The role of cannabinoids in adult neurogenesis

Header: Cannabinoids and Neurogenesis

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Abstract
The processes underpinning post-developmental neurogenesis in the mammalian brain continue to be defined. Such processes involve the proliferation of neural stem cells (NSCs) and neural progenitor cells (NPCs), neuronal migration, differentiation and integration into a network of functional synapses within the brain. Both intrinsic (cell signalling cascades) and extrinsic (neurotrophins, neurotransmitters, cytokines, hormones) signalling molecules are intimately associated with adult neurogenesis and largely dictate the proliferative activity and differentiation capacity of neural cells. Cannabinoids are a unique class of chemical compounds incorporating plant-derived cannabinoids (the active components of *Cannabis sativa*), the endogenous cannabinoids and synthetic cannabinoid ligands, and these compounds are becoming increasingly recognized for their roles in neural developmental processes. Indeed, cannabinoids have clear modulatory roles in adult neurogenesis, likely through activation of both CB_1 and CB_2 receptors. In recent years a large body of literature has deciphered the signalling networks involved in cannabinoid-mediated regulation of neurogenesis. This timely review summarises the evidence that the cannabinoid system is intricately associated with neuronal differentiation and maturation of NPCs, and highlights intrinsic/extrinsic signalling mechanisms that are cannabinoid targets. Overall these findings identify the central role of the cannabinoid system in adult neurogenesis in the hippocampus and the lateral ventricles, and hence provide insight into the processes underlying post-developmental neurogenesis in the mammalian brain.

The abbreviations used are:
2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; ACEA, arachidonyl-2'-chloroethylamide; AEA, anandamide; APP, amyloid precursor protein; BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; BrdU, 5-bromo-2'deoxyuridine; CB, cannabinoid receptor; CBC, cannabichromene; CBD, cannabidiol; CREB, cAMP response element-binding protein; DAGL, diacylglycerol lipase; DCX, double cortin; Dlx2, distal-less; DRG, dorsal root ganglion; EGFR, epidermal growth factor receptor; EPSCs, excitatory postsynaptic currents; ERK, extracellular signal-regulated kinase; FAAH, fatty acid amide hydrolase; FGF, Fibroblast growth factor-2; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; IGF-1, insulin-like growth factor-1; IL, interleukin; LTP, long-term potentiation; MAGL, monoacylglycerol lipase; mTORC1, mammalian target of rapamycin complex 1; NeuN, Neuronal Nuclei; NGF, nerve-growth factor; NPC, neural progenitor cell; NSC, neural stem cells; PI3K, phosphoinositide-3 kinase; PKC, Protein kinase C; PS1, presenilin protein 1; Ptc1, patched 1; RMS, rostral migratory stream; SGZ, subgranular zone; Shh, sonic hedgehog; Smo receptor, smoothened receptor; SVZ, subventricular zone; TACE, TNF-α-converting enzyme; TGF-β, transforming growth factor-beta; THC, Δ^9-tetrahydrocannabinol; TNF, tumour necrosis factor; $TRPV_1$, transient receptor potential cation channel subfamily V member 1; VEGF, vascular endothelial growth factor;

Introduction
For decades the true plasticity of the mammalian central nervous system (CNS) was underestimated and the adult brain was long considered to be a post-mitotic organ incapable of self-regeneration. However, pioneering work in the 1960's by Joseph Altman and colleagues challenged this long-standing dogma (Altman *et al.*, 1965). In this ground breaking publication Altman provided the first evidence that new neurons were generated in the adult rat

hippocampus. Subsequent experiments demonstrated that adult neurogenesis was not specific to the hippocampus, with the adult olfactory bulb identified as another brain region where new neurons are added to existing circuitry throughout life (Altman, 1969). In spite of this work, the concept of post-developmental neurogenesis in the mammalian brain was subject to contemporary scepticism; currently however, the phenomenon of adult neurogenesis is widely studied and research in the intervening years has confirmed adult neurogenesis in the murine hippocampus (Cameron *et al.*, 1993; Kempermann *et al.*, 1997), while the lateral ventricles (Lois *et al.*, 1993), regions adjacent to the ventricles (such as striatum and septum), as well as the thalamus and hypothalamus (Pencea *et al.*, 2001) have been shown to be capable of generating new neurons during adulthood. In the human brain, evidence continues to mount to support the absence of neurogenesis in the adult human neocortex (Rakic, 2006). However, adult neurogenesis has been described in the hippocampus (Eriksson *et al.*, 1998), the lateral ventricles (Sanai *et al.*, 2004) and more recently the striatum (Ernst *et al.*, 2014).

Cannabinoids incorporate the active components of the hemp plant *Cannabis sativa* (the plant-derived cannabinoids), the endogenous cannabinoids (endocannabinoids) produced in humans and animals and the synthetic cannabinoid compounds. The cannabinoid system is linked with all aspects of human physiology and elicits diverse effects by activating the G protein-coupled cannabinoid receptor (CB) type 1 (CB_1) and type 2 (CB_2) subtypes, the expression of which has been localized on glia, immune cells, and neurons throughout the CNS (Downer, 2011). A body of data indicate that cannabinoid ligands control cell genesis in the adult brain, regulating cell proliferation and overall neurogenesis in the mammalian brain (Kochman *et al.*, 2006; Mackowiak *et al.*, 2007). Furthermore, neural progenitor cells (NPCs) express a functional endocannabinoid system (Aguado *et al.*, 2005; Compagnucci *et al.*, 2013) and are producers of endogenous cannabinoids (Butti *et al.*, 2012). Such findings, alongside a number of knockout studies targeting enzymes involved in the biosynthesis and degradation of endocannabinoids (Aguado *et al.*, 2005; Gao *et al.*, 2010), in addition to CB_1 (Jin *et al.*, 2004) and CB_2 (Palazuelos *et al.*, 2006), place the cannabinoid system as a key player in the processes underlying adult neurogenesis.

