

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)

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

43

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

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48

49 1. INTRODUCTION

50 Neurosteroids have been studied for over the past 20 years (reviewed in Balthazart et al.,
51 2018¹; Tomaselli & Vallée, 2019²; Vallée 2014³) and were discovered in the 80' as steroids
52 synthesized by the brain for the brain⁴. One of their main characteristics is their *de novo*
53 production on-demand in the brain, which is common to all species, all the way from
54 amphibians to mammals, up to and including humans⁵⁻⁹. The first step, which can be defined
55 as the rate-limiting step in their synthesis, involves the transport of cholesterol into the
56 mitochondria-mediated by the action of StAR (steroidogenic acute regulatory protein) and
57 TSPO (a translocator protein of 18 kDa)¹⁰⁻¹², and its conversion to pregnenolone catalyzed by
58 CYP11A1 (a P450 side-chain cleaving enzyme, *P450scc*)¹³⁻¹⁵. Subsequently, several
59 pathways of pregnenolone metabolism, firstly in the endoplasmic reticulum, trigger the
60 synthesis of steroid families, including progestagens, corticoids, androgens, and estrogens
61 (reviewed in Mellon & Griffin, 2002¹⁶; Raux et al., 2022¹⁷). Neurosteroid production occurs
62 primarily in neurons and astrocytes in several brain areas, as first indicated by P450scc
63 regionalization for pregnenolone synthesis^{16,18,19}. In addition to the localization of their
64 production, some neurosteroids have aroused scientific interest for their action, not on their
65 receptors, but on neuromodulator receptors, mainly γ -aminobutyric acid (GABA)_A and the N-

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

68 Another family of lipid neuromodulators that has gained the interest of the scientific
69 community over the last 20 years arises from the discovery that the brain can produce
70 endogenous analogs of phytocannabinoids, a class of naturally occurring molecules derived
71 from the *Cannabis Sativa* plant, of which Δ^9 -Tetrahydrocannabinol (THC) is the major
72 psychoactive component²⁵. Those messengers, named “Endocannabinoids” (short for
73 endogenous cannabinoids), (eCBs), were identified in the 90’ as endogenous ligands of the
74 two main cannabinoid receptors, the type 1 (CB1R) and type 2 (CB2R) cannabinoid
75 receptors²⁶, formerly described for binding exogenous cannabinoids, such as THC²⁵.

76 Then, the discovery of the on-demand synthesis of eCBs in the postsynaptic membrane
77 and their action on retrograde synaptic signaling led to a new perspective on how messengers
78 can modulate neuronal activity²⁷ and opened up new fields of cannabinoid research, from the
79 functioning of synaptic plasticity to cognition and motivation functions. The two main studied
80 eCBs include N-Arachidonylethanolamide (anandamide, AEA) and 2-Arachidonoylglycerol
81 (2-AG), which are synthesized from phospholipid precursors and are rapidly degraded into the
82 cell by distinct enzymes, the fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase
83 (MAGL), respectively^{28,29}. The endocannabinoid system (ECS) thus includes the CB1R and
84 CB2R, the eCBs, as well as their synthesis and degradation enzymes. ECS function is a
85 phylogenetically widespread feature in vertebrates³⁰ and has been described in specific
86 regions of the rodent brain and in different (sub)cellular sites, which accounts for its broad
87 involvement in brain function (reviewed in Busquets-Garcia et al., 2018³¹).

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

92 **2. NEUROSTEROIDS & ENDOCANNABINOIDS AS NEUROMODULATORS: NEW** 93 **PERSPECTIVES**

94 The connection between the external environment and the internal state sets up a dynamic
95 modulation orchestrated by chemical substances called neuromodulators. Neuromodulators
96 can be defined as endogenous molecules or neuronal messengers that modulate neuronal
97 activity upon stimulation. Neuromodulators have been observed in several species, from the
98 nematode *Caenorhabditis Elegans* to mammals^{32,33}. Both intrinsic and extrinsic types of
99 neuromodulation have been described³⁴. Intrinsic neuromodulation involves the same
100 neuronal circuit that is both the origin of the modulator release and the target of neuromodulator

101 action, while extrinsic neuromodulation engages separate systems implying neuroactive
102 substances that can be transported into the extracellular compartment. Neuromodulators,
103 therefore, act either in a local autocrine/paracrine mode or in a hormonal mode on neuronal
104 targets distant from their release site³⁵.

