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Unveiling the role of adenosine A_{2A} receptors on neurogenesis induced by activation of cannabinoid receptors in rat SVZ and DG stem/progenitor cell cultures

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Sara Xapelli (Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa) e do Professor Doutor Carlos Duarte (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra)

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“Ever tried. Ever failed. No matter. Try again. Fail again. Fail better.”

(Samuel Beckett)

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Abbreviations

Δ9-THC Δ9-tetrahydrocannabinol

2-AG 2-arachidonoylglycerol

AEA Arachidonoylethanolamide

ARs Adenosine receptors

A_1Rs, A_{2A}Rs, A_{2B}Rs, A_3Rs Adenosine A_1, A_{2A}, A_{2B} and A_3 receptors

BDNF Brain-derived neurotrophic factor

CA3 Cornu Ammonis 3 area

CBRs Cannabinoid receptors

CB_1Rs, CB_2Rs Cannabinoid receptors type 1 and type 2

CNS Central nervous system

CSF Cerebrospinal fluid

DG Dentate gyrus

EC Entorhinal cortex

ECS Endocannabinoid system

EGF Epidermal growth factor

FGF-2 Fibroblast growth factor-2

GABA Gamma-aminobutyric acid

GCs Granule cells

GCL Granular cell layer

GDNF Glial-derived neurotrophic factor

GL Glomerular layer

GPCRs G protein-coupled receptors

HPA Hypothalamic-pituitary-adrenal axis

IGCs Immature granule cells

IPCs Intermediate progenitor cells

LV Lateral ventricle

MAPKs Mitogen-activated protein kinases

NPCs Neural progenitor cells

NSPCs Neural stem/progenitor cells

OB Olfactory bulb

PLC Phospholipase C

RMS Rostral migratory stream

SVZ Subventricular zone

SGZ Subgranular zone

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Abstract

Over the past years, the idea that neurogenesis, the process by which new functional neurons are produced, does not occur in the adult matured brain has considerably changed. In fact, neurogenesis actively occurs mainly in two distinct neurogenic niches, the subventricular zone (SVZ), along the lateral walls of the lateral ventricles, and the subgranular zone (SGZ), in the dentate gyrus (DG) of the hippocampus, where newly formed neurons, derived from neural stem/progenitor cells (NSPCs), have a role in olfaction/odor discrimination and in learning/memory, respectively.

Adenosine, which is a widespread neuromodulator in the brain, greatly influences synaptic neurotransmission and is involved in many physiological and neuropathological processes. Moreover, the endocannabinoid system, which activates primarily type 1 and 2 cannabinoid receptors (CB_1R and CB_2R), is a key regulator of synaptic function, especially by inhibiting neurotransmitter release. Furthermore, it has been shown that CB_1R and CB_2R activation modulates neurogenesis by promoting neural stem cell proliferation, differentiation and maturation. Importantly, an interaction between A_{2A} receptors ($A_{2A}Rs$) and CB_1Rs was reported and shown to allow the control of key modulatory effects on neuronal function and transmission. However, to date, no study has directly evaluated the effects of the crosstalk between $A_{2A}Rs$ and CBRs on neurogenesis.

Therefore, in this work, the putative role of $A_{2A}Rs$ on neurogenesis induced by the activation of CB_1Rs and CB_2Rs was investigated by looking at different stages of the neurogenic process.

Results show that CB_1R, CB_2R or $A_{2A}R$ activation did not change the SVZ cell-fate. Moreover, activation of CB_2R or $A_{2A}R$ did not induce SVZ cell proliferation. However, CB_1R activation promoted cell proliferation, an effect that was blocked by an $A_{2A}R$ selective antagonist. Interestingly, activation of either CB_1R or CB_2R promoted an increase in SVZ neuronal differentiation that is impaired by $A_{2A}R$ blockade, although $A_{2A}R$ activation *per se* had no effect on neuronal differentiation.

Concerning the DG neurogenic niche it was observed that while CB_1R activation had no effect on the cell fate of DG cells, activation of CB_2Rs or $A_{2A}Rs$ promoted self-renewing divisions. Furthermore, $A_{2A}R$ selective antagonist blocked the effect mediated by activation of CB_2Rs in the self-renewal of DG cells. Moreover, $A_{2A}R$-mediated effect on self-renewal was blocked either by CB_1R or CB_2R selective antagonists. Moreover, although activation of CB_1R, CB_2R or $A_{2A}R$ *per se* did not induce DG cell proliferation, an increase in the number of proliferating cells was observed upon CB_1R or CB_2R co-activation with $A_{2A}Rs$. Lastly, CB_1R, CB_2R and $A_{2A}R$ activation promoted DG neuronal differentiation. Interestingly, the effect

mediated by CB_1Rs or CB_2Rs was blocked by an A_{2A}R selective antagonist, while the effect mediated by A_{2A}Rs was impaired in the presence of CB_1R or CB_2R selective antagonists.

Taken together, these results suggest that a possible crosstalk between the adenosinergic and endocannabinoid systems may exist, either by a structural interaction (formation of heterodimers) or by crosstalk at downstream signaling, that ultimately contributes to the control of neurogenesis.

Keywords: Neurogenesis, Neurogenic niches, Adenosine A_{2A} receptors, Cannabinoid receptors

Resumo

A ideia de que neurogénese, o processo pelo qual são produzidos novos neurónios funcionais, não ocorre no cérebro adulto foi alterada consideravelmente ao longo dos anos. Efetivamente, a neurogénese ocorre em dois nichos neurogénicos distintos, a zona subventricular (SVZ), que percorre a parede lateral dos ventrículos laterais, e a zona subgranular (SGZ, no giro dentado (DG) do hipocampo, onde neurónios recém-formados, descendentes de células estaminais/progenitoras neurais, apresentam papéis no olfato/discriminação de odores e em aprendizagem/memória, respetivamente.

A adenosina, um neuro-modulador amplamente presente no cérebro, tem um papel preponderante na transmissão sináptica e está envolvida na regulação de muito processos fisiológicos e neuropatológicos. O sistema endocanabinóide, onde são principalmente ativados os recetores de canabinóides do tipo 1 e 2 (CB_1R e CB_2R), é um regulador-chave da função sináptica, especialmente pela inibição da libertação de neurotransmissores. Além disso, processos essenciais na neurogénese como proliferação, diferenciação e maturação neuronal são modulados pela ativação de recetores de canabinóides. Importantemente, foi demonstrada a interação os entre recetores A_{2A} de adenosina ($A_{2A}Rs$) e CB_1Rs que é especialmente importante no controlo da função neuronal e transmissão sináptica. Contudo, até à data, nenhum estudo avaliou diretamente os efeitos desta possível interação entre os recetores A_{2A} de adenosina e recetores de canabinóides na neurogénese.

Assim, neste trabalho, foi investigado o possível papel dos $A_{2A}Rs$ na neurogénese induzida pela ativação dos recetores CB_1Rs e CB_2Rs, olhando para diferentes estádios do processo neurogénico.

Os resultados mostram que ativação dos $A_{2A}Rs$, CB_1Rs ou CB_2Rs não altera a divisão celular no sentido de promover um aumento ou diminuição da capacidade de auto-renovação na SVZ. Além disso, ativação dos $A_{2A}Rs$ e CB_2Rs não promove proliferação das células SVZ. Contudo, a ativação dos CB_1Rs promove um aumento na proliferação das células SVZ, um efeito bloqueado pela presença de um antagonista dos $A_{2A}Rs$. Interessantemente, a ativação de ambos CB_1Rs ou CB_2R induz um aumento na diferenciação neuronal das células SVZ e este efeito é comprometido pelo bloqueio dos $A_{2A}Rs$, apesar de *per se* a ativação dos $A_{2A}Rs$ não ter nenhum efeito na diferenciação neuronal.

Em relação ao nicho neurogénico DG, foi observado que, apesar da ativação dos CB_1Rs não promover nenhum efeito no destino celular das células derivadas do DG, ativação dos CB_2R e $A_{2A}Rs$ promove a perpetuação do *pool* estaminal das células de DG. O efeito promovido pelos CB_2R é bloqueado pela presença de antagonista seletivo de $A_{2A}Rs$ e semelhantemente, o efeito promovido pelos $A_{2A}Rs$ é bloqueado pela presença de antagonistas seletivos para CB_1R ou CB_2R. Adicionalmente, apesar da ativação dos CB_1Rs,

CB_2Rs ou A_{2A}Rs *per se* não produzir qualquer efeito na proliferação das células DG, um aumento no número de células em proliferação é visto quando há co-ativação de CB_1Rs ou CB_2Rs com A_{2A}Rs. Por fim, a ativação dos CB_1Rs, CB_2Rs e A_{2A}Rs promove um aumento na diferenciação neuronal das células DG. Interessantemente, o efeito promovido pelos CB_1R ou CB_2R é bloqueado pela presença de um antagonista seletivo dos A_{2A}Rs assim como o efeito mediado pelos A_{2A}Rs é bloqueado pela presença de antagonistas seletivos para CB_1R ou CB_2R.

Em resumo, os resultados levam a acreditar que possa existir uma interação entre os sistemas adenosinérgico e endocanabinóide, provavelmente a nível estrutural (com a formação de heterómeros) ou interação ao nível das vias de sinalização, o que contribuirá em última instância para o controlo da neurogénese.

Palavras-chave: Neurogénese, Nichos neurogénicos, Recetores A_{2A} para adenosina, Recetores para canabinóides

CHAPTER 1 Introduction & Aims

1.1. The nervous system & brain, neurogenesis, adenosine and cannabinoids

In this thesis four different subjects of high complexity will be highlighted: (a) neurogenesis, which is an intricate process occurring throughout adulthood in two main neurogenic niches; (b) adenosine, which is ubiquitously present in all cells and is a neuromodulator enrolled in several physiological functions, with receptors distributed throughout all brain areas; (c) the endocannabinoid system, composed of cannabinoid receptors, endogenous ligands and proteins responsible for synthesis, reuptake and degradation, which inhibits neurotransmission and plays an important role in synaptic function and many physiological functions and, finally, (d) the putative crosstalk between adenosine A_{2A} receptors and cannabinoid receptors.

1.2. Neurogenesis

Neurogenesis, generally defined as the process by which new functional neurons are generated from neural stem/progenitor cells was first believed to be a process exclusively occurring during pre-natal development[1–3]. The adult mature brain was supposedly incapable of producing new neurons. This dogmatic view began to change in the 60's because of Smart's and Altman's pioneering studies with [^{3}H]-thymidine autoradiography[4–6]. They showed, for the first time, evidence for the generation of new dentate granule neurons in postnatal rat hippocampus. Subsequently, the first reports suggesting the existence of postnatal neurogenesis occurred in the late 70's and 80's[2,7]. Later, technical advances revolutionized this field of research with the introduction of bromodeoxyuridine (BrdU), a synthetic thymidine analogue used as an S-phase marker of the cell cycle. This labelling technique allowed, along with other immunocytochemical and histological methods, the demonstration and validation that a great portion of newly generated cells in the adult brain were indeed neurons[2,8–11]. Therefore, neural stem/progenitor cells are capable of dividing themselves and then differentiating into newborn neurons that can migrate to pre-existing circuitries[10]. Rapid and significant progress in the field has led to the overall acceptance of neurogenesis being one of the many phenomena occurring in the adult brain that contributes to brain plasticity, synaptic function and circuit dynamics[10,12].

Neurogenesis also occurs in the complex and highly evolved human brain, where new neurons continue to be added in a series of highly regulated and coordinated physiological mechanisms and by activity[9,13,14]. However, this postnatal addition of newborn neurons slowly decreases with age[15,16]. Importantly, this continual addition of neurons over a lifetime implies substantial structural changes and constant remodeling of brain circuits, which implicates a

tremendous impact in central nervous system (CNS) functioning, including, for instance, short- and long-term learning and memory[10,12].

In fact, neurogenesis is a wide spread phenomenon across many mammalian species (including rodents, rabbits, monkeys and humans)[2,17,18] with a high degree of evolutionary conservation, suggesting that it is a fundamental biological mechanism – neurons generated and maintained in these specific regions during adulthood contribute to normal brain function and plasticity[11,16,18–20]. This functional contribution is not just a restorative mechanism but also a way to respond to constant and dynamic challenges applied by environmental stimuli or internal variations, representing a unique feature worth the exceptional effort of brain continuously reshaping itself[14].

Neural stem/progenitor cells (NSPCs) are present in the adult nervous system and have the ability to self-renew their own pool through cell proliferation and/or to generate cells from the neural lineage (neurons, astrocytes and oligodendrocytes)[21,22]. Moreover, NSPCs pass through sequential developmental stages that show structurally and functionally distinct cellular properties and dynamics in order to generate the newborn neurons[9,23]. Specifically, at the level of spatial organization, the presence or absence of specific cell constituents seems to be crucial for the self-renewal of NSPCs and neuronal differentiation[13].

In the adult brain, the neurogenic process occurs, mainly in two restricted areas, the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus. NSPCs are abundant in these areas which have high rates of proliferation physiologically[14,24–27]. New neurons generated at these sites then migrate toward their final destinations, where they differentiate into mature neurons and are integrated into the neuronal circuitry[20,28,29].

Adult neurogenesis is affected by several factors, such as physical activity, environmental enrichment, stress and aging and by various pathological conditions[30,31]. Extrinsic and intrinsic elements largely regulate and influence the rate of proliferation, maturation, survival and integration of newborn neurons, including niche factors/receptors, cytoplasmic factors, transcriptional factors, and epigenetic regulators. Predominantly, neurotransmitters, neural peptides, adhesion molecules, growth factors, neurotrophins, cytokines, cell-cycle regulators, transcription factors and other key components are the major players involved in the regulation of adult neurogenesis[2,22,25–27]. Epigenetically, many mechanisms perform diverse roles in regulating adult neurogenesis, such as DNA methylation, histone modifications, chromatin remodeling, and non-coding RNAs[23,25,32,33]. In response to injury or pathological stimuli, such as ischemic stroke, neurodegenerative diseases (Parkinson's, Huntington's, Alzheimer's diseases) and brain trauma, the newborn neurons that are generated following the loss of neurons, migrate to atypical or lesioned areas (reactive neurogenesis)[2,9,20,24,27,29].

1.2.1. Neurogenic niches

A neurogenic niche is, by definition, a stem cell rich microenvironment that anatomically and functionally controls their development *in vivo*. Additionally, these restricted areas of the mammalian brain have unique structural architectures that allow specific properties at the molecular, cellular and circuitry levels thus contributing to constitutive generation of new functional neurons throughout life[9,25,27].

The major cellular components of adult neurogenic niches are NSPCs, endothelial cells, astrocytes, microglia, mature neurons, and intermediate neural precursors. They play an important role regulating proliferation and fate specification of adult neural precursors as well as in neuronal migration, maturation, targeting and synaptic integration[9,25,26,30].

In fact, SVZ and SGZ are regions rich in NSPCs that originate neuroblasts that migrate and reach their final target where they mature and integrate the preexisting neuronal network[29].

Moreover, recent studies have shown the generation of matured neuronal cells in other non-traditional neurogenic brain regions[34]. In fact, several studies reported the occurrence of adult neurogenesis in the striatum, neocortex, piriform cortex (part of the olfactory cortex) and within the limbic system, primarily at the amygdala and hypothalamus[17]. Particularly in adult humans, the striatum is a well-developed structure in which there is a higher expression of genes associated with neuronal migration as compared to other brain regions[15,18]. This neurogenesis in non-traditional regions is predominantly enhanced after certain physiological or pathological conditions[35,36].

1.2.1.1. Subventricular zone (SVZ)

SVZ is a thin cell layer located alongside the lateral walls of the lateral ventricles (LV) of the adult brain[20]. This region exhibits a highly organized cytoarchitecture[9,37]. The SVZ high density of NSPCs present in the dorsolateral and rostral sides of the ventricles is correlated with the rostrocaudal gradient of proliferative activity[30,38].

SVZ is, predominantly, constituted of four cell types, different in their morphological nature and ultrastructure (Figure 1)[20,30,37,39–41]:

- Type E cells: nondividing ependymal cells, which are multiciliated cells facing the lumen of the ventricle and that are in close contact with the cerebrospinal fluid (CSF);

-Type B cells: astrocytic-like cells, which constitute the neural stem foundation and are responsible for the dividing/proliferative feature of SVZ; these astrocyte-like type B cells can be subdivided into two subtypes based on differences in their location and morphology:

-type B1 cells are generally closely associated with ependymal cells;

-type B2 cells are more frequently located close to the underlying striatal parenchyma;

Type B cells can also be grouped into 3 main domains according to their position in the niche and the surrounding cells (proximal, intermediate and distal)[28,42];

-Type C cells: transit-amplifying cells which are the immediate progeny of B cells, characterized by being highly proliferative intermediate progenitor cells (IPCs) that usually are close to blood vessels and form clusters interspaced among chains throughout the SVZ;

-Type A cells: young migrating neurons, called neuroblasts, that migrate through sheathed chains of astrocytes towards the olfactory bulb.

In summary, the type B1 astrocyte-like cells of radial glia origin (with unique functional characteristics between those of astrocytes and radial glia) are a quiescent stem cell-like subpopulation[43]. B1 cells divide at a continuous and slow rate and are responsible for the long-term maintenance of the stemness/multipotency state of the niche. These cells generate a different pool composed of rapidly proliferating progenitor cells – type C transit amplifying cells –, committed to the neuronal lineage. These type C cells will, in turn, rapidly divide and generate type A cells[20,30,38,44].

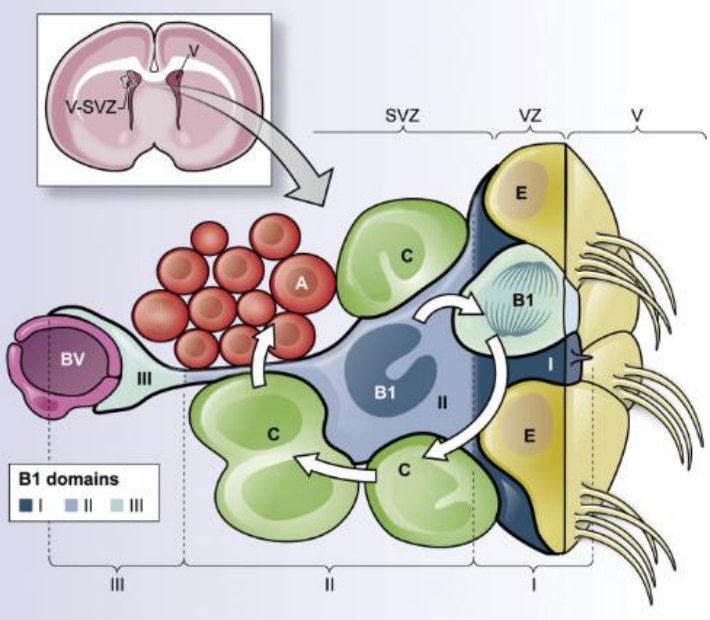


Figure 1 – Cellular organization of the SVZ niche. Cross-section showing the location of the SVZ niche; enlarged representative scheme of SVZ niche and how the process of neurogenesis occurs: NSPCs (type B1 cells, blue), which are surrounded by ependymal cells (type E cells, yellow), give rise to transit amplifying cells (type C cells, green), that divide and generate neuroblasts (type A cells, red); B1 cells domains are also represented as I, II and III according to their position in the niche (adapted from [28]Fuentealba et al, 2012).

Therefore, the process of adult SVZ neurogenesis can be simplified into the following linear, but multifaceted, sequence of events: 1) glial fibrillary acidic protein (GFAP)-expressing B-type astrocytes function as NSPCs and 2) produce transiently amplifying progenitors (type C cells) that 3) differentiate into neuroblasts (type A cells), which then 4) migrate anteriorly to the olfactory bulb (OB) via the rostral migratory stream (RMS)[25,28,45,46]. In the RMS, neuroblasts generated in the SVZ migrate tangentially to the OB by forming elongated cell aggregates, known as "chains", surrounded by a sheath of astrocytes called the glial tube[20]. Once reaching the core of the olfactory bulb, immature neurons detach from the RMS and migrate radially along blood vessels toward glomeruli where they differentiate into different subtypes of olfactory interneurons[27]. This terminal differentiation occurs in different layers of the OB, namely the granular cell layer (GCL), located in the deeper layer of the OB, and glomerular layer (GL), located in the most superficial layer. In these layers the neuroblasts will differentiate into two types of olfactory neurons, the granular cells (the majority) and the periglomerular cells (a small percentage)[47]. Given the fact that the majority of interneurons are either gamma-aminobutyric acid (GABA)ergic or dopaminergic, the newly born granular and periglomerular neurons are synaptically integrated into the existing circuitry, exhibiting initially GABA and then glutamate receptors becoming sensitive to stimuli from the olfactory nerve layer of the OB[26,48].

As previously mentioned numerous factors are involved in the regulation of the neurogenic process. In fact, in the SVZ molecules mediating cell-to-cell and cell-to-substrate interactions, chemoattraction and chemorepulsion mechanisms, extracellular matrix remodeling and other key elements modulate neurogenesis[25,28,30,40]. These factors are implicated in the control of different stages of cell development, namely in the maintenance, proliferation, differentiation, migration, survival, maturation and integration of functional new neurons in the given circuitry[28,47]. Specifically, these factors include context-dependent release of neurotransmitters, neurotrophic/growth factors, morphogens, transcription factors and other extrinsic factors, like epigenetic regulators derived from sensory, motor or social stimuli (extensively reviewed in Lledo et al., 2006)[26,27,37,40].

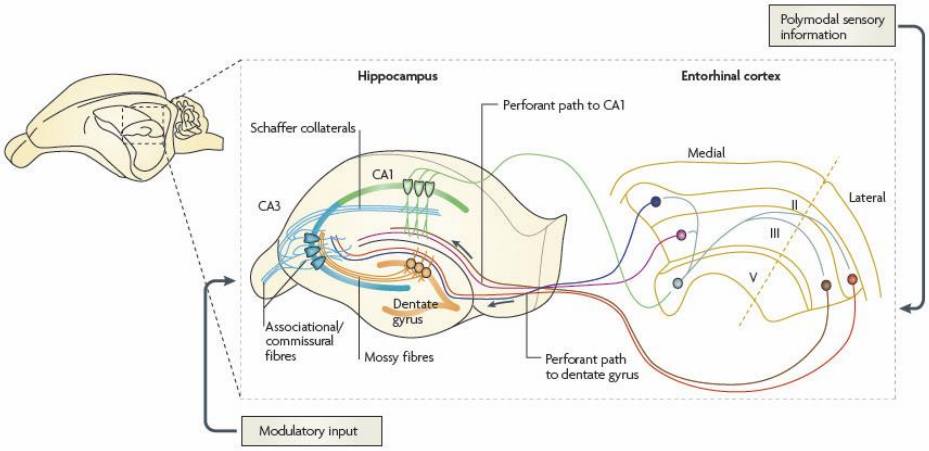
Therefore, the extracellular matrix, endothelial cells and vascular contacts associated with the adult SVZ, have also a direct impact on adult neurogenesis by constituting potential sources of signals regulating progenitor cell behavior and proliferative patterns[26]. The SVZ area is extensively established on extracellular matrix which provides a platform for the presentation of components that affect proliferation and progenitor activity within the neurogenic niche[9,37]. Notably, blood vessels-released factors and endothelial-derived factors may act as neurogenic signals that interact with NSPCs and stimulate stem cell self-renewal and transit-amplifying cells generation and proliferation[26,37,47]. Furthermore, blood vessels may also be important to serve as a scaffold for neuronal migration and neuronal integration[37] and ependymal-derived factors can provide instructive cues to sustain new neuronal production

and to redirect niche responses to locally reintegrate neurons in damaged tissues (whenever in situations of brain injury or pathological conditions)[46,49].

Taking into consideration that the OB is the first processing center of odor information, the addition of new interneurons into this region is likely responsible for the plasticity of the olfactory system, which is especially important for rodents[20]. The real functional significance is still unclear and relatively debatable but evidences point out that survival and proper synaptic integration of these newly-born neurons into the OB is highly correlated with odor acquisition and discrimination and short- and long-term odor memory[3,26,39,47]. Additionally, SVZ neurogenesis may contribute to the regulation of pheromone-related events, such as mating and social recognition[25]. Recent studies also suggest that SVZ-derived NSPCs may have non-neurogenic functions, such as regulating immune trafficking and maintaining CNS homeostasis[42,50,51].

1.2.1.2. Subgranular zone (SGZ)

The hippocampus, the brain region associated with learning and the memory formation, is composed of 3 distinct regions, the DG, the Cornu Ammonis 3 area (CA3) and the CA1 area, which form the trisynaptic hippocampal circuit (or loop)[19,52,53]. The degree of complexity within the hippocampus is enormous but, to simplify the trisynaptic loop, this circuit can be fairly comprehended as: existence of synapses between the entorhinal cortex (EC) and the dendrites of the granule cells of the DG (1), between the mossy fibers (the axons of the granule cells) and the pyramidal neurons of CA3 (2) and between the axons of the CA3 pyramidal neurons and the pyramidal neurons of CA1 (3), which then again project out to the subiculum and the EC (Figure 2)[19,54–56]. SGZ is located at the interface between the granule cell layer (GCL) and the hilar layer of the hippocampal DG[20,30]. Unlike SVZ, the SGZ neurogenic niche is structurally less recognizable and NSPCs are not in direct contact with CSF[9,28].

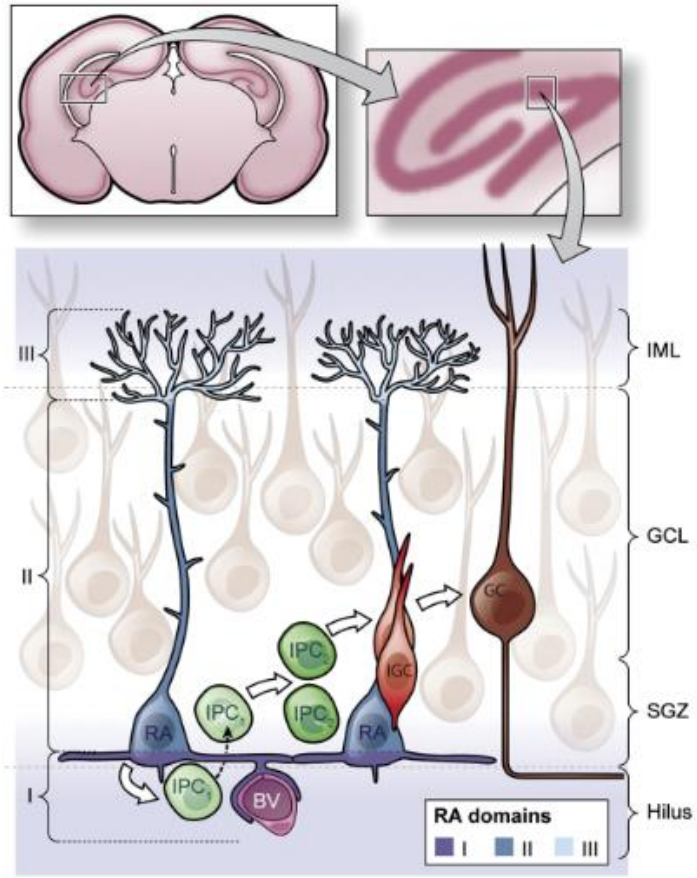


◄ **Figure 2 – Neuroanatomy of the hippocampus.** The trisynaptic arrangement of the hippocampus is due to the existence of 3 distinct zones: CA1, CA3 and DG. Excitatory projections connect the entorhinal cortex with the granule cells of the DG through the performant path, which in turn connects, through mossy fibers, with the pyramidal cells of the CA3 area. The CA3 area connects through Schaffer collaterals with the CA1 area. Entorhinal cortex also connects with the CA1 area (adapted from [56]Neves et al., 2008).

The SGZ niche is mainly represented by four different (regarding morphology and antigen properties) types of cells (Figure 3)[22,26,28,42,57,58]:

-Type I cells, radial astrocytes, which are predominantly present and highly polarized cells with astrocytic properties and contribute to maintain niche stemness. These astrocytes, similarly to SVZ type B cells, can also be grouped into 3 main domains according to their position and neighboring cells (proximal, intermediate and distal)[28];

-Type II cells, intermediate progenitor cells (IPCs), which lack glial features and are thought to be the most active cells in terms of proliferation, and by giving rise to progenitor cells can be subdivided into ‘‘type-II a’’ or ‘‘type-II b’’ cells, according to the presence or absence of doublecortin, respectively;



◄ **Figure 3 – Cellular organization of the SGZ niche.** Cross-section showing the location of the SGZ niche in the hippocampus; enlarged representative scheme of SGZ niche and how the process of neurogenesis occurs: NSPCs, represented as radial astrocytes (RA, type I cells, blue), give rise to transit amplifying cells which are intermediate progenitor cells (IPCs, type II cells, green), that divide asymmetrically and generate neuroblasts, immature granule cells (IGCs, type III cells, red); RA domains are also represented as I, II and III according to their position in the niche (adapted from [28]Fuentealba et al, 2012).

-Type III cells, immature granule cells (IGCs) or neuroblasts, which are immediate precursors of granule cells and can be subdivided into immature (D_1) or more differentiated neuroblasts (D_2);

-Mature granule cells (GCs), which express specific neuronal markers and are functionally integrated into the circuit.

In summary, the process of adult SGZ neurogenesis can be simplified into the following sequence of events: 1) type I radial astrocytes function as NSPCs and 2) generate type II intermediate progenitor cells that progressively 3) differentiate into type III immature granule cells (IGCs), which then 4) migrate a short distance to the granule cell layer (GCL)[20,25,27,59]. This migration to the GCL promotes the local maturation of newly generated immature cells and integration into the neural network as functional granule neurons of the DG. Compared to mature granule cells, newborn neurons exhibit hyperexcitability and enhanced synaptic plasticity during specific developmental stages. As they differentiate and progressively become more specialized granule cells, they develop axonal projections to CA3 area and the dendritic arborization becomes increasingly more complex and extends deeper into the molecular cell layer[2,20,22,27]. After a prolonged maturation phase, adult-born neurons exhibit similar basic electrophysiological properties as mature neurons, such as firing behavior and the kinetics of excitatory and inhibitory inputs[25,54,59]. Unlike SVZ, where NSPCs give rise to two distinct interneuron lineages in the OB, adult NSPCs in the SGZ of the hippocampus predominantly give rise to functional glutamatergic excitatory neurons that integrate DG circuitry[28,60].

Nonetheless, similarly to SVZ, the SGZ microenvironment is intimately associated with other cells, namely endothelial cells, glial cells and other neuronal types, that structurally and biochemically support and regulate the niche and contribute significantly to adult neurogenesis[28]. Particularly, the vasculature, especially capillaries, plays an important role in regulating the proliferation of adult NSPCs in SGZ[9].

Diverse niche components, signaling pathways and external stimuli differentially modulate NSPCs behavior and the course of adult hippocampal neurogenesis. These factors are involved in the control of stem cell behavior, cell fate determination, maintenance of

stemness, as well as the regulation of all different stages of the neurogenic process (differentiation, maturation, migration and integration)[27,28,40]. They include hormones, neurotransmitters, growth factors, transcription factors and other activity/plasticity-related players in response to environmental stimuli (extensively reviewed in Lledo et al., 2006)[25–27,40,61].

In addition, the hippocampus, specifically the SGZ of the DG, is targeted by many inputs and neurotransmitter systems[48] which causes SGZ neurogenesis to be particularly sensitive to the surrounding neuronal activity. In fact, neurons at the neurogenic site may provide spatiotemporal regulation of adult neurogenesis in response to hippocampal-dependent neuronal activity[9]. SGZ neurogenesis is also known to be influenced by neuropathological states, such as epilepsy, where newborn neurons are incorrectly reorganized into aberrant synapses/network connections[2,25].

Importantly, adult hippocampal neurogenesis may contribute to a diverse number of functions. It is believed that immature granule cells, by being electrophysiologically more excitable, have more potential to modulate activity-dependent plasticity of the DG circuit processing[55,59,62]. This continuous rewiring of the brain is particularly important in cognition, learning and memory formation[26,55,63]. The addition of new neurons in the adult SGZ is critical during specific time windows and occurs to reorganize the circuit networks in order to process information in hippocampus-dependent learning activities, contributing to memory formation[9,17]. Specifically, tasks like spatial-navigation learning, long-term spatial memory retention, and spatial pattern discrimination may be dynamically handled by the addition of new neurons to the hippocampal circuitry[25,52,63].