Adult neurogenesis

Adult neurogenesis can be loosely divided into four stages: proliferation of neural stem cells (NSCs) and NPCs, migration, neuronal differentiation and finally integration into functional synaptic networks. The two regions in which adult neurogenesis has been most extensively studied are the dentate gyrus of the hippocampus and the lateral ventricles. NSCs in the dentate gyrus reside predominantly in the subgranular zone (SGZ) where four types (Type I, Type IIa, Type IIb and Type III) have been characterised based upon proliferation rate, protein expression and morphology. In the murine forebrain all newborn neurons are derived from Type I NPCs, these cells possess a glial-like radial process, although are predominantly unipolar/bipolar in contrast to multipolar astrocytes, express glial fibrillary acidic protein (GFAP) and the intermediate filament protein nestin (Garcia *et al.*, 2004). Type I NSCs are characterised by a low rate of proliferation (Ahn *et al.*, 2005). In contrast, Type IIa cells are non-radial, do not express GFAP, and exhibit a considerably higher proliferation rate compared with the relatively quiescent Type I cells. Type IIa cells maintain nestin expression and both cell types are positive for the Sox gene family (Suh *et al.*, 2007). Type IIb cells maintain important properties of stem cells as they uphold expression of nestin and Sox, but begin to express markers of neuronal committed progenitors, in particular the microtubule-associated protein double cortin (DCX). If

local conditions are favourable type IIb cells can mature to the nestin negative/DCX positive early neuronal type III cell (Kronenberg *et al.*, 2003).

In lateral ventricles the subventricular zone (SVZ) contains the majority of ventricular NSCs and is one of the key regions of the brain where neurogenesis occurs throughout adulthood (Curtis *et al.*, 2007). Three cell types have been discovered in the SVZ: Type B cells much resemble Type I cells in the SGZ; they are GFAP positive, possess a radial process and have a relatively low proliferation rate. Type C cells in the SVZ are reminiscent of Type II cells in the SGZ as they are GFAP negative, non-radial and highly proliferative. Both cell types express nestin and Sox (Doetsch *et al.*, 1997). Type A cells represent a population of neuroblasts which migrate at a rate of 30,000 per day along the rostral migratory stream (RMS) to the olfactory bulb (Alvarez-Buylla *et al.*, 2001).

NSCs in both the dentate gyrus and the lateral ventricles have the capacity to produce cells that differentiate to neurons, astrocytes and oligodendrocytes (Gage, 2000). Neuroblasts originating in the SVZ primarily differentiate into olfactory bulb interneurons (Luskin, 1993). Under the right conditions, NSCs in the dentate gyrus can migrate to the granular cell layer and give rise to granular cells that integrate into the hippocampal circuitry forming glutamatergic synapses with granular neurons, interneurons and pyramidal cells in *Cornu Ammonis region 3* (CA3) (Toni *et al.*, 2008). It has been suggested that these new born granular cells begin to resemble mature neurons, with regard to both their morphology and electrophysiological properties after approximately four weeks, although the maturation process continues for several months (Suh *et al.*, 2009). In the young adult rat hippocampus approximately 9,000 new cells are generated each day with 50% of these cells expressing neuronal markers within 5-12 days. Although survival rate is low, it has been estimated that each month the number of new granular cells generated equates to about 6% of the total granular cell number (Cameron *et al.*, 2001).

***Extrinsic signals* in adult neurogenesis**

NSC/NPCs are highly sensitive to their microenvironment (i.e. their stem cell niche) and extrinsic signalling molecules largely dictate the proliferative activity and differentiation capacity of these cells. The functions of neurotrophic factors as extrinsic signalling molecules in adult neurogenesis continues to be unravelled, with strong evidence indicating that Trk receptors (and p75NTR co-receptor) are abundant on dividing progenitor cells in the adult primate SVZ/SGZ (Tonchev *et al.*, 2007), with a body of literature indicating that brain-derived neurotrophic factor (BDNF) is a central player in adult neurogenesis. A common method of labelling proliferating cells in the dentate gyrus is to administer the thymidine analogue 5-bromo-2'deoxyuridine (BrdU), which incorporates into the DNA of cells during the S-phase of the cell cycle thus allowing the post-mortem identification of cells that have undergone proliferation. Intra-hippocampal infusion of BDNF has been shown to increase the number of cells positive for BrdU and the neuron-specific protein Neuronal Nuclei (NeuN) in adult rats (Scharfman *et al.*, 2005), while dentate gyrus-specific BDNF RNA interference reduces net-neurogenesis in rats by impairing the survival of immature neurons (Taliaz *et al.*, 2010). Similarly, NPC-specific deletion of the high affinity BDNF receptor TrkB in mice compromises dendritic development and the survival capacity of immature neurons (Bergami *et al.*, 2008), while BDNF-TrkB signalling has been shown to be imperative for hippocampal NSC proliferation in mice (Li *et al.*, 2008). Of note, two other neurotrophic factors have been implicated in the regulation of adult neurogenesis; nerve-growth factor (NGF) has been shown to increase cell proliferation (Birch *et al.*, 2013) and immature neuron survival (Frielingsdorf *et al.*,

2007) in the rat dentate gyrus, while vascular endothelial growth factor (VEGF) has also been shown to induce cell proliferation (Jin *et al.*, 2002) and promote immature neuron survival (Schanzer *et al.*, 2004) in the SVZ and SGZ of the adult rat.

In addition to neurotrophic factors, data indicate that several growth factors, including Insulin-like growth factor-1 (IGF-1) and Fibroblast growth factor-2 (FGF-2) are extrinsic factors involved in the regulation of adult neurogenesis. Indeed, subcutaneous or intraventricular infusion of IGF-1 enhances neurogenesis in the adult rat hippocampus (Aberg *et al.*, 2000), while data from Zhao *et al*., (2007) demonstrate that conditional deletion of *FGFR1* impairs the proliferation of NPCs in the dentate of adult mice (Zhao *et al.*, 2007).

Neurotransmitters are also important regulators of neurogenesis in the adult brain. In particular, Bolteus *et al*., (2004) demonstrated that γ-aminobutyric acid (GABA) has a direct effect on migrating neuroblasts in the adult mouse SVZ (Bolteus *et al.*, 2004), while many other studies have delineated the role of GABA in the regulation of NSC proliferative activity, fate decision and synaptic integration of immature neurons (Pallotto *et al.*, 2014). Similarly, glutamate can influence both proliferation and survival of NPCs; activation of the NMDA glutamate receptor has an inhibitory effect on cell proliferation and net-neurogenesis in the rat (Cameron *et al.*, 1995) and, in a somewhat paradoxical fashion, induction of long-term potentiation (LTP) at the perforant path-dentate gyrus pathway in rats increases proliferation and survival of NPCs/immature neurons via a NMDA receptor-dependent mechanism (Bruel-Jungerman *et al.*, 2006). Furthermore, the NMDA receptor has been shown to regulate survival of neuroblasts migrating from the mouse SVZ (Platel *et al.*, 2010). Taken together this suggests a complex role for glutamate in neurogenesis regulation. Additionally, monoamine neurotransmitters such as serotonin, noradrenaline and dopamine have also been identified as neurogenic modulators, either via direct links in the case of dopamine (Van Kampen *et al.*, 2004) or due to the fact that antidepressants and antipsychotics targeting these systems can affect neurogenesis (Dranovsky *et al.*, 2006).

