New perspectives on the role of the neurosteroid pregnenolone as an endogenous regulator of type-1 cannabinoid receptor (CB1R) activity and function

Pregnenolone : endogenous allosteric signaling specific inhibitor of CB1R

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Abstract

Pregnenolone is a steroid with specific characteristics, being the first steroid synthesized from cholesterol at all sites of steroidogenesis, including the brain. For many years, and even currently, pregnenolone was defined as an inactive precursor of all steroids, as no specific target had been discovered. But, over the last decade, it has become a steroid of interest since it has been recognized as being a biomarker for brain-related disorders through the development of metabolomic approaches and advanced analytical methods. In addition, physiological roles for pregnenolone emerged when specific targets were discovered.

In this review, we will highlight the discovery of the selective interaction of pregnenolone with the type-1 cannabinoid receptor (CB1R). After describing the specific characteristic of CB1Rs, we will discuss the newly discovered mechanisms of their regulation by pregnenolone. In particular, we will describe the action of pregnenolone as a negative allosteric modulator (NAM) and a specific signaling inhibitor of the CB1R. These particular characteristics of pregnenolone provide a great strategic opportunity for therapeutic development in CB1-related

disorders. Finally, we will outline new perspectives using innovative genetic tools for the discovery of original regulatory mechanisms of pregnenolone on CB1-related functions.

Key words: Pregnenolone, Type-1 cannabinoid receptor (CB1R), GPCR, Allosteric regulation, Biased signaling, Neurosteroids.

Introduction

Pregnenolone is defined as the "mother hormone" being the precursor of all the so-called steroid hormones, including progesterone and hydroxylated metabolites (such as allopregnanolone), dehydroepiandrosterone (DHEA), corticoids and the sex hormones, androgens and estrogens (Figure 1). The biosynthesis of specific steroids matches the repertoire of expressed steroidogenic enzymes in a steroidogenic tissue or cell[1].

De novo synthesis of pregnenolone from cholesterol, and its downstream steroid hormones, is a typical feature of all steroidogenic sources, including the classical steroidogenic tissues, such as the gonads, adrenals and placenta. However, other tissues can also be classified as steroidogenic tissues since they express the enzyme (CYP11A1) at least, which initiates steroid synthesis by converting cholesterol to pregnenolone. This is the case for adipocytes[2,3], gastro-intestinal tissue[4,5], retina[6], and skin with subcutis[7]. Furthermore, it has been well documented that the brain is a steroidogenic tissue, for which the concept of neurosteroids has emerged as one of the most innovative and pioneering in the field of neuroendocrinology.

The initial discovery of the neurosteroid concept originated in the detection of steroids in the rodent brain with no peripheral sources of steroids[8,9]. This concept was further validated by studies on the expression of steroidogenic enzymes for synthesis and metabolism, and on neurosteroid content in brain tissues and cells. In this context, *de novo* steroid synthesis in the brain has been reported to be well conserved across species, from amphibians to mammals, including humans[10–13]. The biosynthesis of steroid hormones requires the transfer of cholesterol from multiple sources to the inner mitochondrial membrane, where steroidogenesis begins with the conversion of cholesterol to pregnenolone. The brain can regulate the amount of cholesterol by *de novo* synthesis and express steroidogenic enzymes, confirming that it is an independent steroidogenic organ[14]. In addition, the mitochondria in the brain, as in the other steroidogenic cells, the adrenals, gonads and placenta, are important delivery sites for intracellular cholesterol and cholesterol transport to the mitochondrial inner membrane which is the rate-limiting step for steroid synthesis, with the production of pregnenolone being critical for the subsequent production of all steroid hormones[15,16].

It has been suggested that steroids act as neuromodulators to regulate brain-related functions[17,18]. Further challenges lie ahead in proving these regulations, such as detecting

neurosteroids in discrete brain areas linked to brain function (for review[19]), as well as fully characterizing steroid action on specific receptor functions in the brain (for review[20]). In particular, neurosteroids could bind as neuromodulators to G-protein-coupled receptors (GPCRs), which are most commonly involved in neuromodulation and constitute the largest, most ubiquitous family of membrane receptors. Here, we reviewed recent findings on the discovery of pregnenolone function as a negative allosteric modulator of the type-1 cannabinoid receptor (CB1R), the major GPCR in the mammalian brain, and the subsequent regulation of the homeostasis of the endocannabinoid system (ECS), providing a new translational potential for pregnenolone[21].

Pregnenolone: a neurosteroid with unique characteristics

Neurosteroids synthesis: an overview

Mitochondrial production of pregnenolone from cholesterol

Cholesterol, intercalated between the phospholipid molecules of the lipid membrane is an essential and tightly regulated constituent of membranes maintaining membrane fluidity. Cholesterol surplus to the complexing capacity of the membrane lipids is the "active" or "free" cholesterol, which can then move to intracellular membranes, restoring plasma membrane cholesterol to its resting level. The pool of "active" or "free" cholesterol constitutes the substrate for steroidogenesis[22].

In order to initiate and sustain steroidogenesis, a constant supply of cholesterol must be available within the cell and it has to be delivered to the cleavage site in the inner mitochondrial membrane. The cholesterol used in steroidogenic cells, such as adrenals and gonads, derives from a number of sources including *de novo* synthesis of cellular cholesterol, lipoprotein-derived cholesteryl esters, and hydrolysis of cholesteryl esters stored in lipid droplets, free cholesterol being produced from cholesterol esters by hormone-sensitive lipase (HSL), encoded by the *LIPE* gene (for review[7,14]). Brain cholesterol, however, comes from local synthesis in neural and glial cells since the blood-brain barrier prevents the entry of circulating lipoproteins carrying cholesterol[23].

