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Crosstalk between neurosteroid and endocannabinoid systems in cannabis addiction

The pregnenolone-CB1R regulatory loop in homeostasis

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Abstract

Steroids and endocannabinoids are part of two modulatory systems and some evidence has shown their interconnections in several functions. Homeostasis is a common steady-state described in the body, which is settled by regulatory systems to counterbalance deregulated or allostatic set points towards an equilibrium. This regulation is of primary significance in the central nervous system to maintain neuronal plasticity and prevent brain-related disorders. In this context, the recent discovery of the shutdown of the endocannabinoid system (ECS)

overload by the neurosteroid pregnenolone has highlighted new endogenous mechanisms of ECS regulation related to cannabis-induced intoxication. These mechanisms involve a regulatory loop mediated by overactivation of the central type-1 cannabinoid receptor (CB1R), which triggers the production of its own regulator, pregnenolone. Therefore, this highlights a new process of regulation of steroidogenesis in the brain. Pregnenolone, long considered an inactive precursor of neurosteroids, can then act as an endogenous negative allosteric modulator (NAM) of CB1R. The present review aims not to extend our knowledge of ECS in cannabis addictive functions, which has been widely examined but to provide a new framework with a novel endogenous mechanism of ECS regulation involving the neurosteroid pregnenolone. In addition, this new endogenous regulatory loop could provide a relevant therapeutic model in the current context of increasing recreational and medical use of cannabis.

Keywords: Neurosteroids, Pregnenolone, Endocannabinoid system (ECS), Type-1 cannabinoid receptor (CB1R), Endocannabinoids (eCBs), Neuromodulation, Cannabis addiction

1. INTRODUCTION

Neurosteroids have been studied for over the past 20 years (reviewed in Balthazart et al., 2018[1]; Tomaselli & Vallée, 2019[2]; Vallée 2014[3]) and were discovered in the 80' as steroids synthesized by the brain for the brain'[4]. One of their main characteristics is their *de novo* production on-demand in the brain, which is common to all species, all the way from amphibians to mammals, up to and including humans[5–9]. The first step, which can be defined as the rate-limiting step in their synthesis, involves the transport of cholesterol into the mitochondria-mediated by the action of StAR (steroidogenic acute regulatory protein) and TSPO (a translocator protein of 18 kDa)[10–12], and its conversion to pregnenolone catalyzed by CYP11A1 (a P450 side-chain cleaving enzyme, *P450scc*)[13–15]. Subsequently, several pathways of pregnenolone metabolism, firstly in the endoplasmic reticulum, trigger the synthesis of steroid families, including progestagens, corticoids, androgens, and estrogens (reviewed in Mellon & Griffin, 2002[16]; Raux et al., 2022[17]). Neurosteroid production occurs primarily in neurons and astrocytes in several brain areas, as first indicated by P450scc regionalization for pregnenolone synthesis[16,18,19]. In addition to the localization of their production, some neurosteroids have aroused scientific interest for their action, not on their receptors, but on neuromodulator receptors, mainly γ-aminobutyric acid $(GABA)_A$ and the N-

methyl-D aspartate (NMDA) receptors[20–22], which has conferred them a neuromodulator status and thus a crucial role in the regulation of brain functions[18,23,24].

Another family of lipid neuromodulators that has gained the interest of the scientific community over the last 20 years arises from the discovery that the brain can produce endogenous analogs of phytocannabinoids, a class of naturally occurring molecules derived from the *Cannabis Sativa* plant, of which Δ^9-Tetrahydrocannabinol (THC) is the major psychoactive component[25]. Those messengers, named "Endocannabinoids" (short for endogenous cannabinoids), (eCBs), were identified in the 90' as endogenous ligands of the two main cannabinoid receptors, the type 1 (CB1R) and type 2 (CB2R) cannabinoid receptors[26], formerly described for binding exogenous cannabinoids, such as THC[25].