The immune system can also heavily influence the fate of NSCs/NPCs with the anti-proliferative and anti-neuronal differentiative effects of inflammatory cytokines such as interleukin (IL)-6, IL-1β and tumour necrosis factor (TNF)-α well documented (Kohman *et al.*, 2013). Elsewhere the pro-neurogenic effects of the anti-inflammatory cytokine IL-10 has been demonstrated in the amyloid precursor protein (APP)/presenilin protein 1 (PS1) transgenic mouse (Kiyota *et al.*, 2012). Importantly, a body of data indicates that cross-talk may exist between inflammatory mediators (particularly TNF-α) and NSCs/NPCs, that may have important consequences for neural development and repair in disease states. Indeed, central administration of TNF-α to rats increases BrdU incorporation in SVZ cells (Wu *et al.*, 2000), while inhibiting endogenous TNF-α signalling regulates the proliferative capacity of mouse neural precursor cells (Rubio-Araiz *et al.*, 2008). In support of this, clear evidence indicates that this cytokine is upregulated in the mouse brain during demyelination and remyelination, enhancing the proliferative capacity of oligodendrocyte progenitor cells (Arnett *et al.*, 2001). Furthermore, Katakowski and colleagues (2007) have shown that TNF-α-converting enzyme (TACE) proteolysis promotes stroke-induced SVZ progenitor cell neurogenesis in rats (Katakowski *et al.*, 2007), indicating that TNF-α signalling may intricately impact neural development and brain repair, particularly in stroke pathogenesis.

Finally, several hormones including thyroid hormones (Remaud *et al.*, 2014), glucocorticoids and perhaps more speculatively oxytocin (Schoenfeld *et al.*, 2012) have been linked to neurogenesis regulation.

***Intrinsic signals* in adult neurogenesis**

A large body of research has delineated the multiple mechanisms regulating events associated with adult neurogenesis, including cell proliferation, differentiation, maturation, migration, and integration of neural cells into neuronal networks (Gage, 2000). Furthermore, through studies predominantly performed in rodents, the complexity of the cellular and molecular signalling processes regulating neurogenesis in the mammalian brain continues to be deciphered. It is now clear that key intrinsic signalling pathways involving Sonic Hedgehog (Shh), Wnt, bone morphogenetic protein (BMP), Notch and transcription factors are intimately associated with adult neurogenesis (Faigle *et al.*, 2013).

Shh is a signalling glycoprotein which acts through the Patched 1 (Ptc1)–Smoothened (Smo) receptor complex to activate intricate signal transduction pathways involved in the development of the CNS (Ruiz i Altaba *et al.*, 2002). Indeed, Ptc and Smo are expressed in the adult hippocampus (Traiffort *et al.*, 1998) and conditional deletion of Smo reduces the proliferation of progenitor cells in the postnatal hippocampus and SVZ (Machold *et al.*, 2003). In support of this, pharmacological inhibition of Shh signalling has been shown to reduce granule cell proliferation in the adult rat dentate gyrus (Lai *et al.*, 2003). More recent evidence also indicates that Shh signalling mediates cellular migration in the adult mouse mammalian brain (Balordi *et al.*, 2007), indicating the multifaceted role of Shh signalling in neurogenesis.

The Wnt signalling pathway is a long-standing player in the regulation of adult neurogenesis (McMahon *et al.*, 1990). Wnt ligands are a family of glycoproteins that play a role in the maturation of neurons, remodelling of axons and the maintenance of adult tissue homeostasis (Clevers *et al.*, 2012). Indeed, Wnt signalling, via β-catenin, mediates cellular differentiation in adult-derived mouse hippocampal progenitor cells (Lie *et al.*, 2005), and data elsewhere indicates that Wnt-mediated neurogenesis requires NeuroD1 in adult mouse hippocampal NPCs (Gao *et al.*, 2009). Overall, loss of function of Wnt signalling is strongly associated with determining the development of CNS disorders (De Ferrari *et al.*, 2000; Lovestone *et al.*, 2007).

BMPs are members of the transforming growth factor-beta (TGF-β) superfamily and consist of at least 20 growth factors that act as key regulators of axonal growth in a number of neuronal populations (Hegarty *et al.*, 2013). Indeed, clear evidence indicates that BMPs act as potent inhibitors of neuronal differentiation in the adult mouse SVZ (Lim *et al.*, 2000), while Mira and colleagues (2010) have demonstrated that inhibition of BMP signalling in adult mouse SGZ neural precursor cells differentially regulates neurogenesis (Mira *et al.*, 2010).

The components of the Notch signalling pathway are expressed in the SVZ and SGZ of the adult mammalian brain and data indicates that this pathway, through the inhibition of pro-neural genes, is a key regulator of neurogenesis in the CNS (Irvin *et al.*, 2004). Indeed, Notch signalling is associated with reducing the adult mouse neural progenitor pool (Hitoshi *et al.*, 2002) and promoting the self-renewal of nestin expressing cells in the adult mouse SGZ (Ables *et al.*, 2010). Interestingly, recent evidence indicates that cross-talk between Notch and epidermal growth factor receptor (EGFR) signalling exist, with downstream consequences on NSCs/NPCs in the adult mouse SVZ (Aguirre *et al.*, 2010). Furthermore, Notch 1 knock-out mice demonstrate a reduction in dendritic trees associated with granule cells in the mouse dentate gyrus (Ables *et al.*, 2010), highlighting the intrinsic role of Notch signalling in an array of neurodevelopmental cellular processes.

Recently, several transcription factors have been highlighted for their role in adult neurogenesis. In addition to the long-standing role of cAMP response element-binding protein (CREB) in regulating cell development (Finkbeiner *et al.*, 1997), more recent data indicate that CREB phosphorylation robustly enhances progenitor cell proliferation and controls the survival of new neurons in the adult mouse hippocampus *in vivo* (Jagasia *et al.*, 2009). Interestingly, overexpression of Ascl1 transcription factor regulates the fate of oligodendrocytes in the mouse SGZ *in vivo* (Jessberger *et al.*, 2008), and both the orphan nuclear receptor Tlx (Zhang *et al.*, 2008) and Sox2 gene family (Ferri *et al.*, 2004) are central in regulating NSC proliferation in the mouse hippocampus. In support of this data indicating that transcription factors are strongly linked to neural differentiation in the rodent brain *in vivo*, further evidence has identified that Tbr2 (Hodge *et al.*, 2012) and Distal-less (Dlx2) (Brill *et al.*, 2008) are also associated with neural differentiation in the mouse dentate gyrus and olfactory bulb, respectively.