Two transport processes can occur for intracellular cholesterol supplies. First, mobilization of cholesterol from cellular stores such as lipid droplets or other cellular membranes to the outer mitochondrial membrane and second, the transfer of this cholesterol from the outer to the inner mitochondrial membrane[24]. The factors and processes responsible for the mobilization of cholesterol in steroidogenic cells to the outer mitochondrial membrane are thought to involve changes in cellular architecture and putative transport proteins. The mechanisms by which cholesterol is transported to the mitochondrial outer and inner membranes may involve the steroidogenic acute regulatory (StAR) protein in conjunction with

the translocator protein (18-kDa) TSPO (formerly known as peripheral benzodiazepine receptor) together with TSPO-associated proteins[25]. StAR is widely distributed in specific populations of neurons and glial cells. StAR expression in the brain has been found in pyramidal, granule cells and in the dentate gyrus of the hippocampus. A much broader distribution of StAR-positive cells was detected with neuronal and glial staining in the striatum, cerebellum, pons and thalamus. StAR protein was also detected, to a lesser extent, in neurons in the preoptic area and the arcuate nucleus of the hypothalamus[14]. Moreover, the coexpression of StAR and the cholesterol side-chain cleavage enzyme, P450scc encoded by the *CYP11A1* gene, has been established in neuronal subpopulations in mouse and human brains[26]. Since StAR-mediated transport seems to be low under basal conditions, it was proposed that plasma membrane cholesterol may also be transported via START (StAR-related lipid transfer) proteins that contain a lipid-binding domain homologous to the C terminus of StAR[27]. TSPO is abundant in all steroidogenic cells and it has been shown that TSPO drug ligands stimulate the formation of steroids[25].

Although these proteins in the mitochondrial membrane play a key role in neurosteroidogenesis, regulatory mechanisms underlying the neurosteroid biosynthesis in the brain remain unclear.

Neurosteroidogenesis enzymes

Evidence has emerged of the existence of steroid synthesis mechanisms in the brain that are similar to, but independent of, those present in peripheral tissues. Over time, a broader distribution of the enzymatic machinery for steroidogenesis has been identified, providing evidence for local, paracrine actions of brain-derived steroids in brain tissues[17,28].

Many of the enzymes necessary for steroidogenesis occur in brain neurons and glia cells in the central and peripheral nervous systems, with astrocytes being the most active steroidogenic cells in the brain [29–32]. 21 carbon pregnenolone is produced from 27 carbon cholesterol that is converted to 20α, 22 (α or β)-dihydroxycholesterol through two NADPH-dependent hydroxylases. Then, the cytochrome side chain cleavage enzyme P450scc, or CYP11A1 enzyme, catalyzes the C20-C22 bond-cleavage in the 20, 22-hydroxylated cholesterol, leading to pregnenolone. Brain astrocytes and neurons express the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) or CYP11A1 enzyme, which converts cholesterol to pregnenolone[33,34]. In particular, P450scc has been reported within the hippocampal neurons of rats[35]. Pregnenolone can then be processed through multiple metabolic pathways, depending on the demands and needs of the cells. First, pregnenolone can be transformed either to progesterone or to 17-hydroxy pregnenolone and dehydroepiandrosterone (DHEA). Pregnenolone and DHEA, may be metabolized into 7α-hydroxylated derivatives[36,37]. Another alternative is sulfation of the C3 hydroxyl group of

pregnenolone into 3β-sulfated derivative by sulfotransferase enzymes in the rodent and human brain[38]. However, the presence of sulfated pregnenolone, which has been reported in postmortem human brain tissue, is a matter of debate in the rodent brain[39]. It is unlikely that the sulfated form of pregnenolone crosses the blood-brain barrier (BBB), which would require the presence of transporter proteins. Moreover, although pregnenolone sulfate could be uptaken across the BBB in rodents, it would be rapidly metabolized in favor of free pregnenolone[40], which is concordant with the low levels of pregnenolone sulfate measured in rodents brain with highly sensitive mass spectrometry methods[41–44]. The 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme required for the conversion of pregnenolone to progesterone is expressed in the rat and human brain[45–48]. Two enzymes, 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) or 3α-hydroxysteroid oxido-reductase (3α-HSOR) that convert progesterone to its active metabolite, allopregnanolone were then localized in principal glutamatergic output neurons in the cortex, hippocampus, and amygdala of mice, and in the human brain[29,49]. The conversion in the brain of pregnenolone into DHEA by 17-hydroxylase/17,20 lyase (P450c17) is challenging since no expression of P450c17 has been detected in rat brain glia[50], but adult rat brain, and neurons and astrocytes in culture have been shown to convert pregnenolone to DHEA (for review[39]). In the human brain, postmortem studies report that P450c17 was not expressed in the temporal lobe and limbic system of the adult human[51], but was present in the developing human fetal nervous system, suggesting that DHEA may be synthesized locally[52]. Finally, DHEA can be converted, in the rat and human brain, into androstenedione and then into testosterone, which can be metabolized into estradiol by aromatase[49,53,54]. Similarly, evidence has shown that estradiol can be synthesized from pregnenolone in the rat hippocampus[55].

In summary, the tissue-specific expression of steroidogenic enzymes allows the modulation of active steroid levels locally, thus measuring local steroid concentrations rather than the circulating levels would seem to be a more accurate indicator of the steroid action within a specific tissue.

Local measurement of neurosteroids and pregnenolone

Steroid metabolome approaches have recently been promoted with the development of state-of-the-art quantification methods. Metabolomics can be defined as the study of the global metabolite profile in a system (cell, tissue, or organism) and its application to research into mental disorders has become prominent in recent years[56]. In metabolomic research, one of the most widely used analytical techniques is mass spectrometry (MS), which focuses on identifying selected metabolites known to be involved in a particular metabolic pathway, such as steroid metabolites[57].

Validation of chromatography coupled with MS methods have been successful in the simultaneous quantification of steroid metabolites in different biological matrices, including plasma, serum, saliva, urine, hair, cerebrospinal fluid and brain regions in animals and/or humans. The development of these methods has been extensively published and reviewed (see as recent examples among others[58–67]). Local measurement of steroid metabolites in human fluids and in specific areas of the rodent brain has helped to uncover the role of some of them in physiological functions and brain-related diseases. The comparison between local brain and circulating concentrations further emphasizes the concept of neurosteroids.