Then, the discovery of the on-demand synthesis of eCBs in the postsynaptic membrane and their action on retrograde synaptic signaling led to a new perspective on how messengers can modulate neuronal activity[27] and opened up new fields of cannabinoid research, from the functioning of synaptic plasticity to cognition and motivation functions. The two main studied eCBs include N-Arachidonoylethanolamide (anandamide, AEA) and 2-Arachidonoylglycerol (2-AG), which are synthesized from phospholipid precursors and are rapidly degraded into the cell by distinct enzymes, the fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively[28,29]. The endocannabinoid system (ECS) thus includes the CB1R and CB2R, the eCBs, as well as their synthesis and degradation enzymes. ECS function is a phylogenetically widespread feature in vertebrates[30] and has been described in specific regions of the rodent brain and in different (sub)cellular sites, which accounts for its broad involvement in brain function (reviewed in Busquets-Garcia et al., 2018[31]).

Both the neurosteroid and endocannabinoid systems are involved in brain-modulating processes, and their imbalance can lead to brain dysfunction. In the present study, we address an unlikely and unexpected interaction between neurosteroids and the endocannabinoid system, focusing on pregnenolone and CB1R function in cannabis addictive-like behavior.

2. NEUROSTEROIDS & ENDOCANNABINOIDS AS NEUROMODULATORS: NEW PERSPECTIVES

The connection between the external environment and the internal state sets up a dynamic modulation orchestrated by chemical substances called neuromodulators. Neuromodulators can be defined as endogenous molecules or neuronal messengers that modulate neuronal activity upon stimulation. Neuromodulators have been observed in several species, from the nematode *Caenorhabditis Elegans* to mammals[32,33]. Both intrinsic and extrinsic types of neuromodulation have been described[34]. Intrinsic neuromodulation involves the same neuronal circuit that is both the origin of the modulator release and the target of neuromodulator

action, while extrinsic neuromodulation engages separate systems implying neuroactive substances that can be transported into the extracellular compartment. Neuromodulators, therefore, act either in a local autocrine/paracrine mode or in a hormonal mode on neuronal targets distant from their release site[35].

Two examples of well-described neuromodulatory systems are the GABAergic and glutamatergic neuronal systems, which underlie the excitability properties of neurons and control the cellular inhibitory and excitatory balance, respectively (reviewed in Sears & Hewett, 2021[36]). These two systems work together to shape adaptive responses to external stimuli and are involved in the maintenance of physiological balance. Hence, deregulation of this equilibrium can contribute to the pathophysiology of brain disorders.

In this context, some neurosteroids are referred to as paracrine and/or autocrine neuromodulators[37] and they have been shown to modulate $GABA_A$ and NMDA receptor activity with a similar affinity to that of the endogenous neurotransmitter ligands GABA and glutamate, respectively (reviewed in Ratner et al., 2019[38]). This is the case for the sulfated derivative of pregnenolone, but not pregnenolone, which modulates $GABA_A$ and NMDA receptors negatively and positively, respectively[39]. In addition, the progesterone metabolites allopregnanolone and pregnanolone act as positive allosteric modulators of the $GABA_A$ receptor by facilitating the effects of GABA[40].

On the other hand, the neuromodulatory actions of eCBs involve mainly a retrograde signaling mechanism, (i.e. release from the post-synaptic site to act on the pre-synaptic compartment) which can result in the inhibition of the transmitter release at both excitatory and inhibitory synapses. Indeed, the eCBs, by acting on CB1Rs localized on presynaptic GABAergic and glutamatergic neurons[26,41] can modulate synaptic plasticity by fine-tuning the activity of the aforementioned neurons, a process referred to as depolarisation-induced suppression of inhibition (DSI) or excitation (DSE), respectively[27,42–44]. The CB1Rs are among the most abundant G-protein coupled receptors (GPCRs) in the brain[45] and are expressed in the brain to similar protein amounts as $GABA_A$ and NMDA receptors. CB1Rs are highly present in sensory and motor brain areas, including the basal ganglia, substantia nigra, globus pallidus, cerebellum, and hippocampus, which underlies their role in cognition and motivation[45]. In addition, eCBs interact with other neuromodulators, such as dopamine (DA) and serotonin (5-HT) (reviewed in Peters et al., 2021[46]). This interaction can occur through the release of eCBs from DA and 5-HT neurons, which regulates glutamatergic and GABAergic afferents. This has been documented, for example, for the mesolimbic dopaminergic system, in which DA neurons in the ventral tegmental area (VTA) projecting to the nucleus accumbens (NAc) receive GABAergic inputs and 5-HT/Glutamate inputs from the dorsal raphe nucleus (DRN). As a result, eCBs can fine-tune the activity of the mesolimbic DA system by acting on presynaptic

CB1Rs in GABAergic and glutamatergic neurons, thereby modulating excitatory and inhibitory signaling, respectively[47,48]. This illustrates that eCBs play a crucial role in controlling adaptive behaviors and maintaining reward system homeostasis, positioning the ECS as a key modulatory network in the brain (reviewed in De Melo Reis, 2021[49]; Lutz, 2020[50]).