Cannabinoids

The *Cannabis* plant has been utilised by humans in several capacities for thousands of years and Western medicine has recognised its therapeutic potential since the late 1800's (Reynolds, 1890). Today, this potential is still recognised (Robson, 2014) and the properties of the endocannabinoid system continue to be deciphered.

The CB_1 receptor was first described and cloned in the early 1990's (Gerard *et al.*, 1991; Matsuda *et al.*, 1990); it was found to be abundantly expressed throughout the CNS, and, in particular in areas associated with learning and memory including the hippocampus (Herkenham *et al.*, 1990). A second cannabinoid receptor, the CB_2 receptor, was also cloned in the 1990's (Munro *et al.*, 1993) where it was initially thought to be localised to the periphery, however, its expression in the CNS has been described (Gong *et al.*, 2006). Shortly after the identification of these receptors (receptor nomenclature follows (Alexander *et al.*, 2013)) their endogenous ligands, known as endocannabinoids, were discovered. The two endocannabinoids that have been studied in most detail are N-arachidonoylethanolamide (also known as anandamide; AEA) (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995). AEA is a phospholipid-derived molecule that is an agonist at the CB_1 and CB_2 receptor; it is detectable peripherally in the plasma and throughout the mammalian brain; in particular it is found at high concentrations in the hippocampus, cerebellum and cortex (Felder *et al.*, 1998). AEA is rapidly synthesised in neurons following depolarisation and subsequent Ca^{2+} influx (Dimarzo *et al.*, 1994). 2-AG, like AEA, is synthesised in an activity-dependent manner, is ubiquitously found in the CNS and is both a CB_1 and CB_2 receptor agonist, however the concentration of 2-AG is up to 1000 times that of AEA (Sugiura *et al.*, 1995). In neuronal signalling endocannabinoids function as retrograde neurotransmitters; they are synthesised and released by a post-synaptic neuron and activate receptors on presynaptic neurons (Wilson *et al.*, 2001). Deactivation of endocannabindoids occurs through specific enzymatic reactions. Fatty acid amide hydrolase (FAAH) is an intracellular membrane bound enzyme that degrades fatty-acid amides and it is responsible for inactivating AEA by catalysing its breakdown to arachidonic acid (AA) and ethanolamine (Cravatt *et al.*, 1996). Deactivation of 2-AG is primarily achieved by the enzyme monoacylglycerol lipase (MAGL) again producing AA (Dinh *et al.*, 2002).

In addition to endogenous cannabinoid receptor ligands, other classes of cannabinoids have been identified. The identification of *Cannabis* plant-derived cannabinoids, or phytocannabinoids, including cannabinol (CBN), cannabidiol (CBD) and the main psychoactive component of the plant Δ^9-tetrahydrocannabinol (THC), preceded the discovery of

endocannabinoids by several decades (Mechoulam *et al.*, 2014). To date it has been suggested that there is over 100 phytocannabinoids and novel cannabinoids continue to be isolated from the *Cannabis sativa* plant (Radwan *et al.*, 2009). Moreover, many synthetic agonists, inverse agonists and antagonists of the cannabinoid receptors have been produced. The synthetic cannabinoids HU-210 and *R*-(+)-WIN55212 show a high affinity for both the CB_1 and CB_2 receptor (Rinaldi-Carmona *et al.*, 1994), while selective agonists have also been identified including the CB_1 selective agonist arachidonyl-2'-chloroethylamide (ACEA) (Hillard *et al.*, 1999) and the CB_2 selective agonist JWH-133 (Huffman *et al.*, 1999). Other synthetic ligands that bind cannbinoid receptors but evoke inhibitory effects include SR141716A and SR144528 which exert CB_1 and CB_2 selectivity respectively (Rinaldi-Carmona *et al.*, 1994; Rinaldi-Carmona *et al.*, 1998), as well as the high affinity CB_1 ligand AM251 (Gatley *et al.*, 1996) and the high affinity CB_2 ligand AM630 (Ross *et al.*, 1999). Several lines of evidence suggest that theses ligands not only result in receptor antagonism but also inverse agonism (Pertwee, 2005).

In vivo effect of cannabinoids on adult neurogenesis

In addition to the various neurogenesis regulators discussed above, there is considerable evidence to suggest that both exogenous and endogenous cannabinoids can control cell genesis in the adult brain, although the effects can vary considerably according to the cannabinoid, dose and duration of administration (see Table. 1). What appears to be a common characteristic of both synthetic (Mackowiak *et al.*, 2007) and plant-derived (Kochman *et al.*, 2006) cannabinoids is that acute administration has no effect on cell proliferation or overall neurogenesis in the hippocampus, however, chronic administration of exogenous cannabinoids has been shown to affect the process. For example, chronic treatment with the potent synthetic cannabinoid HU-210, a drug that has a high affinity for both CB_1 and CB_2, enhances both proliferation and survival of cells in the rat dentate gyrus (Jiang *et al.*, 2005). Similarly, chronic administration of the CB_2 selective agonist HU-308 also exhibits proliferative-enhancing affects (Palazuelos *et al.*, 2012), raising the possibility that these effects may be mediated, at least in part, by CB_2 receptor signalling. This is supported by evidence that number of $BrdU^+$ cells in the dentate gyrus is reduced in CB_2 deficient mice (Palazuelos *et al.*, 2006). In contrast to this, chronic administration of another synthetic CB_1/CB_2 agonist WIN55,212-2 to rats during adulthood was found to have no effect on the number of immature neurons in the dentate gyrus, however, interestingly, administration during adolescence decreased the number of immature neurons, an affect that is attributed to selective suppression of dorsal but not ventral hippocampal neurogenesis (Abboussi *et al.*, 2014). Further contrasting effects are observed in the aged brain where WIN55,212-2 administration partially restored age-related deficits in hippocampal neurogenesis in rats (Marchalant *et al.*, 2009), suggesting a unique temporal role for cannabinoid receptors in the regulation of neurogenesis throughout the lifespan. The effects of the phytocannabinoid Δ^9-THC appear to be dose and/or time dependent; three week oral administration of a weekly escalating dose of Δ^9-THC was found to have no affect on cell proliferation in the mouse dentate gyrus (Kochman *et al.*, 2006), whereas, six week oral administration of a static dose of Δ^9-THC has been shown to decrease cell proliferation without having an affect on overall neurogenesis in mice (Wolf *et al.*, 2010). Interestingly, the study by Wolf and colleagues (2010) found that chronic administration of another phytocannabinoid CBD also decreased proliferation but, strikingly, and perhaps appearing somewhat counter-intuitive, is that CBD induced a substantial increase in net-neurogenesis, in a CB_1 receptor-dependent mechanism (Wolf *et al.*, 2010). This data is supported by evidence that repeated administration of CBD to wild type mice increases

hippocampal NPC proliferation via CB_1, which may underlie the anxiolytic effect of CBD in chronic stress (Campos *et al.*, 2013).