Pregnenolone was detected in human post-mortem brain tissue at considerably higher concentrations than those typically observed in serum or plasma[68,69]. Moreover, in response to stress challenges, higher levels of brain pregnenolone than those in the blood of adult rodents have been measured using gas chromatography coupled with MS[70,71]. Similarly, acute administration of drugs - including cocaine, amphetamine, alcohol, nicotine and the psychoactive component of cannabis, Δ^9-tetrahydrocannabinol - induced a specific increase in pregnenolone in brain areas to a much greater extent than in rodent plasma[72]. Furthermore, alterations in pregnenolone concentration related to pathological aspects of the central nervous system (CNS) have been reported in animals and humans (see as reviews[73,74]). Briefly, in humans, increased levels of postmortem pregnenolone have been reported in brain regions of patients with Alzheimer's disease compared to non-cognitively impaired control subjects[69,75], as well as in subjects with schizophrenia and bipolar disorder compared to control subjects[68]. In addition, an increase in pregnenolone levels, associated with a decrease in progesterone metabolites, was observed in cerebrospinal fluid (CSF) and plasma of male subjects with multiple sclerosis, an inflammatory and demyelinating disease of the CNS with relevant neurodegenerative aspects[76]. In contrast, decreased pregnenolone has been found in the CSF of patients suffering from anxiety and depressive disorders[77] or postmenstrual syndrome[78], and in the serum of subjects diagnosed with schizophrenia[79,80].

In terms of animal research, although many studies have reported neurosteroid alterations under various circumstances involving stress and drug challenges, as well as in relation to cognitive and/or anxiety/depression states[74], few studies have focused on pregnenolone. One of the main reasons for this lack of attention to pregnenolone is our limited knowledge of its cellular and molecular targets.

Cellular and molecular targets of pregnenolone

Pregnenolone is inactive in modulating the ionotropic receptors, $GABA_A$ and NMDA receptors that are the classical targets of neurosteroids, such as allopregnanolone and the sulfated forms of pregnenolone and DHEA[81–85] (Table 1).

Pregnenolone, in contrast to its sulfate derivative, has been described as being inactive or as having a very low affinity on sigma-1 (σ1) receptor[86,87]. σ1 receptors are expressed in many organs including the nervous system, and located in the specific microdomains of the endoplasmic reticulum (ER) called mitochondrial associated membranes (MAM)[88]. σ1 receptor can influence synaptic functions through modulation of NMDA receptor activity and can remove the negative-regulation of NDMARs by CB1R[89]. Moreover, pregnenolone, along with DHEA, can interact with the microtubule associated proteins (MAPs), in particular MAP2 and CLIP-170[90,91]. Pregnenolone or DHEA are able to bind, with affinity in the nanomolar range, with the amino-terminal region of MAP2 protein, which stimulates MAP2-driven microtubule assembly. Finally, a novel mechanism of action of pregnenolone involving the GPCR, CB1R, has recently been demonstrated[72]. In cell models expressing human CB1R (hCB1R), pregnenolone (from 10 nM to 1 µM) inhibited the increase in P-Erk1/2^{MAPK} and the decrease in cellular and mitochondrial respiration induced by the CB1 agonist Δ^9-THC. By using the Forced-Biased Metropolis Monte Carlo simulated annealing program (MMC)[92], a potential binding pocket for pregnenolone was found in the lipid facing TMH1/TMH7/Hx8 region of the CB1R. This binding pocket was confirmed, since pregnenolone lost its inhibitory effects on THC-induced decrease in cellular respiration in cells transfected with a mutant hCB1R that contained a point mutation that forbid the binding of the ketone end of pregnenolone to the CB1R[72].

CB1R activity and function

Brief description of the endocannabinoid system (ECS)

The discovery of the principal psychoactive constituent of *Cannabis sativa*, Δ^9-tetrahydrocannabinol (Δ^9-THC)[93] in the mid-60s led to the identification of the CB1R. Although it was initially believed that Δ^9-THC worked via nonspecific mechanisms, this assumption was refuted by the discovery of transmembrane proteins able to bind cannabinoids and therefore called "cannabinoid receptors" [94]. The CB1R was first identified in the rat brain and is dominantly expressed in the central nervous system[94,95], whereas the type-2 cannabinoid receptor (CB2R) was identified at the periphery where it is contained in immune cells and tissues[96,97].

Together, CB1R and CB2R belong to the family of G-protein coupled receptors (GPCRs) and are activated by endogenous lipid compounds, endocannabinoids (eCBs), of which the two best described are N-arachidonoylethanolamine (AEA, Anandamide)[98] and 2-Arachidonoylglycerol (2-AG)[99,100]. The ECS, formed by eCBs, their synthesis and degradation enzymes, and target receptors, is a signaling system involved in the fine-tuning of many physiological functions. Therefore, deregulation of eCBs-mediated signaling may contribute to the etiology of disorders such as neuropsychiatric and metabolic disorders[101–104]. In this

context, unraveling the endogenous regulatory mechanisms that help to maintain the physiological activity of the ECS is highly relevant for the development of promising therapeutic approaches.

CB1R distribution

CB1R is considered the most abundant GPCR in the mammalian brain. Its distribution across brain regions is highly heterogeneous and parallels the well-known effects of cannabinoids on motor functions, cognition, emotional behaviors, and reward processing[97]. The highest CB1R expression levels are observed in the basal ganglia, the cerebellum, and corticolimbic areas. Lower yet physiologically relevant CB1R levels are also detected in the thalamus, the hypothalamus, the brainstem, and the spinal cord[105–109].

Central CB1Rs are primarily contained in neurons[110], mainly in cortical GABAergic interneurons and cortical glutamatergic neurons[111]. Nevertheless, CB1R is associated with other neurotransmitter systems (e.g. serotoninergic, cholinergic, noradrenergic, and dopaminergic systems)[110,112,113] and non-neuronal cell types (astrocytes, oligodendrocytes, microglia)[114–116]. In addition to being a "central receptor", CB1R is also largely expressed in peripheral tissues and organs such as the terminals of sensory nerves, the gastro-intestinal tract, the pancreas, the muscles, the liver, and the adipose tissue[117–121].