Stage specific markers can be used to understand how the entire neurogenic process is displayed. Developmental stages like cell fate, differentiation and neuronal maturation can be precisely detected because newly born neurons express a series of transient markers, such as GFAP, Sex determining region Y-box 2 (Sox2), Nestin, Doublecortin (DCX) and Neuronal nuclei (NeuN). Developmental stages are likely to reflect a continuum rather than discrete steps. Therefore, the entire neurodevelopmental progression of neurogenesis, from precursor cells to mature neurons, can be followed and dissected using these specific markers, as illustrated in Figure 4.

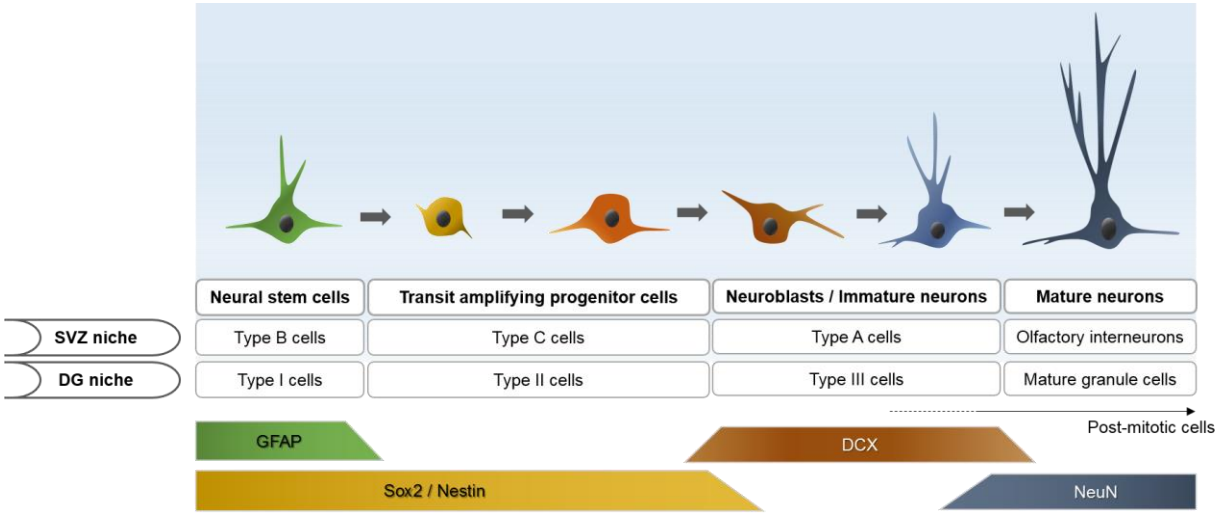


Figure 4 – Adult neurogenesis in the SVZ and DG niches and stage-specific markers. Summary of the 4 main developmental stages in adult neurogenesis both in SVZ and DG niches: neural stem cells divide into transit amplifying progenitor cells which give rise to neuroblasts/immature neurons that develop into mature neurons. GFAP: glial fibrillary acidic protein; Sox2: sex determining region Y-box 2; Nestin; DCX: doublecortin; NeuN: neuronal nuclei.

Overall, the SVZ and SGZ neurogenic niches exhibit key similarities and differences that seem important to mention. Overall, **similarities** between both neurogenic regions are displayed regarding niche composition (highly specialized with an heterogeneous population of cells), signaling pathways maintaining precursor pools, temporal sequence of new neurons' integration, critical periods of survival (the same regulatory factors act on specific time windows of stage development) and enhanced plasticity, and contribution to circuit dynamics. On the contrary, these regions show **differences** in specific features, specifically in niche organization, SGZ is a compacted region overflowed with different neuronal inputs and neurotransmitters which promote continuous interplay and plasticity, whereas SVZ is a rich vascular microenvironment and is not interconnected with such dense neuronal network, in neuronal subtype differentiation, and in migration of newborn neurons[25,29,42,64,65].

1.2.1.3. Non-traditional neurogenic regions

The presence of "local" progenitor cells in various brain regions outside the "standard" neurogenic niches, has been shown[24,66,67]. These areas, where it still remains controversial whether adult neurogenesis indeed occurs, include the neocortex, piriform cortex, striatum, cerebellum, hypothalamus, amygdala and substancia nigra[15,66,68–72].

According to Bonfanti & Peretto, 2011, we can include neurogenesis in the mammalian CNS into 2 distinct groups: a 'complete' neurogenesis, greatly limited to SVZ and SGZ neurogenic sites; and a rather 'incomplete' neurogenesis, generally occurring in the parenchyma of the adult brain (non-neurogenic regions)[73]. This "incomplete" definition of non-neurogenic regions results from several characteristics: parenchymal regions lack a well-defined functional and structural niche organization; the source and nature of the NSPCs vary from region to region; these regions are not directly related with germinal layers. Importantly, neurogenesis is interrupted at various intermediate levels in these non-neurogenic regions, possibly because they are more sensitive to modulation by external regulatory mechanisms and for not being well established[35,69]. In addition, Bonfanti & Nacher, 2012, suggested, that immature neurons stay in the CNS for indeterminate time and might be recruited into preexisting circuits under certain stimuli, representing a new form of plasticity between synaptic remodeling and adult neurogenesis[36].

Although many studies exist concerning neurogenesis in the adult neocortex, amygdala and striatum[15,74–77], the most studied non-traditional neurogenic region, to date, is the hypothalamus. Recent evidences suggest that the adult hypothalamus may, indeed, be a neurogenic niche because of its localization in the brain (adjacent to the third ventricle) and its neurogenic potential, with two neurogenic regions: hypothalamic ventricular zone and hypothalamic proliferating zone[34,69,71,78]. Given the critical role of hypothalamic neural circuitry in maintaining physiological homeostasis via the HPA axis, functional integration of newborn neurons may result in atypical effects in physiology and behavior[67,79]. Possibly may serve as a compensatory mechanism contributing to the plastic control of energy balance and flexibility to adapt to metabolic challenges[80–82].

1.3. Adenosine and adenosine receptors

The purinergic neurotransmission was introduced in 1972 after it was shown that adenosine 5'-triphosphate (ATP) was a transmitter in non-adrenergic, non-cholinergic inhibitory nerves in the guinea-pig *taenia coli*[83–85]. ATP is one of the most important molecules for human biology because it acts as an energy exchange coin for almost every metabolic reaction[86]. Particularly in the CNS, ATP acts both as a fast excitatory neurotransmitter and as a neuromodulator and has powerful long-term effects in the developing brain and in disease[83].

Adenosine, formed from the catabolism of ATP, is a ubiquitous purine ribonucleoside and signaling molecule/messenger with important functions at maintaining energetic homeostasis and other physiological processes for mammalians, particularly in excitable tissues like the heart and the brain[86–88].

Generally, many of its actions involve reducing the activity of these excitable tissues or increase the delivery of metabolic substrates, thus, regulating metabolic dynamics[86]. In fact, adenosine can induce vasodilation in most vascular areas, reduce blood pressure or heart rate and regulate activity in the sympathetic nervous system[87].

Particularly in the brain, which highly expresses adenosine receptors, purinergic fast transmission is involved in a multitude of physiological processes, including regulation of sleep, arousal, mood, motivation and neuroprotection[83,86,89,90]. Also, it is involved in the control of innate and adaptive physiological systems, being related with most neuropathological disorders of the CNS, ranging from neurodegenerative diseases, such as Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) diseases to multiple sclerosis (MS), epilepsy, schizophrenia, cerebral ischemia and mood disorders (depression, anxiety), as well as brain cancer (glioma)[83,84,86,89,91–94].

The role of adenosine in the CNS can be seen as an action on two distinct frontlines: first, as a neuromodulator, interfering with neuronal circuitry dynamics and, second, as a homeostatic modulator, coordinating metabolic activity[89,95,96].

Extracellular adenosine modulates neuronal activity and many other physiological mechanisms operating via metabotropic adenosine receptors (ARs). This modulation happens through the activation of a seven transmembrane G-protein-coupled receptor (GPCR) that lead to changes in the intracellular levels of second messengers and ion channels (Ca^{2+} and K^{+} channels), thus influencing pre- and post-synaptically the entire neuronal network[92,97]. ARs are present throughout all brain areas and can be divided into four subtypes (A_1, A_{2A}, A_{2B} and A_3), based on their unique pharmacological profile, tissue distribution and transducing signaling pathways[92,98–102]. These four receptors can be divided according to their affinity to adenosine in high affinity (A_1 and A_{2A} - K_m < 30 nM) and low affinity (A_{2B} – K_m 1–20 μM) receptor subtypes; the affinity of A_3 receptor is species-dependent, and is low in rodents and high in humans[88,103,104]. These receptors are also known as P1 receptors, when named according to the terminology of purinoceptors: P1 - adenosine-sensitive and P2 - ATP-sensitive[92,97,105].

Typically, ARs can also be divided into subcategories according to their associated signaling pathways: A_1 and A_3 receptors are coupled to G_i proteins, having an inhibitory effect on adenylate cyclase, therefore decreasing cyclic adenosine monophosphate (cAMP) levels whereas A_{2A} and A_{2B} receptors are coupled to G_s proteins, having an stimulatory effect on adenylate cyclase, thus increasing cAMP levels (Figure 5)[85,106]. Furthermore, ARs can be involved in the activation of other pathways, namely phospholipase C (PLC), Ca^{2+} signaling and mitogen-activated protein kinases (MAPKs)[88,100,106].

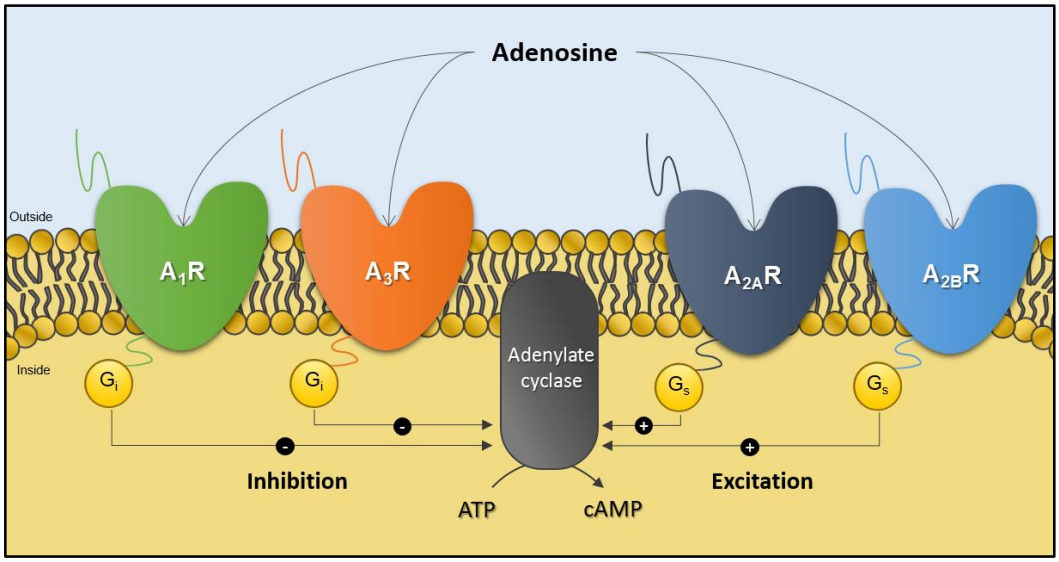
The distribution of ARs throughout the entire CNS is not homogeneous. A_1Rs are most abundant in the neocortex, limbic system, dorsal horn of spinal cord, basal ganglia and cerebellum whereas A_{2A}Rs are highly expressed in the basal ganglia and olfactory bulb.

Subcellularly, A_1Rs are predominantly located in axons whereas A_{2A}Rs have a broader localization, whether dendritically in basal ganglia neurons or pre-synaptically in cortical neurons[97]. A_1Rs and A_{2A}Rs are also located in astrocytes, microglia and oligodendrocytes and A_{2A}Rs in blood vessels[101]. In opposition, A_{2B}Rs and A_3Rs are expressed in low levels in the brain, being the expression of the latter moderate in the human cerebellum and hippocampus (extensively reviewed in the book 'Adenosine Receptors in Health and Disease' chapter Adenosine Receptors and the Central Nervous System[104], 2009).

Several key physiological and pathological effects of ARs have been described: e.g. A_1Rs are involved in the regulation of sleep and inhibition of neurotransmitter release; A_{2A}Rs are involved in the control of wakefulness and locomotion and play a role in neurodegeneration; A_{2B}Rs are linked with cardiac preconditioning and pro-inflammatory (acute injury) and anti-inflammatory (some chronic disease states) responses; A_3Rs mediate inflammatory responses and are involved in chronic neuropathic pain relief[97,100,104,107].

Although in a general sense many of adenosine effects are inhibitory, it is commonly accepted that activation of A_1R is translated into a neuroprotective function: by acting on A_1Rs it promotes the decrease of glutamate release (avoiding glutamate excitotoxicity conditions) and hyperpolarizes neurons, improving brain repair mechanisms[95]. On the other hand, both stimulation or blockage of A_{2A}Rs under specific conditions was found to promote brain protection[101,108,109].

In particular, A_{2A}Rs modulatory role of neuronal activity seems to be especially important because of their numerous actions in the CNS, ability to "fine-tune" the functioning of other neurotransmitter modulatory systems and neuroprotective contribution on several brain diseases[101,110,111].



◄ **Figure 5 – Adenosine receptors signaling.** Adenosine activates four types of receptors: A_1R & A_3R, inhibitory receptors of adenylate cyclase; $A_{2A}R$ & $A_{2B}R$, excitatory receptors of adenylate cyclase (re-illustrated from [103]Ham & Evans, 2012).

1.3.1. A_{2A} receptor

Adenosine A_{2A} receptor ($A_{2A}R$) neuromodulatory role can specifically comprehend the regulation of sleep-wake cycle, neuronal death, motor activity and psychiatric behaviors, inflammation, blood flow, angiogenesis and oxygen consumption[95,101,107]. In addition to crucial roles in physiologic mechanisms, $A_{2A}Rs$ are also involved in pathophysiological conditions of the CNS[90,106,112,113].

Structurally, $A_{2A}Rs$ are organized, like every other GCPR, in seven transmembrane α-helices (7TM, helices 1–7) followed by one short membrane-associated helix, three extracellular loops (ECL1-3), three intracellular loops (ICL1-3), an extracellular amino-terminus (N-terminus) and a cytosolic carboxyl terminus (C-terminus). Particularly, their helical core, which constitutes the binding pocket, together with the extracellular loops and the four disulfide bridges of the extracellular N-terminal domain are especially important in ligand recognition and binding[114–116].

Regarding downstream signaling and second messengers (Figure 6), $A_{2A}R$ interaction with the trimeric G-protein alpha-s/beta/gamma causes the exchange of GDP to GTP, which is transduced into stimulation of adenylyl cyclase, increasing cAMP levels. $A_{2A}R$ activation can, therefore, regulate MAPK activity, Ca^{2+} and K^+ levels and other signaling pathways in a cAMP-dependent or -independent manner. In detail, MAPK activity is enhanced upon $A_{2A}R$ activation by activating extracellular signal-regulated protein kinases 1/2 (ERK1/2) pathway. Furthermore, ERK1/2 pathway activation involves the activation of protein kinase A (PKA) which in turn phosphorylates and stimulates cAMP responsive binding element (CREB). Multiple PKA-related downstream mechanisms can also be triggered, like the Cdc42, which enhances PKC activity, ultimately, promoting cell survival. Moreover, $A_{2A}R$ activation inhibits Ca^{2+} influx (PKA-dependent) and inhibits voltage-dependent Na^+ channels (cAMP-dependent)[97,100,101,117–121].

Along with the complexity of $A_{2A}R$ signaling, there is interplay between $A_{2A}R$ receptors and receptors for other neurotransmitters and/or neuromodulators that enhances exponentially $A_{2A}Rs$-associated complexity and allows the modulation and “fine-tuning” of several systems. It can be detected either by $A_{2A}Rs$ forming heteromers with other receptors or by targeting common intracellular transducing cascades[92,110,111,121]. This interaction with other receptors (homo-, heteromerization or receptor mosaics), which can be direct or via intermediary adapter

proteins, may have an effect on communication networks intrinsic to the receptor complex, downstream signaling, ligand sensitivity/functionality, and compartmentalization of a given receptor. This interaction with other receptors and their signal transduction pathways may have an impact at the functional level and, ultimately, on phenotypical manifestation and behavioral output and responses[122–126].

Notably, strong evidences show that receptor-receptor interaction of $A_{2A}R$ can occur with other G protein-coupled receptors (GPCRs), such as metabotropic glutamate receptor mGluR5[127], dopamine D_2 receptors[128] and cannabinoid CB_1 receptors (CB_1Rs)[129].

Interaction with other receptors has also been shown, namely with A_1R, with ionotropic receptors, like NMDA receptors or nicotinic cholinergic receptors (nAChR) and with receptors for neurotrophic factors, namely receptors for brain-derived neurotrophic factor – BDNF (TrkB) and glial-derived neurotrophic factors – GDNF (Ret and/or GFRα1) (extensively reviewed in the book 'Adenosine Receptors in Health and Disease' chapter Adenosine Receptors and the Central Nervous System[104], 2009).

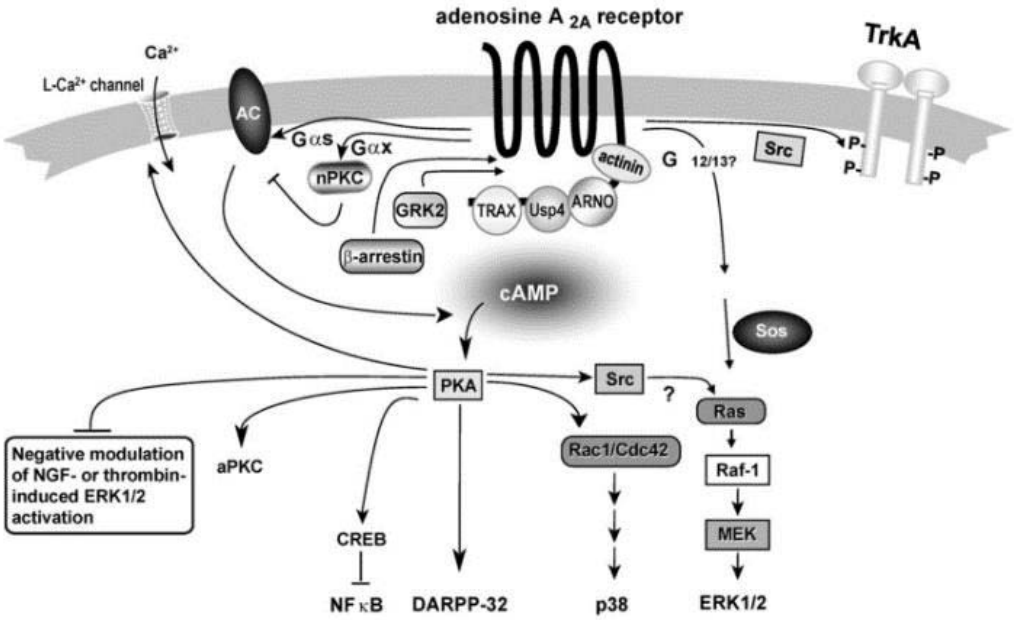


Figure 6 – A_{2A} receptor signaling pathways. $A_{2A}R$ is a G protein alpha s (Gs) receptor. When $A_{2A}R$ is activated, it activates adenylate cyclase which leads to production of cAMP. This induces the activation of cAMP-dependent kinase (PKA) pathway which triggers a sequence of downstream signaling events. Other kinases (e.g. nPKC) and G protein-independent mechanisms (e.g. coexistence and transactivation of TrkA receptors via Src pathway) are also illustrated as being part of $A_{2A}R$ signaling (adapted from [121]Fredholm et al., 2007).

The effects of agonists and antagonists of A_{2A}Rs (see Table 1) have been widely studied through crystallography and computational approaches in order to understand their potential as therapeutic modulators. Most of the A_{2A}Rs agonists are simply derivatives of adenosine modified at the 5'-position of the ribose and N^6 positions of the purine. The most known A_{2A}R-selective agonist is CGS21680. On the contrary, A_{2A}Rs antagonists can be classically divided in xanthines or non-xanthine derivatives. Natural occurring xanthines like caffeine or theophylline generally have great affinity for ARs, with the highest affinity being at the A_{2A}R, constituting the most known typical antagonists for A_{2A}Rs[92].

Table 1 – A_{2A}R main agonists/antagonists. Affinity for each AR type is represented by K_i values of agonists/antagonists (resulted from binding assays using either recombinant human ARs or rat ARs or from cAMP functional assays) (adapted from [106]Jacobson and Gao, 2006)

Ligand	A_1R K_i value (nM)	A_{2A}R K_i value (nM)	A_{2B}R K_i value (nM)	A_3R K_i value (nM)
A_{2A}R agonists				
NECA	14	20	140	25
CGS21680	289	27	>10000	67
DPMA	168	153	>10000	106
Binodenoson	48000	270	430000	903
ATL-146e	77	0.5	N.D.	45
CV-3146	>10000	290	>10000	>10000
A_{2A}R antagonists				
KW6002	2,830	36	1800	>3000
CSC	28,000	54	N.D.	N.D.
SCH58261	725	5.0	1,110	1,200
SCH442416	1,110	0.048	>10000	>10000
ZM241385	774	1.6	75	743
VER 6947	17	1.1	112	1470
VER 7835	170	1.7	141	1931
'Schering compound'	82	0.8	N.D.	N.D.

N.D., not determined or not disclosed. Other A_{2A}R ligands may be considered, namely partial agonists, synthetic ligands or other derivatives, but are not represented. For references and for further details on the structures of the compounds listed in this table, see [92]Cristalli et al, 2009.

Importantly, recent evidences show the involvement of A_{2A}Rs in the regulation of specific stages of neurogenesis, such as cell proliferation and neuritogenesis. Particularly:

- Impaired neuritogenesis caused by p53 blockage was rescued by activation of the A_{2A}R (designated the A_{2A} rescue effect) via KIF2A, a kinesin family member[130];

- Long-term administration of low dose of caffeine, an antagonist of A_{2A}Rs, was shown to inhibit hippocampal neurogenesis and hippocampus-dependent learning and memory[131];
- Also, caffeine was shown to alter, by a 7-day administration, the proliferation of adult hippocampal neuronal precursors in mice in a dose dependent manner – moderate doses lead to a decrease in proliferation whereas supra-physiological doses increase proliferation of neural precursors[132];
- Sleep deprivation-induced decline of neuronal proliferation and differentiation was rescued by the 48h treatment with caffeine[133];
- Unpublished data from our lab shows that A_{2A}R activation induces neurogenesis from DG neurosphere cultures.

1.4. Endocannabinoids and cannabinoid receptors

Endocannabinoids and their receptor signaling system (ECS - endocannabinoid system), are highly common across vertebrates and invertebrate species[134]. In fact, the ECS is an important intrinsic mechanism of the human biology that is known to affect both CNS and peripheral processes[135].

Specifically, it consists of a family of lipid signaling molecules referred as endocannabinoids (eCBs), their receptor(s), bioactive intermediaries, downstream signaling pathways, uptake mechanism and specific metabolic synthesizing and degrading enzymes[136].

The two main characterized eCBs are anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), which are widely distributed in the brain and specifically target cannabinoid receptors[135,137]. 2-AG concentration is 200-fold higher than that of AEA in the brain tissue[134]. Other eCBs have also been identified, such as 2-arachidonylglyceryl ether (noladin ether), N-arachidonoyl-dopamine (NADA) and O-arachidonoyl-ethanolamine (virodhamine)[138,139].

Cannabinoid receptors (CBRs) constitute specific binding sites for endo- and exocannabinoids[140]. CB_1 and CB_2 receptors, the most described CB receptors, belong to the superfamily of GPCRs and are physiologically different, exhibiting only 48% similarity in their amino acid sequences and 68% in the transmembrane domains[135]. Their tissue distribution and downstream signaling mechanisms are distinct, which suggests their physiological divergent importance in mammalian biology[138,141,142].

CB_1 receptor is considered as the neuronal receptor whereas CB_2 receptor is considered as the receptor of the immune system[122,138,141]. Although these two receptors are the best characterized to date, other putative CBRs can be modulated by eCBs, CBR agonists

and/or antagonists: GPR55 activity can be modulated by certain eCBs and phytocannabinoids but its pharmacology remains puzzling; 5 transient receptor potential (TRP) cation channels are also activated by eCBs, specifically TRPV1 (transient receptor potential vanilloid 1); peroxisome proliferator activated receptors (PPARs) have been shown to weakly bind eCBs as well as phytocannabinoids and synthetic cannabinoids[138,143,144]. All the more, despite CB_1R is the most associated to the actions of endo- and exocannabinoids in the brain, increasing evidence shows that CB_2Rs, TRPV1Rs are present in the brain[145].

In view of that, the main eCBs 2-AG and AEA target CB receptors differently: 2-AG acts as a full agonist at CB_1R and CB_2R and AEA acts as partial agonist for CB_1, CB_2, GPR55 and TRPV1 receptors[138,144,146–148] (see Table 2 for CB_1R and CB_2R agonists and antagonists).

Exocannabinoids are, in general, referred as the group of exogenous substances that are structurally related to Δ^9-tetrahydrocannabinol (Δ^9-THC) – the main psychoactive cannabinoid found in cannabis – and that bind to CBRs[135,138,149]. These usually include plant-derived cannabinoids (phytocannabinoids) and synthetic cannabinoids[139].

Table 2 – CB_1R and CB_2R main agonists/antagonists. Affinity for either CB_1R or CB_2R is represented by K_i values of selective agonists and antagonists (resulted from *in vitro* binding assays using [^{3}H]CP55940, [^{3}H]HU243 or [^{3}H]BAY-38-7271 for CB_1R-and CB_2R-specific binding sites) (adapted from [150]Pertwee, 2008)

Ligand	CB_1R K_i value (nM)	CB_2R K_i value (nM)
CB_1R selective agonists		
ACEA (arachidonyl-2'-chloroethylamide)	1.4, 5.29	195, >2000
O-1812	3.4	3870
ACPA (arachidonylcyclopropylamide)	2.2	750
2-arachidonylglyceryl ether (noladin ether)	21.2	>3000
R-m-AEA (R-(+)-methanandamide	17.9 to 28.3	815 to 868
CB_2R selective agonists		
AM1241	280	3.4
JWH-133	677	3.4
L-759633	1043, 15850	6.4, 20
L-759656	529 to >20 000	11.8 to 57
JWH-015	383	13.8
HU-308	>10000	22.7
Agonists without significant selectivity for CB_1R and CB_2R		
HU-210	0.06 to 0.73	0.17 to 0.52
CP55940	0.5 to 5.0	0.69 to 2.8

R-(+)-WIN55212	1.89 to 123	0.28 to 16.2
Nabilone	1.84	2.19
(–)-Δ^9-THC ((–)-*trans*-Δ^9-tetrahydrocannabinol)	5.05 to 80.3	3.13 to 75.3
(–)-Δ^8-THC ((–)-*trans*-Δ^8-tetrahydrocannabinol)	44, 47.6	39.3, 44
Anandamide	61 to 543	279 to 1940
2-AG (2-arachidonoylglycerol)	58.3, 472	145, 1400
CB_1R selective antagonists/inverse agonists		
SR141716A	1.8 to 12.3	514 to 13 200
AM281	12	4200
AM251	7.49	2290
CB_2R selective antagonists/inverse agonists		
SR144528	50.3 to > 10 000	0.28 to 5.6
AM630	5152	31.2

Other CB_1R and CB_2R ligands may be considered, namely synthetic ligands or other derivatives, but are not represented. For references and for further details on the structures of the compounds listed in this table, see [151]Pertwee, 2005.

eCBs are not stored in vesicles, like most neurotransmitters, but rather are synthesized and released in postsynaptic neurons upon demand ('on-demand' model), by neural stimulation, (depolarization followed by increasing Ca^{2+} levels). eCBs activate mainly CB_1Rs localized in presynaptic neurons and are reuptaked (travel backwards across the synapse through a endocannabinoid membrane transporter – EMT) and degraded by specific hydrolyzing enzymes[135,143,152–154]. They serve as fast retrograde messengers that actively suppress neurotransmitter release in presynaptic terminals in a phasic or long-lasting manner (depending on the type of stimulus or the action of factors that control their levels) in both excitatory or inhibitory neurons throughout the CNS[143]. The resulting effect of eCBs is an inhibition of presynaptic terminals called 'depolarization-induced suppression of inhibition' (DSI) or 'excitation' (DSE) depending if it occurs at GABA or glutamate synapses, respectively[152,155]. Whether this presynaptic inhibition is mainly due to the action of AEA or 2-AG retrograde signaling it still remains unclear[135,152]. However, there is also evidence suggesting that eCBs can also act in a non-retrograde manner, modulating postsynaptic activity direct (autocrine CBR activation) or indirectly (via gliotransmission to astrocytes that can modulate pre- and post-synaptic terminals), revealing that it is an evolved multidimensional process[143,155].

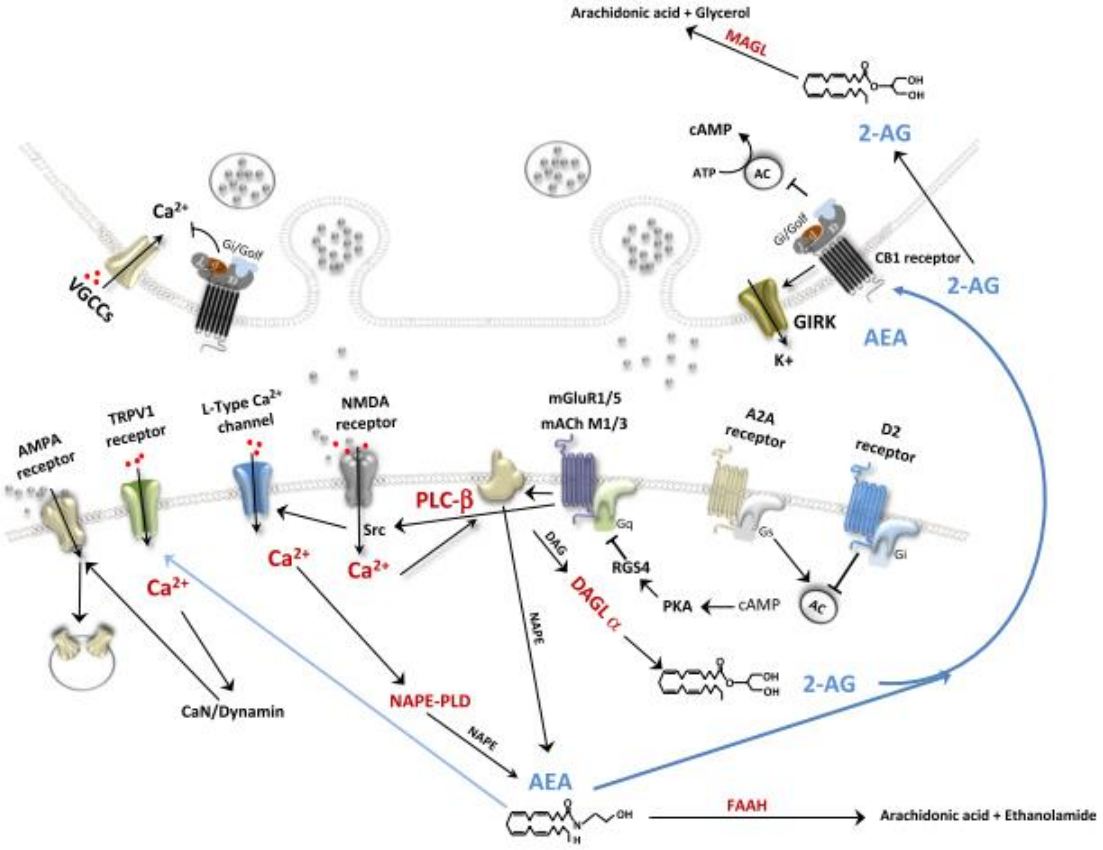
Synaptic eCB signaling is highly complex and involves many metabolic pathways, activation of CB receptors and crosstalk of intrinsic mechanisms (summarized in Figure 7)[145]. eCBs spatiotemporal controlled synthesis is performed through the coordinated expression of

metabolic enzymes specialized for the process: 2-AG is synthesized in the brain by the enzyme diacylglycerol lipase (DAGL)α, which converts diacylglycerols (DAGs) into 2-AG upon stimulation; AEA synthesis is rather more complex and is still unclear but the most suitable candidate as a synthesizing enzyme is N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase-D (NAPE-PLD)[122,137,140]. AEA and 2-AG degradation, on the other hand, is performed by the degrading enzymes fatty acid amide hydrolases 1/2 (FAAH) and monoacylglycerol lipases 1/2 (MAGLs), respectively; in addition, oxidizing enzymes like cyclooxygenases (COXs) and lipoxygenase can also degrade these 2 substrates[135].