In this neuromodulation framework, the putative interactions between neurosteroids modulating $GABA_A$ and NMDA receptors together with ECS could prove relevant to maintaining the activity of the neuronal networks involved in reward homeostasis. Here, though, we propose the neurosteroid pregnenolone as a key player in the interconnection between steroid and endocannabinoid systems, with the discovery of a regulatory loop between pregnenolone and the CB1R.

3. DISCOVERY AND EVIDENCE OF THE REGULATORY LOOP BETWEEN PREGNENOLONE AND CB1R

3.1 CB1 activation and pregnenolone production

3.1.1 The actors of brain pregnenolone production

General steroid biosynthesis derives from about 2% of the daily cholesterol pool in humans, acquired from the diet from *de novo* synthesis from acetyl-CoA[51]. In the brain, it has been proposed that the source of cholesterol rather originates from *de novo* synthesis, as the blood-brain barrier is not permeable to cholesterol lipoproteins[52]. One of the rate-limiting factors for steroidogenesis is the available amount of cholesterol in astrocytes and neurons. First, the free cholesterol pool in the cytosol is related to the action of the hormone-sensitive lipase/cholesteryl esterase (HSL), encoded by the LIPE gene, which is responsible for hydrolyzing cholesteryl esters located in lipid droplets[53]. HSL protein and its enzymatic activity have been detected in the mouse brain at neuronal synapses[54]. It was then described that the proteins StAR and TSPO may be respectively involved in the delivery of cholesterol and its transport from the outer membrane to the inner membrane of mitochondria (reviewed in Selvaraj et al., 2018[55]). StAR mRNA and protein were detected in brain glial and neuronal cells and co-localization with P450scc was observed in the mouse and human cerebral cortex[56].

In steroidogenic tissues, including the brain, cholesterol is the unique substrate of the P450scc (CYP11A1) enzyme, which catalyzes its conversion to pregnenolone. The structural organization of cholesterol, in particular the C20-C22 region of its side chain, allows the recognition and binding to the catalytic domain of the enzyme. CYP11A1 is involved in three consecutive reactions from cholesterol to pregnenolone, including the hydroxylation of C22 and C20, and then the cleavage of the C22-C20 bond. The expression of P450scc mRNA has been detected in several rodent brain areas, including the cortex, amygdala, hippocampus, and, midbrain[57], and also in humans (reviewed in Lin & Papadopoulos, 2021[58]). Human

P450scc is encoded by a single gene on chromosome 15, the CYP11A1 gene[59]. Its expression has been reported in the human temporal lobe, frontal lobe, and hippocampus, but is much lower than in adrenals, expressing high CYP11A1 mRNA levels[60,61].

3.2 Effect of acute THC on steroidogenesis

We found in rodents that acute THC, through a CB1-dependent mechanism, dramatically and dose-dependently (with a plateau at 9 mg/kg in rats and 12 mg/kg in mice) increased pregnenolone levels in several brain areas and peripheral tissues, without altering downstream steroidal metabolites, suggesting that pregnenolone is the specific steroid target of THC[62]. This effect also appeared to be drug-specific, as other drugs of abuse (such as cocaine, nicotine, amphetamine, and alcohol) induced a much smaller increase in pregnenolone. Pregnenolone levels were especially high in CB1-enriched areas of the brain, including the frontal cortex, nucleus accumbens, dorsal striatum, thalamus, hippocampus, and cerebellum (reviewed in Tomaselli & Vallée, 2019[2]).

By screening the phosphorylation status of proteins involved in steroidogenesis, we identified that acute THC administration was able to promote steroid synthesis in the nucleus accumbens (NAc) of the adult male rat by increasing the phosphorylated amount of HSL and P450scc, without affecting the StAR or TSPO proteins[62].