Besides its systemic and cellular localization, CB1R is also distributed in several subcellular compartments where it plays distinct roles. Central CB1Rs are mainly detected in presynaptic terminals where they control the retrograde suppression of neurotransmission and ensure the excitatory/inhibitory balance within the brain. Briefly, eCBs are synthesized *de novo* in postsynaptic cells upon intracellular Ca2+ increase and activate presynaptic CB1Rs leading to the transient or long-lasting suppression of neurotransmitter release (*i.e.* GABA or Glutamate) [122].

Several lines of evidence indicate that CB1Rs are not solely involved in retrograde signaling. It has been reported that CB1Rs are present in endosomal compartments in the context of internalization and axonal trafficking and, more recently, at the outer mitochondrial membrane[113,123]. Indeed, mitochondrial CB1Rs (mtCB1Rs) can inhibit the soluble adenylate cyclase and the OXPHOS mitochondrial respiratory chain, thereby regulating bioenergetic processes within the brain[123]. While the discovery of mtCB1Rs provides new insights into the behavioral effects of cannabinoids, it will also undoubtedly shed light on new mechanisms of action of eCBs-mediated signaling at the subcellular level[124,125] (Figure 2A).

Endocannabinoids: endogenous ligands of CB1R

CB1R are activated by endogenous lipid messengers called eCBs, which are typically produced *de novo* upon neuronal activation rather than stored in secretory vesicles, and can

signal in a retrograde manner (*i.e.,* from postsynaptic neurons to presynaptic neurons)[122]. Their biosynthesis occurs through the cleavage of phospholipid precursors, and their lipid nature allows them to move quickly within biological membranes. However, their diffusion within aqueous environments (e.g., cytosol and extracellular space) requires efficient transport systems to activate distant cannabinoid receptors and for their intracellular inactivation[126].

The cellular reuptake of endocannabinoids is also facilitated by uncharacterized eCBs membrane transporters (EMT) that participate in the termination of eCBs-mediated signaling[127,128]. The two main eCBs are AEA and 2-AG, which are partial and full agonists of CB1Rs, respectively[129]. Although they are both derived from arachidonic acid, AEA and 2-AG display distinct biosynthesis and degradation pathways[126].

AEA formation in neurons is initiated by the enzyme *N*-acyltransferase (NAT) to yield *N*-arachidonoyl-phosphatidylethanolamine (NAPE) from phospholipid precursors and enriches membrane pools thereof. NAPE is then hydrolyzed into AEA by a NAPE-specific phospholipase D (NAPE-PLD)[130]. The inactivation of AEA is primarily catalyzed by the enzyme fatty acid amine hydrolase (FAAH), located on intracellular membranes of postsynaptic neurons. However, multiple oxidation pathways may participate in the intracellular deactivation of AEA[131–133].

2-AG is the most abundant eCB in the brain and an intermediate in lipid metabolism[126,134]. It is primarily involved in the retrograde inhibition of neurotransmission and is synthesized upon depolarization of postsynaptic neurons. Specifically, phosphatidylinositol (PI) is hydrolyzed by a phospholipase C (PLC) into diacylglycerol (DAG). The hydrolysis of DAG by diacylglycerol lipase (DAGL) then leads to the formation of 2-AG[126,135–137]. 2-AG travels to presynaptic neurons retrogradely and is hydrolyzed by the monoacylglycerol lipase (MAGL). While MAGL is primarily responsible for the termination of 2AG signaling, the α/β-hydrolase domain 6 (ABHD6) and domain 12 (ABHD12) enzymes can degrade 2AG to avoid its excessive accumulation in postsynaptic neurons and the extracellular space, respectively[138–140]. As for AEA, alternative pathways may participate in the inactivation of 2AG[141].

CB1R activity

The CB1R displays both basal and agonist-induced signaling and internalization. Basal activity of CB1Rs has been mainly attributed to constitutive (agonist-independent) receptor activity, however, studies in neurons have suggested a role for postsynaptic endocannabinoid (eCB) release in the persistent activity of presynaptic CB1R[142]. Moreover, the tonic activity of ECS may arise from the endogenous release of eCBs onto CB1Rs but also from the presence of CB1Rs in a constitutively active state[143]. In addition, CB1 activity is mediated by ligand-receptor interaction and the specificity of the CB1 GPCR which involves biased signaling will

be addressed, as well as the different functions induced by ligand binding to CB1R orthosteric or allosteric sites.

Constitutive activity

Distinguishing between endogenous ligand activity and the constitutive activity of a receptor in an intact biological system is quite challenging[144,145]. However, it is generally accepted that the CB1 GPCR behaves constitutively in most systems[142,143,146,147]. The ability of the GPCR to mediate a signal response in the absence of an agonist will define its constitutive activity[148].

A constitutive activity is based on a two-state model of a receptor, including two interchangeable conformations, a constitutively active "on" state and a constitutively inactive "off" state. In this model, an agonist will increase the number of receptors in the active state, while an inverse agonist will shift the equilibrium from the active to the inactive state, and a neutral antagonist will not affect any state in the model[143]. CB1R constitutive activity was first described by comparing 'basal' G protein activation and G protein regulated signal transduction in cells expressing recombinant CB1Rs compared with CB1R-deficient host cells[149–151].

Then, further identification of *in vivo* and *in vitro* effects of several inverse agonists (including Rimonabant or SR141716A, AM251, AM281 or LY320135) on CB1Rs confirmed the constitutive activity for CB1Rs[143]. However, the interpretation of these studies must take into consideration the local production of eCBs, which may exert autocrine and paracrine stimulation of CB1Rs. Thus, the term 'basal endocannabinoid system tone' or signal transduction 'in the absence of exogenously applied agonists' has been put forward as being more accurate than 'constitutive activity'[148].