Therefore, the activation of CBRs protects the nervous system from overstimulation or over-inhibition that may be caused by other neurotransmitters, regulating neurocircuitry dynamics, thereby having a role in anxiety, depression, cognition, addiction, motor function, feeding behavior, immune responses, inflammation/neuroinflammation and pain[122,138,156–160].

Additionally, growing evidence shows that eCBs shape neuronal connectivity and have been implicated as modulators of synaptic transmission and plasticity, being involved in processes like homeostatic, short- and long-term plasticity (short- and long-term depression or potentiation)[143,154,155,161–163].

During development of the CNS, eCBs play a key role in the regulation of proliferation, differentiation, fate specification, migration, synaptic establishment and survival of neural progenitors. eCBs also regulate neuritogenesis, axonal growth and guidance and synaptogenesis in differentiated neurons[136,137,142,164].



◄ **Figure 7 – Synaptic eCB signaling.** Main pathways associated with synaptic eCB signaling. Both pre- and postsynaptic signaling cascades are involved (adapted from [145]Melis et al., 2014)**.**

1.4.1. CB_1 receptor

CB_1 receptor (CB_1R) is among the most abundant GPCRs in the CNS (equivalent densities as for GABA and glutamate-gated ion channels)[135,165]. It is also present in numerous peripheral tissues (although with a lower expression) like the heart, spleen, reproductive tissues, endocrine glands and intestinal tracts, etc[138,166]. In the CNS, there is a wide distribution of CB_1Rs (see Table 3). The high expression of CB_1Rs in motor/sensory regions correlates with the prominent effects observed by stimulation with cannabinoids, namely in motor coordination and perception, pain modulation, cognition, memory and learning and regulation of emotional states[124,135,156,167]. Additionally, CB_1Rs are mainly confined at presynaptic central and peripheral nerves, and the majority is expressed on GABAergic terminals and, in less concentrations, in glutamatergic terminals[138,155].

Importantly, these receptors are also expressed at low levels by astrocytes, oligodendrocytes, and neural stem cells, highlighting the importance of this receptor as a neuromodulator in the CNS internetworks[168,169].

Structurally, the CB_1R is organized, like other GPCRs, in seven α-helical transmembrane domains (7TMs) with an extracellular glycosylated amino-terminus and a cytosolic carboxyl-terminus and intra- and extracellular loops. Particularly, the cytoplasmatic region – the carboxyl-terminus – is responsible for G protein-binding, desensitization and cellular trafficking of the receptor and the extracellular loop is involved in ligand binding and receptor localization[170–172].

Both AEA and 2-AG seem to bind to CB_1Rs as full agonists (or nearly full agonists), triggering specific downstream signaling pathways[173]. Predominantly, by coupling to $G_{i/o}$ proteins, CB_1R regulates, through several signaling machineries, the activity of many proteins, ion channels, enzymes, second messengers and kinases (Figure 8)[169]. Specifically, CB_1R activation:

- inhibits adenylyl cyclase, which decreases cAMP levels and downregulates the cAMP/ PKA pathway[124];
- activates all 3 families of functional MAPKs – p44/42 MAPK, p38 kinase and JNK-terminal kinase and also PI3K pathway[174];
- regulates voltage-gated Ca^{2+} channels (VGCCs)[148];
- inhibits presynaptic Ca^{2+} influx and inward rectifying K^+ channels (responsible for K^+ influx)[153];

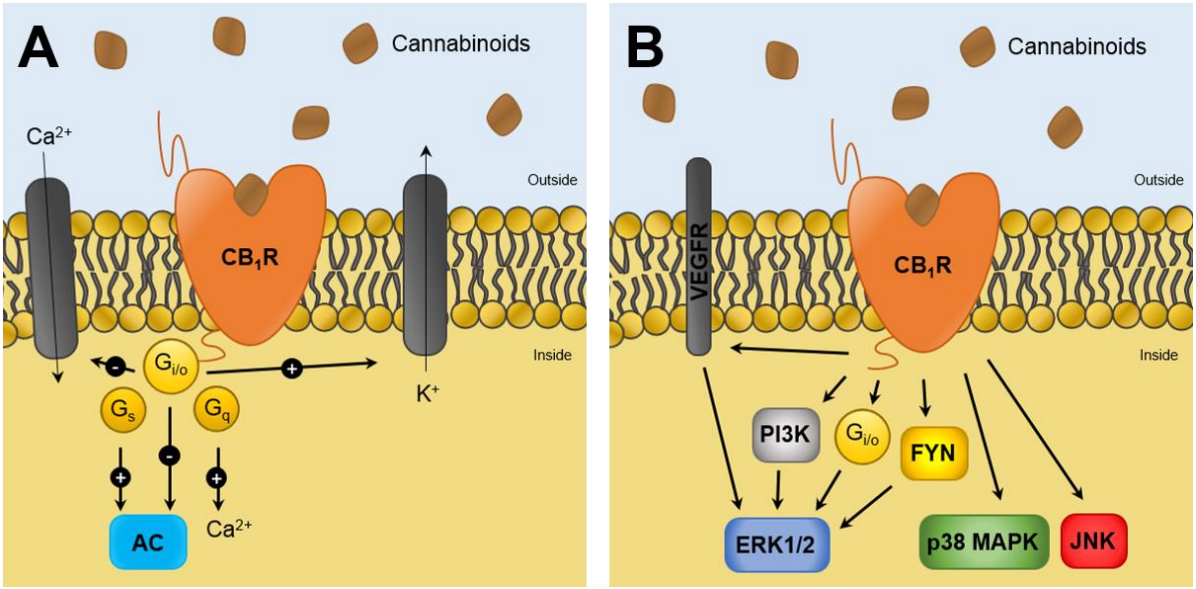


Figure 8 – CB_1R main signaling pathways. A. Activation of CB_1R activates $G_{i/o}$ proteins inhibiting adenylate cyclase and cAMP levels and modulates ion channels. **B.** CB_1R activation can also activate different MAPK pathways (PI3K, ERK, p38 MAPK, JNK pathways) (re-illustrated from [174]Turu & Hunyady, 2010).

CB_1Rs can also modulate the release of several neuromodulators including serotonin, acetylcholine, dopamine, opioids, norepinephrine (among others) by interacting with other GPCRs, either by intracellular crosstalk of transduction signaling or by forming heteromers[124,143,148,174].

Evidence shows that heterodimerization occurs between CB_1R and other CB receptors, opiate δ receptors, dopamine D_1 and D_2 receptors and, with high relevance for this work, with adenosine A_{2A} receptors promoting alterations at the functional and signal transduction levels[129,148,174–178].

Importantly, in the brain, CB_1 and CB_2 receptors can form heteromers, demonstrating a bidirectional cross-antagonism phenomenon in which CB_1R antagonists have the ability to block the effect of CB_2Rs agonists and vice-versa[179].

Cannabinoid-based therapy, although particularly important due to the abundance of CB_1Rs throughout multiple brain areas and their complex interactions with other neuromodulatory systems, still remains controversial because the use of CB_1R agonists may lead to psychoactive side effects and craving consequences, as well as problems such as dosage concentrations and short window of beneficial actions may arise[174,180,181].

Table 3 – CB_1R expression in the mammalian CNS (adapted from [182]Svízenská et al., 2008)

Cannabinoid receptor	Localization	Intensity
CB_1R	– Telencephalon: Layers II, III, IV of the somatosensory cortex, layer II of the cingulate cortex, layers II and IV of the entorhinal cortex, layer III of the piriform cortex, association cortical regions of the frontal lobe; Molecular layer of the dentate area, CA1, CA2 and CA3 fields of Ammon's horn, subicular complex; Ependymal and subependymal zones of the olfactory bulb, anterior olfactory nuclei, olfactory part of the anterior commissure; Amygdala; Internal segment of the globus pallidus, caudate nucleus and putamen; Striatonigral pathway; Entopeduncular nucleus; – Brainstem: Substancia nigra (SN) *pars reticulata*; Periaqueductal gray area (PAG); Gray matter around 4th ventricle; Spinal trigeminal tract and nucleus; – Cerebellum: Molecular layer – Spinal cord: Dorsal horn and lamina X – Dorsal root ganglia (DRG): Medium and large-sized neurons	Dense
	– Telencephalon: Layer V of the somatosensory cortex, temporal association cortex, secondary somatosensory and motor cortex, visual and auditory cortex; Polymorphic layer of the dentate area; Basal forebrain and septum; External segment of the globus pallidus, ventral pallidum, claustrum, and stria terminalis – Diencephalon: Anterior, mediodorsal, medioventral and intralaminar thalamic nucleus; Habenular nucleus; Lateral and paraventricular nucleus of the hypothalamus, infundibular stem – Brainstem: Solitary tract nucleus; Ambiguus nucleus; Inferior olive – Spinal cord: Deep dorsal horn; Thoracic intermediolateral nucleus	Moderate
	– Telencephalon: Primary motor and somatosensory, visual and auditory cortex; Granule cell layer of the dentate gyrus; Olfactory tubercle; Ventral pallidum; Nucleus Accumbens – Diencephalon: Sensory and motor thalamic nuclei; Subthalamic nucleus – Brainstem: Ventral tegmental area; SN pars compacta	Low

1.4.1.2. CB_1R role in neurogenesis

Cannabinoids, besides their neuromodulatory role, constitute a group of signaling cues that regulate neurogenesis at the levels of NSPCs proliferation, differentiation and migration[136,139,141,183]. Importantly, CB_1R signaling influences the identity and cell features by regulating neuronal differentiation because its expression is not only increased with progressive differentiated stage but also because it is associated with proliferative and/or pro-survival cascades that allow the regulation of cell-cycle [139,184,185].

Growing evidences show that eCBs and CBRs have a huge impact on the regulation of the neurogenic process[142,186,187]. Notably, CB_1R contribution to neurogenesis has been shown[139,141,168] (for extensive review see Prenderville et al.[187]). Specifically:

- Evidences shows that CB_1R knockout (KO) in mice results in impaired neurogenesis, suggesting a regulatory role of CB_1R in neurogenesis[188];

- 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 eCBs (both AEA and 2-AG) increase the number of BrdU (a marker for proliferation)-positive NPCs from dissociated neurospheres[168];
- CB_1R agonist ACEA was shown to promote murine neural precursor differentiation toward a neuronal lineage via CB_1R-dependent mechanism, suggesting that CB_1R activation may represent a pro-neuronal differentiation signal[189];
- Both CB_1R (ACEA) and CB_2R (JWH-056) agonists have been shown to stimulate the proliferation of primary murine cortical neurospheres[190];
- CB_1R activation (with R-m-AEA, a CB_1R agonist) was demonstrated to induce proliferation, self-renewal and neuronal differentiation in mouse neonatal SVZ cell cultures[191];
- Treatment with CB_1R antagonist AM251 abrogated an exercise-induced increase of cell proliferation in the hippocampus, suggesting that endogenous cannabinoid signaling is required for this increase in cell proliferation[192];
- eCBs via CB_1R activation exerted a modulatory role on NPC proliferation and differentiation, under an excitotoxicity-induced neurogenesis context[193];
- AEA exposure was shown to affect murine NPCs cell fate determination; AEA treatment promoted an increase in glial differentiation followed by an increase in neuronal differentiation rates[194].

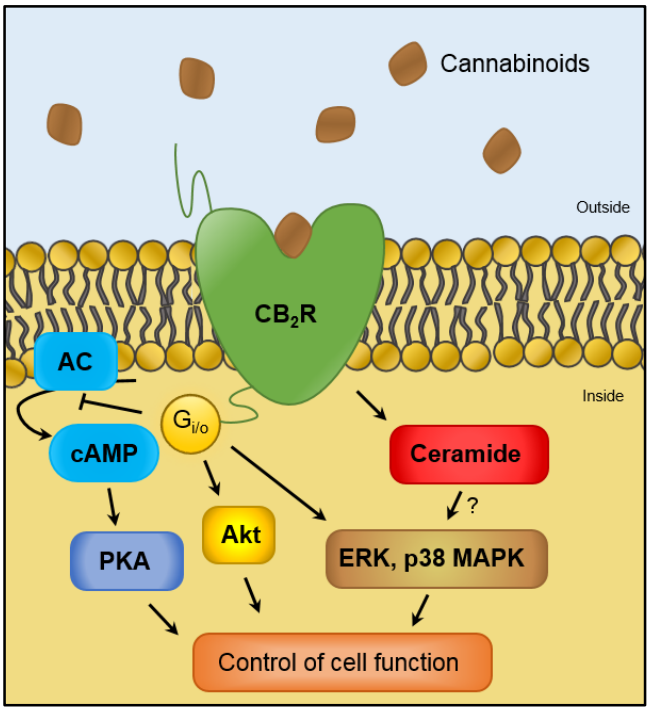
1.4.2. CB_2 receptor

CB_2 receptor (CB_2R) is also part of the receptor superfamily of GPCR and its localization is strikingly different from that of CB_1R[167]. It is mainly present in cells of the immune and hematopoietic systems and other peripheral tissues[124,180,195]. Specifically, it is present in specific tissues of immune cell production and regulation, the spleen, tonsils, thymus[165]. In humans, CB_2Rs have important functions in the immune system by modulating the release of cytokines, molecules responsible for the regulation of immune function and inflammatory responses[195,196]. The localization of these receptors in immune tissues suggests their role in key immunomodulatory function, particularly in the brain, where they are involved in immune surveillance through microglia (which are morphologically, phenotypically and functionally related to macrophages)[124,148,196,197]. Nevertheless, and most importantly, CB_2Rs were also identified in the adult CNS particularly in glial cells, including microglia and astrocytes, neural and oligodendroglial progenitors, and neuronal subpopulations of certain brain structures[195,198,199] (see Table 4 for CB_2R expression in the CNS). The multifocal existence of

functional CB_2Rs at CNS synapses implies their neuro/immunomodulatory action in brain's neurobiological processes associated with the stated regions like the control of pain, brain reward, cognition, emotion and others[200].

As a GPCR, this receptor shares the same structural features as the CB_1R, characterized by seven transmembrane spanning domains, extracellular N-terminus, involved in ligand binding, 3 extracellular loops (EC1-3), 3 intracellular loops (IC1-3) and an intracellular C-terminal domain, which is involved in signal transduction and coupling to G proteins[195,201].

Different eCBs activate CB_2R signaling mechanisms differently. 2-AG was demonstrated to be a selective agonist whereas AEA was shown not to significantly bind to CB_2Rs[198]. Similarly to CB_1R signaling, CB_2Rs can modulate adenylyl cyclase and MAP kinase activity, through their ability to couple to $G_{i/o}$ proteins[124,153]. This coupling triggers primarily the activation of canonical/classical intracellular responses that lead to the inhibition of adenylyl cyclase and subsequently to an impairment of cAMP/PKA short- and long-term effects. Since PKA modulates the expression of CREB, which is a transcription factor involved in the regulation of a variety of 'pro-survival-proliferation-differentiation' genes, the overall result of PKA inhibition is reduced cell maintenance. Additionally, there is stimulation of several MAPK cascades, namely ERK1/2 and p38 MAPK cascades and also PI3/Akt pathway, linked to pro-survival effects[124,153,195,198]. However, in contrast to CB_1R, CB_2R stimulation is believed not to modulate ion channel function (Ca^{2+} and K^+ channels), but has an impact in intracellular stores of Ca^{2+} from InsP3-sensitive stores by activation of PLC (Figure 9)[124,195].



◄ **Figure 9 – CB_2 receptor main signaling pathways.** CB_2Rs activation exerts an effect on adenylate cyclase and consequently on cAMP levels and protein kinase A (PKA) pathway; other pathways can also be activate, namely MAPK cascades (ERK, p38 MAPK) and Akt pathway (re-illustrated from [202]Fernández-Ruiz et al., 2007)**.**

Like CB_1Rs, cross-talk between CB_2Rs and other GPCRs is known to occur, but the molecular and cellular basis for the interactions, the extent to which they occur and the impact on CNS function is still not fully understood[179,195].

Overall, the neuroprotective role of CB_2Rs comes from their ability to predominantly mediate anti-inflammatory and immunomodulatory actions. This represents a crucial feature to specifically target the neuroinflammatory component of acute brain injuries (brain trauma or cerebral ischemia) or some neurodegenerative diseases, such as multiple sclerosis, Alzheimer's disease or amyotrophic lateral sclerosis, and consequently delaying brain damage[149,181,198].

All together, these evidences suggest that CB_2Rs play an important role in neuro-immunomodulatory responses and, because they do not have, unlike CB_1Rs, any psychoactive effect, they represent perfect targets for the development of new therapies based on CB_2R-agonist properties and actions[148,149].

Table 4 – CB_2R expression in the mammalian CNS (adapted from [182]Svízenská et al., 2008)

Cannabinoid receptor	Localization	Intensity
CB_2R	– Telencephalon: – Neurons of the layers III and V of the orbital, visual, auditory, motor and piriform cortex; Island of Calleja; Pyramidal neurons of the hippocampal CA2 and CA3 areas; Anterior olfactory nucleus; Striatum; Amygdala; – Diencephalon: Ventral and lateral posterior, posterior, and paracentral thalamic nuclei; Retina – Brainstem: Dorsal cochlear nucleus; Facial nucleus – Cerebellum: Purkinje cell bodies; Cerebellar granule cells – DRG: Neurons of a neonatal rat	Dense
	– Diencephalon: Geniculate body nuclei – Brainstem: SN *pars reticulata* – neurons larger than 20 μm; PAG; Inferior colliculus, interpeduncular, paratrochlear, and red nuclei; Paralemniscal nucleus, dorsal nucleus of lateral lemniscos; Pontine nuclei; Paratrochlear nucleus, medial and lateral vestibular nuclei; Parvocellular reticular nucleus; The spinal trigeminal tract nucleus – Cerebellum: Dendrites of Purkinje cells in the molecular layer	Moderate
	– Diencephalon: Paraventricular and mediodorsal thalamic nuclei; Ventromedial and arcuate hypothalamic nuclei	Low

1.4.2.1. CB_2R role in neurogenesis

Gathering evidences show the implication of the CB_2R in processes related to the control of proliferation, differentiation and survival of neural cells[198,202,203]. Fairly recent evidences show that CB_2Rs have a role in neurogenesis by promoting NSPCs proliferation or by reestablishing neurogenic properties (for extensive review see Prenderville et al.[187]). Specifically:

- Evidences show that CB_2R-KO reduces the self-renewal of murine embryonic cortical NPCs, while both HU-308 and JWH-133 (CB_2R agonists) increase both primary neurosphere generation and neural progenitor self-renewal *in vitro*[204];
- Activation of CB_2Rs was shown to promote mouse NPCs proliferation and an increase in neurosphere formation through a mechanism dependent on the phosphoinositide-3 kinase/Akt pathway[205];
- Treatment with a CBR agonist (WIN55,212-2) or with a CB_2R-selective agonist (JWH-133) in an *in vivo* study showed an increased NPCs proliferation on the SVZ of mice, via an autocrine DAGL-CB_2R signalling arrangement, with this effect being more pronounced in aged mice[206];
- The administration of HU-308 (a CB_2R selective agonist) was shown to induce an increase in proliferation of NSPCs via PI3K/Akt/mTORC1-dependent signaling both in vitro and in vivo[207];
- More recently, CB_2R agonist AM1241 has been shown to promote the proliferation/differentiation of primary normal human NSPCs (hNSCs) in the presence of the HIV-1 neurotoxic glycoprotein Gp120; Gp120-induced DNA fragmentation was reduced by the administration of AM1241 as well as astroglyosis and gliogenesis, which suggests a neuroprotective role of CB_2Rs against impaired neurogenesis[208];

Accordingly, the fact that eCBs and exocannabinoids regulate NSPCs proliferation and differentiation constitutes a potential mechanism for the treatment of adult brain disorders, especially because 1) the ECS is activated to counteract/alleviate neuronal damage and neuroinflammation and 2) some symptoms associated with adult brain disorders appear to be correlated with dysregulation of eCBs[135,141,156,169].

By intervening on a pathophysiological context, cannabinoids may modulate adult neurogenesis and, ultimately, contribute for the treatment of a wide variety of disorders, such as anxiety, depression and neurodegenerative diseases like stroke, ALS, multiple sclerosis, Alzheimer's, Parkinson's and Huntington's disease[156,180,181,209–213].

1.5. Crosstalk between A_{2A} receptors and cannabinoid receptors in the CNS

A_{2A} and CB_1 receptors are highly expressed in the CNS, whereas CB_2Rs are expressed in low concentrations.

Notably, A_{2A}R crosstalk with CBRs (mainly CB_1R) is specifically significant because it modulates CB_1R actions which in turn has major repercussions on the motor depressant and rewarding effects of cannabinoids[129,214]. Additionally, this interaction between A_{2A}Rs and CBRs is particularly important because it was shown to activate key modulatory effects on synaptic function and transmission[92,215–217].

Evidence for a structural and functional cross-talk between these receptors has been reported by several authors:

- In a human neuroblastoma cell line, CB_1R signaling was completely dependent on A_{2A}R activation. Moreover, on a behavioral perspective, blockage of A_{2A}Rs with selective antagonist ZM241385 counteracted the depreciating effects on motor control produced by the intrastriatal administration of a cannabinoid CB_1R agonist, suggesting that this was due to the formation of functional heteromeric complexes[214];
- A_{2A}R-CB_1R heterodimerization was shown to occur in living cells (human embryonic kidney cells, HEK-293T cells), as observed by bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) – sequential BRET-FRET (SRET); additionally, the occurrence of A_{2A}R-D_2R, CB_1R-D_2R and A_{2A}R-CB_1R as well the existence of D_2R-A_{2A}R-CB_1R hetero-oligomers was also demonstrated[175,177];
- The specific involvement of A_{2A}R in the addictive-related properties of cannabinoids was demonstrated by the reduction of THC-induced rewarding and aversive effects in mice lacking A_{2A}Rs compared to wild-type[218];
- A control of CB_1R function by A_{2A}Rs in glutamatergic terminals in the striatum was shown in which it is was suggested an inhibitory presynaptic interaction between CB_1Rs and A_{2A}Rs[219];
- Detailed presynaptic interactions between A_{2A}R–CB_1R in glutamatergic nerve terminals of corticostriatal synapses in which presynaptic A_{2A}R activation dampened CB_1R-mediated inhibition of corticostriatal terminals was also demonstrated[220];
- The activation of A_{2A}Rs was found to directly regulate the synaptic effects of CB_1Rs or indirectly, through activation of mGlu5Rs in the rodent striatum[221];

- Presynaptic CB_1Rs that interact with A_{2A}Rs were shown to be involved in the motor depressant and addictive effects of cannabinoids and that postsynaptic CB_1Rs interacting with A_{2A}Rs and D_2Rs are responsible for the cataleptogenic effects of cannabinoids[129];
- Blockade of CB_1Rs (either with antagonists or in CB_1R-KO mice) reduced the locomotor-activating effects of A_{2A}R antagonists, which, on the other hand, were able to increase endocannabinoid-dependent LTD[222];
- Cannabidiol can induce robust neuroprotection (anti-inflammatory effects) mediated by CB_2Rs and adenosine receptors (mainly A_{2A}Rs) in an *in vitro* model of newborn hypoxic-ischemic brain [223];
- Administration with an A_{2A}Rs-antagonist alters the reinforcing effects of cannabinoids (induced by CB_1R-agonists) by preferentially acting at presynaptic A_{2A}R sites[224];
- A tight control of CB_1R function by presynaptic A_{2A}Rs in the inhibition of striatal glutamatergic terminals was described[219];
- Interestingly, the administration of A_{2A}Rs antagonists, in the presence of a sub-threshold dose of cocaine, was demonstrated to modulate synaptic activity and enhance locomotion and this enhanced activity required activation of CB_1Rs[225];
- Receptor heteromers have also been demonstrated in native brain samples from parkinsonian rats and primates: in a rodent hemiparkinsonian model, the acute or chronic administration of L-DOPA was found to disrupt the A_{2A}-CB_1–D_2 receptor heteromers crosstalk[226] and also L-DOPA treatment disrupts D_2R-A_{2A}R-CB_1R heteromers in the caudate nucleus of primates[227];
- In a rodent model of Parkinson disease, single administration of A_{2A}R and CB_1R antagonists promoted dopaminergic survival whereas combined administration had a weakened effect, suggesting that the functional crosstalk between the adenosine and cannabinoid system may explain differences among single versus combined treatments[228].

Overall, these studies suggest that the adenosinergic tone is important for cannabinoid-mediated effects and that A_{2A}Rs could exert a role on CBRs-mediated function via receptor heterodimerization[216].

Taking into account the differential effects of A_{2A} and CBRs agonists and antagonists in the brain it is possible to predict that this crosstalk may be involved in the regulation of the neurogenic process. It is also possible to infer that eCBs may play a role in regulating neurogenesis through interaction with A_{2A}Rs.

1.6. Main objective and specific aims

Growing evidences suggest that there is contribution of A_{2A}Rs and CB_1Rs and CB_2Rs to neurogenesis. Nevertheless, most of these studies only evaluate the individual effect of CBRs agonists and antagonists in neurogenesis whereas A_{2A}Rs role remains to be fully established. In fact, to date, the crosstalk between A_{2A}Rs and CBRs in neurogenesis has not been evaluated. Therefore, we proposed to evaluate this putative crosstalk based on 2 guiding criteria:

- Characterize the crosstalk of A_{2A} receptors with CB_1Rs/CB_2Rs in both neurogenic niches (SVZ and SGZ/DG);
- Understand how the crosstalk between these receptors can affect the following neurogenic properties: cell-fate, proliferation and neuronal differentiation;

Considering the widespread brain distribution of A_{2A}Rs and the ECS, a better understanding of this possible interaction between CBRs and A_{2A}Rs, specifically at neurogenic niches, could contribute to the development of therapeutic alternatives to several brain disorders, either by potentiating endogenous repair mechanisms or preventing further brain damage. Consistently, both systems may, in the long run, represent promising pharmacological platforms for developing therapeutic proneurogenic compounds that could act upon these platforms and enhance neurogenesis.

CHAPTER 2 Material & Methods

2.1. Ethics Statement

All experiments were performed in accordance with the European Community (86/609/EEC; 2010/63/EU; 2012/707/EU) guidelines. The work was performed with biological material obtained from rat pups and subsequently maintained *in vitro*. The pups were handled according to standard and humanitarian procedures to reduce animal suffering.

2.2. SVZ and DG Cell Cultures

SVZ and DG neurospheres were prepared from early postnatal (P1-3) Sprague-Dawley rats. After sacrificing the animal and removing its brain, SVZ and DG fragments were dissected out from 450 µm-thick coronal brain slices, digested with 0.05% Trypsin-EDTA (Life Technologies, Carlsbad, CA, USA) in Hank's balanced saline solution (HBSS, Life Technologies), and mechanically dissociated with a P1000 pipette. The originated cell suspension was then diluted in serum-free medium (SFM), composed of Dulbecco's modified Eagle's medium/Ham's F-12 medium with glutaMAX (DMEM+GlutaMAX, Life Technologies) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Pen/Strep; Life Technologies), 1% B27 (Life Technologies) and growth factors (for SVZ cells: 20 ng/mL epidermal growth factor (EGF; Life Technologies); for DG cells: 20 ng/mL epidermal growth factor (EGF; Life Technologies) and 10 ng/mL fibroblast growth factor-2 (FGF-2; Life Technologies) (*proliferative conditions*). SVZ cells were then plated on uncoated Petri dishes and allowed to develop for six days, whereas DG cells were allowed to develop for twelve days, both in a 95% air-5% CO2 humified atmosphere at 37 °C.

Six and twelve days after plating SVZ and DG cells, respectively, the resulting neurospheres were adhered for 24h onto glass coverslips coated with 0,1 mg/mL poly-D-lysine (PDL, Sigma-Aldrich, St. Louis, MO, USA) in SFM devoid of growth factors (*differentiative conditions*). One day after plating, the medium was renewed with or without (control) a range of pharmacological treatments for $A_{2A}R$, CB_1R and CB_2R ligands (illustrated in Figure 10).

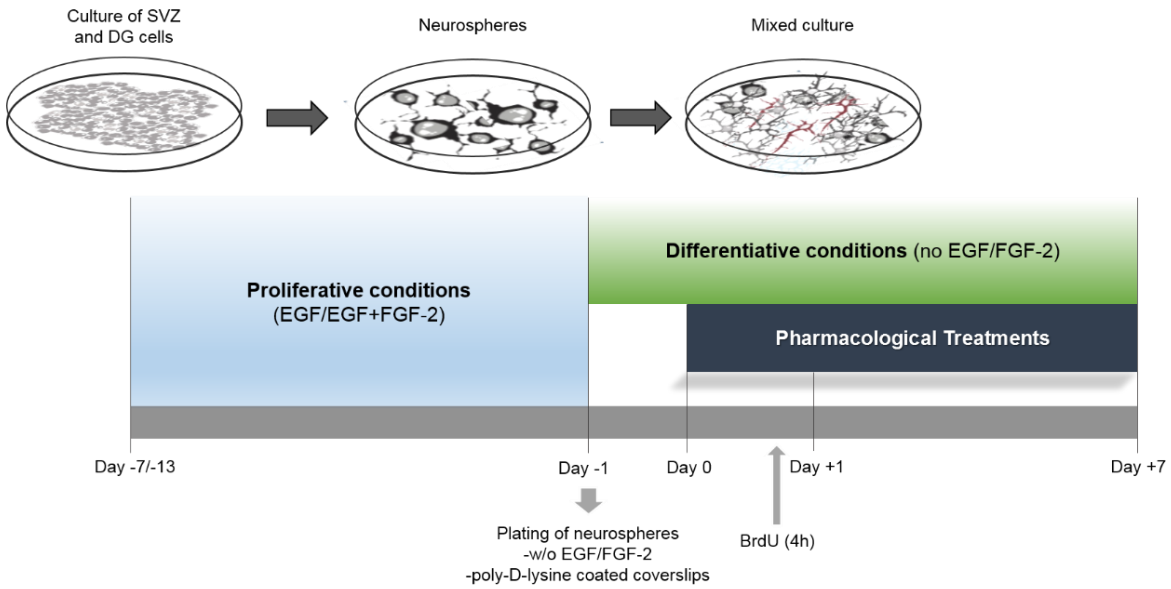


Figure 10 – SVZ and DG cell culture scheme. SVZ and DG cells were allowed to develop in proliferative conditions for 6 or 12 days, respectively. After that time period, the resulting neurospheres were plated onto PDL-coated coverslips in differentiative conditions without growing factors. Day 0 represents the day in which pharmacological treatments were executed.

2.3. Pharmacological Treatments

To study cell-fate, a Sox2 cell-pair assay was performed as described by Xapelli et al.[191], where dissociated SVZ and DG cell suspensions obtained during the cell culture procedure were plated on poly-D-lysine coated glass coverslips at a density of 12800 cells/cm^2 and 19200 cells/cm^2, respectively. After seeding, SVZ and DG cells were grown, respectively, in SFM supplemented with 10 ng/mL EGF (low EGF) and in SFM supplemented with 10 ng/mL EGF and 5 ng/mL FGF-2 (low EGF/FGF-2). Moreover, plated cells were treated or not (control) with selective agonists and/or antagonists for A_{2A}Rs, CB_1Rs and CB_2Rs for 24h (Table 5). Thereafter, cells were processed for immunocytochemistry against Sox2, a marker of neural stem cells with the ability to self-renew.

To study cell proliferation, plated neurospheres were allowed to develop for 24h in the absence (control) or presence of the A_{2A}Rs, CB_1Rs and CB_2Rs ligands (Table 5). A thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU, 10 µM, Sigma-Aldrich) was added for the last 4h of the culture session.

To study neuronal differentiation, neurospheres were allowed to develop for 7 days in the absence (control) or presence of the aforementioned ligands and an immunocytochemistry (ICC) for NeuN was performed.

Whenever cultures needed to be co-treated with a combination of drugs, SVZ or DG cells were primarily treated with CB_1R (ACEA) or CB_2R (HU-308) selective agonists for 30 minutes prior to $A_{2A}R$ selective agonist (CGS21680) treatment and then grown for 24h in the case of cell-fate or proliferation studies, or 7 days, in the case of neuronal differentiation studies, in the presence of the ligands. Similarly, treatment with selective antagonists for CB_1Rs (AM251) and CB_2Rs (AM630) or $A_{2A}Rs$ (ZM241385) was performed for 30 minutes prior to the treatment with CB_1Rs, CB_2Rs or $A_{2A}Rs$ selective agonists and then co-incubated for further 24h in the case of cell-fate and proliferation studies, or 7 days in the case of neuronal differentiation studies.

Table 5 – Pharmacological treatments used.