105 Two examples of well-described neuromodulatory systems are the GABAergic and
106 glutamatergic neuronal systems, which underlie the excitability properties of neurons and
107 control the cellular inhibitory and excitatory balance, respectively (reviewed in Sears & Hewett,
108 2021³⁶). These two systems work together to shape adaptive responses to external stimuli and
109 are involved in the maintenance of physiological balance. Hence, deregulation of this
110 equilibrium can contribute to the pathophysiology of brain disorders.

111 In this context, some neurosteroids are referred to as paracrine and/or autocrine
112 neuromodulators³⁷ and they have been shown to modulate GABA_A and NMDA receptor activity
113 with a similar affinity to that of the endogenous neurotransmitter ligands GABA and glutamate,
114 respectively (reviewed in Ratner et al., 2019³⁸). This is the case for the sulfated derivative of
115 pregnenolone, but not pregnenolone, which modulates GABA_A and NMDA receptors
116 negatively and positively, respectively³⁹. In addition, the progesterone metabolites
117 allopregnanolone and pregnanolone act as positive allosteric modulators of the GABA_A
118 receptor by facilitating the effects of GABA⁴⁰.

119 On the other hand, the neuromodulatory actions of eCBs involve mainly a retrograde
120 signaling mechanism, (i.e. release from the post-synaptic site to act on the pre-synaptic
121 compartment) which can result in the inhibition of the transmitter release at both excitatory and
122 inhibitory synapses. Indeed, the eCBs, by acting on CB1Rs localized on presynaptic
123 GABAergic and glutamatergic neurons^{26,41} can modulate synaptic plasticity by fine-tuning the
124 activity of the aforementioned neurons, a process referred to as depolarisation-induced
125 suppression of inhibition (DSI) or excitation (DSE), respectively^{27,42–44}. The CB1Rs are among
126 the most abundant G-protein coupled receptors (GPCRs) in the brain⁴⁵ and are expressed in
127 the brain to similar protein amounts as GABA_A and NMDA receptors. CB1Rs are highly present
128 in sensory and motor brain areas, including the basal ganglia, substantia nigra, globus pallidus,
129 cerebellum, and hippocampus, which underlies their role in cognition and motivation⁴⁵. In
130 addition, eCBs interact with other neuromodulators, such as dopamine (DA) and serotonin (5-
131 HT) (reviewed in Peters et al., 2021⁴⁶). This interaction can occur through the release of eCBs
132 from DA and 5-HT neurons, which regulates glutamatergic and GABAergic afferents. This has
133 been documented, for example, for the mesolimbic dopaminergic system, in which DA neurons
134 in the ventral tegmental area (VTA) projecting to the nucleus accumbens (NAc) receive
135 GABAergic inputs and 5-HT/Glutamate inputs from the dorsal raphe nucleus (DRN). As a
136 result, eCBs can fine-tune the activity of the mesolimbic DA system by acting on presynaptic

137 CB1Rs in GABAergic and glutamatergic neurons, thereby modulating excitatory and inhibitory
138 signaling, respectively^{47,48}. This illustrates that eCBs play a crucial role in controlling adaptive
139 behaviors and maintaining reward system homeostasis, positioning the ECS as a key
140 modulatory network in the brain (reviewed in De Melo Reis, 2021⁴⁹; Lutz, 2020⁵⁰).