Interestingly, the development of neutral competitive CB1R antagonists (such as AM4113, NESS0327, LH21, O-2050) and the comparison of their effects with those of inverse agonists, should allow for the distinction between the tonic activity of endocannabinoid release at CB1Rs and the presence of CB1Rs in a constitutively active state. In this context, it has been revealed that constitutive CB1 activity has a pivotal function in the tonic control of hippocampal GABA release[152]. Moreover, constitutive CB1 activity also regulates GABAergic and glutamatergic neurotransmission in the ventral tegmental area and basolateral amygdala of mouse brain, suggesting that this constitutive activity is significantly involved in anxiety and motivation-related functions such as for reward[153]. These findings indicate that constitutive CB1 activity does not just occur in artificial systems, but can also regulate neurotransmission in native brain tissue.

In addition, it has been demonstrated that the effect of inverse agonists, such as rimonabant (SR141716A) can be attributed to a mechanism of action involving the suppression of constitutive CB1R activity. Thus, the negative modulation of the constitutive CB1 activity is

certainly linked to the serious adverse psychiatric effects (anxiety, depression and suicidality) associated with inverse agonists like rimonabant[154,155].

It is noteworthy that the constitutive activity of CB1 certainly depends on the experimental models used but also on the expression of the gene coding for the CB1R. Thus, the regional distribution of CB1R expression explains the great diversity of constitutive responses.

Biased signaling

Most GPCRs have long been thought to couple with multiple Gα proteins (e.g., Gαi/o. Gαs, Gαq, or Gα12/13) and β-arrestins, thereby triggering multiple intracellular signaling pathways in parallel and/or sequentially through different transduction mechanisms. Accumulated evidence has now established that distinct GPCR agonists have the potential to selectively activate one specific signaling cascade over another, a phenomenon termed "biased agonism" or "functional selectivity," and trigger distinct physiological responses[156].

GPCRs interact with a host of elements in their environment that modify the specificity, selectivity, and time course of signaling. So, the Ligand-Receptor-Environment complex will dictate the signaling capacity of the system[157]. The expression of each actor in this complex will engage a single receptor to differentially activate multiple signaling pathways in a brain structure-selective manner. In this paradigm, the agonist ligands will favor a conformation of receptor that will be engaged in specific signaling[158]. This functional selectivity or ligand bias has clearly been demonstrated for CB1 signaling where the CB1R can adopt multiple conformations depending on agonist occupancy. One of the first series of studies revealing this ligand bias identified a ligand preferentially coupling to Gα proteins at CB1R in Sf9 insect cell membrane preparations, demonstrating agonist-selective G protein signaling by the CB1R[159]. Further investigations demonstrated the existence of functional selectivity *in vitro* as well as *in vivo*, by showing that distinct cannabinoid agonists can display different abilities to regulate diverse components of dopamine neurotransmission system *in vivo*[160]. Interestingly, the expression level of the CB1R has recently been identified as a novel determinant of the signaling outcome, and this effect has been shown to be dependent on the CB1 agonist used and the expression level of the Gαi protein[161].

Apart from G proteins, arrestin proteins are essential components of multiple GPCR signaling cascades involving kinases or phosphatases[162]. CB1Rs can thus recruit β-arrestin-1 (Arrestin-2) and β-arrestin-2 (arrestin-3) for signaling, and the coupling of CB1R to β -arrestin may affect the localization of receptors at the surface, their internalization, recycling, and degradation[163].

Studies of GPCRs and arrestin proteins may present conflicting results due to the different methodological approaches used, and research on structural features associated with

cannabinoid receptor-β-arrestin interactions is limited, although several mutational studies have explored the importance of C-terminal residues for binding of β-arrestins to CB1R[164].

Modulation of CB1 activity via allosteric sites (PAM, NAM)

The study of allosteric modulation is a relatively new concept in GPCR research that emerged in the early 1990s. Two binding sites on the GPCR were then defined for a ligand. First, the orthosteric binding site, which is defined as the primary binding site recognized by the endogenous ligand, and second, the allosteric sites, which are topographically and structurally distinct from the orthosteric sites.

From a mechanistic point of view, allosteric ligands potentiate (positive allosteric modulators; PAM) or inhibit (negative allosteric modulators; NAM) receptor activation by an orthosteric ligand[165]. From a quantitative perspective, allosteric ligands can alter the binding affinity and signaling efficiency of orthosteric ligands, and allow for receptor subtype selectivity, receptor trafficking, and/or even cause signaling in the absence of an orthosteric ligand[166]. The combination of these effects leads to the overall outcome of allosteric modulation on receptor function[167].

Several classes of allosteric modulators, which bind to CB1R allosteric binding sites, have been discovered. CB1R allosteric modulators have been recently and widely reviewed elsewhere[21,167,168]. To date, approximately nine classes of CB1R allosteric modulators have been identified, including the lipoxin A4, ZCZ011, pepcan-12, Org27569, and PSNCBAM-1, and more recently pregnenolone. Among them, several have been endogenously detected such as lipoxin A4 first described as a PAM[169] but also NAM[170], and NAMs pepcan-12[171] and pregnenolone[72] (Figure 2B). Here we will focus more particularly on pregnenolone in modulating CB1R activity and functions.

As previously described, the endogenous neurosteroid pregnenolone can bind to a specific binding pocket on the CB1R[72]. From a mechanistic perspective, pregnenolone did not alter (up to 100 µM) the equilibrium binding of the radiolabeled CB1 receptor agonists [3H]CP55,940 and [3H]WIN 55,212-2, and did not decrease (up to 1 µM) Δ^9-THC-induced cAMP reduction while inhibiting CB1 signaling in a manner that was non-competitive for ERK1/2 phosphorylation[72] (Figure 2C). These effects are consistent with a mechanism of action as a NAM for the CB1R. Furthermore, given that pregnenolone does not have a similar biochemical profile to Org27569 and PSNBCAM-1, namely no increase in agonist binding to CB1, it can be hypothesized that pregnenolone binds in a signaling-selective conformation of a distinct allosteric site[72].