The CB_1 receptor inverse agonist AM 251 is often used to oppose the effects of endocannabinoids at the receptor and acute administration of this drug increases cell proliferation in the SGZ 24 h post-treatment (Hill *et al.*, 2006; Wolf *et al.*, 2010). However, this increase reverts to a decrease from 48 h onwards (Wolf *et al.*, 2010), again suggesting a complex temporal role for cannabinoid signalling in NSC fate. Chronically, the same inverse agonist was found to have no effect (Rivera *et al.*, 2011), however it has been shown to block the proliferative-enhancing affects of aerobic exercise (Hill *et al.*, 2010). This raises the possibility that endocannabinoid signalling via the CB_1 receptor may not be important for basal regulation of NPCs, but rather is essential for mediating the effects of exercise which is well established as a potent neurogenesis stimulator (van Praag, 2009). Another drug used to inhibit endocannabinoid activity, the CB_1 and transient receptor potential cation channel subfamily V member 1 ($TRPV_1$) antagonist SR141716A, has been shown to increase cell proliferation in the dentate gyrus and the lateral ventricles of mice (Jin *et al.*, 2004). This effect was observed in both wild type and CB_1, but not $TRPV_1$, knockout mice. Furthermore, Aguado and colleagues (2006) have observed reduced astrogliogenesis and increased neurogenesis in CB_1-deficient mice (Aguado *et al.*, 2006). These findings illustrate that multiple receptors are responsible for the effects of cannabinoids on neurogenesis, which may account for the complexity of the results observed.

Studies utilising gene knockdown technology to limit activity of the endocannabinoid system have provided compelling evidence linking cannabinoids and neurogenesis in the adult brain. Knockdown of the enzyme responsible for AEA hydrolysis, FAAH, increases cell proliferation in the dentate gyrus of adult mice (Aguado *et al.*, 2005), while Goncalves and colleagues (2008) have demonstrated that chronic inhibition of the enzyme responsible for production of 2-AG almost completely abolished cell proliferation in the mouse SVZ, while inhibiting FAAH also increased neurogenesis (Goncalves *et al.*, 2008). These findings illustrate the importance of basal endocannabinoid tone in maintaining neurogenesis. Elsewhere, complete knockdown of the α subtype of the Diacylglycerol lipase α (DAGLα) enzyme reduces brain 2-AG and AEA levels by approximately 80% and 40% respectively, and furthermore leads to a decrease in cell proliferation rate and a 50% reduction in immature DCX positive neurons in the mouse hippocampus (Gao *et al.*, 2010). The same study shows that a reduction in central 2-AG alone can also interfere with neurogenesis; knockdown of the DAGLβ subtype reduces 2-AG levels in the brain without significantly affecting AEA, and results in a decrease in cell proliferation in the hippocampus. Further evidence supporting a role for endocannabinoid signalling in adult hippocampal neurogenesis can be found in studies involving cannabinoid receptor knock-out animals; a $CB_1^{-/-}$ genotype is accompanied by a 50% decrease in proliferating cells in the dentate gyrus (Jin *et al.*, 2004; Kim *et al.*, 2006). Furthermore, Aguado and colleagues (2007) have demonstrated that kainic acid-induced hippocampal NPC proliferation is attenuated in $CB_1^{-/-}$ mice, indicating the role of CB_1 in neurogenesis induced by excitotoxicity (Aguado *et al.*, 2007). Intricate data from the same group indicates that $CB_1^{-/-}$ mice have reduced cortical thickness at post-natal day 2, indicating the integral role of CB_1 in controlling the specification of upper- and deep-layer cortical neurons (Diaz-Alonso *et al.*, 2012). Finally, $CB_2^{-/-}$ animals also exhibit a decreased proliferation rate illustrating the importance of both the CB_1 and CB_2 receptors (Palazuelos *et al.*, 2006). Taken together these studies suggest that the

endocannabinoid system, acting via multiple complex mechanisms, is a key player in the regulation of adult neurogenesis *in vivo*.

In vitro effect of cannabinoids on adult neurogenesis

It is known that NPCs (Aguado *et al.*, 2005) express a functional endocannabinoid system and are targeted by cannabinoids to promote neurosphere generation and NPC proliferation (see Table. 2). In addition, endocannabinoids are central in regulating neural differentiation and migration. Indeed, in embryonic murine precursor's derived from the cortex, AEA enhances cell differentiation toward a neuronal lineage via a CB_1-dependent mechanism (Compagnucci *et al.*, 2013). Furthermore, using freshly dissected RMS tissue from the postnatal brain, Oudin and colleagues (2011) have shown that endocannabinoid tone is central in controlling neuroblast migration from RMS explants (Oudin *et al.*, 2011). Elsewhere, Butti and colleagues (2012) demonstrate that SVZ adult mouse NPCs are producers of AEA, and that AEA regulates spontaneous excitatory postsynaptic currents (EPSCs) in medium spiny neurons (Butti *et al.*, 2012). Furthermore, the synthetic cannabinoid WIN-55,212-2, in addition to the selective FAAH inhibitor, URB597, have been shown to promote neurosphere generation, while WIN-55,212-2, URB597 and endocannabinoids (both AEA and 2-AG) increase the number of $BrdU^+$ NPCs from dissociated neurospheres (Aguado *et al.*, 2005). In further experiments from this group using postnatal rat cortical neural progenitors, WIN-55,212-2, URB597, AEA and 2-AG increased the number of $GFAP^+$ cells with a concomitant decrease in β-tubulin III^+ cells after differentiation for 2 days, indicating the pro-gliogenic action of synthetic and endogenous cannabinoids during the differentiation process (Aguado *et al.*, 2006). Elsewhere, the CB_2 specific agonist AM1241 has been shown to promote the proliferation/differentiation of human NSCs in the presence of the HIV-1 glycoprotein Gp120, and furthermore AM1241 prevents DNA fragmentation induced by administration of Gp120, which suggests a neuroprotective role of CB_2 against impaired neurogenesis, with relevance to the cognitive deficits seen in HIV-1 patients (Avraham *et al.*, 2014). Indeed, CB_2 knockout reduces the self-renewal (as determined by neurosphere generation *in vitro*) of murine embryonic cortical NPCs (Palazuelos *et al.*, 2006), while both HU-308 and JWH-133 increase both primary neurosphere generation and neural progenitor self-renewal *in vitro* (Palazuelos *et al.*, 2006). Rubio-Araiz and co-workers demonstrated that both CB_1 (ACEA) and CB_2 (JWH-056) agonists stimulate the proliferation of primary murine cortical neurospheres (Rubio-Araiz *et al.*, 2008) and recently it has also been demonstrated that hemopressin (a CB_1 inverse agonist) promotes oligodendroglial differentiation within SVZ NSC/NPC cultures derived from neonatal mice (Xapelli *et al.*, 2014). In support of this, the CB_1 receptor agonist ACEA promotes murine neural precursor differentiation via CB_1, with the CB_2 receptor agonist JWH-133 being ineffective (Compagnucci *et al.*, 2013).