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

147 **3. DISCOVERY AND EVIDENCE OF THE REGULATORY LOOP BETWEEN** 148 **PREGNENOLONE AND CB1R**

149 3.1 CB1 activation and pregnenolone production

150 3.1.1 The actors of brain pregnenolone production

151 General steroid biosynthesis derives from about 2% of the daily cholesterol pool in humans,
152 acquired from the diet from *de novo* synthesis from acetyl-CoA⁵¹. In the brain, it has been
153 proposed that the source of cholesterol rather originates from *de novo* synthesis, as the blood-
154 brain barrier is not permeable to cholesterol lipoproteins⁵². One of the rate-limiting factors for
155 steroidogenesis is the available amount of cholesterol in astrocytes and neurons. First, the free
156 cholesterol pool in the cytosol is related to the action of the hormone-sensitive
157 lipase/cholesteryl esterase (HSL), encoded by the LIPE gene, which is responsible for
158 hydrolyzing cholesteryl esters located in lipid droplets⁵³. HSL protein and its enzymatic activity
159 have been detected in the mouse brain at neuronal synapses⁵⁴. It was then described that the
160 proteins StAR and TSPO may be respectively involved in the delivery of cholesterol and its
161 transport from the outer membrane to the inner membrane of mitochondria (reviewed in
162 Selvaraj et al., 2018⁵⁵). StAR mRNA and protein were detected in brain glial and neuronal cells
163 and co-localization with P450scc was observed in the mouse and human cerebral cortex⁵⁶.

164 In steroidogenic tissues, including the brain, cholesterol is the unique substrate of the
165 P450scc (CYP11A1) enzyme, which catalyzes its conversion to pregnenolone. The structural
166 organization of cholesterol, in particular the C20-C22 region of its side chain, allows the
167 recognition and binding to the catalytic domain of the enzyme. CYP11A1 is involved in three
168 consecutive reactions from cholesterol to pregnenolone, including the hydroxylation of C22
169 and C20, and then the cleavage of the C22-C20 bond. The expression of P450scc mRNA has
170 been detected in several rodent brain areas, including the cortex, amygdala, hippocampus,
171 and, midbrain⁵⁷, and also in humans (reviewed in Lin & Papadopoulos, 2021⁵⁸). Human

172 P450scc is encoded by a single gene on chromosome 15, the CYP11A1 gene⁵⁹. Its expression
173 has been reported in the human temporal lobe, frontal lobe, and hippocampus, but is much
174 lower than in adrenals, expressing high CYP11A1 mRNA levels^{60,61}.

175

176 3.2 Effect of acute THC on steroidogenesis

177 We found in rodents that acute THC, through a CB1-dependent mechanism, dramatically
178 and dose-dependently (with a plateau at 9 mg/kg in rats and 12 mg/kg in mice) increased
179 pregnenolone levels in several brain areas and peripheral tissues, without altering downstream
180 steroidal metabolites, suggesting that pregnenolone is the specific steroid target of THC⁶². This
181 effect also appeared to be drug-specific, as other drugs of abuse (such as cocaine, nicotine,
182 amphetamine, and alcohol) induced a much smaller increase in pregnenolone. Pregnenolone
183 levels were especially high in CB1-enriched areas of the brain, including the frontal cortex,
184 nucleus accumbens, dorsal striatum, thalamus, hippocampus, and cerebellum (reviewed in
185 Tomaselli & Vallée, 2019²).

186 By screening the phosphorylation status of proteins involved in steroidogenesis, we
187 identified that acute THC administration was able to promote steroid synthesis in the nucleus
188 accumbens (NAc) of the adult male rat by increasing the phosphorylated amount of HSL and
189 P450scc, without affecting the StAR or TSPO proteins⁶².