Overall, these data demonstrate that the activation of CB1R by THC, stimulates the two key steps of neurosteroidogenesis, i.e. the availability of free cholesterol and the synthesis of pregnenolone from cholesterol, which result in increased pregnenolone brain levels.

3.3 Effects of pregnenolone on CB1-mediated addiction-related functions

Pregnenolone has been shown to prevent the effect of CB1 activation at several stages that underlie the mechanisms of cannabis-related addictive behavior. From a neuromodulatory perspective, *ex-vivo* electrophysiology experiments have shown that pregnenolone alters the effects of CB1 activation on glutamate synaptic plasticity. Pregnenolone, acting at the level of presynaptic glutamatergic neurons, attenuated the effect of THC inhibition on glutamate release as determined by the measurement of excitatory postsynaptic currents (EPSC) in rodent brain (NAc) slices[62]. This downward effect was about 34%, while another study reported a smaller decrease (~15%)[63]. In addition, pregnenolone also down-regulates the glutamatergic response to a synthetic CB1 agonist in CA1 hippocampal slices, since pregnenolone can block the WIN55,512-2-induced decrease in fEPSCs[64].

At the cellular and neurochemical scales, the regulatory action of pregnenolone was assessed on the stimulatory effect of THC on striatal DA neurotransmission. The activation of CB1R by endocannabinoids and exogenous cannabinoids, including THC, has been shown in

animals to enhance the firing of mesolimbic DA neurons and ventral striatal DA levels[65–68]. THC also increased DA levels in the ventral striatum of humans as shown by positron emission tomography (PET) scans in healthy subjects[69]. By using *in vivo* microdialysis coupled to electrophysiology[70], acute pregnenolone inhibited the THC-induced increase in the firing activity of DA neurons located in the VTA and the associated increase in DA output in the NAc of adult mice[62]. In addition, subacute treatment with pregnenolone in postnatal rats (PN 5-13) subjected to prenatal THC exposure has been shown to prevent hyperdopaminergic alterations[66]. Therefore, pregnenolone may modulate the effects of THC on the electrical and neurochemical activities of striatal dopaminergic neurons, suggesting that pregnenolone may restore the balance of the mesolimbic DA network, conceivably by counteracting the inhibition of GABA release induced by CB1 activation on presynaptic GABAergic neurons (reviewed in Tomaselli & Vallée, 2019[2]).

From a behavioral perspective, THC and CB1 agonists exhibit reward properties by implementing self-administration behavior in animals (reviewed in Tanda & Goldberg, 2003[71]). In a mouse model of self-administration of the CB1 agonist, WIN 55,212-2[72], pregnenolone significantly reduced the amount of drug intake and motivation for the drug[62]. Furthermore, to address the action of pregnenolone on cannabis use disorders (CUD), the models of cannabis-induced acute psychotic states (CIAPS) in mice[73] and prenatal cannabis exposure (PCE) in rats[66] have been studied. The CIAPS and PCE models are of clinical relevance as exposure to THC acutely during the teenage years or throughout the pre-birth period may increase the likelihood of developing subsequent psychotic-like symptoms and cognitive deficits[74–76]. In the CIAPS or PCE model, pregnenolone inhibits several THC-induced psychotic-like symptoms including somatosensory gating and deficits in memory[66,73].

Together, these data strongly suggest that pregnenolone may reduce the development of cannabis abuse by acting on cannabis-related behavioral alterations and underlying mechanisms.

3.4 Pregnenolone action on CB1R

3.4.1 Pregnenolone acts as an endogenous signaling-specific negative allosteric modulator on CB1R

One general consequence of allosteric modulation is the stabilization of the receptor in a given conformation, which can lead to a change in protein-protein interactions and thus affect receptor activation. In general, negative and positive allosteric modulators (NAMs and PAMs, respectively) bind to an allosteric site on the receptor that is distinct from the endogenous ligand-binding site (orthosteric site) and may influence the binding of the ligand and/or signaling pathways, resulting in the altered affinity and/or efficacy for the agonist (reviewed in

Kenakin, 2009[77]). If we consider the case of NAMs, they have specific properties that differ from orthosteric antagonists and give them benefits for therapeutic development. This includes a probe-specific effect, which means that NAMs produce different effects depending on the ligands of the receptor. Furthermore, NAMs do not completely block the binding of the natural agonist. This feature may be an advantage from a therapeutic point of view, as it allows the maintenance of normal receptor activity towards endogenous ligands.