Mechanisms of cannabinoid-induced regulation of intrinsic/extrinsic signalling in adult neurogenesis

The cellular signalling events orchestrated by cannabinoids in NPCs continue to be elucidated, with particular roles for extracellular signal-regulated kinase (ERK), phosphoinositide-3 kinase (PI3K) and Akt pathways suggested (see Figure. 1). In particular, CB_2 couples to the ERK and PI3K/Akt cascades (Molina-Holgado *et al.*, 2007; Palazuelos *et al.*, 2006; Palazuelos *et al.*, 2012), and the CB_2 agonist HU-308 promotes the proliferation of NPCs via ERK and PI3K/Akt signalling (Palazuelos *et al.*, 2006). In support of this, HU-308 is a robust activator of the PI3K/Akt pathway in the HiB5 hippocampal progenitor cell line (Palazuelos *et al.*, 2012).

Interestingly, mammalian target of rapamycin complex 1 (mTORC1) signalling is a target of the PI3K/Akt pathway and hence is central in neural cell survival/death decision, and mTORC signalling also contributes to CB_2 regulated NPC proliferation. Indeed, HU-308 induces cell proliferation in both embryonic organotypic cortical slices and in adult hippocampal NPCs via an mTORC1-dependent mechanism (Palazuelos *et al.*, 2012). Elsewhere, both CB_1 (ACEA) and CB_2 (JWH-056) agonists have been shown to stimulate the proliferation of mouse neural precursor cells via PI3K/Akt pathways (Molina-Holgado *et al.*, 2007) and TNF-α signalling mechanisms (Rubio-Araiz *et al.*, 2008). Both the synthetic cannabinoid HU210, and AEA, promote the proliferation of cultured embryonic hippocampal NPCs in a concentration dependent manner involving $G_{i/o}$ proteins and the ERK signalling pathways (Jiang *et al.*, 2005). Further *in vitro* evidence indicates that ACEA enhances murine neural precursor differentiation to neurons by targeting ERK signalling (Compagnucci *et al.*, 2013). In addition, ACEA reduces ERK phosphorylation in neural precursor cells and this reduction promotes neuronal differentiation. Using neurogenesis and PCR arrays, Compagnucci *et al*., (2013) recently demonstrated that CB_1 activation promotes the expression of genes involved in neuronal maturation and commitment to a neuronal lineage (Compagnucci *et al.*, 2013). In contrast, the endogenous cannabinoid AEA has been shown to inhibit cortical neuron progenitor differentiation to mature neuronal phenotype, decrease the proliferation of primary postnatal murine NPCs (Soltys *et al.*, 2010) and inhibit the differentiation of the human NSC line, HNSC.100 (Rueda *et al.*, 2002). These events are CB_1 receptor-dependent and since AEA inhibits NGF-induced ERK activation in PC12 cells via CB_1, this suggests that AEA inhibits NPC differentiation through attenuation of the ERK pathway (Rueda *et al.*, 2002).

Further data elsewhere indicate that signalling involving CREB transcription factor may govern cannabinoid-induced regulation of NPCs. Indeed, exposure of murine NPCs to AEA promotes glial and neuronal differentiation, with a possible role for CREB (Soltys *et al.*, 2010). Much data indicate that CREB is a cannabinoid target, with recent evidence indicating that CB_2 agonists target CREB signalling in the rat cortex after subarachnoid hemorrhage (Fujii *et al.*, 2014) and cerebral ischemia (Choi *et al.*, 2013). In support of this, THC (Casu *et al.*, 2005) and AEA (Isokawa, 2009) administration has been shown to regulate the expression of phosphorylated CREB in the rat cerebellum and hippocampus, respectively, while the CB_2 receptor agonist, *trans*-caryophyllene, promotes the phosphorylation of neural CREB (Choi *et al.*, 2013).

The Sox2 gene family regulate NSC proliferation in the hippocampus, and recent evidence indicates that CB_1 activation enhances the number of $Sox2^+$ cells via Notch signalling in cultured mouse SVZ cells, suggesting that CB_1 activation promotes the self-renewal of SVZ cultures (Xapelli *et al.*, 2013). Cannabinoids also regulate the expression of the T-box transcription factor, Tbr, which may be central in mediating the neurogenic effects of cannabinoids. Indeed, Saez *et al*., (2014) has recently demonstrated that prenatal exposure of rats to WIN-55,212-2 differentially regulates the number of glutamatergic intermediate progenitors ($Tbr2^+$) and post-mitotic neurons ($Tbr1^+$) during embryonic development in the cortex (Saez *et al.*, 2014). Interestingly, this indicates that prenatal exposure to WIN-55,212-2 impacts the differentiation of glutamatergic neurons in the developing cerebral cortex. In support of this, data from CB_1-deficient murine embryos indicate that there is a decrease in $Tbr2^+$ cells in the SVZ (Diaz-Alonso *et al.*, 2014) while $Tbr1^+$ post-mitotic cells accumulate abnormally during embryogenesis in deep bins of the cortical plate of CB_1-deficient mice when compared with wild type littermates (Diaz-Alonso *et al.*, 2012).

Neurotrophic factors are strongly linked to adult neurogenesis and recent evidence suggests that there is functional interplay between BDNF and CB_1 receptors in the brain (De Chiara *et al.*, 2010). In support of this, Maison and colleagues (2009) demonstrated that BDNF increases CB_1 expression in cultured rat cerebellar granule neurons (Maison *et al.*, 2009), while BDNF can also promote the production of cortical endocannabinoids (Lemtiri-Chlieh *et al.*, 2010). In human studies, D'Souza and colleagues (2009) demonstrated that intravenous administration of THC enhances the expression of peripheral BDNF in serum (D'Souza *et al.*, 2009), and this is supported by evidence that CB_2 stimulation promotes BDNF expression in rat neurons (Choi *et al.*, 2013). Recent evidence also suggests that CB_1 can cross-talk with NGF signalling in adult mouse dorsal root ganglion (DRG) neurons (Wang *et al.*, 2014). In addition, intricate new data from Keimpema and co-workers (2013) indicates that NGF impacts endocannabinoid signalling to promote cholinergic differentiation in mice (Keimpema *et al.*, 2013).