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

193 3.3 Effects of pregnenolone on CB1-mediated addiction-related functions

194 Pregnenolone has been shown to prevent the effect of CB1 activation at several stages that
195 underlie the mechanisms of cannabis-related addictive behavior. From a neuromodulatory
196 perspective, *ex-vivo* electrophysiology experiments have shown that pregnenolone alters the
197 effects of CB1 activation on glutamate synaptic plasticity. Pregnenolone, acting at the level of
198 presynaptic glutamatergic neurons, attenuated the effect of THC inhibition on glutamate
199 release as determined by the measurement of excitatory postsynaptic currents (EPSC) in
200 rodent brain (NAc) slices⁶². This downward effect was about 34%, while another study reported
201 a smaller decrease (~15%)⁶³. In addition, pregnenolone also down-regulates the glutamatergic
202 response to a synthetic CB1 agonist in CA1 hippocampal slices, since pregnenolone can block
203 the WIN55,512-2-induced decrease in fEPSCs⁶⁴.

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

207 animals to enhance the firing of mesolimbic DA neurons and ventral striatal DA levels^{65–68}.
208 THC also increased DA levels in the ventral striatum of humans as shown by positron emission
209 tomography (PET) scans in healthy subjects⁶⁹. By using *in vivo* microdialysis coupled to
210 electrophysiology⁷⁰, acute pregnenolone inhibited the THC-induced increase in the firing
211 activity of DA neurons located in the VTA and the associated increase in DA output in the NAc
212 of adult mice⁶². In addition, subacute treatment with pregnenolone in postnatal rats (PN 5-13)
213 subjected to prenatal THC exposure has been shown to prevent hyperdopaminergic
214 alterations⁶⁶. Therefore, pregnenolone may modulate the effects of THC on the electrical and
215 neurochemical activities of striatal dopaminergic neurons, suggesting that pregnenolone may
216 restore the balance of the mesolimbic DA network, conceivably by counteracting the inhibition
217 of GABA release induced by CB1 activation on presynaptic GABAergic neurons (reviewed in
218 Tomaselli & Vallée, 2019²).

219 From a behavioral perspective, THC and CB1 agonists exhibit reward properties by
220 implementing self-administration behavior in animals (reviewed in Tanda & Goldberg, 2003⁷¹).
221 In a mouse model of self-administration of the CB1 agonist, WIN 55,212-2⁷², pregnenolone
222 significantly reduced the amount of drug intake and motivation for the drug⁶². Furthermore, to
223 address the action of pregnenolone on cannabis use disorders (CUD), the models of cannabis-
224 induced acute psychotic states (CIAPS) in mice⁷³ and prenatal cannabis exposure (PCE) in
225 rats⁶⁶ have been studied. The CIAPS and PCE models are of clinical relevance as exposure
226 to THC acutely during the teenage years or throughout the pre-birth period may increase the
227 likelihood of developing subsequent psychotic-like symptoms and cognitive deficits^{74–76}. In the
228 CIAPS or PCE model, pregnenolone inhibits several THC-induced psychotic-like symptoms
229 including somatosensory gating and deficits in memory^{66,73}.

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

233 3.4 Pregnenolone action on CB1R

234 3.4.1 Pregnenolone acts as an endogenous signaling-specific negative allosteric 235 modulator on CB1R

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

242 Kenakin, 2009⁷⁷). If we consider the case of NAMs, they have specific properties that differ
243 from orthosteric antagonists and give them benefits for therapeutic development. This includes
244 a probe-specific effect, which means that NAMs produce different effects depending on the
245 ligands of the receptor. Furthermore, NAMs do not completely block the binding of the natural
246 agonist. This feature may be an advantage from a therapeutic point of view, as it allows the
247 maintenance of normal receptor activity towards endogenous ligands.