Drug	Biological activity	Concentration used	Catalog number	Company
ACEA [*N*-(2-Chloroethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide]	Cannabinoid CB_1 receptor agonist	1 µM	1319	Tocris, Bristol, UK
HU-308 [4-[4-(1,1-Dimethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-methanol]	Cannabinoid CB_2 receptor agonist	1 µM	3088	
CGS21680 [4-[2-[[6-Amino-9-(*N*-ethyl-β-*D*-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride]	Adenosine A_{2A} receptor agonist	30 nM	1063	
AM251 [*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide]	Cannabinoid CB_1 receptor antagonist	1 µM	1117	
AM630 [6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone]	Cannabinoid CB_2 receptor antagonist	1 µM	1120	
ZM241385 [4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol]	Adenosine A_{2A} receptor antagonist	50 nM	1036	

2.4. Immunocytochemistry (ICC)

Cells were fixed for 30 minutes in 4% PFA in PBS, and permeabilized and blocked for non-specific binding sites for 1h30 with 0,5% Triton X-100 (Sigma-Aldrich) dissolved in PBS. Cells were then incubated overnight at 4°C with the primary antibodies (see Table 6) and 0.1% Triton X-100 and BSA 0.3% (w/v) in PBS, and for 1h at RT with the appropriate secondary antibodies in PBS as follows: donkey anti-rabbit Alexa Fluor 568, donkey anti-goat Alexa Fluor

568 or donkey anti-rat Alexa Fluor 488 (all 1:200 and all from Life Technologies). Nuclei were stained with Hoechst 33342 (6 µg/mL in PBS, Life Technologies). The final preparations were mounted using Mowiol fluorescent medium. Fluorescence images were recorded using an Axioskop 2 Plus fluorescent microscope (Carl Zeiss Inc., Göttingen, Germany).

2.5. Cell fate studies (Sox2 cell-pair assay)

SVZ and DG neurosphere-derived cells were stained for Sox2, a marker of NSPCs with the ability to self-renewal (Figure 11A). Cell pairs resulting from the division of a single NSPC were counted and categorized in 3 groups according to their Sox 2 expression: in both daughter cells (Sox 2 +/+), in only one of the daughter cell (Sox 2 +/-) and no expression (Sox2 -/-) (Figure 11B). Sox2 expression in the daughter cells characterizes the response of cells to the pharmacological treatment applied, ultimately reflecting the cell-fate of the pool of NSPCs, namely expansion (symmetrical self-renewal), maintenance (asymmetrical self-renewal) or extinction (symmetrical commitment).

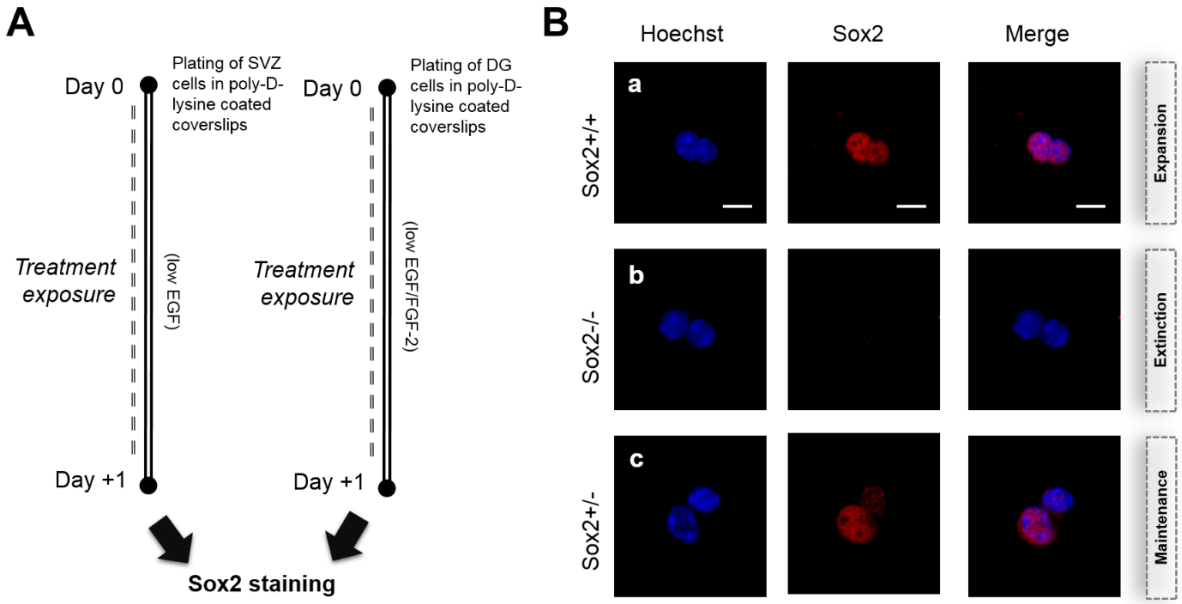


Figure 11 – Sox2 cell-pair assay. A. Schematic representation of the experimental protocol for studying SVZ and DG cell-fate. Day 0 represents the day of cultures in which cells were exposed to pharmacological treatments for the following 24h. **B.** Representative confocal images of cell pairs obtained following (a) the symmetrical division of a SVZ cell into two Sox2 + cells (Sox2 +/+), (b) the symmetrical terminal division into two Sox2- progenitors (Sox2 -/-) and (c) the asymmetrical division into a Sox2+ and a Sox2- progenitor (Sox2 +/-). Scale bars 20 µm. Sox2: sex determining region Y-box 2 (adapted from Xapelli et al.[191]).

2.6. Cell Proliferation Studies

To investigate the effect of the different pharmacological treatments on cell proliferation, SVZ and DG cells were exposed to 10 µM 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich), a synthetic thymidine analogue able to substitute thymidine in the DNA double chain synthesis occurring in dividing cells, for the last 4h of each specific pharmacological treatment (24h). Then, SVZ and DG cells were fixed in 4% PFA for 30 min and rinsed with PBS at RT. Subsequently, BrdU was unmasked by permeabilizing cells in PBS 1% Triton X-100 at RT for 30 min and DNA was denaturated in 1 M HCl for 40 min at 37ºC. Following incubation in PBS with 0.5% Triton X-100 and 3% BSA to block nonspecific binding sites, cells were incubated overnight with the anti-BrdU antibody (see Table 6). After an additional rinse in PBS, nuclei counterstaining and mounting were performed as described previously.

Table 6 – Primary antibodies used for immunocytochemistry.

Antigen	Company	Catalog number	Host	Dilution
Sox2 (a marker of neural stem cells with the ability to self-renew)	Santa Cruz Biotechnology (Dallas, TX, USA)	sc-17320	Goat	1:100
BrdU (5-bromo-2'-deoxyuridine)	AbD Serotec, Bio-Rad Laboratories (Oxford, UK)	OBT00306	Rat	1:200
Neuronal Nuclei (NeuN) (mature neuronal marker)	Cell Signaling Technology (Danvers, MA, USA)	12943	Rabbit	1:200

2.7. Statistical Analysis

In all ICC experiments, measurements were performed at the border of SVZ and DG neurospheres, where migrating cells form a pseudo-monolayer of cells. In every independent experiment, each condition was measured in three different coverslips.

Percentages of Sox2 cell pairs were obtained from counting about 60 cell pairs in triplicate coverslips obtained from 3-6 independent cultures.

Percentages of BrdU and NeuN immunoreactive cells were calculated from cell counts in five independent microscopic fields in each triplicated coverslip with a 40x objective (aproximately 200-300 cells per field).

Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance followed by Bonferroni's-multiple comparison test, with $p<0.05$ considered to represent statistical significance. All graphs and statistical analysis were performed in Graph Pad Prism version 6.01 software (GraphPad Software, San Diego, California).

CHAPTER 3 Results & Discussion

3.1. Results Overview

The role of A_{2A}Rs on neurogenesis induced by activation of CB_1Rs/CB_2Rs in rat SVZ and DG stem/progenitor cell cultures was studied. In fact, using SVZ and DG neurospheres as a model, three distinct stages of neurogenesis were evaluated: cell-fate, proliferation and neuronal differentiation.

To have a clear picture of data, the results were displayed concerning SVZ and DG niches and then, within each niche, the three evaluated stages (cell-fate, proliferation and neuronal differentiation).

3.2. SVZ neurogenic niche

3.2.1.1. CB_1Rs or CB_2Rs or A_{2A}Rs activation induce no significant effects in SVZ cell-fate

In order to investigate the capacity of A_{2A}R, CB_1R and CB_2R ligands of modulating cell-fate of SVZ cells, a Sox2 cell-pair assay was performed as described by Xapelli et al.[191], where SVZ cells were plated for 24h in medium complemented or not (control) with the ligands (Figure 12A).

It was observed that SVZ cells treated with CB_1R (ACEA, 1 µM) or CB_2R (HU-308, 1 µM) selective agonists or with A_{2A}Rs selective agonist (CGS21680, 30 nM) induced no significant changes in the percentages of either Sox2+/+ cell pairs (control: 50.93±0.84%; ACEA 1 µM: 53.76±3.00%; HU-308 1 µM: 53.19±3.64%; CGS21680 30 nM: 51.57±3.17%; N = 3) or Sox2-/- cell pairs (control: 46.90±0.93%; ACEA 1 µM: 43.86±4.54%; HU-308 1 µM: 44.61±3.55%; CGS21680 30 nM: 47.66±3.06%; N = 3) when compared to control conditions (Figure 12B).

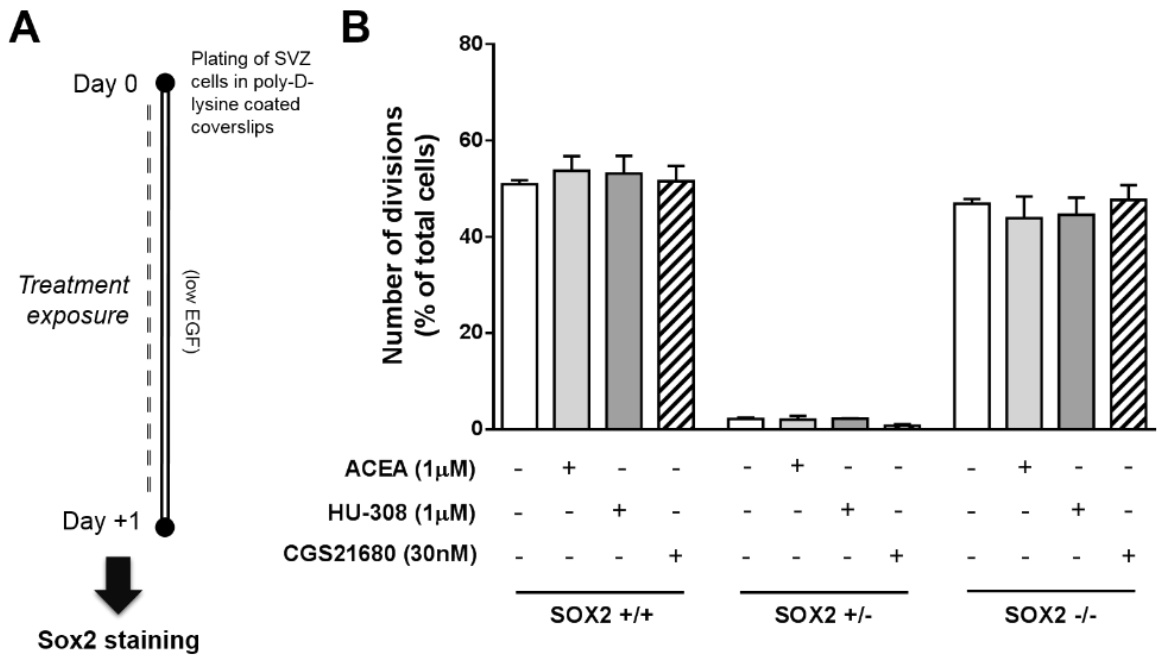


Figure 12 – CB_1R, CB_2R or $A_{2A}R$ activation *per se* do not change the SVZ cell-fate. A. Schematic representation of the experimental protocol for studying SVZ cell-fate. Day 0 represents the day of cultures in which cells were exposed to pharmacological treatments for the following 24h. **B.** Bar graphs represent the number of each type of cell divisions counted (60 pairs counted *per* condition *per* experiment). Data are expressed as the percentage of total cell pairs and are represented as the mean ± SEM. N=3.

3.2.1.2. Co-activation of CB_1Rs or CB_2Rs with $A_{2A}Rs$ induces no significant changes on SVZ cell-fate

It was next assessed the effect of a co-administration of both CB_1Rs and CB_2Rs selective agonists with the $A_{2A}R$ selective agonist on the SVZ cell-fate. SVZ cells were treated with either ACEA (1 μM) or HU-308 (1 μM) for 30 minutes prior to CGS21680 (30 nM) treatment and then grown for 24h in the presence of the ligands.

As expected, the exposure to both CB_1R selective agonist (ACEA, 1 μM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM) induced no significant changes in the percentages of either Sox2+/+ cell pairs (control: 50.93±0.84%; ACEA 1 μM+CGS21680 30 nM: 49.83±1.27% N = 3) or Sox2-/- cell pairs (control: 46.90±0.93%; ACEA 1 μM+CGS21680 30 nM: 48.21±1.05%; N = 3) as compared to control conditions (Figure 13A). Similarly, the co-administration of CB_2R selective agonist (HU-308, 1 μM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM) induced no significant changes in the percentages of either Sox2+/+ cell

pairs (control: 50.93±0.84%; HU-308 1 µM+CGS21680 30 nM: 48.38±3.70% N = 3) or Sox2-/- cell pairs (control: 46.90±0.93%; HU-308 1 µM+CGS21680 30 nM: 50.04±3.66%; N = 3) when compared to control (Figure 13B).

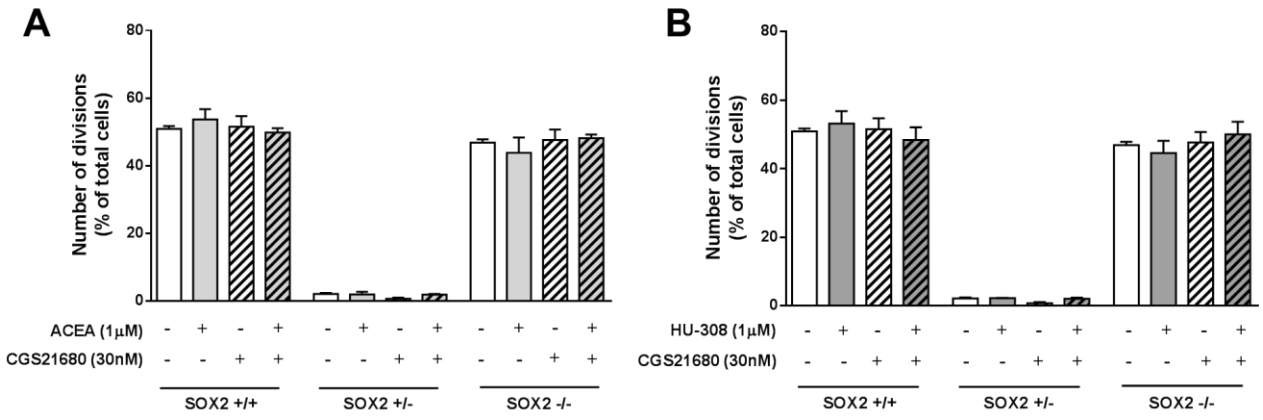


Figure 13 – Co-activation of CB_1Rs or CB_2Rs with $A_{2A}Rs$ promotes no changes on SVZ cell-fate. Bar graphs represent the number of each type of cell divisions counted (60 pairs counted *per* condition *per* experiment) in cultures treated with CB_1Rs agonist ACEA (1 µM) and $A_{2A}R$ agonist CGS21680 (30 nM) **(A)** or in cultures treated with HU-308 (1 µM) and $A_{2A}R$ agonist CGS21680 (30 nM) **(B)**. Data are expressed as the percentage of total cell pairs and are represented as the mean ± SEM. N=3.

3.2.2.1. CB_1R agonist ACEA stimulates SVZ cell proliferation

Next it was investigated whether $A_{2A}R$, CB_1R and CB_2R ligands modulate SVZ cells proliferation. For that purpose, SVZ cells were treated with selective agonists for $A_{2A}Rs$, CB_1Rs and CB_2Rs for 24 days. BrdU, a thymidine analogue, was added during the last 4h of the culture to label SVZ cells that went through S-phase. After fixation, incorporated BrdU was immunolabeled and positive nuclei were counted (Figure 14A).

Treatment of SVZ cells with CB_1R agonist ACEA (1 µM) promoted a substantial increase in the number of BrdU-positive cells when compared to control cultures (control: 100.0±0.01%; ACEA 1 µM: 151.3±11.58%; N = 8, ***p<0.001) whereas treatment with CB_2R agonist HU-308 (1 µM) and $A_{2A}R$ selective agonist CGS21680 (30 nM) induced no significant alterations in the number of BrdU-positive cells when compared to control cultures (control: 100.0±0.01%; HU-308 1 µM: 99.75±12.43; CGS21680 30 nM: 105.3±19.81%; N = 8) (Figure 14B, C).

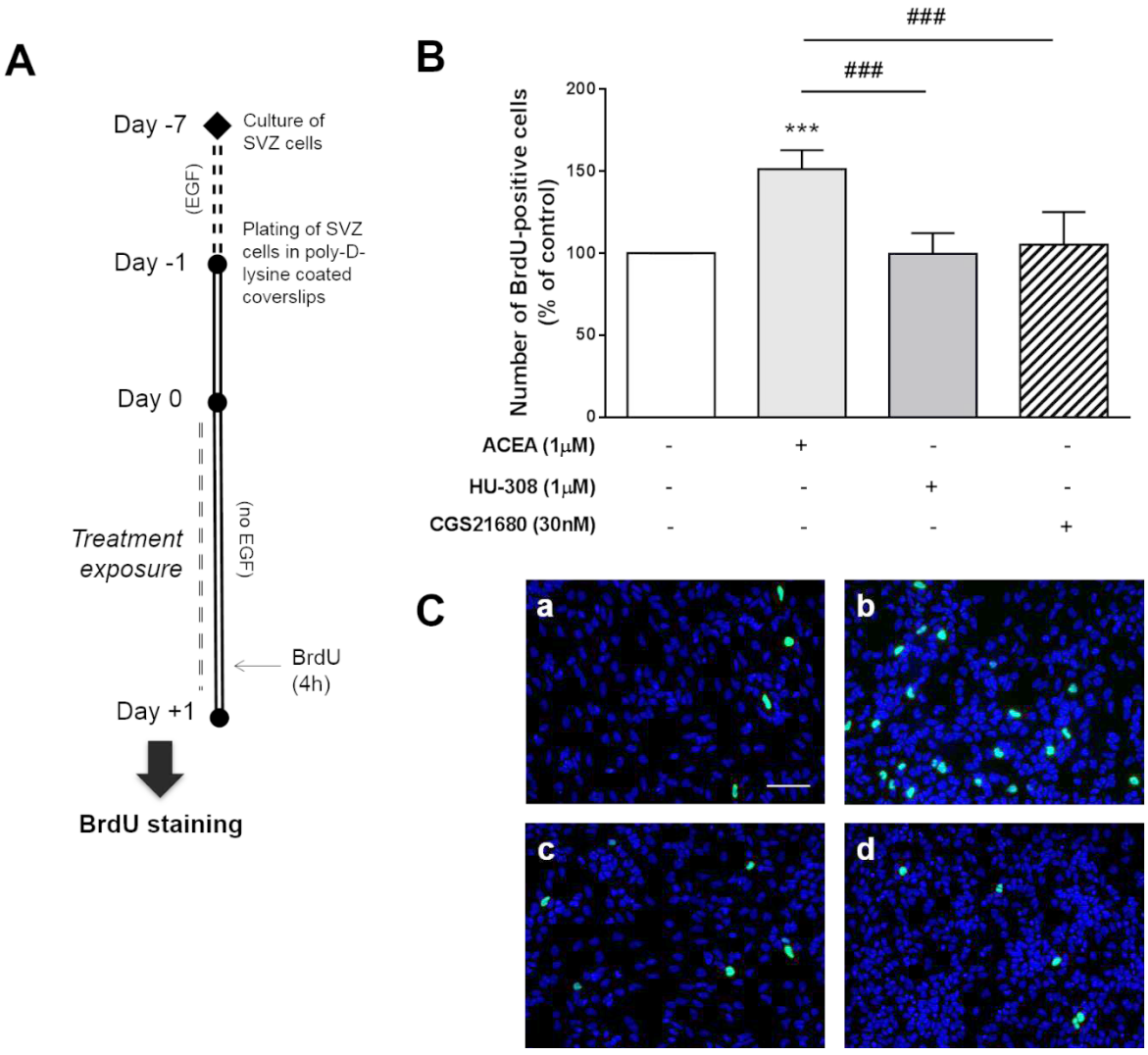


Figure 14 – CB_1R activation promotes SVZ cell proliferation while CB_2R and $A_{2A}R$ activation does not. A. Schematic representation of the experimental protocol. Day 0 represents the day in which neurosphere-derived cells were exposed to pharmacological treatments for the following 24h. **B.** Bar graph depicts the number of BrdU-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=8. ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control; ###p<0.001, by ANOVA using Bonferroni's post test for comparison with ACEA. **C.** Representative fluorescent digital images of BrdU-positive cell nuclei (green) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to ACEA 1 μM (b), HU-308 1 μM (c) and CGS21680 30 nM (d). Scale bar = 50 μm.

3.2.2.2. CB_1R activation coupled with $A_{2A}R$ activation preserves the increase on SVZ cell proliferation induced by CB_1R *per se*

In order to assess if co-incubation with the CB_1Rs or CB_2Rs selective agonists with the $A_{2A}R$ selective agonist could induce any changes in SVZ cell proliferation, SVZ cells were treated with either ACEA (1 µM) or HU-308 (1 µM) for 30 minutes prior to CGS21680 (30 nM) treatment for 24h.

In the presence of both CB_1R selective agonist (ACEA, 1 µM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM), there was a significant increase in the number of BrdU-positive cells when compared to control cultures (control: 100.0±0.01%; ACEA 1 µM+CGS21680 30 nM: 163.8±27.63%; N = 8, ***p<0.001), while it was similar to ACEA exposure alone (ACEA 1 µM: 151.3±11.58%) (Figure 15). On the contrary, the co-administration of CB_2R selective agonist (HU-308, 1 µM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM) promoted, as expected, no alterations in the number of BrdU-positive cells as compared to control cultures (control: 100.0±0.01%; HU-308 1 µM+CGS21680 30 nM: 100.7±17.27%; N = 8) (Figure 16).

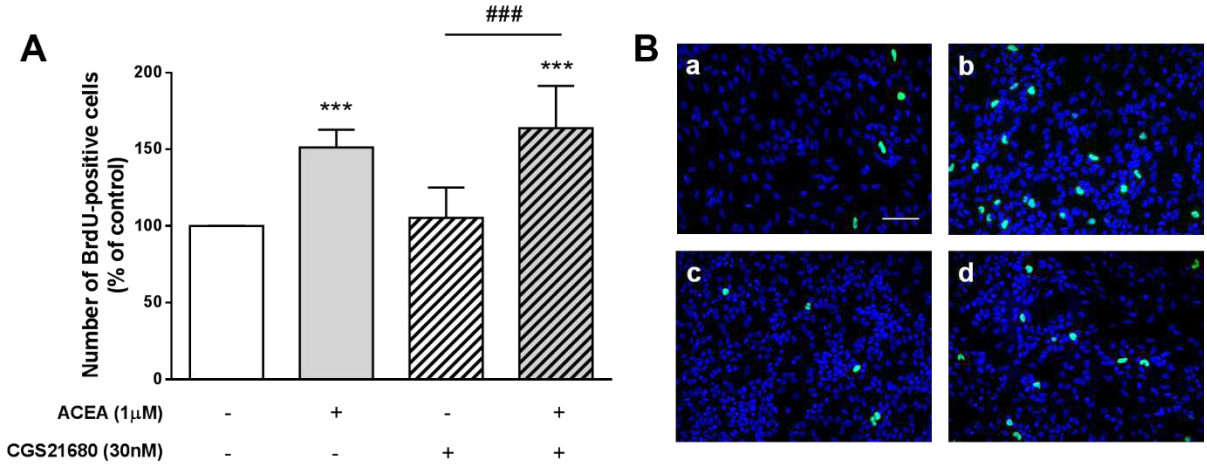


Figure 15 – Increased SVZ cell proliferation via CB_1R activation is maintained with $A_{2A}R$ co-activation. A. Bar graph depicts the number of BrdU-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=8. ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control; ###p<0.001, by ANOVA using Bonferroni's post test for comparison with CGS21680. **B.** Representative fluorescent digital images of BrdU-positive cell nuclei (green) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to ACEA 1 µM (b), CGS21680 30 nM (c) and ACEA 1 µM+CGS21680 30 nM (d). Scale bar = 50 µm.

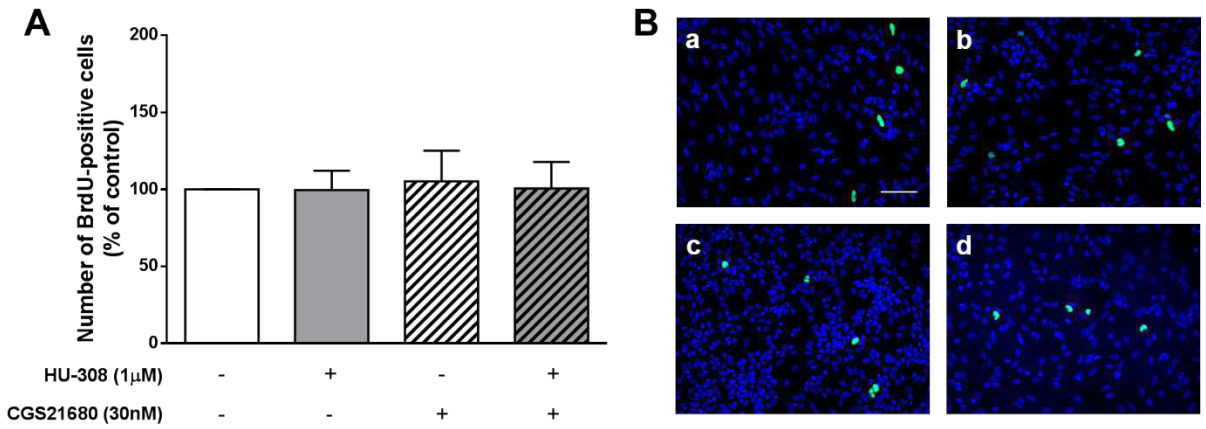


Figure 16 – SVZ cell proliferation is not affected by CB_2R and $A_{2A}R$ co-activation. A. Bar graph depicts the number of BrdU-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=8. **B.** Representative fluorescent digital images of BrdU-positive cell nuclei (green) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to HU-308 1 μM (b), CGS21680 30 nM (c) and HU-308 1 μM+CGS21680 30 nM (d). Scale bar = 50 μm.

3.2.2.3. $A_{2A}R$ is required for CB_1R-mediated stimulation of SVZ cell proliferation

I further wanted to study the putative role of $A_{2A}Rs$ in CB_1R mediated increase in SVZ proliferation. For that purpose, SVZ cells were treated with $A_{2A}R$ antagonist ZM241385 (50 nM) for 30 minutes prior to treatment with the CB_1R selective agonist ACEA (1 μM) and then co-incubated for further 24h.

In fact, the increase in the number of BrdU-positive SVZ cells promoted by CB_1R activation was blocked by the presence of an $A_{2A}R$ selective antagonist (ZM241385, 50 nM) (control: 100.0±0.01%; ACEA 1 μM: 151.3±11.58%; ACEA 1 μM+ZM241385 50 nM: 100.3±14.64%; ZM241385 50 nM: 96.13±10.80%; N= 4-8, $^{###}p<0.001$) (Figure 17).

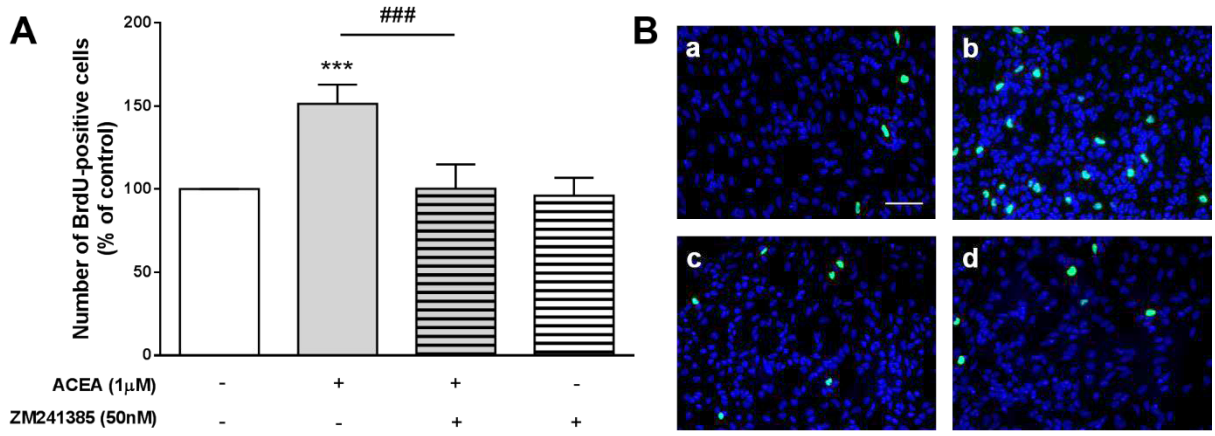


Figure 17 – $A_{2A}R$ blockade impairs CB_1R-mediated increase in SVZ cell proliferation. A. Bar graph depicts the number of BrdU-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=4-8, ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control; ###p<0.001, by ANOVA using Bonferroni's post test for comparison with ACEA. **B.** Representative fluorescent digital images of BrdU-positive cell nuclei (green) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to ACEA 1 μM (b), ACEA 1 μM+ZM241385 50 nM (c) and ZM241385 50 nM (d). Scale bar = 50 μm.

3.2.3.1. CB_1R and CB_2R activation induces SVZ neuronal differentiation

Thereafter, the role of $A_{2A}R$, CB_1R and CB_2R ligands on SVZ neuronal differentiation was studied. For this, SVZ cells were treated with selective agonists for $A_{2A}Rs$, CB_1Rs and CB_2Rs for 7 days and then fixed and stained for NeuN (marker for mature neurons) (Figure 18A).

Treatment of SVZ cells with CB_1R (ACEA, 1 μM) or with CB_2R (HU-308, 1 μM) selective agonists induced an increase in the number of NeuN-positive cells when compared to control cultures (control: 100.0±0.02%; ACEA 1 μM: 141.8±16.59%; HU-308 1 μM: 128.6±12.36; N = 6, **p<0.01 and ***p<0.001) whereas treatment with $A_{2A}R$ selective agonist (CGS21680, 30 nM) induced no significant changes (control: 100.0±0.02%; CGS21680: 111.6±11.87%; N = 6) (Figure 18B, C).