A body of literature indicates that signalling involving adenosine, Protein kinase C (PKC), growth factors and IL-1 receptor may govern cannabinoid-induced regulation of NPCs. Indeed, using adult neural precursor cells prepared from the whole brains of 8-week old mice, Shinjo *et al*., (2013) recently demonstrated that the major non-THC phytocannabinoid, cannabichromene (CBC), promotes cell survival during differentiation while blunting cell differentiation into astroglia. The authors suggest the involvement of ERK, ATP and adenosine signalling cascades in mediating the effects of CBC on neural cells (Shinjyo *et al.*, 2013). Recent evidence also indicates that cannabinoids can target the actin-bundling protein fascin, which plays a role in the migration of neuroblasts and neural development (Sonego *et al.*, 2013). Indeed, the CB_1 agonist ACEA controls the interaction between fascin and PKC, which indicates that CB_1-dependent signalling may regulate actin-bundling activity, with subsequent impact on neuroblast migration (Sonego *et al.*, 2013). EGFR signalling is key in controlling NSC survival, and using the Cor-1 NSC line, data from Sutterlin and colleagues (2013) demonstrate that CB_1 and CB_2 cooperate with EGFR in the regulation of NSC expansion (Sutterlin *et al.*, 2013). Similarly, CB_1 has been shown to couple activated FGF receptor to an axonal growth in rat cerebellar granule neurons (Williams *et al.*, 2003). Finally, Garcia-Ovejero and colleagues (2013) have demonstrated that both CB_1 and CB_2 are co-expressed with IL-1R1 and IL-1R2 in mouse brain neurospheres, and both ACEA and JWH-133 impact IL-1 signalling in primary cultures of mouse brain-derived neurospheres, increasing IL-1β, while decreasing IL-1Ra production by neurospheres. This is significant given that IL-1β negatively regulates neurosphere proliferation (Garcia-Ovejero *et al.*, 2013).

Concluding remarks

While much progress has been made in recent decades in understanding the process of adult neurogenesis, the underlying mechanisms have yet to be fully elucidated. As highlighted in this review, the microenvironment clearly determines the rate of proliferation of NSCs and NPCs, their survival and their differentiation into mature neurons that are integrated into functional networks. Endocannabinoids may play pivotal roles in at least some of these phases of neurogenesis. Of particular interest are the varying temporal effects of synthetic, endogenous and plant-derived cannabinoids on the proliferation and survival phases of neurogenesis, indicating complex physiological regulation of this process that may be modulated by drugs that target the endocannabinoid system. The functional importance of neurogenesis has yet to be clarified, however the weight of evidence indicates that impaired neurogenesis is associated with

depression and cognitive impairment. Pharmacological targeting of the cannabinoid system as a regulator of neurogenesis may prove a fruitful strategy in the prevention or treatment of mood or memory disorders.

Figure. 1
Endocannabinoid signalling regulates NPCs in the adult brain. Endocannabinoids acting in an autocrine and paracrine fashion may activate CB_1 and/or CB_2 receptors. CB_1 and CB_2 activity can induce both PI3K/Akt/mTORC and MEK/MAPK/CREB signalling pathways that influence cell proliferation, differentiation and survival, while also promoting integration of immature neurons into existing circuitry. In addition, CREB can induce transcription of BDNF that can directly influence cell fate and may also increase CB_1 expression and endocannbinoid production, possibly leading to positive feedback within the signalling system.

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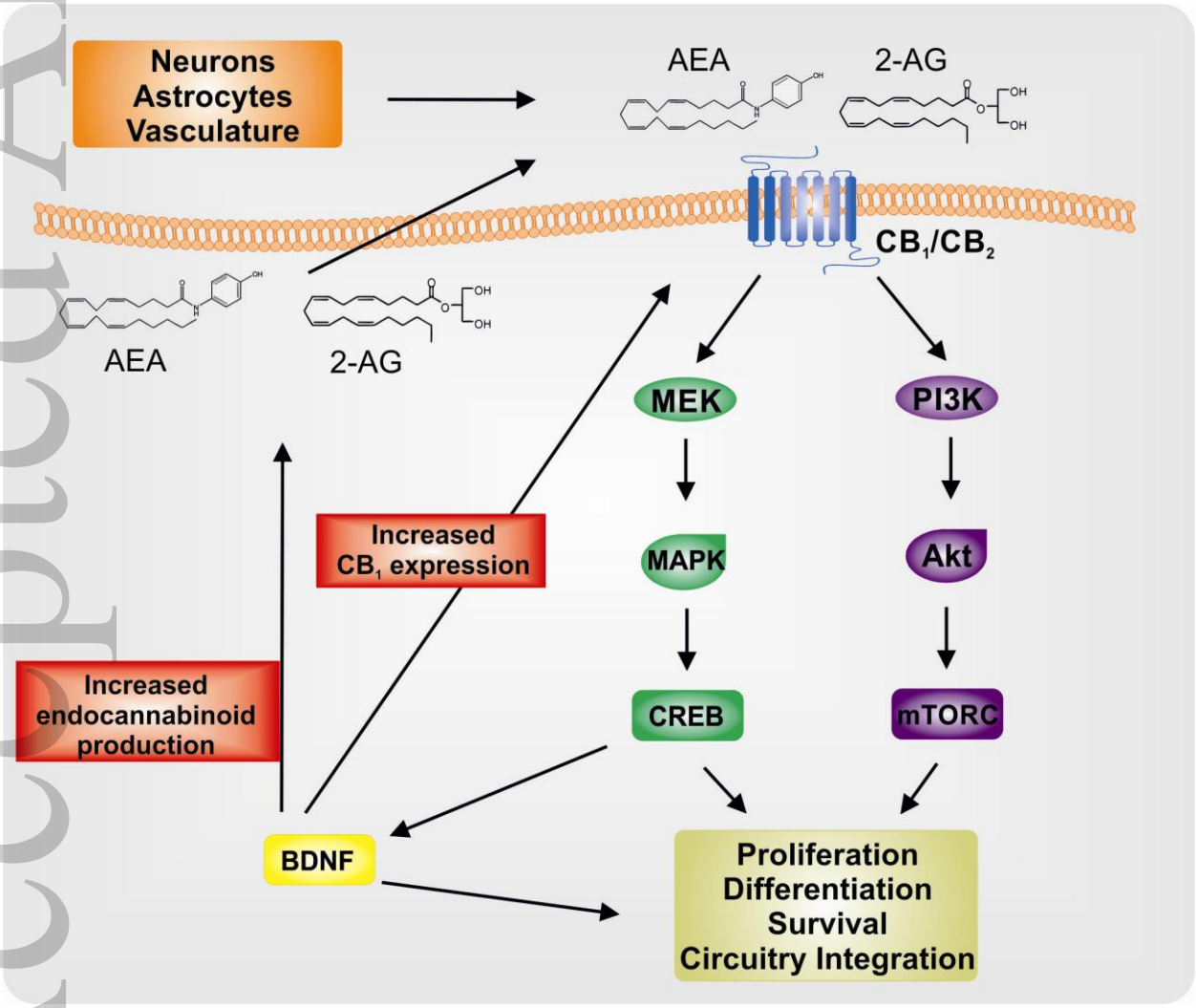