A subsequent study, characterizing pregnenolone action on CB1, found that pregnenolone induced a concentration-dependent decrease in [3H]SR141716A binding, but due to the incomplete displacement of [3H]SR141716A by pregnenolone at the maximum concentration

that could be used in the assay, it was not determined whether pregnenolone acted in a competitive or allosteric manner[172]. Moreover, contrary of the previously described data[72], pregnenolone did not modulate CB1 agonist-mediated ERK1/2 phosphorylation in CHO-hCB1 cells[172]. Furthermore, it was also found that pregnenolone did not attenuate the suppression of depolarization-induced excitation (DSE) induced by 2-AG in autaptic hippocampal neurons[170]. However, in this model, Straiker and colleagues also showed that Lipoxin A4 acted as a NAM on CB1Rs rather than a PAM. Since it was previously reported that Lipoxin A4 exhibited probe-dependence favoring AEA over 2AG[169], it is possible that the above reported discrepancies arise from the probe-dependence of pregnenolone. Finally, although one study has reported the lack of pregnenolone effects on cannabinoid-induced attenuation of GABAergic and glutamatergic transmission *ex vivo*[173], a subsequent survey has shown that pregnenolone (10µM) could modulate CB1-mediated suppression of neurotransmission through the ERK1/2^{MAPK} signaling cascade[174].

The property of pregnenolone as an endogenous NAM of the CB1R was further confirmed through *in vivo* studies. Namely, pregnenolone was able to attenuate a complete spectrum of cannabinergic effects induced by the CB1 agonist Δ^9-THC in mice[72,175].

In conclusion, modulation of CB1R by pregnenolone offers interesting potential for the relief of CB1-related diseases, but the mechanism of action of pregnenolone on CB1 requires further exploration. It should also be noted that pregnenolone binds to a specific CB1R site in the TMH1/TMH7/Hx8 transmembrane region, located in the C-terminal region of the CB1 receptor[72]. In this region it has been proposed that cannabinoid receptor interaction protein 1a (CRIP1a) could interact with CB1R[176] suggesting potential functional interactions of pregnenolone with CRIP1a[21].

CB1R function modulation and therapeutic opportunity

Advantages of biased signaling and allosteric modulation

The insight that G protein- and arrestin-dependent signals can be dissociated using pathway-selective "biased" agonists is gaining importance in the field of drug discovery for GPCRs[157]. This biased signaling represents an interesting therapeutic opportunity to target specific pathways that only bring about the desired effects. Indeed, the basic principle of ligand bias is the selective activation of signaling pathways that mediate therapeutic effects and are free of adverse effects, which relies on the fact that these pathways are distinct [164].

Historically, drug discovery approaches have focused on identifying ligands that can compete with endogenous ligands at the orthosteric sites. However, compared to orthosteric ligands, allosteric ligands present several advantages for clinical research and drug discovery[177]. Allosteric ligands exhibit increased selectivity of receptor subtypes and modulate

specific signaling pathways (biased-signaling) that reduce off-target and on-target side effects from interferences. They also have a maximum ("ceiling") effect, so that increasing the dose does not enhance the allosteric response, thus avoiding overdose. Finally, allosteric modulators of CB1R should induce fine-tuning of CB1 signaling, in conditions where endocannabinoids are produced and released "on demand". Thus, the functional selectivity of CB1Rs might generate a pharmacologically improved therapeutic effect, with reduced on-target adverse effects compared to the orthosteric ligand.

In summary, ligand-mediated biased signaling and allosteric modulation of CB1Rs offer pharmacological approaches that could potentially be used to develop improved CB1 drugs by modulating only therapeutically relevant CB1 signaling pathways[172]. In line with this, the discovery that pregnenolone is a biased endogenous allosteric signaling modulator is highly relevant to CB1-dependent functions and affords new and exciting opportunities for the development of pregnenolone analogues with strong therapeutic benefits.

Pregnenolone function and proof of concept studies

As mentioned previously, the CB1R is widely distributed within the body, thus highlighting its pivotal role as a modulator of physiological functions. Such functions include cognition and memory[178], emotional behaviors[179], energy metabolism[101], motor functions[180], thermoregulation, and pain processing[181]. Despite the wide range of functions involving CB1Rs, one core feature of CB1-mediated signaling is responsiveness to external and internal stimuli. It is generally accepted that CB1 activation functions as an adaptive response to preserve homeostasis[103]. However, repeated challenges (e.g., chronic stress, high calorie diet) may contribute to the deregulation of CB1 activity and the onset and etiology of various disorders[101,102]. Moreover, the acute or chronic disruption of endogenous CB1 signaling by exogenous cannabinoids may produce deleterious effects due to the receptor's over-activation[182].

Our research group first demonstrated that pregnenolone was able to attenuate the complete spectrum of tetrad cannabinergic effects (locomotor suppression, hypothermia, catalepsy, and analgesia) induced by the CB1 agonist Δ^9-THC in mice. Furthermore, pregnenolone was able to normalize the release of dopamine (DA) induced by Δ^9-THC in the nucleus accumbens shell (NAcS) of rats, a feature shared by all drugs of abuse[183], and to reduce motivation for the self-administration of the CB1 agonist WIN 55,512-2 in an operant conditioning paradigm[72]. The action of pregnenolone on CB1Rs was related to a drastic increase in pregnenolone production by CB1 agonists[72], resulting in unforeseen feedback control of CB1 activity in a paracrine/autocrine manner.

It has also been proposed that pregnenolone could compensate for some mechanisms of THC addiction, such as restoring the activity of CB1Rs located on GABAergic interneurons of the

ventral tegmental area (VTA), leading to the normalization of DA release [184]. In particular, the reported alleviating effects on THC-intoxication were specific to pregnenolone and not its downstream steroids[72].

Together, these data highlight the therapeutic potential for pregnenolone against cannabis use disorder (CUD), for which no pharmacological treatment was available[185] until the recent development of C3-17 pregnenolone synthetic analogs, named AEF compounds, by the biotechnology company *Aelis Farma* (WO2012/160006Al; WO2014/083068Al; WO2019/162328Al patents). Specifically, those compounds display a better therapeutic profile than pregnenolone, with increased half-life, no conversion into steroids and good bioavailability and administration *per os*. One of the lead AEF compounds is currently in a phase II clinical trial for CUD indications and shows very promising results (ClinicalTrials.gov; Identifier NCT03717272; Effect of AEF0117 on Subjective Effects of Cannabis in CUD Subjects).