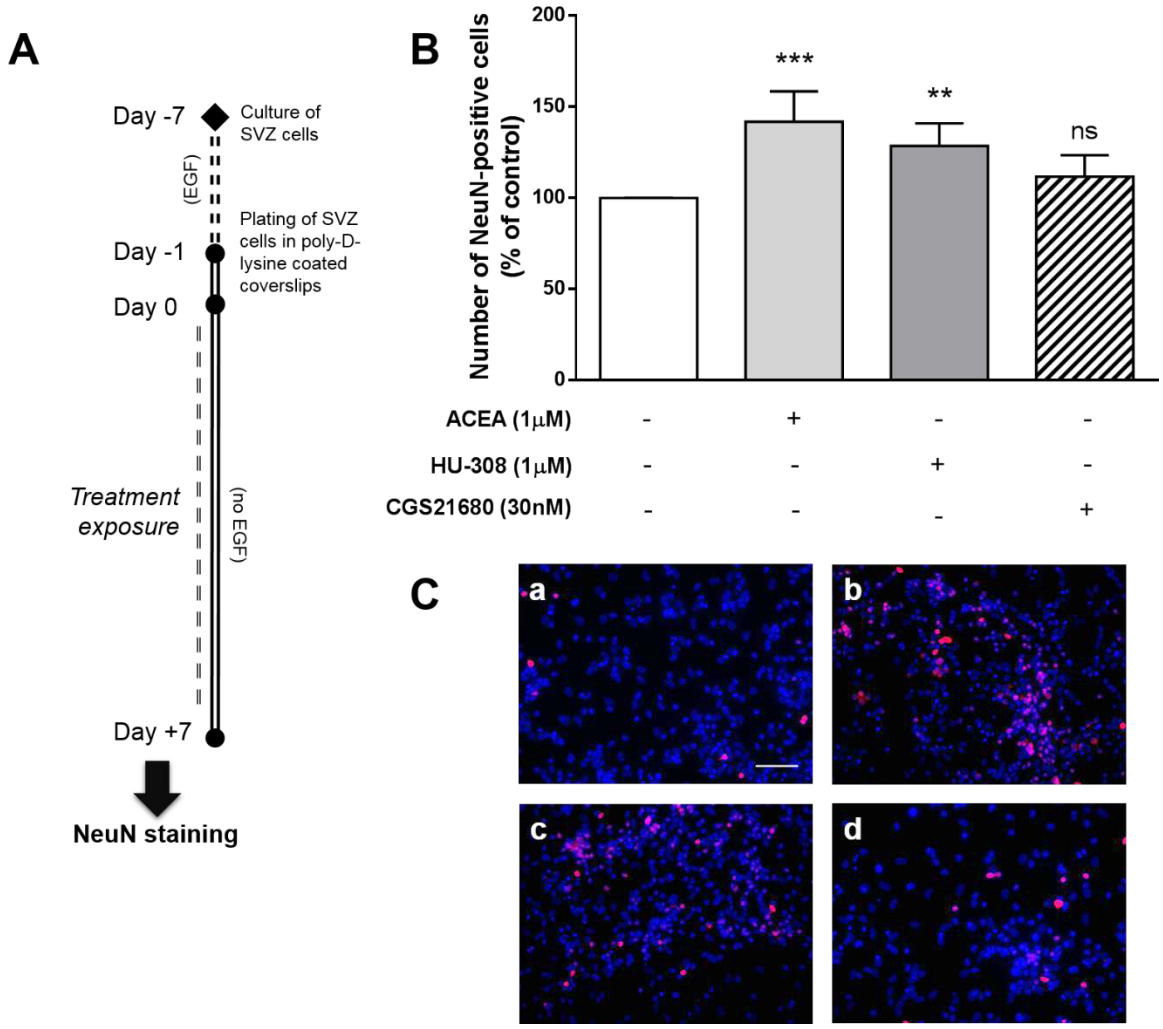


Figure 18 – CB_1R and CB_2R activation induces SVZ neuronal differentiation but not $A_{2A}R$ activation. A. Schematic representation of the experimental protocol. Day 0 represents the day in which neurosphere-derived cells were exposed to pharmacological treatments for the following 7 days. **B.** Bar graph depicts the number of NeuN-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=6. **p<0.01 and ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control. ns: non-significant. NeuN: Neuronal Nuclei. **C.** Representative fluorescent digital images of NeuN-positive neurons (red) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to ACEA 1 μM (b), HU-308 1 μM (c) and CGS21680 30 nM (d). Scale bar = 50 μm.

3.2.3.2. SVZ neuronal differentiation promoted by CB_1R and CB_2R activation is not changed with $A_{2A}R$ co-activation

In order to assess if a combined administration of both CB_1R and CB_2R selective agonists with the $A_{2A}R$ selective agonist promoted any alteration on SVZ neuronal differentiation, SVZ cells were treated with either ACEA (1 µM) or HU-308 (1 µM) for 30 minutes prior to CGS21680 (30 nM) treatment and then grown for further 7 days in the presence of the ligands.

The results show that, in the presence of both CB_1R selective agonist (ACEA, 1 µM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM), there was a significant increase in the number of NeuN-positive cells when compared to control cultures (control: 100.0±0.02%; ACEA 1 µM+CGS21680 30 nM: 129.7±23.66%; N = 6, *p<0.05), while it was similar to ACEA exposure alone (ACEA 1 µM: 141.8±16.59%) (Figure 19). Also, the co-administration of CB_2R selective agonist (HU-308, 1 µM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM) induced an increase in the number of NeuN-positive cells as compared to control cultures (control: 100.0±0.02%; HU-308 1 µM+CGS21680 30 nM: 141.0±18.37%; N = 6, ***p<0.001), with a similar effect when comparing with HU-308 exposure alone (HU-308 1 µM: 128.6±12.36) (Figure 20).

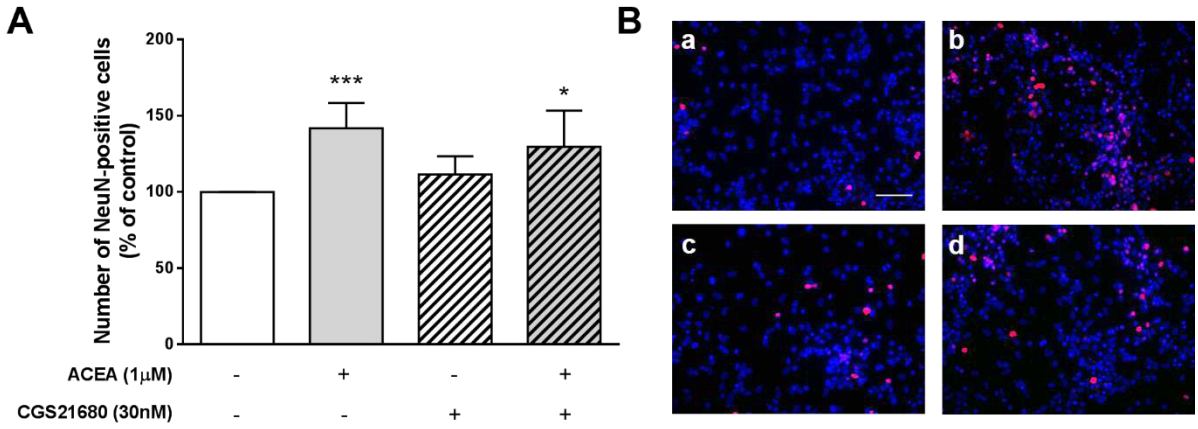


Figure 19 – Induced SVZ neuronal differentiation via CB_1R activation is not changed with $A_{2A}R$ co-activation. A. Bar graph depicts the number of NeuN-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=6. *p<0.05 and ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control. **B.** Representative fluorescent digital images of NeuN-positive neurons (red) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to ACEA 1 µM (b), CGS21680 30 nM (c) and ACEA 1 µM+CGS21680 30 nM (d). Scale bar = 50 µm.

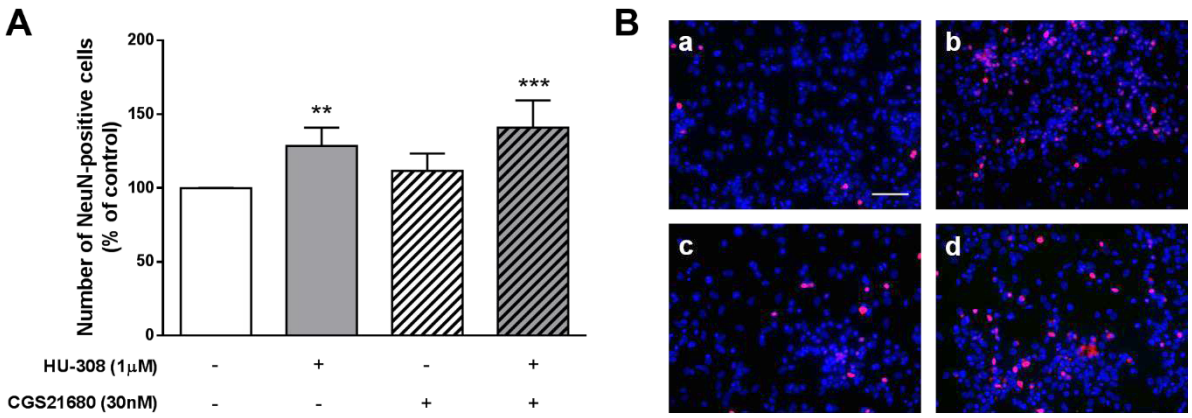


Figure 20 – Induced SVZ neuronal differentiation via CB_2R activation is not changed with $A_{2A}R$ co-activation. A. Bar graph depicts the number of NeuN-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=6. **p<0.01 and ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control. **B.** Representative fluorescent digital images of NeuN-positive neurons (red) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to HU-308 1 µM (b), CGS21680 30 nM (c) and HU-308 1 µM+CGS21680 30 nM (d). Scale bar = 50 µm.

3.2.3.3. $A_{2A}R$ activation is necessary for SVZ neuronal differentiation promoted by CB_1Rs and CB_2Rs

In line with the previous results we wanted to clarify the role of $A_{2A}R$ in CB_1R and CB_2R activation-induced SVZ neuronal differentiation. For that purpose, SVZ cells were treated, as before, with $A_{2A}R$ antagonist ZM241385 (50 nM) for 30 minutes prior to treatment with CB_1R or CB_2R selective agonists, ACEA (1 µM) or HU-308 (1 µM), respectively.

Interestingly, the results show that in the presence of an $A_{2A}R$ selective antagonist (ZM241385, 50 nM), the increase in the number of NeuN-positive SVZ cells promoted by CB_1R activation is lost, being similar to control cultures (control: 100.0±0.02%; ACEA 1 µM: 141.8±16.59%; ACEA 1 µM+ZM241385 50 nM: 91.34±15.51%; ZM241385 50 nM: 106.4±8.53%; N = 6, $^{###}$p<0.001) (Figure 21A, C). In the same way, CB_2R activation coupled with $A_{2A}R$ blockade induced no increase in the number of NeuN-positive SVZ cells, further suggesting the involvement of $A_{2A}R$ on SVZ neuronal differentiation mediated by CB_2R activation (control: 100.0±0.02%; HU-308 1 µM: 128.6±12.36; HU-308 1 µM+ZM241385: 89.66±6.85%; ZM241385 50 nM: 106.4±8.53%; N = 6, $^{###}$p<0.001) (Figure 21B, D).

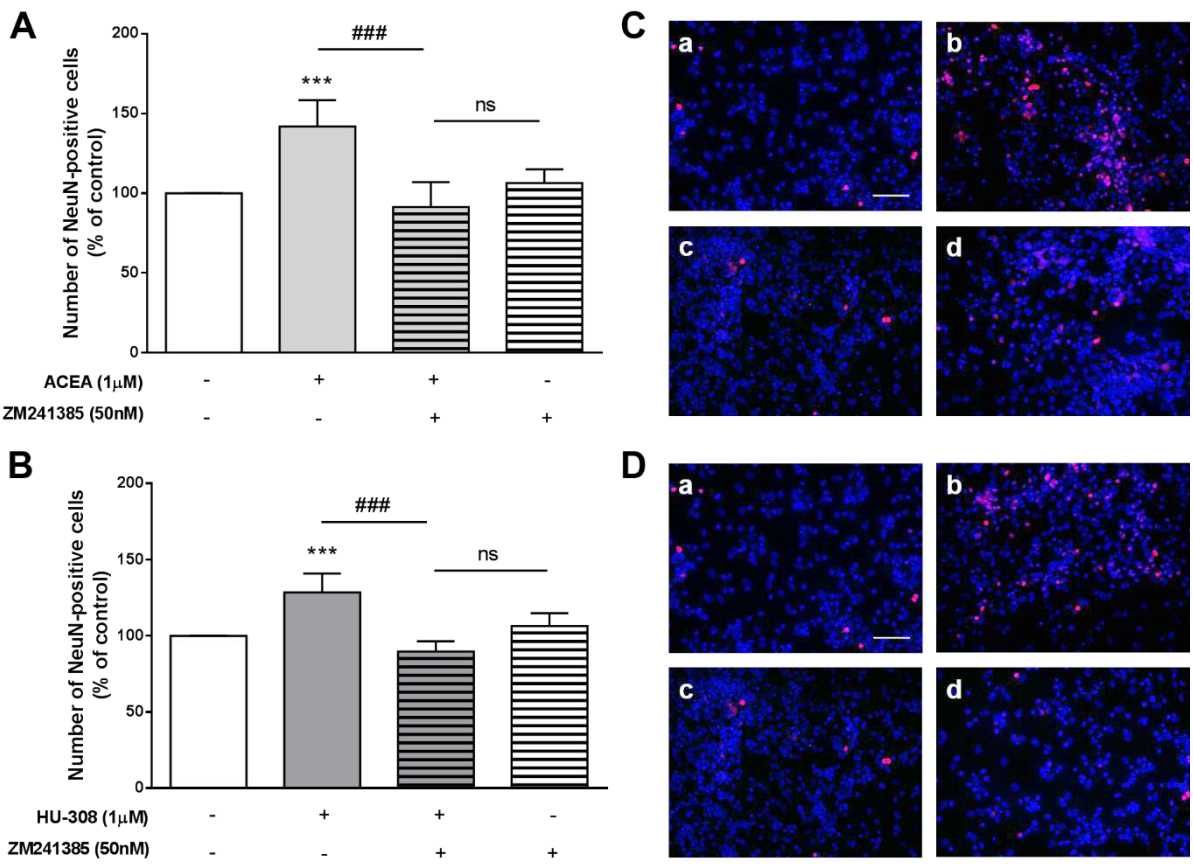


Figure 21 – $A_{2A}R$ blockade impairs the CB_1R- and CB_2R-induced SVZ neuronal differentiation. A, B. Bar graph depicts the number of NeuN-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=6. ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control; $^{###}$p<0.001, by ANOVA using Bonferroni's post test for comparison with ACEA and HU-308, respectively. ns: non-significant. **C, D.** Representative fluorescent digital images of NeuN-positive neurons (red) and Hoechst staining (blue nuclei), in control cultures (C,D-a) and in cultures exposed to ACEA 1 μM (C-b), ACEA 1 μM+ZM241385 50 nM (C-c) and ZM241385 50 nM (C-d); HU-308 1 μM (D-b), HU-308 1 μM+ZM241385 50 nM (D-c) and ZM241385 50 nM (D-d) Scale bar = 50 μm.

3.3. DG neurogenic niche

3.3.1.1. CB_2R and $A_{2A}R$ activation promotes self-renewal of DG cells

In order to investigate the ability of $A_{2A}R$, CB_1R and CB_2R ligands of modulating cell-fate of DG cells, a Sox2 cell-pair assay was performed as previously described (Figure 22A).

We observed that individual treatment of DG cells for 24h with CB_2R selective agonist HU-308 (1 µM) and $A_{2A}R$ selective agonist CGS21680 (30 nM) induced a significant increase in the percentages of either Sox2+/+ cell pairs (control: 49.87±2.45%; HU-308 1 µM: 59.11±3.48%; CGS21680 30 nM: 57.66±4.03%; N = 6, **p<0.01 and ***p<0.001) with a concomitant decrease in the percentage of Sox2-/- cell pairs (control: 48.54±2.20%; HU-308 1 µM: 39.24±3.02%; CGS21680 30 nM: 41.51±3.18%; N = 6, **p<0.01 and ***p<0.001) when compared to control (Figure 22B).

On the contrary, treatment with CB_1R agonist ACEA (1 µM) promoted no significant changes in the percentages of Sox2+/+ cell pairs (control: 49.87±2.45%; ACEA 1 µM: 49.53±1.43%; N = 6) and Sox2-/- cell pairs (control: 48.54±2.20%; ACEA 1 µM: 49.24±1.22%; N = 6) when compared to control conditions (Figure 22B).

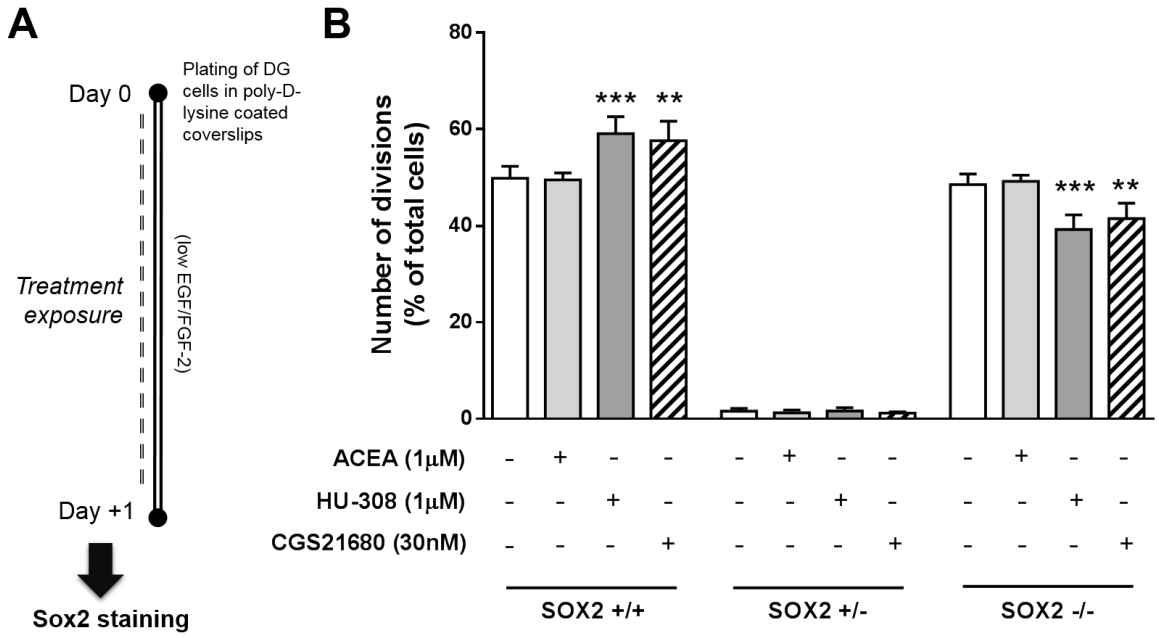


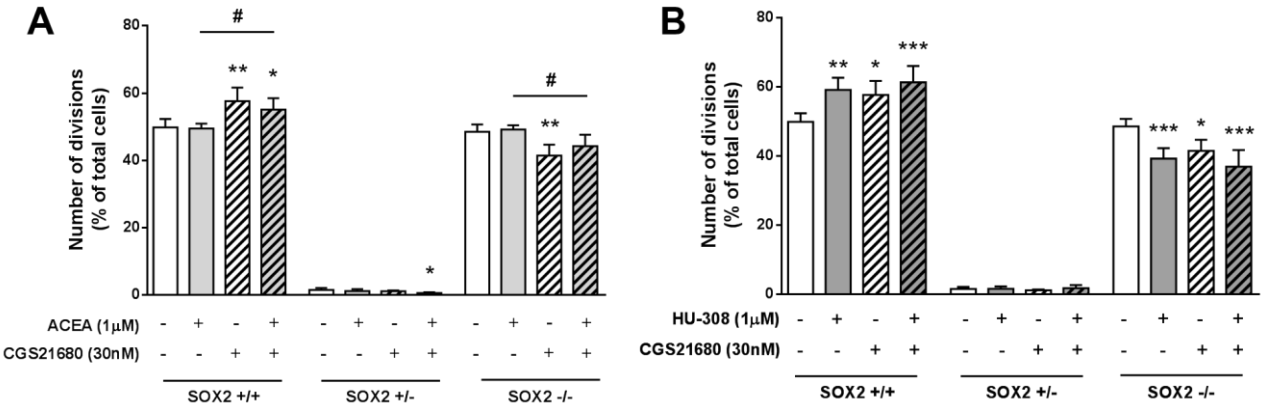
Figure 22 – CB_2R and $A_{2A}R$ activation *per se* induce an increase in self-renew divisions on DG, whereas CB_1R activation does not. A. Schematic representation of the experimental protocol for studying DG cell-fate. Day 0 represents the day of cultures in which cells were exposed to pharmacological treatments for the following 24h. **B.** Bar graphs represent the number of each type of cell divisions counted (60 pairs counted *per* condition *per* experiment). Data are expressed as the percentage of total cell pairs and are represented as the mean ± SEM. N=6. **p<0.01 and ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control.

3.3.1.2. Self-renewal of DG cells is maintained when both CB_1Rs and CB_2Rs are co-activated with A_{2A}Rs

It was next assessed the role of a combined administration of both CB_1R and CB_2R selective agonists with the A_{2A}R selective agonist on DG cell-fate. Similarly to the SVZ treatment, DG cells were treated with either ACEA (1 µM) or HU-308 (1 µM) for 30 minutes prior to CGS21680 (30 nM) treatment and then grown for further 24h in the presence of the ligands. Considering that A_{2A}R activation had an effect on DG cell-fate *per se*, as previously presented, we expected this effect to be maintained when we combined ACEA or HU-308 with CGS21680 treatment, respectively.

In agreement with this hypothesis, the results demonstrate that, in the presence of both CB_1R selective agonist (ACEA, 1 µM) and A_{2A}R selective agonist (CGS21680, 30 nM), there is a significant increase in the percentage of Sox2+/+ cell pairs similar to CGS21680 exposure alone (control: 49.87±2.45%; CGS21680 30 nM: 57.66±4.03%; ACEA 1 µM+CGS21680 30 nM: 55.04±3.42%; N = 6, *p<0.05), as compared with control, with a tendency to decrease in the percentage of Sox2-/- cell pairs (control: 48.54±2.20%; ACEA 1 µM+CGS21680 30 nM: 44.24±3.39%; N = 6) (Figure 23A).

In the same way, co-administration of CB_2R selective agonist (HU-308, 1 µM) and A_{2A}R selective agonist (CGS21680, 30 nM) induced a significant increase in the percentage of Sox2+/+ cell pairs (control: 49.87±2.45%; HU-308 1 µM: 59.11±3.48%; HU-308 1 µM+CGS21680 30 nM: 61.27±4.75% N = 6, ***p<0.001) with a concomitant decrease in the percentage of Sox2-/- cell pairs (control: 48.54±2.20%; HU-308 1 µM: 39.24±3.02%; HU-308 1 µM+CGS21680 30 nM: 36.09±4.81%; N = 6, ***p<0.001) (Figure 23B).



◄ Figure 23 – Co-activation of CB_1Rs or CB_2Rs with $A_{2A}R$ promotes an increase in self-renew divisions on DG cell-fate. Bar graphs represent the number of each type of cell divisions counted (60 pairs counted *per* condition *per* experiment) in cultures treated with CB_1Rs agonist ACEA (1 μM) and $A_{2A}R$ agonist CGS21680 (30 nM) **(A)** or in cultures treated with HU-308 (1 μM) and $A_{2A}R$ agonist CGS21680 (30 nM) **(B)**. Data are expressed as the percentage of total cell pairs and are represented as the mean ± SEM. N=6. *p<0.05, **p<0.01 and ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control. #p<0.05, by ANOVA using Bonferroni's post test for comparison with ACEA.

3.3.1.3. $A_{2A}R$ activation is necessary for DG self-renewing promoted by CB_2R activation

In line with the previous results we wanted to clarify the role of $A_{2A}Rs$ in DG cell-fate. Hence, in order to test this, DG cells were treated with $A_{2A}R$ antagonist ZM241385 (50 nM) for 30 minutes prior to treatment with CB_1R or CB_2R selective agonists and then for further 24h in the presence of the ligands.

The results show that, as expected, in cultures treated with both CB_1R agonist (ACEA, 1 μM) and $A_{2A}R$ antagonist (CGS21680, 30 nM), no changes were observed, when compared to control cultures, in the percentages of Sox2+/+ cell pairs (control: 49.87±2.45%; ACEA 1 μM: 49.53±1.43%; ACEA 1 μM+ZM241385 50 nM: 49.38±1.51%; ZM241385 50 nM: 50.38±3.67%; N = 6) or in the percentages of Sox2-/- cell pairs (control: 48.54±2.20%; ACEA 1 μM: 49.24±1.22%; ACEA 1 μM+ZM241385 50 nM: 48.57±2.14%; ZM241385 50 nM: 50.94±1.40%; N = 6) when compared to control cultures (Figure 24A). Interestingly, in the presence of the $A_{2A}R$ antagonist, the observed increase in self-renewal divisions of DG cells promoted by CB_2R activation was blocked, resulting in the decrease of the percentage of Sox2+/+ cell pairs (control: 49.87±2.45%; HU-308 1 μM: 59.11±3.48%; HU-308 1 μM+ZM241385: 51.80±1.99%; ZM241385 50 nM: 50.38±3.67%; N = 6, ###p<0.001) and in a concomitant increase in the Sox2-/- cell pairs (control: 48.54±2.20%; HU-308 1 μM: 39.24±3.02%; HU-308 1 μM+ZM241385: 46.71±2.01%; ZM241385 50 nM: 50.94±1.40%; N = 6, ##p<0.01), when compared to HU-308 treatment, to similar values as control cultures (Figure 24B).

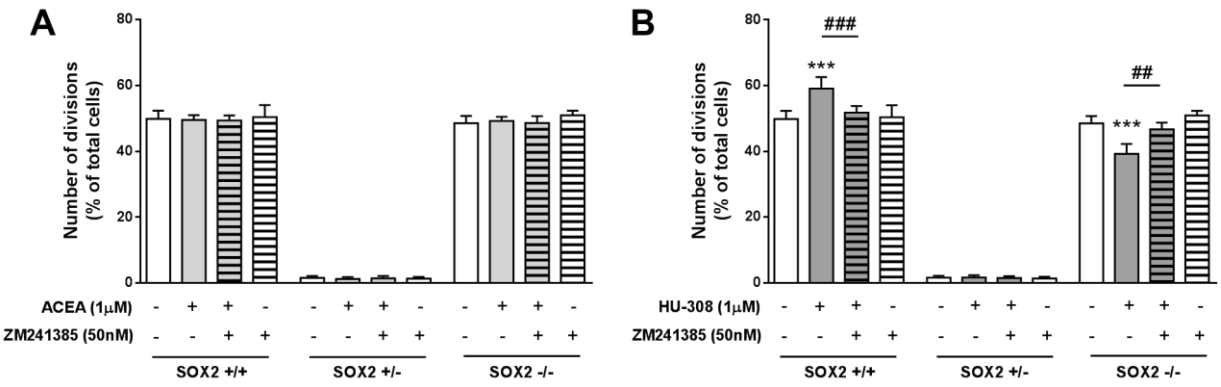


Figure 24 – $A_{2A}R$ blockade impairs the CB_2R-mediated increase in self-renewal divisions of DG cells. Bar graphs represent the number of each type of cell divisions counted (60 pairs counted *per* condition *per* experiment) in cultures treated with CB_1Rs agonist ACEA (1 μM) and $A_{2A}R$ antagonist ZM241385 (50 nM) **(A)** or in cultures treated with HU-308 (1 μM) and $A_{2A}R$ antagonist ZM241385 (50 nM) **(B)**. Data are expressed as the percentage of total cell pairs and are represented as the mean ± SEM. N=6, ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control; $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001, by ANOVA using Bonferroni's post test for comparison with HU-308.

3.3.1.4. DG self-renewing promoted by $A_{2A}R$ activation is dependent on CB_1Rs or CB_2Rs activation

In addition, since both CB_2R and $A_{2A}Rs$ promote an increase in DG self-renewing properties it was further tested if CB_1Rs and CB_2Rs are required for $A_{2A}Rs$ role in DG cell-fate. To address this question, DG cells were treated with either CB_1R selective antagonist AM251 (1 μM) or CB_2R selective antagonist AM630 (1 μM) 30 minutes prior to treatment with $A_{2A}R$ selective agonist CGS21680 (30 nM) and then incubated for further 24h in the presence of the ligands.

The results show that, the effect of $A_{2A}R$ agonist CGS21680 (30 nM) in DG cell-fate is lost when cultures are co-treated with both $A_{2A}R$ agonist (CGS21680, 30 nm) and CB_1R antagonist (AM251, 1 μM), resulting in a decrease in the percentage of Sox2+/+ cell pairs (control: 49.87±2.45%; CGS21680 30 nM: 57.66±4.03%; CGS21680 30 nM+AM251 1 μM: 50.19±2.66%; AM251 1 μM: 49.06±1.25%; N = 6, $^{\#\#}$p<0.01) and an increase in the percentages of Sox2-/- cell pairs (control: 48.54±2.20%; CGS21680 30 nM: 41.51±3.18%; CGS21680 30 nM+AM251 1 μM: 48.62±2.60%; AM251 1 μM: 49.58±2.06%; N = 6, $^{\#\#}$p<0.01) when compared to CGS21680 treatment alone (Figure 25A).

Similarly, the same happened in cultures co-treated with both $A_{2A}R$ agonist (CGS21680 30 nm) and CB_2R antagonist (AM630, 1 µM), where it is observed a decrease in the percentage of Sox2+/+ cell pairs (control: 49.87±2.45%; CGS21680 30 nM: 57.66±4.03%; CGS21680 30 nM+AM630 1 µM: 50.74±3.24%; AM630 1 µM: 46.48±2.47%; N = 6, $^{\#}p<0.05$) and an increase in the percentages of Sox2-/- cell pairs (control: 48.54±2.20%; CGS21680 30 nM: 41.51±3.18%; CGS21680 30 nM+AM630 1 µM: 47.91±3.46%; AM630 1 µM: 52.54±2.58%; N = 6, $^{\#}p<0.05$) when compared to CGS21680 treatment alone (Figure 25B).

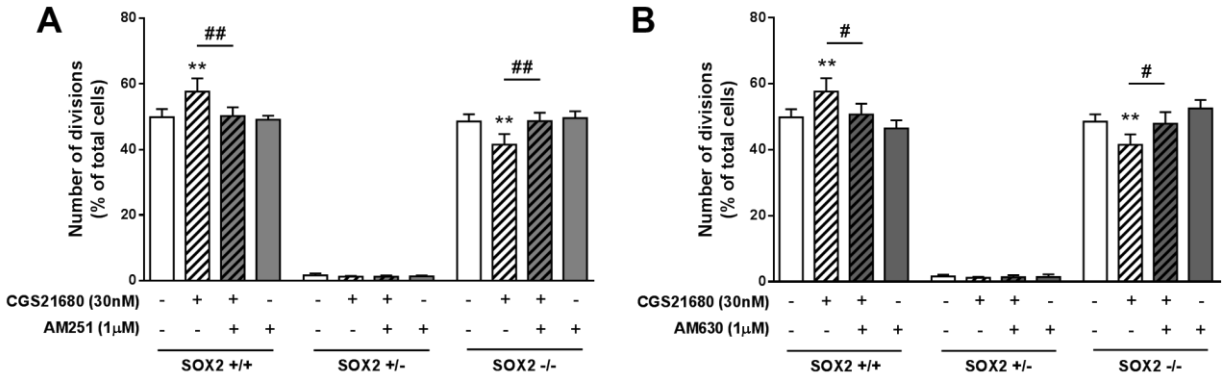


Figure 25 – CB_1Rs or CB_2Rs blockade impairs the $A_{2A}R$-induced self-renewing of DG cells. Bar graphs represent the number of each type of cell divisions counted (60 pairs counted *per* condition *per* experiment) in cultures treated with $A_{2A}Rs$ agonist CGS21680 (30 nM) and CB_1R antagonist AM251 (1 µM) **(A)** or in cultures treated with $A_{2A}Rs$ agonist CGS21680 (30 nM) and CB_2R antagonist AM630 (1 µM) **(B)**. Data are expressed as the percentage of total cell pairs and are represented as the mean ± SEM. N=6, **p<0.01, by ANOVA using Bonferroni's post test for comparison with control; $^{\#}p<0.05$ and $^{\#\#}p<0.01$, by ANOVA using Bonferroni's post test for comparison with CGS21680.

3.3.2.1. CB_1Rs, CB_2Rs or $A_{2A}Rs$ activation induces no significant changes on DG proliferation

We next investigated whether $A_{2A}R$, CB_1R and CB_2R ligands could modulate DG cells proliferation. For that purpose, DG cells were treated with selective agonists for $A_{2A}Rs$, CB_1Rs and CB_2Rs for 24h, as similar to SVZ cells. Incorporated BrdU was immunolabeled and positive nuclei were counted (Figure 26A).

Treatment of DG cells with CB_1R (ACEA, 1 µM) and CB_2R (HU-308, 1 µM) selective agonists and $A_{2A}R$ selective agonist (CGS21680, 30 nM) induced no significant increase in the number BrdU-positive cells when compared to control cultures (control: 100.5±1.22%; ACEA

1 µM: 109.7±18.39%; HU-308 1 µM: 102.8±5.55%; CGS21680 30 nM: 110.7±18.98%; N = 5) (Figure 26B, C).