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Table 1. Literature assessing the *in vivo* effects of cannabinoids in neurogenesis

Treatment	Measurement	Observation	Reference
HU-210	Cell proliferation in the dentate gyrus in adult rats	Enhanced	Jiang *et al.*, 2005
HU-308	Hippocampal progenitor proliferation in adult mice	Enhanced	Palazuelos *et al.*, 2012
WIN55,212-2	Dorsal hippocampal neurogenesis during adolescence	Reduced	Abboussi *et al.*, 2014
WIN55,212-2	Age-related deficits in hippocampal neurogenesis	Partial restoration	Marchalant *et al.*, 2009
Δ^9-THC/CBD	Precursor cell proliferation in the dentate gyrus	Reduced	Wolf *et al.*, 2010
CBD	Cell survival in the dentate gyrus	Enhanced	Wolf *et al.*, 2010
CBD	Number of $BrdU^+$ cells co-localised with $NeuN^+$ cells in hippocampus	Enhanced	Campos *et al.*, 2013
DAGL inhibitor	Cell proliferation in the adult SVZ	Reduced	Goncalves *et al.*, 2008
URB597/AEA/ WIN55,212-2	Adult hippocampal NPC proliferation	Enhanced	Aguado *et al.*, 2005
WIN55,212-2/JWH-133/ URB597	Progenitor cell proliferation in the SVZ	Enhanced	Goncalves *et al.*, 2008
AM 251	Cell proliferation in the SGZ	Enhanced	Hill *et al.*, 2006
AM 251	Cell proliferation in the SGZ	Enhanced at 24h/ reduced at 48h	Wolf *et al.*, 2010
$FAAH^{-/-}$	Cell proliferation in the dentate gyrus of adult mice	Enhanced	Aguado *et al.*, 2005
$DAGL\alpha^{-/-}$	Cell proliferation and number of DCX^+ neurons in the hippocampus	Reduced	Gao *et al.*, 2010
$DAGL\beta^{-/-}$	Cell proliferation in the hippocampus	Reduced	Gao *et al.*, 2010
$CB_1^{-/-}$	Cell proliferation in the dentate gyrus and SVZ	Reduced	Jin *et al.*, 2004 Kim *et al.*, 2006
$CB_1^{-/-}$	Number of $BrdU^+$ cells co-localised with $S100\beta^+$ cells in the SGZ and granule cell layer of the dentate gyrus	Reduced	Aguado *et al.*, 2006
$CB_1^{-/-}$	Number of $BrdU^+$ cells co-localised with $NeuN^+$ cells in the SGZ and granule cell layer of the dentate gyrus	Enhanced	Aguado *et al.*, 2006
$CB_1^{-/-}$	Kainic acid-induced hippocampal NPC proliferation	Reduced	Aguado *et al.*, 2007
$CB_1^{-/-}$	Cortical thickness	Reduced at P2	Diaz-Alonso *et al.*, 2012
SR141716A	Cell proliferation in the SVZ	Enhanced	Jin *et al.*, 2004
JTE-907/AM630	Cell proliferation in the SVZ	Reduced	Goncalves *et al.*, 2008
$CB_2^{-/-}$	Number of $BrdU^+$ cells in dentate gyrus	Reduced	Palazuelos *et al.*, 2006

JTE-907 and AM630 are CB_2 receptor antagonists

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Table 2. Literature assessing the *in vitro* effects of cannabinoids in neurogenesis

Treatment	Measurement	Observation	Reference
HU210/AEA	Proliferation of embryonic hippocampal NPCs/NSCs	Enhanced	Jiang *et al.*, 2005
HU-308	Proliferation of HiB5 NPCs	Enhanced	Palazuelos *et al.*, 2012
HU-308	Proliferation of cortical progenitors in organotypic cultures	Enhanced	Palazuelos *et al.*, 2012
AEA/ACEA	Differentiation of embryonic murine neural precursors derived from the cortex towards neural lineage	Enhanced	Compagnucci *et al.*, 2013
ACEA/JWH-133	Migration of Cor-1 NSC line	Enhanced	Oudin *et al.*, 2011
AM251/JTE-907/ DAGL inhibitors	RMS neuroblast migration	Reduced	Oudin *et al.*, 2011
ACEA/JWH-133	RMS neuroblast migration	Enhanced	Oudin *et al.*, 2011
ACEA/JWH-056	Proliferation of neurospheres	Enhanced	Rubio-Araiz *et al.*, 2008
WIN-55,212-2/URB597	Neurosphere generation	Enhanced	Aguado *et al.*, 2005
WIN-55,212-2/URB597/ AEA/2-AG	Number of $BrdU^+$ NPCs from dissociated neurospheres	Enhanced	Aguado *et al.*, 2005
WIN-55,212-2/URB597/ AEA/2-AG	Number of $GFAP^+$ cells after differentiation of postnatal NPCs for 2 days	Enhanced	Aguado *et al.*, 2006
WIN-55,212-2/URB597/ AEA/2-AG	Number of β-tubulin III^+ cells after differentiation of postnatal NPCs for 2 days	Decreased	Aguado *et al.*, 2006
AM1241	Proliferation/differentiation of human NSCs in presence of Gp120	Enhanced	Avraham *et al.*, 2014
$CB_2^{-/-}$	Neurosphere generation of murine embryonic cortical NPCs	Reduced	Palazuelos *et al.*, 2006
HU-308/JWH-133	Primary neurosphere generation and NPC self-renewal	Increased	Palazuelos *et al.*, 2006
Hemopressin	Oligodendroglial differentiation within SVZ NPC/NSC cultures	Increased	Xapelli *et al.*, 2014

Hemopressin is a CB_1 inverse agonist

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