Along with CUD, cannabinoid intoxication has long been associated with the onset of psychosis. In particular, it has been reported that early-life exposure to cannabis is correlated to a greater risk of developing psychotic disorders during adulthood, while cannabinoids can elicit transient psychotic symptoms in healthy subjects[186]. Although the relationship between cannabis and psychosis is still being debated, CB1Rs have emerged as potential targets for antipsychotic drugs. However, the main concerns remain the severe side effects of CB1 pharmacological blockade[187]. In this context, Busquets-Garcia et al. have reported promising effects of pregnenolone against Δ^9-THC -induced psychotic-like behaviors in mice[175]. In particular, they showed that pregnenolone was able to antagonize a wide range of effects elicited at different doses of Δ^9-THC, including impairments in cognitive functions, somatosensory gating, and social interaction[175].

Furthermore, pregnenolone administration is associated with an amelioration of psychosis-related symptoms in humans and murine models of schizophrenia. However, it is not known whether CB1Rs mediate those effects and whether the high doses of pregnenolone used induced an increase in downstream steroids, such as allopregnanolone, which is a potent modulator of $GABA_A$ receptors[74].

More recently, Frau et al. have reported the neuroprotective effects of pregnenolone against the neurological alterations associated with prenatal cannabis exposure (PCE)[188]. In a rat model of PCE, they showed that the adolescent male offspring of dams exposed to Δ^9-THC display increased behavioral sensitivity to Δ^9-THC-induced somatosensory gating alterations. These behavioral changes were causally linked to a dysfunction in the dopaminergic system, producing increased activity of dopaminergic neurons within the VTA and increased DA release in the NAcS in response to Δ^9-THC. Encouragingly, the chronic administration of

pregnenolone normalized the firing properties of dopaminergic neurons, DA release, and somatosensory gating alterations in PCE animals[188]. Furthermore, the pharmacological inhibition of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) did not impair the neuroprotective action of pregnenolone, hence confirming that pregnenolone but not its metabolites were involved in these effects[188].

Conclusions and perspectives

In conclusion, the above-reported studies encourage the idea that the mechanisms of action of pregnenolone on CB1Rs as a negative "signaling-specific" allosteric modulator can be used as a therapeutic means to block THC-induced addiction and psychotic-like states. Moreover, whereas pregnenolone could antagonize THC-induced hyperdopaminergic states, it did not produce hypodopaminergic phenotypes alone nor did it have any *per se* effects at the doses used *in vivo*[72,175,188]. Instead, the neuroprotective effects of pregnenolone appeared to be state-dependent which may confer significant therapeutic advantages such as a safe profile of action, unlike CB1 antagonists[189]. This strengthens the proof of concept for a suitable therapeutic profile of signal-specific inhibitors of excessive CB1 signaling, such as the recently developed synthetic analogs of pregnenolone.

Further studies are needed to determine whether pregnenolone is a suitable therapeutic candidate for other CB1-related pathologies associated with changes in ECS components, such as bipolar disorders, stress-related disorders, attention-deficit/hyperactivity disorders, and eating disorders[101,102] and whether changes in pregnenolone levels might represent suitable biomarkers for such pathologies.

Finally, whereas the aforementioned *in vivo* studies have examined the ability of pregnenolone to counteract the effects of exogenous cannabinoids, we still do not know how pregnenolone and CB1R interact with one another in physiological conditions and how endogenously-produced pregnenolone may help to maintain ECS-mediated homeostasis (Figure 2C-D). In this specific research frame, pharmacological tools such as P450scc and 3β-HSD inhibitors or pregnenolone synthetic analogs may impede the interpretation of data in the long run, as they will likely produce changes in steroid levels and function in non-physiological ranges. Therefore, the development of a mouse model including a mutated CB1R unable to bind pregnenolone, as shown in our *in vitro* model[72], should allow us to elucidate new endogenous mechanisms of CB1 regulation by pregnenolone. Genetic tools are indeed key to unravelling new CB1-mediated functions, such as exploring the in vivo effects of mtCB1 receptors in DN22-CB1 and DN22-CB1-KI mice[124,190]. Targeting the pregnenolone-CB1 binding pocket may represent an exciting opportunity to shed light on new functions for steroids in regulating GPCR activity.

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Table1. Reported molecular targets for pregnenolone

Targets	Acronym	Models	Reported effect (effective concentration)	References
Ionotropic receptors	$GABA_A$	GABA-mediated synaptic responses in rat hippocampal neurons	Inactive	Park-Chung et al., 1999[83]
	NMDA	NMDA-mediated Ca^{++} influx in rat hippocampal neurons	Inactive	Weaver et al., 2000[85]
Ligand-operated protein	σ1	Binding assay in rodent brain tissue	Inactive or low affinity	Monnet & Maurice, 2006; Su et al., 1988[86,87]
Microtubules	MAPs	Culture of rat neurons	Binding to MAP2 and CLIP-170 (30–60 nM)	Murakami et al., 2000 ; Weng et al., 2013[90,91]
GPCR	CB1R	cell lines expressing the human CB1R	Binding to a specific allosteric site of CB1R (10 nm -1 µM)	Vallée et al., 2014[72]