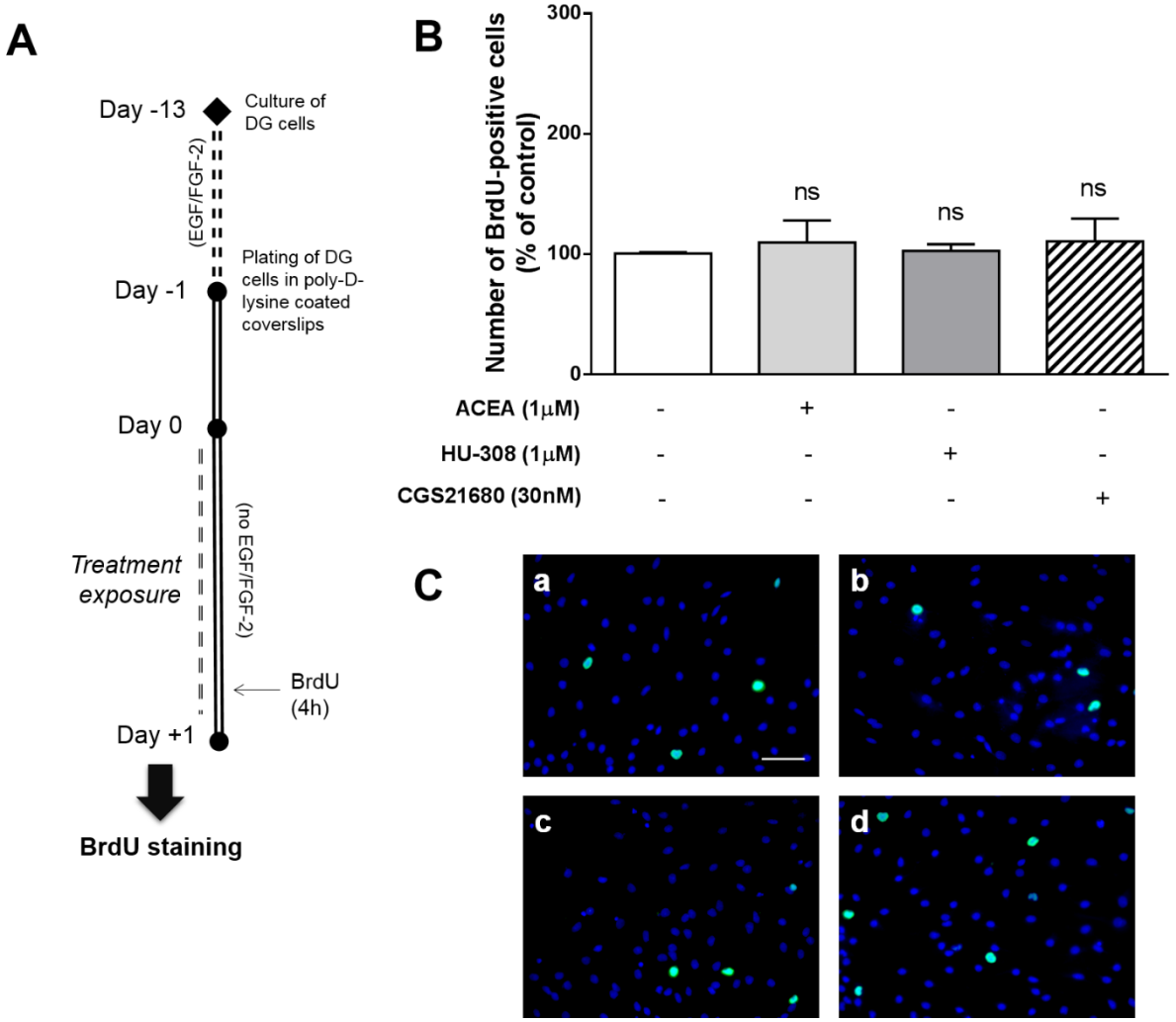


Figure 26 – CB_1R, CB_2R and $A_{2A}R$ activation have no effect on DG cell proliferation. A. Schematic representation of the experimental protocol. Day 0 represents the day in which neurosphere-derived cells were exposed to pharmacological treatments for the following 24h. **B.** Bar graph depicts the number of BrdU-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=5. **C.** Representative fluorescent digital images of BrdU-positive cell nuclei (green) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to ACEA 1 µM (b), HU-308 1 µM (c) and CGS21680 30 nM (d). Scale bar = 50 µm.

3.3.2.2. Co-activation of CB_1Rs or CB_2Rs with $A_{2A}R$ increases DG proliferation

To further understand the role of CB_1Rs, CB_2Rs and $A_{2A}Rs$ in DG proliferation, it was assessed if a combined administration of either CB_1Rs or CB_2Rs selective agonists with the $A_{2A}R$ selective agonist promoted any change in the number of BrdU-positive DG cells. Similar

to SVZ treatment, DG cells were treated with either ACEA or HU-308 for 30 minutes prior to CGS21680 treatment and allowed to grow for further 24h in the presence of the ligands.

Surprisingly, we observed that a significant increase in the number of BrdU-positive nuclei was obtained in cultures incubated with both CB_1R selective agonist (ACEA, 1 μM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM) when compared to control (control: 100.5±1.22%; ACEA 1 μM+CGS21680 30 nM: 204.3±43.98%; N = 5, ***p<0.001) (Figure 27). In the same way, the co-incubation of CB_2R selective agonist (HU-308, 1 μM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM) induced a significant increase in the number of BrdU-positive cells as compared to control cultures (control: 100.5±1.22%HU-308 1 μM+CGS21680 30 nM: 205.5±25.50%; N = 5, ***p<0.001) (Figure 28).

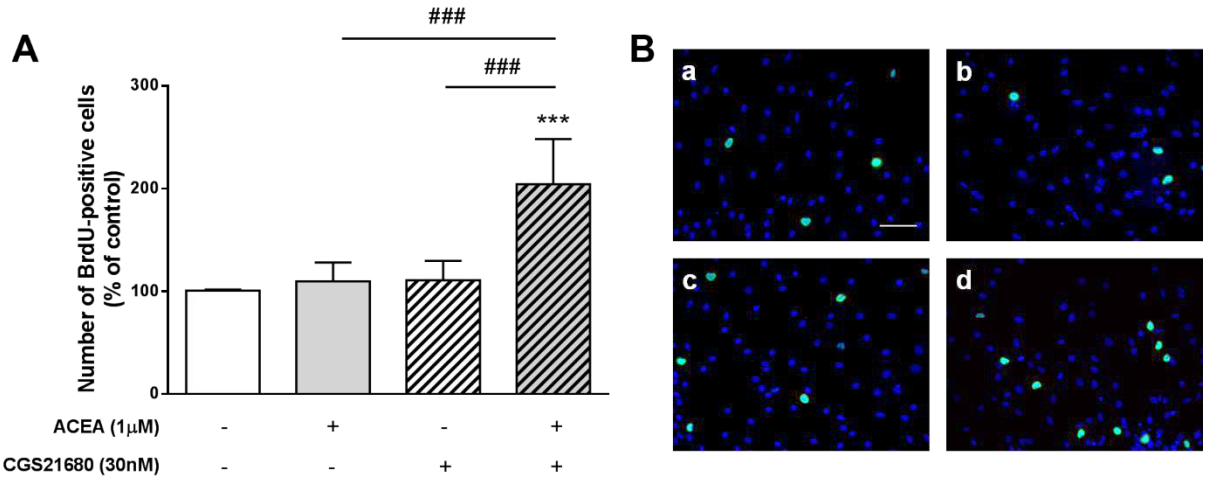


Figure 27 – CB_1R activation coupled with $A_{2A}R$ activation induces DG proliferation. A. Bar graph depicts the number of BrdU-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=5. ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control; ###p<0.001, by ANOVA using Bonferroni's post test for comparison with ACEA+CGS21680. **B.** Representative fluorescent digital images of BrdU-positive neurons (green) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to ACEA 1 μM (b), CGS21680 30 nM (c) and ACEA 1 μM+CGS21680 30 nM (d). Scale bar = 50 μm.

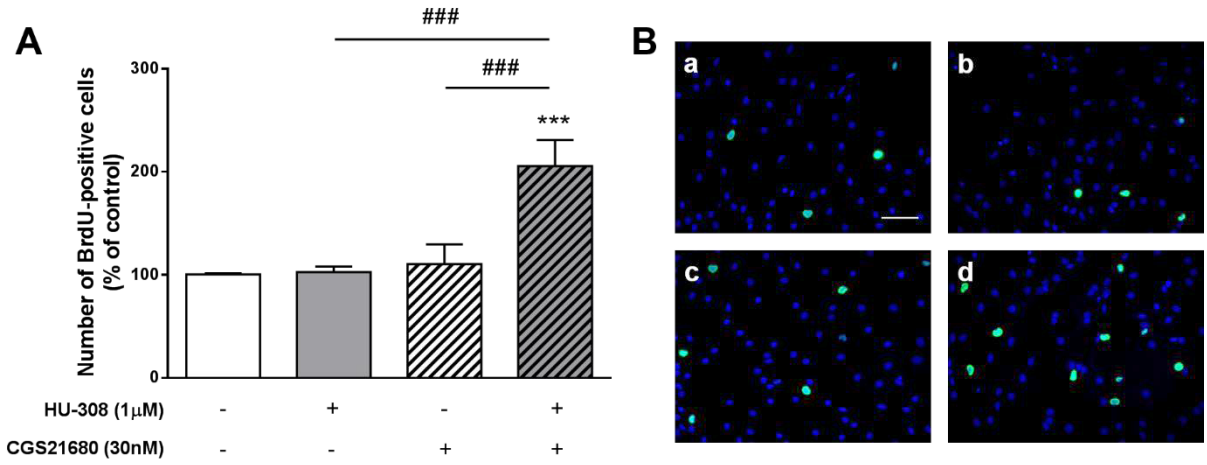


Figure 28 – CB_2R activation coupled with $A_{2A}R$ activation induces DG proliferation. A. Bar graph depicts the number of BrdU-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=5. ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control; ###p<0.001, by ANOVA using Bonferroni's post test for comparison with HU-308+CGS21680. **B.** Representative fluorescent digital images of BrdU-positive neurons (green) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to HU-308 1 μM (b), CGS21680 30 nM (c) and HU-308 1 μM+CGS21680 30 nM (d). Scale bar = 50 μm.

3.3.3.1. CB_1Rs, CB_2Rs or $A_{2A}Rs$ activation promotes DG neuronal differentiation

To determine the role of $A_{2A}R$, CB_1R and CB_2R ligands on DG neuronal differentiation, DG cells were treated with selective agonists for $A_{2A}Rs$, CB_1Rs and CB_2Rs for 7 days. Cells were then stained for NeuN, a marker for mature neurons (Figure 29A).

Treatment of DG cells with CB_1R (ACEA, 1 μM) and CB_2R (HU-308, 1 μM) selective agonists and $A_{2A}R$ selective agonist (CGS21680, 30 nM) induced a significant increase in the number of NeuN-positive cells when compared to control cultures (control: 99.99±0.02%; ACEA 1 μM: 173.6±12.37%; HU-308 1 μM: 151.9±8.16%; CGS21680 30 nM: 158.2±8.69%; N = 5, ***p<0.001) (Figure 29B, C).

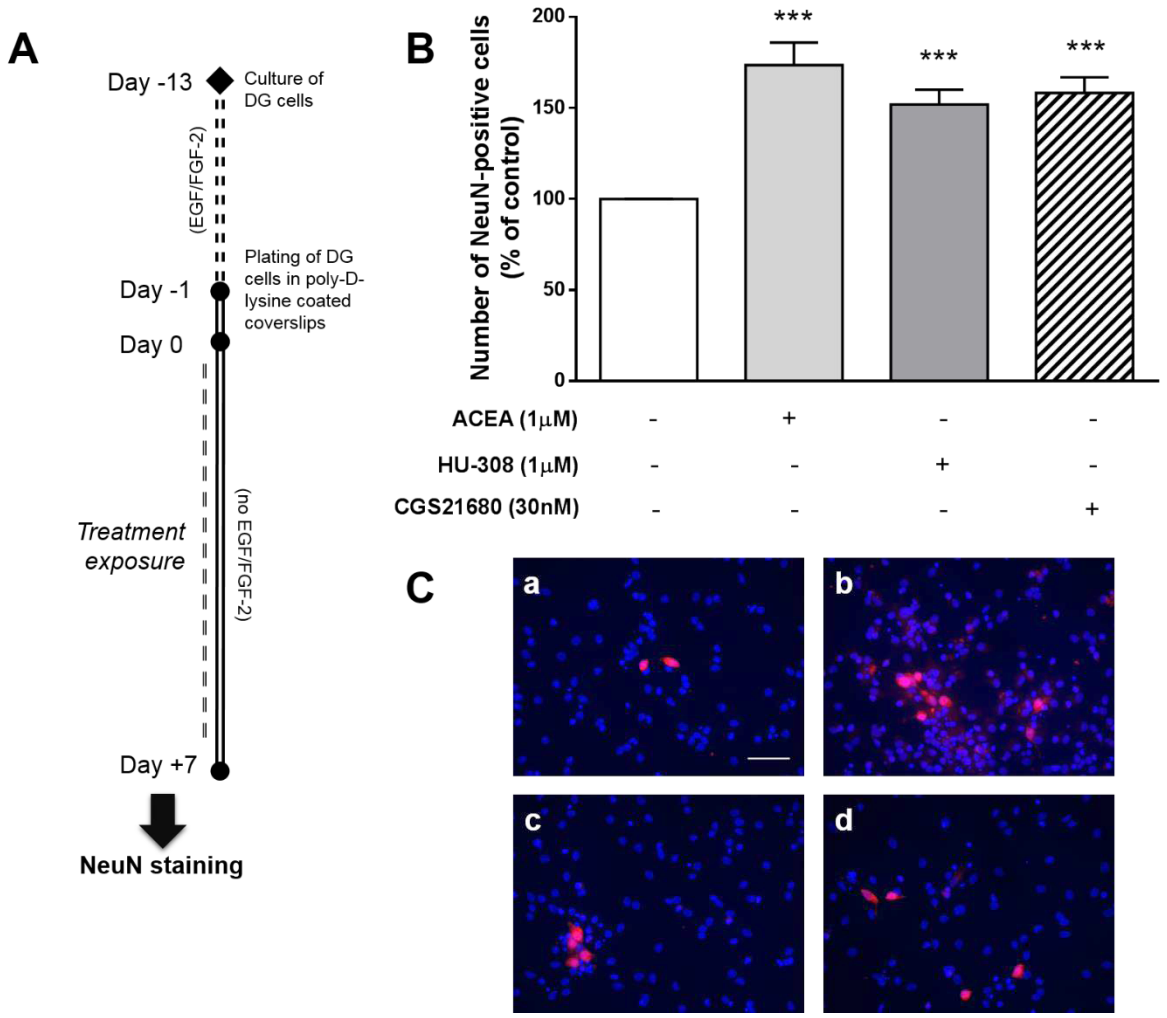


Figure 29 – CB_1R, CB_2R and $A_{2A}R$ activation induces DG neuronal differentiation. **A.** Schematic representation of the experimental protocol. Day 0 represents the day in which cells were exposed to pharmacological treatments for the following 7 days. **B.** Bar graph depicts the number of NeuN-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=5. ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control. NeuN: Neuronal Nuclei. **C.** Representative fluorescent digital images of NeuN-positive neurons (red) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to ACEA 1 μM (b), HU-308 1 μM (c) and CGS21680 30 nM (d). Scale bar = 50 μm.

3.3.3.2. Co-activation of CB_1Rs or CB_2Rs with $A_{2A}R$ increases DG neuronal differentiation

To study whether co-incubation with CB_1Rs or CB_2Rs selective agonists with the $A_{2A}R$ selective agonist could change neuronal differentiation, DG cells were treated with either ACEA (1 µM) or HU-308 (1 µM) for 30 minutes prior to CGS21680 (30 nM) treatment and then cells were grown for 7 days in the presence of the ligands.

It was observed that, in the presence of both CB_1R selective agonist (ACEA, 1 µM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM), there was an increase in the number of NeuN-positive cells when compared to control cultures (control: 99.99±0.02%; ACEA 1 µM+CGS21680 30 nM: 147.8±13.44%; N = 5, ***p<0.001), with no synergistic effect when comparing to ACEA (1 µM) and CGS21680 (30 nM) treatments alone (ACEA 1 µM: 173.6±12.37%; CGS21680 30 nM: 158.2±8.69%) (Figure 30). Similarly, the co-administration of CB_2R selective agonist (HU-308, 1 µM) and $A_{2A}R$ selective agonist (CGS21680, 30 nM) induced an increase in the number of NeuN-positive cells as compared to control cultures (control: 99.99±0.02%; HU-308 1 µM+CGS21680 30 nM: 146.1±28.51%; N = 5, **p<0.01), also, with no synergistic effect when comparing to HU-308 (1 µM) and CGS21680 (30 nM) treatments alone (HU-308 1 µM: 151.9±8.16%; CGS21680 30 nM: 158.2±8.69%) (Figure 31).

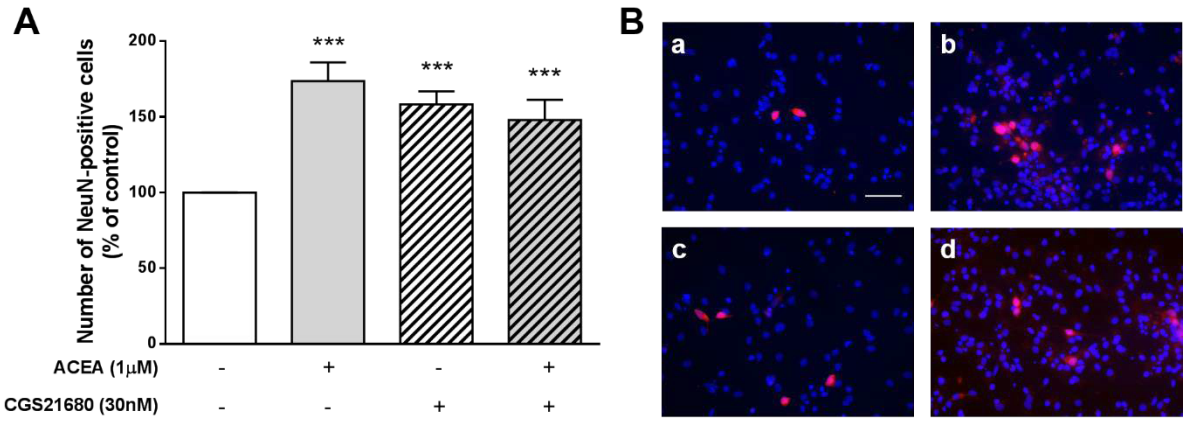


Figure 30 – CB_1R activation coupled with $A_{2A}R$ activation induces DG neuronal differentiation. A. Bar graph depicts the number of NeuN-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=5. ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control. **B.** Representative fluorescent digital images of NeuN-positive neurons (red) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to ACEA 1 µM (b), CGS21680 30 nM (c) and ACEA 1 µM+CGS21680 30 nM (d). Scale bar = 50 µm.

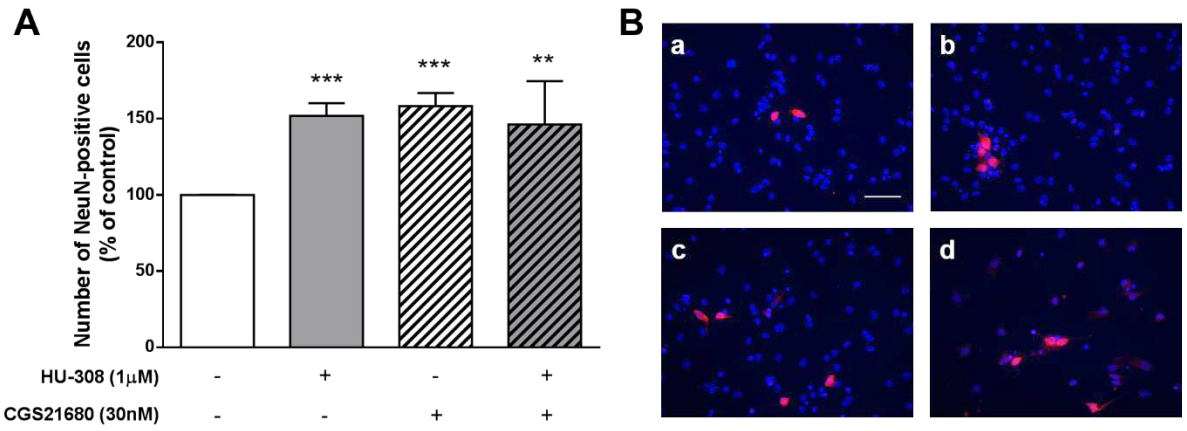


Figure 31 – CB_2R activation coupled with $A_{2A}R$ activation induces DG neuronal differentiation. **A.** Bar graph depicts the number of NeuN-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=6. **p<0.01 and ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control. **B.** Representative fluorescent digital images of NeuN-positive neurons (red) and Hoechst staining (blue nuclei), in control cultures (a) and in cultures exposed to HU-308 1 μM (b), CGS21680 30 nM (c) and HU-308 1 μM+CGS21680 30 nM (d). Scale bar = 50 μm.

3.3.3.3. $A_{2A}R$ role in DG neuronal differentiation promoted by CB_1Rs and CB_2Rs

Considering that treatment with $A_{2A}R$, CB_1R and CB_2R selective agonists promoted DG neuronal differentiation, we further wanted to clarify the putative role of $A_{2A}R$ in DG neuronal differentiation promoted by CB_1Rs and CB_2Rs activation. For that purpose, similar to SVZ cultures, DG cells were treated with $A_{2A}R$ antagonist (ZM241385, 50 nM) for 30 minutes prior to treatment with CB_1R (ACEA, 1 μM) or CB_2R (HU-308, 1 μM) selective agonists, and then further co-incubated for 7 days.

The results show that the increase in the number of NeuN-positive DG cells promoted by CB_1R activation is lost in the presence of the $A_{2A}R$ selective antagonist ZM241385 (50 nM) (control: 99.99±0.02%; ACEA 1 μM: 173.6±12.37%; ACEA 1 μM+ZM241385 50 nM: 117.9±10.42%; ZM241385 50 nM: 102.1±12.96%; N = 5, $^{###}$p<0.001) (Figure 32A, C). In the same way, the increase in the number of NeuN-positive DG cells promoted by CB_2R activation is partially blocked in the presence of an $A_{2A}R$ selective antagonist ZM241385 (50 nM) (control: 99.99±0.02%; HU-308 1 μM: 151.9±8.16%; HU-308 1 μM+ZM241385: 117.4±8.09%; ZM241385: 102.1±12.96%; N = 5, *p<0.05 and $^{###}$p<0.001) (Figure 32B, D).

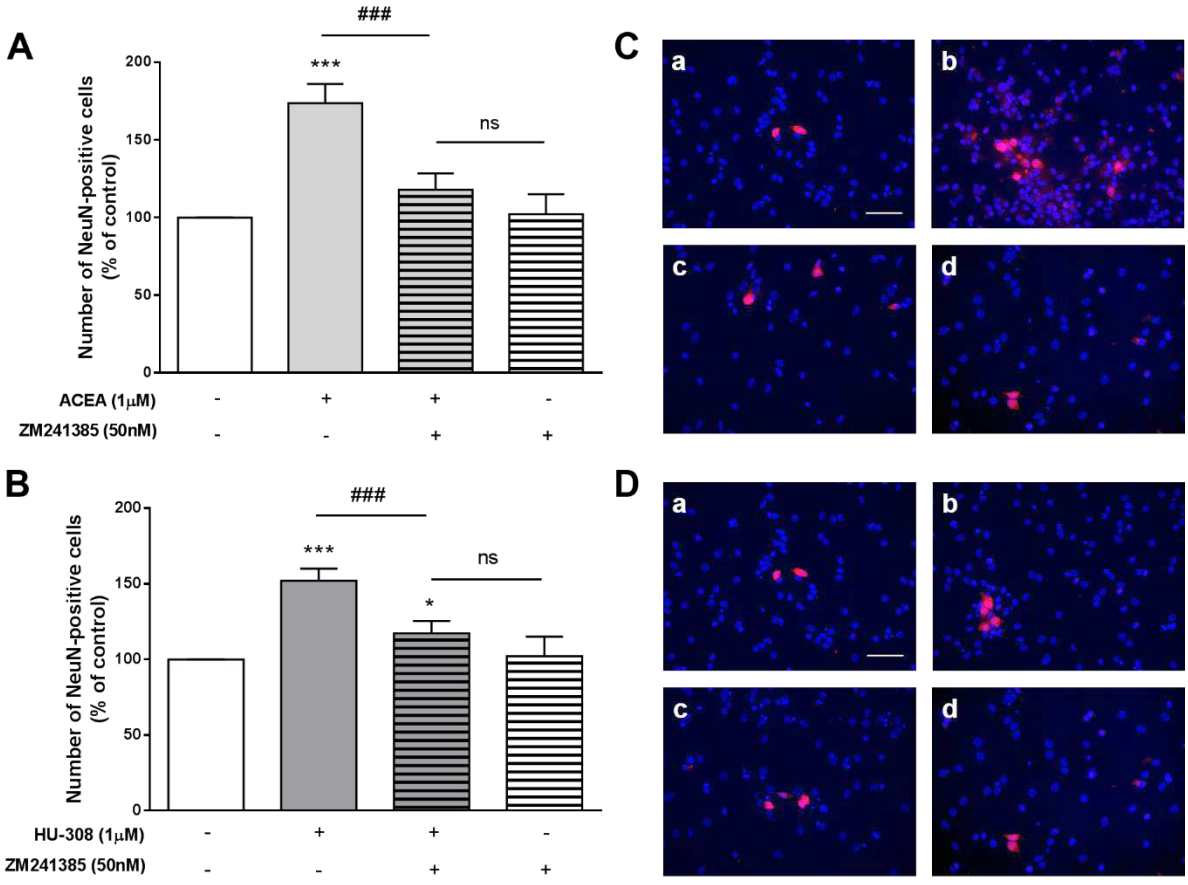


Figure 32 – $A_{2A}R$ blockade impairs the CB_1R- and CB_2R-induced DG neuronal differentiation. A, B. Bar graph depicts the number of NeuN-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=5. *p<0.05 and ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control; ###p<0.001, by ANOVA using Bonferroni's post test for comparison with ACEA and HU-308, respectively. ns: non-significant. **C, D.** Representative fluorescent digital images of NeuN-positive neurons (red) and Hoechst staining (blue nuclei), in control cultures (C,D-a) and in cultures exposed to ACEA 1 μM (C-b), ACEA 1 μM+ZM241385 50 nM (C-c) and ZM241385 50 nM (C-d); HU-308 1 μM (D-b), HU-308 1 μM+ZM241385 50 nM (D-c) and ZM241385 50 nM (D-d) Scale bar = 50 μm.

3.3.3.4. $A_{2A}R$-mediated DG neuronal differentiation is dependent on CB_1Rs and CB_2Rs

In line with the previous results, it was next investigated if CB_1Rs or CB_2Rs blockage coupled with $A_{2A}R$ activation would influence DG neuronal differentiation. To address this question, DG cells were treated with either CB_1R (AM251, 1 μM) or CB_2R (AM630, 1 μM) selective antagonists 30 minutes prior to treatment with $A_{2A}R$ selective agonist CGS21680 (30 nM), and co-incubated for further 7 days.

Remarkably, we found that, treatment with CB_1R antagonist AM251 (1 µM) blocked the effect mediated by $A_{2A}R$ agonist CGS21680 (30 nM) on DG neuronal differentiation, resulting in a decrease number of NeuN-positive cells (control: 99.99±0.02%; CGS21680 30 nM: 158.2±8.69%; CGS21680 30 nM+AM251 1 µM: 91.44±16.67%; AM251 1 µM: 81.30±16.62%; N = 5, $^{###}p<0.001$) when compared to CGS21680 treatment (Figure 33A, C). In the same way, cultures co-treated with both $A_{2A}R$ agonist (CGS21680, 30 nM) and CB_2R antagonist (AM630, 1 µM), it was observed a decrease in the number of NeuN-positive DG cells (control: 99.99±0.02%; CGS21680 30 nM: 158.2±8.69%; CGS21680 30 nM+AM630 1 µM: 102.0±14.43%; AM630 1 µM: 110.0±12.89%; N = 5, $^{###}p<0.001$) when comparing with CGS21680 treatment alone (Figure 33B, D).

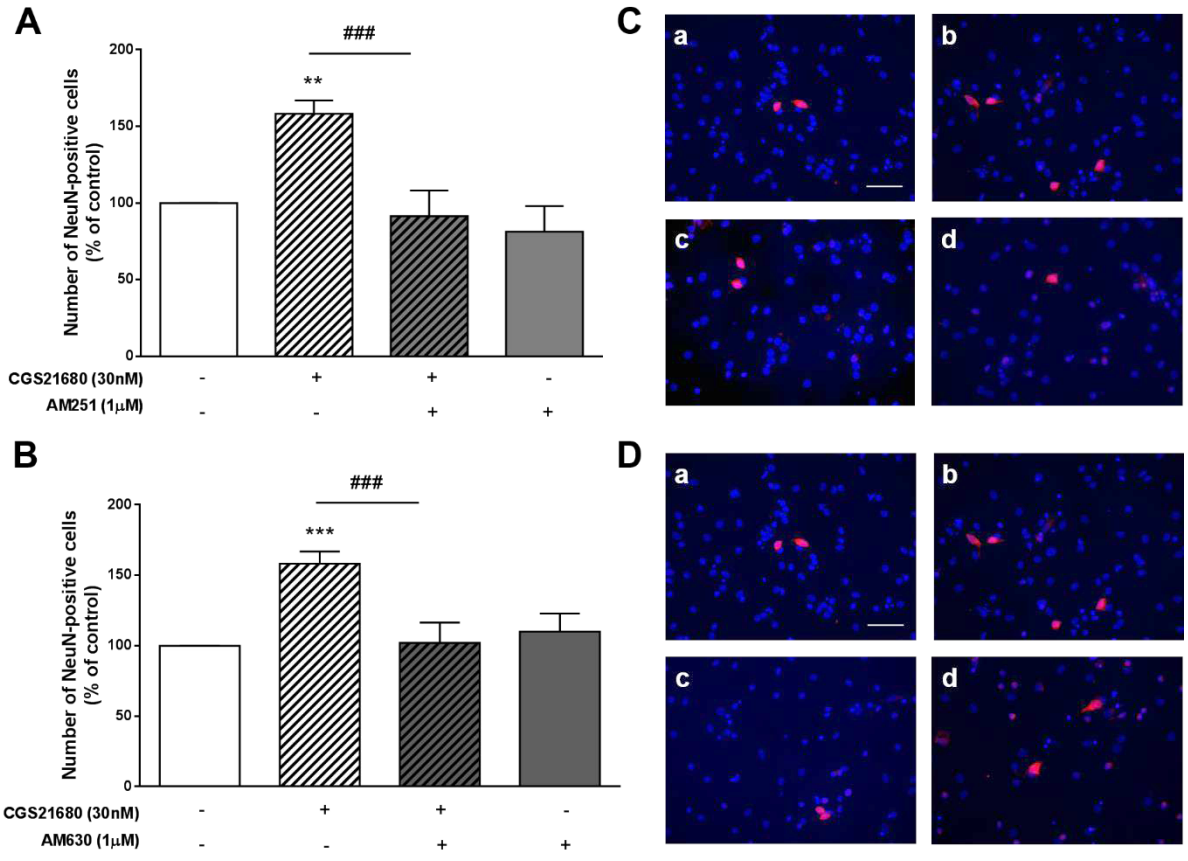


Figure 33 – CB_1Rs and CB_2Rs blockade impairs $A_{2A}R$-induced neuronal differentiation of DG cells. A, B. Bar graph depicts the number of NeuN-positive cells. Values were normalized to the control mean for each experiment and are represented as the mean ± SEM. Control was set to 100%. N=5. **p<0.01 and ***p<0.001, by ANOVA using Bonferroni's post test for comparison with control; $^{###}p<0.001$, by ANOVA using Bonferroni's post test for comparison with CGS21680. **C, D.** Representative fluorescent digital images of NeuN-positive neurons (red) and Hoechst staining (blue nuclei), in control cultures (C,D-a) and in cultures exposed to CGS21680 30 nM (C-b), CGS21680 30 nM+AM251 1 µM (C-c) and AM251 1 µM (C-d); CGS21680 30 nM (D-b), CGS21680 30 nM+AM630 1 µM (D-c) and AM630 1 µM (D-d) Scale bar = 50 µm.

3.4. Discussion

ECS and adenosinergic system are two major players in the CNS. Although several studies have been trying to understand the role of eCBs and adenosine in the regulation of many physiological processes, gaps still persist in our knowledge regarding endocannabinoid action and A_{2A}Rs role in neurogenesis.

Here, it was examined the effects of CB_1R, CB_2R and A_{2A}R activation on SVZ- and DG-derived cells, given that SVZ and DG represent the two main neurogenic niches of the adult brain. These neurogenic niches are packed with NSPCs that can be modulated by a wide array of factors. In fact, stem cell-based therapy is emerging as a thriving area for regenerative medicine with a lot of clinical applications. Nonetheless, this approach is severely hindered by the shortage of knowledge about the proneurogenic potential of certain compounds.