In the case of the CB1R, several allosteric modulators have been described, among which three are classified as endogenous allosteric modulators, including pregnenolone (reviewed in Gado et al., 2019[78]; Khurana et al., 2017[79]; Raux et al., 2022[17]). Several pieces of evidence have demonstrated that pregnenolone behaves as a NAM on CB1R. First, it did not alter the equilibrium binding of CB1R ligands, such as WIN55,212-2 or CP55,940, to hCB1R in CHO cells[62]. In addition, the behavioral effects of THC were increased by blocking the synthesis of pregnenolone by the administration of an inhibitor of P450scc in mice, supporting a decrease in the potency and/or affinity of THC by pregnenolone[62]. Finally, pregnenolone involves biased modulation of CB1 signaling in response to THC on hCB1R in HEK293 cells, specifically by inhibiting the activation of ERK1/2^{MAPK} pathway without altering the G-protein pathway involved in THC-induced cAMP decrease[62].

Further examination of the CB1-mediated biased action of pregnenolone remains to be investigated across multiple signaling cascades, including action on ERK1/2^{MAPK}, as one study reported no effect of pregnenolone on the activation of ERK1/2^{MAPK} induced by THC or WIN55,512-2 in CHO-hCB1R cells[80]. Moreover, the lack of effect of pregnenolone described on DSE induced by 2-AG in hippocampal neurons[81] may suggest a ligand-dependent action of pregnenolone.

3.4.2 Pregnenolone binds to a specific binding site on CB1R

Computational experiments, using the Forced-Biased Metropolis Monte Carlo simulated annealing program (MMC) for ligand-protein binding[82] between pregnenolone and a CB1R model[83], have revealed that pregnenolone binds to a specific binding pocket formed by the transmembrane domains, TMH1 and TMH7, associated with the C-terminal intracellular helix 8 (Hx8)[62]. Each polar group of pregnenolone interacts with residue E.1.49 (for the ketone group) or R7.65 (hydroxyl group), on the TMH1, 7, and Hx8 regions, respectively. The localization of this binding site has been confirmed by *in vitro* experiment measuring mitochondrial respiration in cells transfected with a mutant human CB1R (hCB1R$^{p.E1.49G}$), in which the glutamate (E) residue in position 1.49 has been substituted by a glycine (G). In these cells, the inhibitory effect of pregnenolone on the THC-induced decrease in cellular respiration was absent, in contrast to what was observed in cells transfected with a wild-type hCB1R[62].

Pregnenolone binding to the TMH1/TMH7/Hx 8 site, likely makes rigidity of the intracellular end of TMH7, a region that is required to move upon activation of class A GPCRs[84]. Thus, the identified binding site of pregnenolone is in agreement with its action as a negative allosteric modulator on the CB1R.

3.5 How does the PREG-CB1 regulatory loop work?

The postulated model of the PREG-CB1 regulatory loop involves several steps. When excessive activation of CB1 signaling arises during THC intoxication (as a result of high-dose THC in mice or cannabis use/abuse in humans), an imbalance of brain systems may occur, such as in the DA mesolimbic network, known to be involved in the cannabis addictive process. The CB1R signal may serve as a homeostasis sensor, which activates the production of pregnenolone in several brain areas, including the NAc, via the activation of HSL and P450scc. In turn, a feedback mechanism is engaged via the binding of pregnenolone to CB1R at an allosteric binding pocket, which suppresses the effect of THC on specific CB1 signaling pathways (involving a biased activity at the Erk1/2^{MAPK} pathway), and on THC-mediated outcomes, thereby correcting the homeostatic imbalance. It emerges from this model that the interaction between the neurosteroid pregnenolone and the ECS system orchestrates many behaviors in health and disease that are intrinsic features of body homeostasis. A broad illustration of this model is depicted in ***Figure 1***.

