC, Mackie K, Walker JM (1998) Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83:393-411

Twitchell W, Brown S, Mackie K (1997) Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. J Neurophysiol 78:43-50

Valjent E, Pages C, Rogard M, Besson MJ, Maldonado R, Caboche J (2001) Delta 9-tetrahydrocannabinol-induced MAPK/ERK and Elk-1 activation in vivo depends on dopaminergic transmission. Eur J Neurosci 14:342-352

Valjent E, Pascoli V, Svenningsson P, Paul S, Enslen H, Corvol JC, Stipanovich A, Caboche J, Lombroso PJ, Nairn AC, Greengard P, Herve D, Girault JA (2005) Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. Proc Natl Acad Sci U S A 102:491-496

van der Stelt M, Mazzola C, Esposito G, Matias I, Petrosino S, De Filippis D, Micale V, Steardo L, Drago F, Iuvone T, Di Marzo V (2006) Endocannabinoids and beta-amyloid-induced neurotoxicity in vivo: effect of pharmacological elevation of endocannabinoid levels. Cell Mol Life Sci 63:1410-1424

van der Stelt M, Trevisani M, Vellani V, De Petrocellis L, Schiano Moriello A, Campi B, McNaughton P, Geppetti P, Di Marzo V (2005) Anandamide acts as an intracellular messenger amplifying Ca2+ influx via TRPV1 channels. EMBO J 24:3026-3037

van Praag H, Christie BR, Sejnowski TJ, Gage FH (1999) Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A 96:13427-13431

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030-1034

van Praag H, Shubert T, Zhao C, Gage FH (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. J Neurosci 25:8680-8685

Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD, Sharkey KA (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science 310:329-332

Varma N, Carlson GC, Ledent C, Alger BE (2001) Metabotropic glutamate receptors drive the endocannabinoid system in hippocampus. J Neurosci 21:RC188

Varvel SA, Anum EA, Lichtman AH (2005) Disruption of CB(1) receptor signaling impairs extinction of spatial memory in mice. Psychopharmacology (Berl) 179:863-872

Velasco G, Galve-Roperh I, Sanchez C, Blazquez C, Guzman M (2004) Hypothesis: cannabinoid therapy for the treatment of gliomas? Neuropharmacology 47:315-323

Vemuri GS, McMorris FA (1996) Oligodendrocytes and their precursors require phosphatidylinositol 3-kinase signaling for survival. Development 122:2529-2537

Verhagen JV, Wesson DW, Netoff TI, White JA, Wachowiak M (2007) Sniffing controls an adaptive filter of sensory input to the olfactory bulb. Nat Neurosci 10:631-639

Vigers AJ, Bottger B, Baquet ZC, Finger TE, Jones KR (2003) Neurotrophin-3 is expressed in a discrete subset of olfactory receptor neurons in the mouse. J Comp Neurol 463:221-235

Vyas TK, Shahiwala A, Marathe S, Misra A (2005) Intranasal drug delivery for brain targeting. Curr Drug Deliv 2:165-175

Wachowiak M, Wesson DW, Pirez N, Verhagen JV, Carey RM (2009) Low-level mechanisms for processing odor information in the behaving animal. Ann N Y Acad Sci 1170:286-292

Walter L, Dinh T, Stella N (2004) ATP induces a rapid and pronounced increase in 2-arachidonoylglycerol production by astrocytes, a response limited by monoacylglycerol lipase. J Neurosci 24:8068-8074

Walter L, Franklin A, Witting A, Moller T, Stella N (2002) Astrocytes in culture produce anandamide and other acylethanolamides. J Biol Chem 277:20869-20876

Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G, Mackie K, Stella N (2003) Nonpsychotropic cannabinoid receptors regulate microglial cell migration. J Neurosci 23:1398-1405

Walther C, Guenet JL, Simon D, Deutsch U, Jostes B, Goulding MD, Plachov D, Balling R, Gruss P (1991) Pax: a murine multigene family of paired box-containing genes. Genomics 11:424-434

Wang H, Guo Y, Wang D, Kingsley PJ, Marnett LJ, Das SK, DuBois RN, Dey SK (2004) Aberrant cannabinoid signaling impairs oviductal transport of embryos. Nat Med 10:1074-1080

Wang ZJ, Sun L, Heinbockel T (2012) Cannabinoid receptor-mediated regulation of neuronal activity and signaling in glomeruli of the main olfactory bulb. J Neurosci 32:8475-8479

Warner-Schmidt JL, Duman RS (2006) Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. Hippocampus 16:239-249

Watanabe S, Umehara H, Murayama K, Okabe M, Kimura T, Nakano T (2006) Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. Oncogene 25:2697-2707

Weiler E, Farbman AI (1997a) Proliferation in the rat olfactory epithelium: age-dependent changes. J Neurosci 17:3610-3622