Previous studies have mainly focused their attention on the effects played by cannabinoids in neurogenesis, mostly regarding proliferation experiments, and not many studies have evaluated the adenosinergic action on neurogenesis. In fact, it was already shown that CB_1Rs, CB_2Rs and A_{2A}Rs are expressed in SVZ and DG tissue and neurosphere-derived cells. Particularly, NSPCs express a functional endocannabinoid system and are able to produce both AEA and 2-AG and, also, that are targeted by cannabinoids to promote neurosphere generation and NSPC proliferation[168,204,205]. In the same way, SVZ-derived primary neurospheres were found to express A_{2A}Rs and it is well established that hippocampal neurons express the adenosinergic system[229]. Moreover, purinergic signaling was shown to regulate NSPCs expansion and neurogenesis[230].

It was firstly observed that CB_1R, CB_2R or A_{2A}R agonist treatment had no effect on SVZ cell fate. Also, a combined activation of either CB_1R or CB_2R with A_{2A}R also had no effect on SVZ cell fate, resulting in no changes in the percentages of Sox2+/+ or Sox2-/- cell pairs. These results are not in complete accordance with past studies where it was seen that CB_1R activation altered fate specification to a more stem state either in SVZ-derived cells[191] or in mice cortical NSCPs[231].

Moreover, it was observed that CB_1Rs activation promoted an increase in cell proliferation of SVZ cultures, an effect that persisted when cultures were co-incubated together with A_{2A}Rs selective agonist. In fact, it was previously shown an inhibition of NSPCs proliferation in both hippocampus and SVZ of CB_1R- or CB_2R-KO animals, or in animals treated with CB_1R or CB_2R-selective antagonists[204,232]. Additionally, these data are in accordance with several data showing that CB_1R activation promotes SVZ cell proliferation[184,188,191,233]. However, we found that CB_2R activation did not induce cell proliferation, although others have shown that CB_2R activation could promote proliferation in embryonic cell lines, in SVZ

neurosphere cultures and in the SVZ of young mice[206,207]. Importantly, the effect mediated by CB_1Rs on proliferation was blocked by an A_{2A}R selective antagonist, further indicating a role for A_{2A}Rs on CB_1R-mediated stimulation of SVZ proliferation. In fact, Stafford and colleagues showed that A_{2A}R activation led to cell cycle arrest in primary SVZ-derived NSPCs, therefore inhibiting the proliferation, potentially playing a role in the initiation of neuronal differentiation[229], which strongly correlates with the results previously described.

I have also found that SVZ neuronal differentiation significantly increases upon CB_1Rs or CB_2Rs activation. Regarding the role of CBRs in neuronal differentiation, the existing data also appears to be conflicting. In fact, the work done by Compagnucci et al.[189] and Xapelli et al.[191] also shows that CB_1R activation is important to promote neuronal differentiation, while Aguado et al.[234] and Gomez et al.[235] show that endocannabinoid treatment promotes an astroglial and/or oligodendroglial differentiation rather than neuronal differentiation. Importantly, I observed that the effect mediated by CB_1R or CB_2R activation in neuronal differentiation remained unaltered when CB_1Rs or CB_2Rs activation was coupled with A_{2A}R agonist treatment. On the other hand, this effect was blocked by the presence of an A_{2A}R selective antagonist, although A_{2A}R activation *per se* did not change neuronal differentiation. These data suggests a pivotal role of A_{2A}Rs in SVZ neuronal differentiation promoted by CB_1Rs and CB_2Rs activation.

Regarding DG, it was observed that CB_2R and A_{2A}R activation induced self-renewing divisions, resulting in a significant increase in the percentages of Sox2+/+ daughter cell pairs. This is in accordance with the work of Palazuelos et al.[204] where they show that in murine embryonic cortical NSPCs of CB_2R KO mice a reduced self-renewal rate was observed and that CB_2R activation increased neural progenitor self-renewal *in vitro*. Moreover, I also found that the increase in self-renewal observed in DG cells was maintained when CB_1Rs or CB_2Rs were co-activated with A_{2A}Rs. Importantly, CB_2R-induced increase in self-renewal was blocked with an A_{2A}R selective antagonist. In the same way, the effect mediated by A_{2A}Rs activation on self-renewal was blocked by the presence of CB_1R or CB_2Rs selective antagonists. These very interesting data further suggests evidences for a putative crosstalk between these two systems.

Most studies relating A_{2A}Rs role in neurogenesis generally focus their attention on the action of caffeine (a non-selective A_1R and A_{2A}R antagonist) in hippocampal-related neurogenesis. Several studies show that acute treatment with caffeine has beneficial effects on cell proliferation or overall neurogenesis in the hippocampus, whereas chronic administration has the opposite effect[131–133]. On the other hand, the role of cannabinoids on hippocampal neurogenesis has been intensively studied to try to understand how eCBs can affect neurogenesis and subsequently shape learning and memory processes. Evidences

show that the number of proliferating cells in the DG is reduced in CBRs-deficient mice and that CBRs play major roles in NSPCs proliferation, morphogenesis and differentiation[139,204].

I observed no changes on DG proliferation when cultures were treated with CB_1R, CB_2R and $A_{2A}Rs$ selective agonists. These results are not in complete accordance to past studies where it was seen that chronic administration with HU-210 (a drug that has a high affinity for both CB_1Rs and CB_2Rs) enhanced both proliferation and survival of cells in the rat DG[232] or stimulated BrdU incorporation and neurospheres formation[205]. Rubio-Araiz and co-workers also demonstrated that both CB_1R and CB_2R agonists, stimulate the proliferation of primary murine cortical neurospheres[190]. Aguado and her colleagues showed the involvement of the CB_1R in NSPCs proliferation and neurogenesis induced by excitotoxic injury[193]. Moreover, Palazuelos and his colleagues showed that chronic administration of the CB_2R selective agonist also exhibits proliferative-enhancing affects[207]. Nevertheless, when using combined treatments for DG cells using CB_1R and $A_{2A}R$ selective agonists or CB_2R and $A_{2A}R$ selective agonists a surprisingly significant increase on DG cell proliferation occurred, further indicating a possible synergistic effect between CBRs and $A_{2A}Rs$.

Moreover, DG neuronal differentiation is stimulated by CB_1Rs, CB_2Rs or $A_{2A}R$ agonist treatment and this effect persists when DG cells are treated in a combined manner with the CB_1Rs or CB_2Rs and $A_{2A}Rs$ selective agonists. These results are in accordance with Compagnucci and colleagues work in which AEA enhances cell differentiation toward a neuronal lineage via a CB_1R-dependent mechanism[189] and, also, with Wolf and collaborators work in which, in the absence of CB_1Rs, cell proliferation was increased and neuronal differentiation reduced[236]. Furthermore, Avraham and co-workers showed that administration of CB_2R agonist promoted the differentiation of human NSPCs[208]. Interestingly, a study from Lin and colleagues suggested that activation of the purinergic system may act as a proliferation signal for NSPCs, and thereby serve as a negative regulator for neuronal differentiation[230].

Similarly to what happens in DG cell fate decision, CB_1R- or CB_2R-induced increase in neuronal differentiation was blocked with an $A_{2A}R$ selective antagonist. Likewise, the effect mediated by $A_{2A}R$ activation on neuronal differentiation was blocked with CB_1R or CB_2R antagonists. Since proliferation and differentiation are mutually excluding cellular processes[139], CB_1Rs/CB_2Rs and $A_{2A}R$ signaling may be modulating these two processes in an entirely different fashion, being more active either in the cell cycle or in the differentiation stage. Thus, these data further supports the idea that $A_{2A}Rs$ play a crucial part in the regulation of DG cell proliferation and on the DG neuronal differentiation promoted by CB_1Rs and CB_2Rs activation.

Divergent effects are observed throughout literature concerning the role of eCBs on neurogenesis which may partly be explained from the use of different pharmacologic approaches. These discrepancies can also be explained by differences in the study design (treatment schedule or time-points where measurements are performed) or the animal species

or gender used, which may induce misleading interpretations[191]. So, all these readout parameters may account for the differences seen in this study from the combined activation of CB_1Rs and CB_2Rs with A_{2A}Rs.

In fact, several studies resort to the use of non-selective agonists/antagonists for CBRs to evaluate the effects of eCBs on neurogenesis. For example, Abboussi and colleagues showed that chronic administration of the synthetic CB_1R/CB_2R non-selective agonist WIN55,212-2 to rats during adulthood had no effect on the number of immature neurons in the DG, however, administration during adolescence decreased the number of immature neurons[237]. Using the same compound, Marchalant and colleagues found that a low, continuous administration significantly increased neurogenesis in aged rats[238]. Moreover, Aguado and colleagues, through an *in vitro* approach, also showed that WIN55,212-2 promoted an increase in the number of neural progenitors and generation of neurospheres[168]. Moreover, the use of SR141716A, a CB_1R and TRPV1 antagonist, has been shown to increase cell proliferation in the DG and the in lateral ventricles of mice by Jin and colleagues and this was reproducible in both wild type and CB_1R, but not TRPV1, KO mice[188]. Overall, these findings demonstrate that compound selectivity is also really important when studying neurogenesis and that multiple receptors may be responsible for the cannabinoid-induced effects on neurogenesis, which may account for the complexity/variety of general results.

The adenosinergic signaling was shown to be important for some cannabinoid-mediated effects, either CB_1Rs or CB_2Rs[219,223]. In particular, A_{2A}Rs were shown to be involved in the addictive-related behavior promoted by cannabinoid administration, having a role in the counteraction of the rewarding effects promoted by cannabinoids[218]. In fact, A_{2A}Rs could play a role in CB_1Rs/CB_2Rs functions via receptor heterodimerization or signaling cascades cross-linking[175,177,214]. Certainly, the occurrence of a putative crosstalk between A_{2A}Rs and CB_1Rs and/or CB_2Rs presents as a valid deduction, either by a structural interaction (formation of heterodimers), as others have suggested in other brain areas[175,176], or by crosstalk at downstream signaling[104,179]. Considering that CB_1Rs, CB_2Rs and A_{2A}Rs act through signaling pathways, such as ERK1/2 and p38 MAPK, which are common, heterodimerization of receptors or signaling crossing can emerge as solid hypotheses to explain the differences observed in these experiments, among single versus combined treatments with CB_1Rs or CB_2Rs and A_{2A}Rs selective agonists.

Taking together the current data is, as far as I know, the first study showing a putative crosstalk between A_{2A}Rs and CB_1Rs and/or CB_2Rs in neurogenesis.

CHAPTER 4 Closing Remarks

4. Conclusion and Future Perspectives

Generally, NSPCs are spatially located within very particular regions of the brain (at SVZ and DG regions) sensitive to different stimulus and are endowed with quiescent pools of cells that keep continuously self-renewing. This makes them ideal therapeutical targets for stem cell-based therapies and regenerative medicine platforms in order to enhance brain repair in brain trauma or pathological situations.

There is considerable evidence from previous studies suggesting that eCBs can control a variety of physiological processes in the adult brain. However, the current available data about eCB action on neurogenesis is still quite controversial. Furthermore, the role of adenosine and especially of A_{2A}Rs is not known. In fact, the adenosinergic signaling was shown to be important for some cannabinoid-mediated effects and that A_{2A}Rs could play a role in CBRs functions via receptor heterodimerization or signaling cascades cross-linking.

Altogether, this work dissects the modulatory role of A_{2A}Rs on CBR-mediated SVZ and DG neurogenesis, suggesting a possible crosstalk between the cannabinoid and adenosinergic systems.

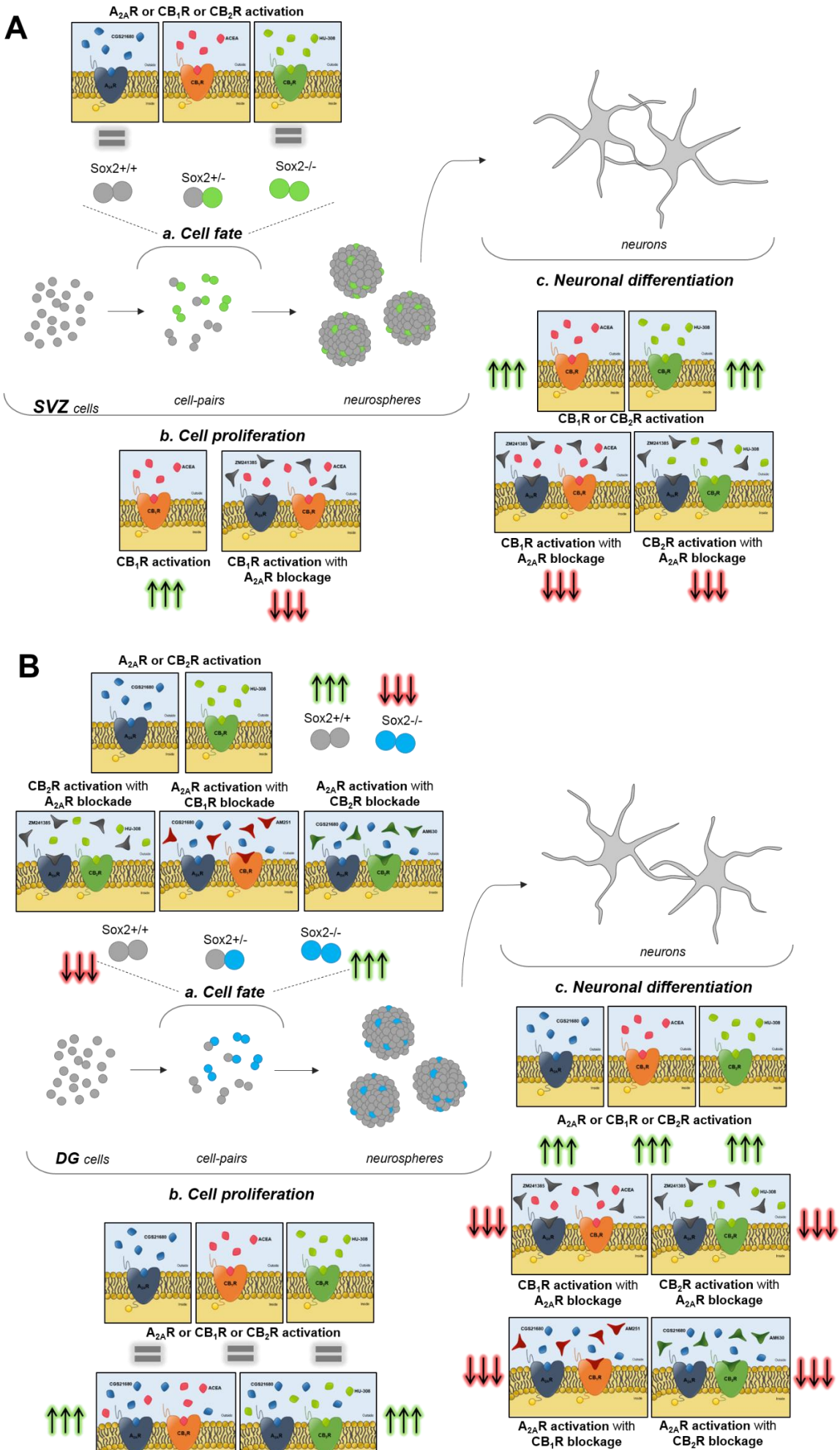
In summary, it was shown that treatment with CB_1R, CB_2R or A_{2A}R selective agonists induces no effect on the SVZ cell-fate. However, in the DG either CB_2R or A_{2A}R activation could promote self-renewing divisions. Moreover, the effect on self-renewal mediated by CB_2R activation in the DG was blocked with an A_{2A}R selective antagonist, while the effect mediated by A_{2A}R activation was blocked with either CB_1R or CB_2R antagonists. Additionally, CB_1R activation was shown to promote SVZ cell proliferation and this effect was blocked by an A_{2A}R selective antagonist. Similarly, treatment with either CB_1R or CB_2R selective agonists promoted an increase in SVZ neuronal differentiation, an effect that was blocked by A_{2A}R blockade. Interestingly, while activation *per se* of CB_1R, CB_2R or A_{2A}R did not induce cell proliferation of DG cells, co-incubation of CB_1R and A_{2A}R selective agonists or with CB_2R and A_{2A}R selective agonists resulted in an increase in DG cell proliferation. Furthermore, CB_1R, CB_2R or A_{2A}R activation promoted DG neuronal differentiation. Finally, the effect mediated by CB_1R or CB_2R was inhibited in the presence of an A_{2A}R selective antagonist, while the effect mediated by A_{2A}R activation was blocked in the presence of CB_1R or CB_2R selective antagonists.

Taking all this into account, it is possible to speculate about the potential regulatory role of A_{2A}Rs in neurogenesis: cross-signaling interaction between A_{2A}Rs and CB_1Rs or CB_2Rs may complement the complexity of endocannabinoid signaling and influence some cell stage-specific features in order to promote neurogenesis. Taken together, a putative crosstalk

between the adenosinergic and cannabinergic systems may indeed exist, either by the formation of heterodimers or by crosstalk at downstream signaling.

Therefore, further analysis is ultimately necessary to define the importance of this putative interaction of A_{2A}Rs and CB_1Rs and/or CB_2Rs, either by forming heterodimers or by interacting at the signaling level, and to clearly define how it may influence the neurogenic process. Indeed, this modulation of A_{2A}Rs and CB_1Rs and/or CB_2Rs of neurogenesis may be occurring at distinct levels of action or at unique temporal time-windows, resulting in different roles depending on which neurogenic stage NSPCs are in (e.g. different modulatory actions on cell fate decisions compared to cell survival upon integration in the new circuitry). In fact, eCBs and A_{2A}Rs may be essential for mediating particular aspects of some neurogenic stages. The role of cannabinoids on neurogenesis is complex and the contribution of A_{2A}Rs action to the net-effect still remains unclear and requires additional investigation to elucidate their full proneurogenic potential. Indeed, further *in vivo* studies will be required to comprehensively understand the role of adenosine, in particular A_{2A}Rs, in the modulation of CBRs-mediated effects on neurogenesis.

Therefore, based on the established knowledge and my data, the main findings of this work can be illustrated as described in Figure 34.


A
A2AR or CB1R or CB2R activation
Sox2+/+
Sox2+/-
Sox2-/-
a. Cell fate
SVZ cells
cell-pairs
neurospheres
neurons
c. Neuronal differentiation
CB1R or CB2R activation
CB1R activation with A2AR blockade
CB2R activation with A2AR blockade
b. Cell proliferation
CB1R activation
CB1R activation with A2AR blockade
B
A2AR or CB2R activation
Sox2+/+
Sox2-/-
CB2R activation with A2AR blockade
A2AR activation with CB1R blockade
A2AR activation with CB2R blockade
Sox2+/+
Sox2+/-
Sox2-/-
a. Cell fate
DG cells
cell-pairs
neurospheres
neurons
c. Neuronal differentiation
A2AR or CB1R or CB2R activation
CB1R activation with A2AR blockade
CB2R activation with A2AR blockade
A2AR activation with CB1R blockade
A2AR activation with CB2R blockade
b. Cell proliferation
A2AR or CB1R or CB2R activation
CB1R activation with A2AR activation
CB2R activation with A2AR activation

◄ **Figure 34 – General schematic representation of the main findings of this work. A.** SVZ neurogenic niche: activation of CB_1Rs, CB_2Rs or A_{2A}Rs induced no changes in the cell fate (**a**); CB_1Rs activation promoted a significant increase in SVZ cell proliferation, an effect blocked by an A_{2A}R selective antagonist (**b**); similarly, activation of either CB_1Rs or CB_2Rs promoted a significant increase in SVZ neuronal differentiation, an effect abolished by A_{2A}R blockade (**c**). **B.** DG neurogenic niche: activation of CB_2Rs or A_{2A}Rs induced a significant increase in self-renewal divisions and the effect mediated by CB_2Rs was blocked by an A_{2A}R selective antagonist and, similarly, the effect mediated by A_{2A}Rs was blocked by CB_1R or CB_2R antagonists (**a**); co-activation of CB_1Rs and A_{2A}Rs or CB_2Rs and A_{2A}Rs promoted an increase on DG cell proliferation, although activation of CB_1Rs, CB_2Rs or A_{2A}Rs *per se* had no effect on cell proliferation (**b**); DG neuronal differentiation was significantly increased by the activation of CB_1Rs, CB_2Rs or A_{2A}Rs; the effect mediated by CB_1Rs or CB_2Rs was blocked by an A_{2A}R selective antagonist and, similarly, the effect mediated by A_{2A}Rs was blocked by CB_1R or CB_2R antagonists (**c**).

CHAPTER 5 References

1. Ramon y Cajal, S. Degeneration and Regeneration of the Nervous System. *New York Oxford Univ. Press. Am. Branch* **1,** 369–769 (1928).

2. Ming, G. & Song, H. Adult neurogenesis in the mammalian central nervous system. *Annu. Rev. Neurosci.* **28,** 223–50 (2005).

3. Sakamoto, M., Kageyama, R. & Imayoshi, I. The functional significance of newly born neurons integrated into olfactory bulb circuits. *Front. Neurosci.* **8,** 1–9 (2014).

4. Smart, I. The subependymal layer of the mouse brain and its cell production as shown by radioautography after thymidine-H3 injection. *J. Comp. Neurol.* **116,** 325–347 (1961).

5. Altman, J. Are new neurons formed in the brains of adult mammals? *Science* **135,** 1127–8 (1962).

6. Altman, J. & Das, G. D. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* **124,** 319–35 (1965).

7. Kaplan, M. S. & Hinds, J. W. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science* **197,** 1092–4 (1977).

8. Eriksson, P. S. *et al.* Neurogenesis in the adult human hippocampus. *Nat. Med.* **4,** 1313–7 (1998).

9. Ma, D. K., Bonaguidi, M. a, Ming, G.-L. & Song, H. Adult neural stem cells in the mammalian central nervous system. *Cell Res.* **19,** 672–82 (2009).

10. Gross, C. G. Neurogenesis in the adult brain: death of a dogma. *Nat. Rev. Neurosci.* **1,** 67–73 (2000).

11. Fuchs, E. & Gould, E. Mini-review: in vivo neurogenesis in the adult brain: regulation and functional implications. *Eur. J. Neurosci.* **12,** 2211–4 (2000).

12. Kaplan, M. S. Environment complexity stimulates visual cortex neurogenesis: death of a dogma and a research career. *Trends Neurosci.* **24,** 617–20 (2001).

13. Götz, M. & Huttner, W. B. The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* **6,** 777–88 (2005).

14. Kempermann, G. The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for? *Trends Neurosci.* **31,** 163–9 (2008).

15. Ernst, A. *et al.* Neurogenesis in the striatum of the adult human brain. *Cell* **156,** 1072–83 (2014).

16. Göritz, C. & Frisén, J. Neural stem cells and neurogenesis in the adult. *Cell Stem Cell* **10,** 657–659 (2012).

17. Quadrato, G., Elnaggar, M. Y. & Di Giovanni, S. Adult neurogenesis in brain repair: cellular plasticity vs. cellular replacement. *Front. Neurosci.* **8,** 17 (2014).

18. Ernst, A. & Frisén, J. Adult Neurogenesis in Humans- Common and Unique Traits in Mammals. *PloS Biol.* **13,** 1–12 (2015).

19. Kempermann, G. New neurons for 'survival of the fittest'. *Nat. Rev. Neurosci.* **13,** 727–36 (2012).

20. Kaneko, N. & Sawamoto, K. Adult neurogenesis and its alteration under pathological conditions. *Neurosci. Res.* **63,** 155–64 (2009).

21. Gage, F. H. Mammalian Neural Stem Cells. *Science (80-.).* **287,** 1433–1438 (2000).

22. Emsley, J. G., Mitchell, B. D., Kempermann, G. & Macklis, J. D. Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. *Prog. Neurobiol.* **75,** 321–41 (2005).

23. Ma, D. K. *et al.* Epigenetic choreographers of neurogenesis in the adult mammalian brain. *Nat. Neurosci.* **13,** 1338–44 (2010).

24. Feliciano, D. M. & Bordey, A. Newborn cortical neurons: only for neonates? *Trends Neurosci.* **36,** 51–61 (2013).

25. Ming, G.-L. & Song, H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* **70,** 687–702 (2011).

26. Zhao, C., Deng, W. & Gage, F. H. Mechanisms and functional implications of adult neurogenesis. *Cell* **132,** 645–60 (2008).

27. Lledo, P.-M., Alonso, M. & Grubb, M. S. Adult neurogenesis and functional plasticity in neuronal circuits. *Nat. Rev. Neurosci.* **7,** 179–93 (2006).

28. Fuentealba, L. C., Obernier, K. & Alvarez-Buylla, A. Adult neural stem cells bridge their niche. *Cell Stem Cell* **10,** 698–708 (2012).

29. Bonfanti, L. *Neural Stem Cells - New Perspectives*. (InTech, 2014). doi:10.5772/56573

30. Abrous, D. N., Koehl, M. & Moal, M. L. E. Adult Neurogenesis : From Precursors to Network and Physiology. *Physiol. Rev.* 523–569 (2005). doi:10.1152/physrev.00055.2003.

31. Duman, R. S., Nakagawa, S. & Malberg, J. Regulation of adult neurogenesis by antidepressant treatment. *Neuropsychopharmacology* **25,** 836–44 (2001).

32. Fujita, S. 50 Years of Research on the Phenomena and Epigenetic Mechanism of Neurogenesis. *Neurosci. Res.* **83,** 43–53 (2014).

33. Covic, M., Karaca, E. & Lie, D. C. Epigenetic regulation of neurogenesis in the adult hippocampus. *Heredity (Edinb).* **105,** 122–34 (2010).

34. Yuan, T.-F. & Arias-Carrión, O. Adult neurogenesis in the hypothalamus: evidence, functions, and implications. *CNS Neurol. Disord. Drug Targets* **10,** 433–9 (2011).

35. Bonfanti, L. The (real) neurogenic/gliogenic potential of the postnatal and adult brain parenchyma. *ISRN Neurosci.* **2013,** 354136 (2013).

36. Bonfanti, L. & Nacher, J. New scenarios for neuronal structural plasticity in non-neurogenic brain parenchyma: the case of cortical layer II immature neurons. *Prog. Neurobiol.* **98,** 1–15 (2012).

37. Ihrie, R. A. & Alvarez-Buylla, A. Lake-front property: a unique germinal niche by the lateral ventricles of the adult brain. *Neuron* **70,** 674–86 (2011).

38. Jankovski, A., Garcia, C., Soriano, E. & Sotelo, C. Proliferation, migration and differentiation of neuronal progenitor cells in the adult mouse subventricular zone surgically separated from its olfactory bulb. *Eur. J. Neurosci.* **10,** 3853–68 (1998).

39. Alvarez-Buylla, A. & Garcia-Verdugo, J. M. Neurogenesis in Adult Subventricular Zone. *J. Neurosci.* **22,** 629–634 (2002).

40. Bovetti, S., Gribaudo, S., Puche, A. C., De Marchis, S. & Fasolo, A. From progenitors to integrated neurons: role of neurotransmitters in adult olfactory neurogenesis. *J. Chem. Neuroanat.* **42,** 304–16 (2011).

41. Curtis, M. a, Faull, R. L. M. & Eriksson, P. S. The effect of neurodegenerative diseases on the subventricular zone. *Nat. Rev. Neurosci.* **8,** 712–23 (2007).

42. Butti, E., Cusimano, M., Bacigaluppi, M. & Martino, G. Neurogenic and non-neurogenic functions of endogenous neural stem cells. *Front. Neurosci.* **8,** 92 (2014).

43. Kriegstein, A. & Alvarez-buylla, A. The Glial Nature of Embryonic and Adult Neural Stem Cells. *Annu Rev Neurosci.* 149–184 (2011). doi:10.1146/annurev.neuro.051508.135600.The

44. Young, K. M., Fogarty, M., Kessaris, N. & Richardson, W. D. Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. *J. Neurosci.* **27,** 8286–96 (2007).

45. Hack, M. A. *et al.* Neuronal fate determinants of adult olfactory bulb neurogenesis. *Nat. Neurosci.* **8,** 865–72 (2005).

46. Paez-Gonzalez, P., Asrican, B., Rodriguez, E. & Kuo, C. T. Identification of distinct ChAT(+) neurons and activity-dependent control of postnatal SVZ neurogenesis. *Nat. Neurosci.* 1–11 (2014). doi:10.1038/nn.3734

47. Whitman, M. C. & Greer, C. a. Adult neurogenesis and the olfactory system. *Prog. Neurobiol.* **89,** 162–75 (2009).

48. Kempermann, G., Wiskott, L. & Gage, F. H. Functional significance of adult neurogenesis. *Curr. Opin. Neurobiol.* **14,** 186–91 (2004).

49. Sawada, M., Matsumoto, M. & Sawamoto, K. Vascular regulation of adult neurogenesis under physiological and pathological conditions. *Front. Neurosci.* **8,** 53 (2014).

50. Martino, G., Butti, E. & Bacigaluppi, M. Neurogenesis or non-neurogenesis : that is the question. *J. Clin. Invest.* **124,** 1–4 (2014).

51. Mosher, K. I. *et al.* Neural progenitor cells regulate microglia functions and activity. *Nat. Neurosci.* **15,** 1485–7 (2012).

52. Vivar, C. & van Praag, H. Functional circuits of new neurons in the dentate gyrus. *Front. Neural Circuits* **7,** 15 (2013).

53. Ribak, C. E. & Shapiro, L. A. Ultrastructure and synaptic connectivity of cell types in the adult rat dentate gyrus. *Prog. Brain Res.* **163,** 155–66 (2007).

54. Wojtowicz, J. M. Adult neurogenesis. From circuits to models. *Behav. Brain Res.* **227,** 490–6 (2012).

55. Deng, W., Aimone, J. B. & Gage, F. H. New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nat. Rev. Neurosci.* **11,** 339–50 (2010).

56. Neves, G., Cooke, S. F. & Bliss, T. V. P. Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. *Nat. Rev. Neurosci.* **9,** 65–75 (2008).

57. Kempermann, G., Jessberger, S., Steiner, B. & Kronenberg, G. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* **27,** 447–52 (2004).

58. Hodge, R. D. & Hevner, R. F. Expression and actions of transcription factors in adult hippocampal neurogenesis. *Dev Neurobiol.* **71,** 680–689 (2011).

59. Mongiat, L. a & Schinder, A. F. Adult neurogenesis and the plasticity of the dentate gyrus network. *Eur. J. Neurosci.* **33,** 1055–61 (2011).

60. Pedroni, A., Minh, D. D., Mallamaci, A. & Cherubini, E. Electrophysiological characterization of granule cells in the dentate gyrus immediately after birth. *Front. Cell. Neurosci.* **8,** 44 (2014).

61. Bonaguidi, M. a, Song, J., Ming, G. & Song, H. A unifying hypothesis on mammalian neural stem cell properties in the adult hippocampus. *Curr. Opin. Neurobiol.* **22,** 754–61 (2012).

62. Aimone, J. B., Wiles, J. & Gage, F. H. Potential role for adult neurogenesis in the encoding of time in new memories. *Nat. Neurosci.* **9,** 723–7 (2006).

63. Stuchlik, A. Dynamic learning and memory, synaptic plasticity and neurogenesis: an update. *Front. Behav. Neurosci.* **8,** 106 (2014).

64. Curtis, M. A., Low, V. F. & Faull, R. L. M. Neurogenesis and progenitor cells in the adult human brain: a comparison between hippocampal and subventricular progenitor proliferation. *Dev. Neurobiol.* **72,** 990–1005 (2012).

65. Ninkovic, J. & Götz, M. Signaling in adult neurogenesis: from stem cell niche to neuronal networks. *Curr. Opin. Neurobiol.* **17,** 338–44 (2007).

66. Gould, E. How widespread is adult neurogenesis in mammals? *Nat. Rev. Neurosci.* **8,** 481–8 (2007).

67. Rojczyk-Gołębiewska, E., Pałasz, A. & Wiaderkiewicz, R. Hypothalamic subependymal niche: A novel site of the adult neurogenesis. *Cell. Mol. Neurobiol.* **34,** 631–642 (2014).

68. Fowler, C. D., Liu, Y. & Wang, U. Estrogen and adult neurogenesis in the amygdala and hypothalamus. *Brain Res Rev* **57,** 342–351 (2008).

69. Migaud, M. *et al.* Emerging new sites for adult neurogenesis in the mammalian brain: a comparative study between the hypothalamus and the classical neurogenic zones. *Eur. J. Neurosci.* **32,** 2042–52 (2010).

70. Chang, G.-Q., Karatayev, O. & Leibowitz, S. F. Prenatal exposure to nicotine stimulates neurogenesis of orexigenic peptide-expressing neurons in hypothalamus and amygdala. *J. Neurosci.* **33,** 13600–11 (2013).