4. PREGNENOLONE-CB1R LOOP IN HOMEOSTASIS PROSPECTIVE

The above findings demonstrate that the production of pregnenolone can be upregulated within the brain. Although all regulatory mechanisms need to be further evaluated, we have identified two effectors/sensors of modulation, including HSL and P450scc. The first involves the availability of free cholesterol required for steroidogenesis with the increase of HSL responsible for the hydrolysis of cholesterol esters. HSL has been reported at neuronal synapses with a wide distribution within the mouse brain and has been proposed to play a key role in the neuroactive lipid homeostasis related to normal brain function[54]. Mitochondrial P450scc enzyme (CYP11A1) is the second target for CB1-mediated pregnenolone increase. P450scc is responsible for the initiation of steroidogenesis with the conversion of cholesterol to pregnenolone. A significant decrease in free GABA levels and a slight reduction in free glutamate levels have been reported in the CSF of subjects with homozygous P450scc disruption[85], suggesting that P450scc may play a role in the inhibitory/excitatory homeostasis process in the brain. Thus, it can be hypothesized that the impact of increased HSL and P450scc may involve the action of pregnenolone on CB1Rs.

Our findings also support a novel role for pregnenolone via its action through the CB1R GPCR. It is more likely that pregnenolone operates as either an autocrine or paracrine neuromodulator than an endocrine one. Indeed, pregnenolone is produced within mitochondria in neurons and astrocytes[86]; yet, the mitochondrial CB1R (mtCB1R) has recently focused all attention with first the discovery of its presence on the outer membrane of mouse brain neuronal mitochondria[87] described as a G protein-coupled receptor containing the first 22 amino acids of the CB1 protein[88]. In addition, mtCB1Rs have been involved in neuronal energy metabolism and CB1-mediated synaptic plasticity such as DSI[87], as well as in the regulation of memory[88]. Finally, mtCB1Rs in astrocytes are involved in glucose metabolism and THC-induced deficits in social behavior[89]. Further attention is indicated to demonstrate a direct action of pregnenolone on mtCB1. We first assessed the action of pregnenolone on *in vitro* mitochondrial respiration using a human CB1R (hCB1R) mutated at the PREG binding site and preventing its binding to the CB1R. In doing so, we found that in cells transfected with intact hCB1R, PREG blocked the THC-induced decrease in cellular and mitochondrial respiration, whereas this effect was abolished in cells transfected with mutated hCB1R[62]. Ongoing *in vivo* experiments translating this genetic strategy into mice are exploring the effects of endogenous pregnenolone on CB1-mediated behavior.

In terms of homeostasis gain and evolutionary purpose, the body has set up an ingenious device such that the disruption of ECS signaling by exogenous THC is attenuated by an endogenous inhibitory modulator produced locally in the brain. The fact that CB1Rs localized in D1 striatal GABAergic neurons have been shown to mediate THC-induced pregnenolone production[62] suggests that pregnenolone serves as a neuroprotective messenger mediated by GABAergic CB1Rs. This has relevance with the regulatory action of GABAergic CB1Rs during excessive ECS stimulation, as provided by the biphasic function of ECS mediated by the balance between glutamatergic and GABAergic CB1Rs in response to low and high doses of THC, respectively[90–93]. Thus, pregnenolone can serve as an endogenous "brake" during uncontrolled ECS activation[94]; however, it is not a brake *stricto sensu*, like orthosteric CB1 antagonist operates (i.e. by completely shutting down the activity of ECS), but rather a "slowing down mechanism", which is more likely to be recruited after high degrees of CB1 activation.

It is noteworthy that pregnenolone, which acts as a NAM by binding to a specific allosteric binding pocket without altering the competitive binding of CB1 ligands, does not tend to alter the equilibrium established by the action of eCBs. Furthermore, inhibition of CB1-specific signaling by pregnenolone, which more likely acts through β-arrestin-1 mediated pathways since it does not change cAMP response mediated by a G protein, is not expected to have a significant impact on the fine-tune of ECS on homeostasis. Together, both properties of pregnenolone may represent promising therapeutic advantages by blunting adverse signaling side effects[50,79,95]. This is critical in the context of the current increase in medical and

recreational cannabis use and CUD while at the same time the perceived risk of cannabis exposure is decreasing[96,97].