Weiler E, Farbman AI (1997b) Proliferation in the rat olfactory epithelium: age-dependent changes. J Neurosci 17:3610-3622

Weiler E, Farbman AI (1998) Supporting cell proliferation in the olfactory epithelium decreases postnatally. Glia 22:315-328

Wesson DW, Donahou TN, Johnson MO, Wachowiak M (2008) Sniffing behavior of mice during performance in odor-guided tasks. Chem Senses 33:581-596

Williams EJ, Walsh FS, Doherty P (2003) The FGF receptor uses the endocannabinoid signaling system to couple to an axonal growth response. J Cell Biol 160:481-486

Wilson RI, Kunos G, Nicoll RA (2001) Presynaptic specificity of endocannabinoid signaling in the hippocampus. Neuron 31:453-462

Wilson RI, Nicoll RA (2002) Endocannabinoid signaling in the brain. Science 296:678-682

Winner B, Cooper-Kuhn CM, Aigner R, Winkler J, Kuhn HG (2002) Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb. Eur J Neurosci 16:1681-1689

Witkin JM, Tzavara ET, Nomikos GG (2005) A role for cannabinoid CB1 receptors in mood and anxiety disorders. Behav Pharmacol 16:315-331

Witt RM, Galligan MM, Despinoy JR, Segal R (2009) Olfactory behavioral testing in the adult mouse. J Vis Exp

Witting A, Walter L, Wacker J, Moller T, Stella N (2004) P2X7 receptors control 2-arachidonoylglycerol production by microglial cells. Proc Natl Acad Sci U S A 101:3214-3219

Wolf SA, Bick-Sander A, Fabel K, Leal-Galicia P, Tauber S, Ramirez-Rodriguez G, Muller A, Melnik A, Waltinger TP, Ullrich O, Kempermann G (2010) Cannabinoid receptor CB1 mediates baseline and activity-induced survival of new neurons in adult hippocampal neurogenesis. Cell Commun Signal 8:12

Wonders CP, Anderson SA (2006) The origin and specification of cortical interneurons. Nat Rev Neurosci 7:687-696

Wu HH, Ivkovic S, Murray RC, Jaramillo S, Lyons KM, Johnson JE, Calof AL (2003) Autoregulation of neurogenesis by GDF11. Neuron 37:197-207

Xapelli S, Agasse F, Sarda-Arroyo L, Bernardino L, Santos T, Ribeiro FF, Valero J, Braganca J, Schitine C, de Melo Reis RA, Sebastiao AM, Malva JO (2013) Activation of type 1 cannabinoid receptor (CB1R) promotes neurogenesis in murine subventricular zone cell cultures. PLoS One 8:e63529

Yang M, Crawley JN (2009) Simple behavioral assessment of mouse olfaction. Curr Protoc Neurosci 8:24.21-24.12

Yoshida R, Ohkuri T, Jyotaki M, Yasuo T, Horio N, Yasumatsu K, Sanematsu K, Shigemura N, Yamamoto T, Margolskee RF, Ninomiya Y (2010) Endocannabinoids selectively enhance sweet taste. Proc Natl Acad Sci U S A 107:935-939

Yoshida T, Fukaya M, Uchigashima M, Miura E, Kamiya H, Kano M, Watanabe M (2006) Localization of diacylglycerol lipase-alpha around postsynaptic spine suggests close proximity between production site of an endocannabinoid, 2-arachidonoyl-glycerol, and presynaptic cannabinoid CB1 receptor. J Neurosci 26:4740-4751

Young JT (1981) Histopathologic examination of the rat nasal cavity. Fundam Appl Toxicol 1:309-312

Yu XH, Cao CQ, Martino G, Puma C, Morinville A, St-Onge S, Lessard E, Perkins MN, Laird JM (2010) A peripherally restricted cannabinoid receptor agonist produces robust anti-nociceptive effects in rodent models of inflammatory and neuropathic pain. Pain 151:337-344

Zeng LH, Rensing NR, Wong M (2009) The mammalian target of rapamycin signaling pathway mediates epileptogenesis in a model of temporal lobe epilepsy. J Neurosci 29:6964-6972

Zhang G, Chen W, Lao L, Marvizon JC (2010) Cannabinoid CB1 receptor facilitation of substance P release in the rat spinal cord, measured as neurokinin 1 receptor internalization. Eur J Neurosci 31:225-237

Zhang L, Xiong W (2009) Modulation of the Cys-loop ligand-gated ion channels by fatty acid and cannabinoids. Vitam Horm 81:315-335

Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645-660

Zimmer A, Zimmer AM, Hohmann AG, Herkenham M, Bonner TI (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proc Natl Acad Sci U S A 96:5780-5785

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, Julius D, Hogestatt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 400:452-457