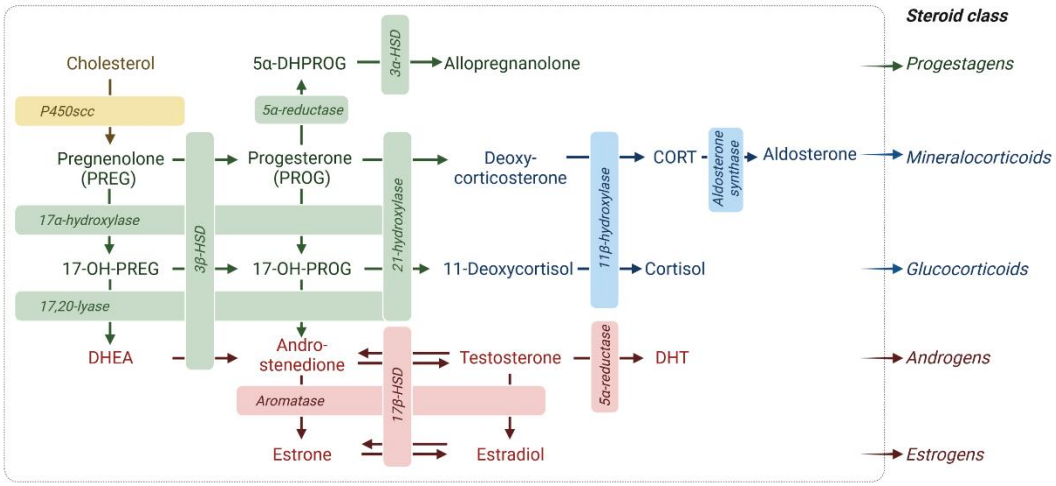
GABA, γ-aminobutyric acid; NMDA, N-methyl-D-aspartate; σ1, sigma-1; MAP, microtubule-associated protein GPCR, G-protein coupled receptor; CB1R, type-1 cannabinoid receptor.

Figure legends

Figure 1. Main steroid synthesis pathways from pregnenolone. Cholesterol is converted into pregnenolone by the cholesterol side-chain cleavage enzyme (P450scc). Pregnenolone then serves as a precursor for the synthesis of other progestagens and other steroids, including mineralocorticoids, glucocorticoids, androgens, and estrogens. Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; 5α-DHPROG, 5α-dihydroprogesterone; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CORT, corticosterone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; PREG, pregnenolone; PROG, progesterone. *Adapted from Hanukoglu, 1992*.

Figure 2. Pregnenolone is an endogenous allosteric modulator of the type-1 cannabinoid receptor (CB1R). (A) Simplified overview of the endocannabinoid system (ECS) in neurons. CB1Rs are present at presynaptic plasma membranes and outer mitochondrial membranes. Presynaptic CB1Rs are primarily involved in the retrograde suppression of neurotransmission. (B) The main endogenous orthosteric ligands of CB1R are AEA and 2-AG, which activate the CB1R via their orthosteric binding site. The endogenous NAMs and PAMs of CB1R can negatively and positively modulate (respectively) CB1 activity via distinct allosteric binding sites. (C) In the presence of high doses of Δ^9-THC, pregnenolone binds to CB1R on a dedicated allosteric pocket and acts as a signal-specific inhibitor of the Erk1/2^{MAPK} pathway, resulting in the blockade of Δ^9-THC-induced toxic outcomes. (D) The role of pregnenolone in regulating the activity of eCBs-mediated CB1 signaling needs to be addressed to depict the physiological functions that pregnenolone-CB1 regulation may fulfill. Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; AC, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CB1, type-1 cannabinoid receptor; eCBs, endocannabinoids; Δ^9-THC, Δ^9-tetrahydrocannabinol; Erk1/2, extracellular signal-regulated kinase 1/2 pathway; GPCR, G-protein coupled receptor; mtCB1, mitochondria-associated CB1; NAM, negative allosteric modulator; NTs, neurotransmitters; PAMs, positive allosteric modulators; PREG, pregnenolone; PCE, prenatal cannabis exposure.

589 **Figure 1**



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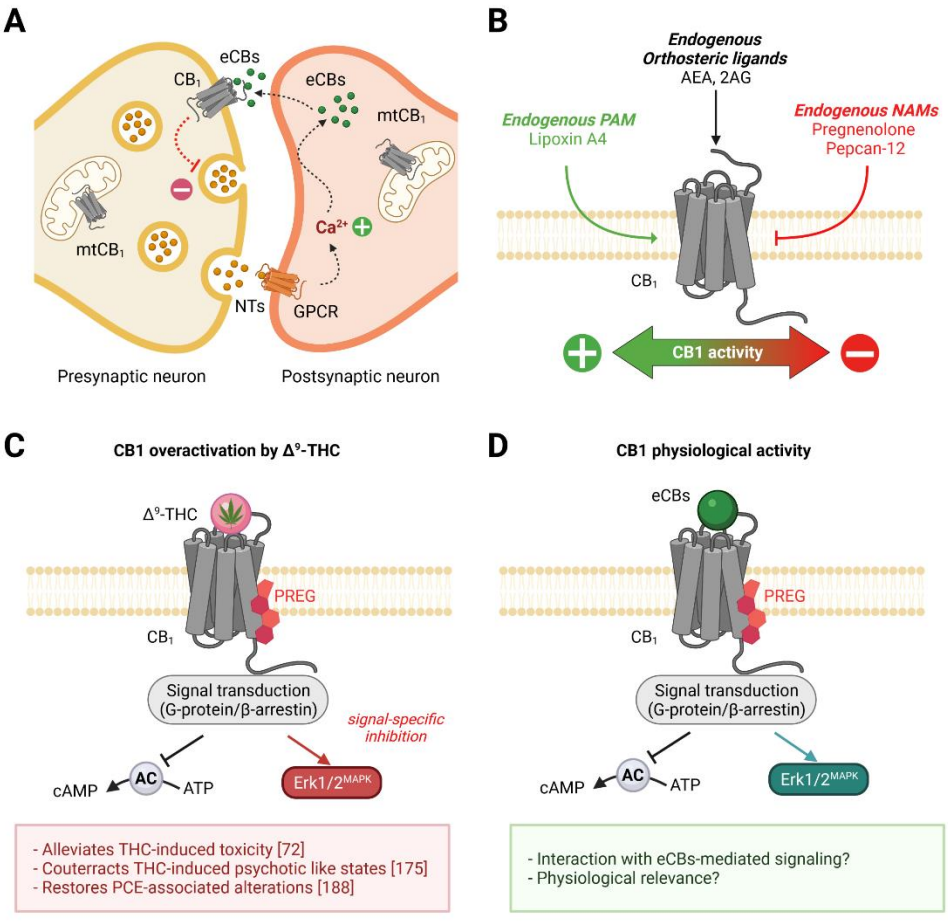
591 **Figure 2**



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