In conclusion, the neurosteroid pregnenolone and eCBs are typical examples of endogenous neuromodulators, binding to the metabotropic GPCR, CB1R, which activates second-messenger signaling cascades resulting in broad and diversified signals. Nevertheless, despite the diversity of CB1 functions that could arise from these signals, it is more likely that the pregnenolone-CB1R regulatory loop is involved in particular functions given the specific brain region and (sub)cellular localization of the CB1R and the functional selectivity of the CB1R that engages a ligand-specific response leading to an allosterically biased signaling response. This review has outlined the role of the PREG-CB1R regulatory loop in CB1-related intoxication and addiction, which can be applied to a broader homeostatic process. Ongoing and future research will help to elucidate whether this cooperative neuromodulation duo participates in fine-tuning ECS function in response to physiological and other pathophysiological inputs.

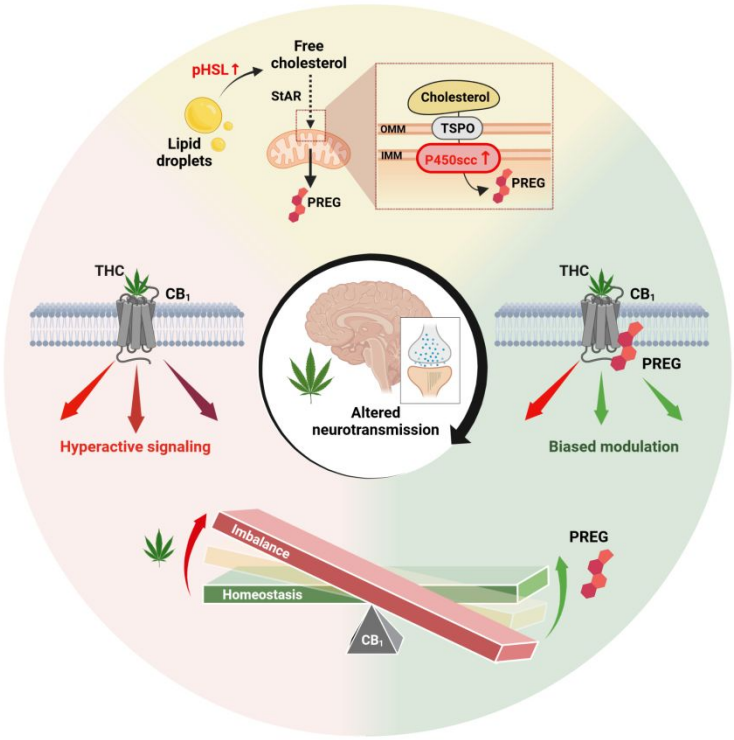

Figure 1: A postulated model for the PREG-CB1 regulatory loop: Over-activation of the CB1 receptor by THC produces an imbalance in CB1-mediated homeostasis leading to hyperactive CB1 signaling and altered neurotransmission. Consequently, THC administration increases the amount of phosphorylated HSL in the brain, acting as the rate-limiting enzyme to mobilize free cholesterol from lipid droplets, then serving as the available pool of cholesterol for *de novo* steroidogenesis. Interaction of cholesterol together with StAR and TSPO allows its transport to the mitochondria and translocation from the outer to the inner mitochondrial membrane for the onset of steroid synthesis. Within the mitochondria, THC administration increases p450scc levels, the enzyme responsible for the synthesis of pregnenolone from cholesterol, hence elevating pregnenolone brain production and levels. Pregnenolone then exerts negative feedback on the CB1 receptor by docking on a dedicated allosteric binding pocket on the receptor. This results in the biased modulation of CB1 intra-cellular signaling pathways, allowing to block THC outcomes and restore CB1-mediated homeostasis. Abbreviations: *CB_1*, Cannabinoid type 1 receptor; *IMM*, inner mitochondrial membrane; *OMM*, outer mitochondrial membrane; *p450scc*, cholesterol side-chain cleavage enzyme; *pHSL*, phosphorylated hormone-sensitive lipase; *PREG*, pregnenolone; *StAR*, steroidogenic acute regulatory protein; *THC*, Δ^9-tetrahydrocannabinol; *TSPO*, translocator protein.

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