71. Cheng, M.-F. Hypothalamic neurogenesis in the adult brain. *Front. Neuroendocrinol.* **34,** 167–78 (2013).

72. Yuan, T.-F., Liang, Y.-X. & So, K.-F. Occurrence of new neurons in the piriform cortex. *Front. Neuroanat.* **8,** 1–5 (2015).

73. Bonfanti, L. & Peretto, P. Adult neurogenesis in mammals - a theme with many variations. *Eur. J. Neurosci.* **34,** 930–50 (2011).

74. Florio, M. & Huttner, W. B. Neural progenitors, neurogenesis and the evolution of the neocortex. *Development* **141,** 2182–2194 (2014).

75. Fowler, C. D., Liu, Y. & Wang, Z. Estrogen and adult neurogenesis in the amygdala and hypothalamus. *Brain Res. Rev.* **57,** 342–51 (2008).

76. Liu, Y. *et al.* Chemosensory cues affect amygdaloid neurogenesis and alter behaviors in the socially monogamous prairie vole. *Eur. J. Neurosci.* **39,** 1632–41 (2014).

77. Luzzati, F., De Marchis, S., Fasolo, A. & Peretto, P. Adult neurogenesis and local neuronal progenitors in the striatum. *Neurodegener. Dis.* **4,** 322–7 (2007).

78. Maggi, R., Zasso, J. & Conti, L. Neurodevelopmental origin and adult neurogenesis of the neuroendocrine hypothalamus. **8,** 1–7 (2015).

79. Lee, D. A. & Blackshaw, S. Functional implications of hypothalamic neurogenesis in the adult mammalian brain. *Int J Dev Neurosci* **30,** 615–621 (2012).

80. Kokoeva, M. V, Yin, H. & Flier, J. S. Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. *Science (80-.).* **310,** 679–83 (2005).

81. Sousa-Ferreira, L., Almeida, L. P. De & Cavadas, C. Role of hypothalamic neurogenesis in feeding regulation. *Trends Endocrinol. Metab.* **25,** 80–8 (2014).

82. Pierce, A. A. & Xu, A. W. De novo neurogenesis in adult hypothalamus as a compensatory mechanism to regulate energy balance. *J. Neurosci.* **30,** 723–30 (2010).

83. Burnstock, G. Introduction to Purinergic Signalling in the Brain. *Glioma Signal. - Adv. Exp. Med. Biol.* **986,** 1–12 (2013).

84. Abbracchio, M. P., Burnstock, G., Verkhratsky, A. & Zimmermann, H. Purinergic signalling in the nervous system: an overview. *Trends Neurosci.* **32,** 19–29 (2009).

85. Burnstock, G., Krügel, U., Abbracchio, M. P. & Illes, P. Purinergic signalling: from normal behaviour to pathological brain function. *Prog. Neurobiol.* **95,** 229–74 (2011).

86. Dunwiddie, T. V & Masino, S. A. The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci* **24,** 31–55 (2001).

87. Layland, J., Carrick, D., Lee, M., Oldroyd, K. & Berry, C. Adenosine: Physiology, Pharmacology, and Clinical Applications. *JACC. Cardiovasc. Interv.* **7,** (2014).

88. Dias, R. B., Rombo, D. M., Ribeiro, J. a, Henley, J. M. & Sebastião, A. M. Adenosine: setting the stage for plasticity. *Trends Neurosci.* **36,** 248–57 (2013).

89. Gomes, C. V, Kaster, M. P., Tomé, A. R., Agostinho, P. M. & Cunha, R. A. Adenosine receptors and brain diseases : Neuroprotection and neurodegeneration. *Biochim. Biophys. Acta* **1808,** 1380–1399 (2011).

90. Tozaki-Saitoh, H., Tsuda, M. & Inoue, K. *Role of purinergic receptors in CNS function and neuroprotection. Advances in pharmacology (San Diego, Calif.)* **61,** (Elsevier Inc., 2011).

91. Boison, D. Role of adenosine in status epilepticus: a potential new target? *Epilepsia* **54 Suppl 6,** 20–22 (2013).

92. Cristalli, G., Müller, C. E. & Volpini, R. *Recent Developments in Adenosine A2A Receptor Ligands*. **193,** (Springer Berlin Heidelberg, 2009).

93. Burnstock, G. Purinergic signalling: past, present and future. *Braz J Med Biol Res* **42,** 3–8 (2009).

94. Rivera-Oliver, M. & Díaz-Ríos, M. Using caffeine and other adenosine receptor antagonists and agonists as therapeutic tools against neurodegenerative diseases: a review. *Life Sci.* **101,** 1–9 (2014).

95. Cunha, R. A. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem. Int.* **38,** 107–25 (2001).

96. Haskó, G., Pacher, P., Sylvester, E. V. & Illes, P. Adenosine receptor signaling in the brain immune system. *Trends Pharmacol Sci.* **26,** 511–516 (2005).

97. Ribeiro, J. a., Sebastião, A. M. & de Mendonça, A. Adenosine receptors in the nervous system: pathophysiological implications. *Prog. Neurobiol.* **68,** 377–392 (2003).

98. Olah, M. E. & Stiles, G. L. Adenosine Receptor Subtypes: Characterization and Therapeutic Regulation. *Annu. Rev. Phamacol. Toxicol* **35,** 581–606 (1995).

99. Rosin, D. L., Robeva, A., Woodard, R. L., Guyenet, P. G. & Linden, J. Immunohistochemical Localization of Adenosine A2A Receptors in the Rat Central Nervous System. **186,** 163–186 (1998).

100. Fredholm, B. B. *et al.* Structure and function of adenosine receptors and their genes. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **362,** 364–374 (2000).

101. Cunha, R. A. Neuroprotection by adenosine in the brain: From A1 receptor activation to A2A receptor blockade. *Purinergic Signal.* **1,** 111–34 (2005).

102. Klotz, K.-N. Adenosine receptors and their ligands. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **362,** 382–391 (2000).

103. Ham, J. & Evans, B. a J. An emerging role for adenosine and its receptors in bone homeostasis. *Front. Endocrinol. (Lausanne).* **3,** 113 (2012).

104. Sebastião, A. M. & Ribeiro, J. A. in *Handbook of experimental pharmacology* (eds. Wilson, C. & Mustafa, S. J.) **193,** 589–615 (2009).

105. Fredholm, B. B. *et al.* International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* **53,** 527–552 (2001).

106. Jacobson, K. a & Gao, Z.-G. Adenosine receptors as therapeutic targets. *Nat. Rev. Drug Discov.* **5,** 247–64 (2006).

107. Chen, J.-F., Eltzschig2, H. K. & Fredholm, B. B. Adenosine receptors as drug targets — what are the challenges. *Nat Rev Drug Discov* **12,** 265–286 (2013).

108. Sebastião, A. M. & Ribeiro, J. a. Triggering neurotrophic factor actions through adenosine A2A receptor activation: Implications for neuroprotection. *Br. J. Pharmacol.* **158,** 15–22 (2009).

109. Paterniti, I. *et al.* Selective adenosine A2A receptor agonists and antagonists protect against spinal cord injury through peripheral and central effects. *J. Neuroinflammation* **8,** 31 (2011).

110. Chen, J.-F. *et al.* Adenosine A2A receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and 'fine tuning' modulation. *Prog. Neurobiol.* **83,** 310–31 (2007).

111. Sebastião, A. M. & Ribeiro, J. A. Fine-tuning neuromodulation by adenosine. *Trends Pharmacol. Sci.* **21,** 341–6 (2000).

112. Burnstock, G. Purinergic Signalling: Pathophysiology and Therapeutic Potential. *Keio J. Med.* **62,** 63–73 (2013).

113. Burnstock, G., Krügel, U., Abbracchio, M. P. & Illes, P. Purinergic signalling: from normal behaviour to pathological brain function. *Prog. Neurobiol.* **95,** 229–74 (2011).

114. Piirainen, H., Ashok, Y., Nanekar, R. T. & Jaakola, V.-P. Structural features of adenosine receptors: from crystal to function. *Biochim. Biophys. Acta* **1808,** 1233–44 (2011).

115. Jaakola, V.-P. & Ijzerman, A. P. The crystallographic structure of the human adenosine A2A receptor in a high-affinity antagonist-bound state: implications for GPCR drug screening and design. *Curr. Opin. Struct. Biol.* **20,** 401–14 (2010).

116. Zhukov, A. *et al.* Biophysical mapping of the adenosine A2A receptor. *J. Med. Chem.* **54,** 4312–23 (2011).

117. D' Ambrosi, N. & Volonte, C. Metabotropic purinergic receptors in lipid membrane microdomains. *Curr. Med. Chem.* **20,** 56–63 (2013).

118. Huang, N. K., Lin, Y. W., Huang, C. L., Messing, R. O. & Chern, Y. *Activation of protein kinase A and atypical protein kinase C by A(2A) adenosine receptors antagonizes apoptosis due to serum deprivation in PC12 cells. The Journal of biological chemistry* **276,** (2001).

119. Feoktistov, I. *et al.* Cyclic AMP and Protein Kinase A Stimulate Cdc42 : Role of A2A Adenosine Receptors in Human Mast Cells. *Mol Pharmacol* **58,** 903–910 (2000).

120. Gsandtner, I. & Freissmuth, M. A Tail of Two Signals : The C Terminus of the A 2A -Adenosine Receptor Recruits Alternative Signaling Pathways. *Mol Pharmacol* 447–449 (2006). doi:10.1124/mol.106.026757.Please

121. Fredholm, B. B., Chern, Y., Franco, R. & Sitkovsky, M. Aspects of the general biology of adenosine A2A signaling. *Prog. Neurobiol.* **83,** 263–76 (2007).

122. Katona, I. & Freund, T. F. Multiple functions of endocannabinoid signaling in the brain. *Annu. Rev. Neurosci.* **35,** 529–58 (2012).

123. Fanelli, F. & Felline, A. Dimerization and ligand binding affect the structure network of A(2A) adenosine receptor. *Biochim. Biophys. Acta* **1808,** 1256–66 (2011).

124. Demuth, D. G. & Molleman, A. Cannabinoid signalling. *Life Sci.* **78,** 549–63 (2006).

125. Fuxe, K., Marcellino, D., Guidolin, D., Woods, A. S. & Agnati, L. F. Heterodimers and receptor mosaics of different types of G-protein-coupled receptors. *Physiology (Bethesda).* **23,** 322–32 (2008).

126. Brugarolas, M. *et al.* G-Protein-Coupled Receptor Heteromers as Key Players in the Molecular Architecture of the Central Nervous System. *CNS Neurosci. Ther.* (2014). doi:10.1111/cns.12277

127. Nishi, A. *et al.* Metabotropic mGlu5 receptors regulate adenosine A2A receptor signaling. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 1322–7 (2003).

128. Ferré, S., Ciruela, F., Woods, A. S., Lluis, C. & Franco, R. Functional relevance of neurotransmitter receptor heteromers in the central nervous system. *Trends Neurosci.* **30,** 440–6 (2007).

129. Ferré, S. *et al.* Adenosine-cannabinoid receptor interactions. Implications for striatal function. *Br. J. Pharmacol.* **160,** 443–53 (2010).

130. Sun, C.-N. *et al.* The A2A adenosine receptor rescues neuritogenesis impaired by p53 blockage via KIF2A, a kinesin family member. *Dev. Neurobiol.* **70,** 604–21 (2010).

131. Han, M.-E. *et al.* Inhibitory effects of caffeine on hippocampal neurogenesis and function. *Biochem. Biophys. Res. Commun.* **356,** 976–980 (2007).

132. Wentz, C. T. & Magavi, S. S. P. Caffeine alters proliferation of neuronal precursors in the adult hippocampus. *Neuropharmacology* **56,** 994–1000 (2009).

133. Sahu, S. *et al.* Caffeine and modafinil promote adult neuronal cell proliferation during 48 h of total sleep deprivation in rat dentate gyrus. *Exp. Neurol.* **248,** 470–81 (2013).

134. Fride, E. Endocannabinoids in the central nervous system--an overview. *Prostaglandins. Leukot. Essent. Fatty Acids* **66,** 221–33 (2002).

135. Mechoulam, R. & Parker, L. a. The endocannabinoid system and the brain. *Annu. Rev. Psychol.* **64,** 21–47 (2013).

136. Harkany, T. *et al.* The emerging functions of endocannabinoid signaling during CNS development. *Trends Pharmacol. Sci.* **28,** 83–92 (2007).

137. Harkany, T., Keimpema, E., Barabás, K. & Mulder, J. Endocannabinoid functions controlling neuronal specification during brain development. *Mol. Cell. Endocrinol.* **286,** S84–90 (2008).

138. Olière, S., Joliette-Riopel, A., Potvin, S. & Jutras-Aswad, D. Modulation of the endocannabinoid system: vulnerability factor and new treatment target for stimulant addiction. *Front. psychiatry* **4,** 109 (2013).

139. Galve-Roperh, I. *et al.* Cannabinoid receptor signaling in progenitor/stem cell proliferation and differentiation. *Prog. Lipid Res.* **52,** 633–50 (2013).

140. Elphick, M. R. The evolution and comparative neurobiology of endocannabinoid signalling. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **367,** 3201–15 (2012).

141. Galve-Roperh, I., Aguado, T., Palazuelos, J. & Guzmán, M. The endocannabinoid system and neurogenesis in health and disease. *Neuroscientist* **13,** 109–14 (2007).

142. Galve-Roperh, I., Aguado, T., Rueda, D., Velasco, G. & Guzmán, M. Endocannabinoids: a new family of lipid mediators involved in the regulation of neural cell development. *Curr. Pharm. Des.* **12,** 2319–25 (2006).

143. Castillo, P. E., Younts, T. J., Chávez, A. E. & Hashimotodani, Y. Endocannabinoid signaling and synaptic function. *Neuron* **76,** 70–81 (2012).

144. Sylantyev, S., Jensen, T. P., Ross, R. A. & Rusakov, D. A. Cannabinoid- and lysophosphatidylinositol-sensitive receptor GPR55 boosts neurotransmitter release at central synapses. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 5193–8 (2013).

145. Melis, M., Greco, B. & Tonini, R. Interplay between synaptic endocannabinoid signaling and metaplasticity in neuronal circuit function and dysfunction. *Eur. J. Neurosci.* **39,** 1189–201 (2014).

146. Ho, W.-S. V, Barrett, D. A. & Randall, M. D. 'Entourage' effects of N-palmitoylethanolamide and N-oleoylethanolamide on vasorelaxation to anandamide occur through TRPV1 receptors. *Br. J. Pharmacol.* **155,** 837–46 (2008).

147. Giuffrida, A. & Seillier, A. New insights on endocannabinoid transmission in psychomotor disorders. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **38,** 51–8 (2012).

148. Dalton, G. D., Bass, C. E., Horn, C. Van & Howlett, A. C. Signal Transduction via Cannabinoid Receptors. *CNS Neurol Disord Drug Targets* **8,** 422–431 (2009).

149. Rom, S. & Persidsky, Y. Cannabinoid receptor 2: potential role in immunomodulation and neuroinflammation. *J. Neuroimmune Pharmacol.* **8,** 608–20 (2013).

150. Pertwee, R. G. Ligands that target cannabinoid receptors in the brain: From THC to anandamide and beyond. *Addict. Biol.* **13,** 147–159 (2008).

151. Pertwee, R. G. Pharmacological actions of cannabinoids. *Handb. Exp. Pharmacol.* **168,** 1–51 (2005).

152. Iremonger, K. J., Wamsteeker Cusulin, J. I. & Bains, J. S. Changing the tune: plasticity and adaptation of retrograde signals. *Trends Neurosci.* **36,** 471–9 (2013).

153. De Petrocellis, L., Cascio, M. G. & Di Marzo, V. The endocannabinoid system: a general view and latest additions. *Br. J. Pharmacol.* **141,** 765–74 (2004).

154. Ohno-Shosaku, T. & Kano, M. Endocannabinoid-mediated retrograde modulation of synaptic transmission. *Curr. Opin. Neurobiol.* **29C,** 1–8 (2014).

155. Cachope, R. Functional diversity on synaptic plasticity mediated by endocannabinoids. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **367,** 3242–53 (2012).

156. Di Iorio, G. *et al.* The endocannabinoid system: a putative role in neurodegenerative diseases. *Int. J. high risk Behav. Addict.* **2,** 100–6 (2013).

157. Kirkham, T. C. Cannabinoids and appetite: food craving and food pleasure. *Int. Rev. Psychiatry* **21,** 163–71 (2009).

158. Rodríguez de Fonseca, F. [The endocannabinoid system and food intake control]. *Rev. Med. Univ. Navarra* **48,** 18–23

159. Wolf, S. A. & Ullrich, O. Endocannabinoids and the brain immune system: new neurones at the horizon? *J. Neuroendocrinol.* **20 Suppl 1,** 15–9 (2008).

160. Luchicchi, A. & Pistis, M. Anandamide and 2-arachidonoylglycerol: pharmacological properties, functional features, and emerging specificities of the two major endocannabinoids. *Mol. Neurobiol.* **46,** 374–92 (2012).

161. Heifets, B. D. & Castillo, P. E. Endocannabinoid signaling and long-term synaptic plasticity. *Annu. Rev. Physiol.* **71,** 283–306 (2009).

162. Wilson, R. I. & Nicoll, R. a. Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature* **410,** 588–92 (2001).

163. Piomelli, D. The molecular logic of endocannabinoid signalling. *Nat. Rev. Neurosci.* **4,** 873–84 (2003).

164. Berghuis, P. *et al.* Hardwiring the brain: endocannabinoids shape neuronal connectivity. *Science* **316,** 1212–6 (2007).

165. Howlett, A. C. *et al.* Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology* **47 Suppl 1,** 345–58 (2004).

166. Pertwee, R. G. The pharmacology of cannabinoid receptors and their ligands: an overview. *Int. J. Obes.* **30 Suppl 1,** S13–8 (2006).

167. Lutz, B. Molecular biology of cannabinoid receptors. *Prostaglandins. Leukot. Essent. Fatty Acids* **66,** 123–42 (2002).

168. Aguado, T. *et al.* The endocannabinoid system drives neural progenitor proliferation. *FASEB J.* **19,** 1704–6 (2005).

169. Micale, V., Mazzola, C. & Drago, F. Endocannabinoids and neurodegenerative diseases. *Pharmacol. Res.* **56,** 382–92 (2007).

170. Shim, J.-Y. & Padgett, L. Functional residues essential for the activation of the CB1 cannabinoid receptor. *Methods Enzymol.* **520,** 337–55 (2013).

171. Ahn, K. H., Bertalovitz, A. C., Mierke, D. F. & Kendall, D. A. Dual role of the second extracellular loop of the cannabinoid receptor 1: ligand binding and receptor localization. *Mol. Pharmacol.* **76,** 833–42 (2009).

172. Stadel, R., Ahn, K. H. & Kendall, D. A. The cannabinoid type-1 receptor carboxyl-terminus, more than just a tail. *J. Neurochem.* **117,** 1–18 (2011).

173. McAllister, S. D. & Glass, M. CB(1) and CB(2) receptor-mediated signalling: a focus on endocannabinoids. *Prostaglandins. Leukot. Essent. Fatty Acids* **66,** 161–71 (2002).

174. Turu, G. & Hunyady, L. Signal transduction of the CB1 cannabinoid receptor. *J. Mol. Endocrinol.* **44,** 75–85 (2010).

175. Navarro, G. *et al.* Detection of heteromers formed by cannabinoid CB1, dopamine D2, and adenosine A2A G-protein-coupled receptors by combining bimolecular fluorescence complementation and bioluminescence energy transfer. *ScientificWorldJournal.* **8,** 1088–97 (2008).

176. Ferré, S., Goldberg, S. R., Lluis, C. & Franco, R. Looking for the role of cannabinoid receptor heteromers in striatal function. *Neuropharmacology* **56 Suppl 1,** 226–34 (2009).

177. Carriba, P. *et al.* Detection of heteromerization of more than two proteins by sequential BRET-FRET. *Nat. Methods* **5,** 727–733 (2008).

178. Mackie, K. Cannabinoid receptor homo- and heterodimerization. *Life Sci.* **77,** 1667–73 (2005).

179. Callén, L. *et al.* Cannabinoid receptors CB1 and CB2 form functional heteromers in brain. *J. Biol. Chem.* **287,** 20851–65 (2012).

180. Pacher, L. & Ba, N. The Endocannabinoid System as an Emerging Target of Pharmacotherapy. *Pharmacol Rev* **58,** 389–462 (2006).

181. Di Marzo, V. Targeting the endocannabinoid system: to enhance or reduce? *Nat. Rev. Drug Discov.* **7,** 438–55 (2008).

182. Svízenská, I., Dubový, P. & Sulcová, A. Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures - a short review. *Pharmacol. Biochem. Behav.* **90,** 501–11 (2008).

183. Zhou, Y., Falenta, K. & Lalli, G. Endocannabinoid signalling in neuronal migration. *Int. J. Biochem. Cell Biol.* **47,** 104–8 (2014).

184. Díaz-Alonso, J., Guzmán, M. & Galve-Roperh, I. Endocannabinoids via CB1 receptors act as neurogenic niche cues during cortical development. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **367,** 3229–41 (2012).

185. Mulder, J. *et al.* Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 8760–5 (2008).

186. Maccarrone, M., Guzmán, M., Mackie, K., Doherty, P. & Harkany, T. Programming of neural cells by (endo)cannabinoids : from physiological rules to emerging therapies. *Nat. Publ. Gr.* **15,** 786–801 (2014).

187. Prenderville, J. a., Kelly, Á. M. & Downer, E. J. The role of cannabinoids in adult neurogenesis. *Br. J. Pharmacol.* n/a–n/a (2015). doi:10.1111/bph.13186

188. Jin, K. *et al.* Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. *Mol. Pharmacol.* **66,** 204–8 (2004).

189. Compagnucci, C. *et al.* Type-1 (CB1) cannabinoid receptor promotes neuronal differentiation and maturation of neural stem cells. *PLoS One* **8,** e54271 (2013).

190. Rubio-Araiz, A. *et al.* The endocannabinoid system modulates a transient TNF pathway that induces neural stem cell proliferation. *Mol. Cell. Neurosci.* **38,** 374–80 (2008).

191. Xapelli, S. *et al.* Activation of type 1 cannabinoid receptor (CB1R) promotes neurogenesis in murine subventricular zone cell cultures. *PLoS One* **8,** e63529 (2013).

192. Hill, M. N. *et al.* Endogenous Cannabinoid Signaling Is Required for Voluntary Exercise-induced Enhancement of Progenitor Cell Proliferation in the Hippocampus. *Hippocampus* **20,** 513–523 (2011).

193. Aguado, T. *et al.* The CB1 cannabinoid receptor mediates excitotoxicity-induced neural progenitor proliferation and neurogenesis. *J. Biol. Chem.* **282,** 23892–23898 (2007).

194. Soltys, J., Yushak, M. & Mao-Draayer, Y. Regulation of neural progenitor cell fate by anandamide. *Biochem. Biophys. Res. Commun.* **400,** 21–6 (2010).

195. Malfitano, A. M., Basu, S., Maresz, K., Bifulco, M. & Dittel, B. N. What we know and do not know about the cannabinoid receptor 2 (CB2). *Semin. Immunol.* **2,** 1–11 (2014).

196. Cabral, G. A., Raborn, E. S., Griffin, L., Dennis, J. & Marciano-Cabral, F. CB2 receptors in the brain: role in central immune function. *Br. J. Pharmacol.* **153,** 240–51 (2008).

197. Miller, A. M. & Stella, N. CB2 receptor-mediated migration of immune cells: it can go either way. *Br. J. Pharmacol.* **153,** 299–308 (2008).

198. Fernández-Ruiz, J., Pazos, M. R., García-Arencibia, M., Sagredo, O. & Ramos, J. a. Role of CB2 receptors in neuroprotective effects of cannabinoids. *Mol. Cell. Endocrinol.* **286,** S91–6 (2008).

199. Gong, J.-P. *et al.* Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res.* **1071,** 10–23 (2006).

200. Morgan, N. H., Stanford, I. M. & Woodhall, G. L. Functional CB2 type cannabinoid receptors at CNS synapses. *Neuropharmacology* **57,** 356–68 (2009).

201. Mnpotra, J. S. *et al. Structural Basis of G Protein-Coupled Receptor- Gi Protein Interaction: Formation of the Cannabinoid CB2 Receptor / Gi Protein Complex. The Journal of biological chemistry* (2014). doi:10.1074/jbc.M113.539916

202. Fernández-Ruiz, J. *et al.* Cannabinoid CB2 receptor: a new target for controlling neural cell survival? *Trends Pharmacol. Sci.* **28,** 39–45 (2007).

203. Downer, E. J. High hopes for CB(2) receptors in neurogenesis. *Br. J. Pharmacol.* **171,** 1345–6 (2014).

204. Palazuelos, J. *et al.* Non-psychoactive CB2 cannabinoid agonists stimulate neural progenitor proliferation. *FASEB J.* **20,** 2405–2407 (2006).

205. Molina-Holgado, F. *et al.* CB2 cannabinoid receptors promote mouse neural stem cell proliferation. *Eur. J. Neurosci.* **25,** 629–34 (2007).

206. Goncalves, M. B. *et al.* A diacylglycerol lipase-CB2 cannabinoid pathway regulates adult subventricular zone neurogenesis in an age-dependent manner. *Mol. Cell. Neurosci.* **38,** 526–536 (2008).

207. Palazuelos, J., Ortega, Z., Díaz-Alonso, J., Guzmán, M. & Galve-Roperh, I. CB2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling. *J. Biol. Chem.* **287,** 1198–209 (2012).

208. Avraham, H. K. *et al.* The cannabinoid CB2 receptor agonist AM1241 enhances neurogenesis in GFAP/Gp120 transgenic mice displaying deficits in neurogenesis. *Br. J. Pharmacol.* **171,** 468–79 (2014).

209. Butti, E. *et al.* Subventricular zone neural progenitors protect striatal neurons from glutamatergic excitotoxicity. *Brain* **135,** 3320–35 (2012).

210. Fogaça, M. V., Galve-Roperh, I., Guimarães, F. S. & Campos, A. C. Cannabinoids, Neurogenesis and Antidepressant Drugs: Is there a Link? *Curr. Neuropharmacol.* **11,** 263–75 (2013).

211. Scotter, E. L., Abood, M. E. & Glass, M. The endocannabinoid system as a target for the treatment of neurodegenerative disease. *Br. J. Pharmacol.* **160,** 480–98 (2010).

212. Skaper, S. D. & Di Marzo, V. Endocannabinoids in nervous system health and disease: the big picture in a nutshell. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **367,** 3193–200 (2012).

213. Pertwee, R. G. The therapeutic potential of drugs that target cannabinoid receptors or modulate the tissue levels or actions of endocannabinoids. *AAPS J.* **7,** E625–54 (2005).

214. Carriba, P. *et al.* Striatal adenosine A2A and cannabinoid CB1 receptors form functional heteromeric complexes that mediate the motor effects of cannabinoids. *Neuropsychopharmacology* **32,** 2249–59 (2007).

215. Schiffmanna, S. N., Fisoneb, G., Morescoc, R., Cunha, R. A. & Ferré, S. Adenosine A2A receptors and basal ganglia physiology. *Prog Neurobiol* **83,** 277–292 (2007).

216. Tebano, M. T., Martire, A. & Popoli, P. Adenosine A(2A)-cannabinoid CB(1) receptor interaction: an integrative mechanism in striatal glutamatergic neurotransmission. *Brain Res.* **1476,** 108–18 (2012).

217. Ferre, S., Justinova, Z., Franco, R. & Goldberg, S. R. Adenosine A2A-cannabinoid CB1 receptor heteromers. Implications for the rewarding effects of cannabinoids. *FASEB J.* **21,** 562.6 (2007).

218. Soria, G. *et al.* Adenosine A2A receptors are involved in physical dependence and place conditioning induced by THC. *Eur. J. Neurosci.* **20,** 2203–13 (2004).

219. Martire, A. *et al.* Pre-synaptic adenosine A2A receptors control cannabinoid CB1 receptor-mediated inhibition of striatal glutamatergic neurotransmission. *J. Neurochem.* **116,** 273–80 (2011).

220. Ferreira, S. G. *et al.* Presynaptic adenosine A2A receptors dampen cannabinoid CB1 receptor-mediated inhibition of corticostriatal glutamatergic transmission. *Br. J. Pharmacol.* **172,** 1074–1086 (2015).

221. Tebano, M. T. *et al.* Adenosine A2A receptors enable the synaptic effects of cannabinoid CB1 receptors in the rodent striatum. *J. Neurochem.* **110,** 1921–30 (2009).

222. Lerner, T. N., Horne, E. A., Stella, N. & Kreitzer, A. C. Endocannabinoid signaling mediates psychomotor activation by adenosine A2A antagonists. *J. Neurosci.* **30,** 2160–2164 (2010).

223. Castillo, A., Tolón, M. R., Fernández-Ruiz, J., Romero, J. & Martinez-Orgado, J. The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB(2) and adenosine receptors. *Neurobiol. Dis.* **37,** 434–40 (2010).

224. Justinová, Z. *et al.* Reinforcing and neurochemical effects of cannabinoid CB1 receptor agonists, but not cocaine, are altered by an adenosine A2A receptor antagonist. *Addict Biol.* **16,** 405–415 (2011).

225. Tozzi, A. *et al.* A2A adenosine receptor antagonism enhances synaptic and motor effects of cocaine via CB1 cannabinoid receptor activation. *PLoS One* **7,** e38312 (2012).

226. Pinna, A. *et al.* L-DOPA disrupts adenosine A(2A)-cannabinoid CB(1)-dopamine D(2) receptor heteromer cross-talk in the striatum of hemiparkinsonian rats: biochemical and behavioral studies. *Exp. Neurol.* **253,** 180–91 (2014).

227. Bonaventura, J. *et al.* L-DOPA-treatment in primates disrupts the expression of A(2A) adenosine-CB(1) cannabinoid-D(2) dopamine receptor heteromers in the caudate nucleus. *Neuropharmacology* **79,** 90–100 (2014).

228. Cerri, S. *et al.* Neuroprotective potential of adenosine A2A and cannabinoid CB1 receptor antagonists in an animal model of Parkinson disease. *J. Neuropathol. Exp. Neurol.* **73,** 414–24 (2014).

229. Stafford, M. R., Bartlett, P. F. & Adams, D. J. Purinergic receptor activation inhibits mitogen-stimulated proliferation in primary neurospheres from the adult mouse subventricular zone. *Mol. Cell. Neurosci.* **35,** 535–548 (2007).

230. Lin, J. H.-C. *et al.* Purinergic signaling regulates neural progenitor cell expansion and neurogenesis. *Dev. Biol.* **302,** 356–366 (2007).

231. Díaz-Alonso, J. *et al.* CB1 Cannabinoid Receptor-Dependent Activation of mTORC1/Pax6 Signaling Drives Tbr2 Expression and Basal Progenitor Expansion in the Developing Mouse Cortex. *Cereb. Cortex* (2014). doi:10.1093/cercor/bhu039

232. Jiang, W. *et al.* Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. *J. Clin. Invest.* **115,** 3104–16 (2005).

233. Trazzi, S., Steger, M., Mitrugno, V. M., Bartesaghi, R. & Ciani, E. CB1 cannabinoid receptors increase neuronal precursor proliferation through AKT/glycogen synthase kinase-3beta/beta-catenin signaling. *J. Biol. Chem.* **285,** 10098–109 (2010).

234. Aguado, T. *et al.* The endocannabinoid system promotes astroglial differentiation by acting on neural progenitor cells. *J. Neurosci.* **26,** 1551–61 (2006).

235. Gomez, O. *et al.* The constitutive production of the endocannabinoid 2-arachidonoylglycerol participates in oligodendrocyte differentiation. *Glia* **58,** 1913–27 (2010).

236. Wolf, S. A. *et al.* Cannabinoid receptor CB1 mediates baseline and activity-induced survival of new neurons in adult hippocampal neurogenesis. *Cell Commun. Signal.* **8,** 12 (2010).

237. Abboussi, O., Tazi, A., Paizanis, E. & El Ganouni, S. Chronic exposure to WIN55,212-2 affects more potently spatial learning and memory in adolescents than in adult rats via a negative action on dorsal hippocampal neurogenesis. *Pharmacol. Biochem. Behav.* **120,** 95–102 (2014).

238. Marchalant, Y., Brothers, H. M. & Wenk, G. L. Cannabinoid agonist WIN-55,212-2 partially restores neurogenesis in the aged rat brain. *Mol. Psychiatry* **14,** 1068–9 (2009).