248 In the case of the CB1R, several allosteric modulators have been described, among which
249 three are classified as endogenous allosteric modulators, including pregnenolone (reviewed in
250 Gado et al., 2019⁷⁸; Khurana et al., 2017⁷⁹; Raux et al., 2022¹⁷). Several pieces of evidence
251 have demonstrated that pregnenolone behaves as a NAM on CB1R. First, it did not alter the
252 equilibrium binding of CB1R ligands, such as WIN55,212-2 or CP55,940, to hCB1R in CHO
253 cells⁶². In addition, the behavioral effects of THC were increased by blocking the synthesis of
254 pregnenolone by the administration of an inhibitor of P450_{scc} in mice, supporting a decrease
255 in the potency and/or affinity of THC by pregnenolone⁶². Finally, pregnenolone involves biased
256 modulation of CB1 signaling in response to THC on hCB1R in HEK293 cells, specifically by
257 inhibiting the activation of ERK1/2^{MAPK} pathway without altering the G-protein pathway involved
258 in THC-induced cAMP decrease⁶².

259 Further examination of the CB1-mediated biased action of pregnenolone remains to be
260 investigated across multiple signaling cascades, including action on ERK1/2^{MAPK}, as one study
261 reported no effect of pregnenolone on the activation of ERK1/2^{MAPK} induced by THC or
262 WIN55,512-2 in CHO-hCB1R cells⁸⁰. Moreover, the lack of effect of pregnenolone described
263 on DSE induced by 2-AG in hippocampal neurons⁸¹ may suggest a ligand-dependent action
264 of pregnenolone.

265

266 3.4.2 Pregnenolone binds to a specific binding site on CB1R

267 Computational experiments, using the Forced-Biased Metropolis Monte Carlo simulated
268 annealing program (MMC) for ligand-protein binding⁸² between pregnenolone and a CB1R
269 model⁸³, have revealed that pregnenolone binds to a specific binding pocket formed by the
270 transmembrane domains, TMH1 and TMH7, associated with the C-terminal intracellular helix
271 8 (Hx8)⁶². Each polar group of pregnenolone interacts with residue E.1.49 (for the ketone
272 group) or R7.65 (hydroxyl group), on the TMH1, 7, and Hx8 regions, respectively. The
273 localization of this binding site has been confirmed by *in vitro* experiment measuring
274 mitochondrial respiration in cells transfected with a mutant human CB1R (hCB1R^{ΔE1.49G}), in
275 which the glutamate (E) residue in position 1.49 has been substituted by a glycine (G). In these
276 cells, the inhibitory effect of pregnenolone on the THC-induced decrease in cellular respiration
277 was absent, in contrast to what was observed in cells transfected with a wild-type hCB1R⁶².

278 Pregnenolone binding to the TMH1/TMH7/Hx 8 site, likely makes rigidity of the intracellular
279 end of TMH7, a region that is required to move upon activation of class A GPCRs⁸⁴. Thus, the
280 identified binding site of pregnenolone is in agreement with its action as a negative allosteric
281 modulator on the CB1R.

282 3.5 How does the PREG-CB1 regulatory loop work?

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

296

297 4. PREGNENOLONE-CB1R LOOP IN HOMEOSTASIS PROSPECTIVE

298 The above findings demonstrate that the production of pregnenolone can be upregulated
299 within the brain. Although all regulatory mechanisms need to be further evaluated, we have
300 identified two effectors/sensors of modulation, including HSL and P450scc. The first involves
301 the availability of free cholesterol required for steroidogenesis with the increase of HSL
302 responsible for the hydrolysis of cholesterol esters. HSL has been reported at neuronal
303 synapses with a wide distribution within the mouse brain and has been proposed to play a key
304 role in the neuroactive lipid homeostasis related to normal brain function⁵⁴. Mitochondrial
305 P450scc enzyme (CYP11A1) is the second target for CB1-mediated pregnenolone increase.
306 P450scc is responsible for the initiation of steroidogenesis with the conversion of cholesterol
307 to pregnenolone. A significant decrease in free GABA levels and a slight reduction in free
308 glutamate levels have been reported in the CSF of subjects with homozygous P450scc
309 disruption⁸⁵, suggesting that P450scc may play a role in the inhibitory/excitatory homeostasis
310 process in the brain. Thus, it can be hypothesized that the impact of increased HSL and
311 P450scc may involve the action of pregnenolone on CB1Rs.

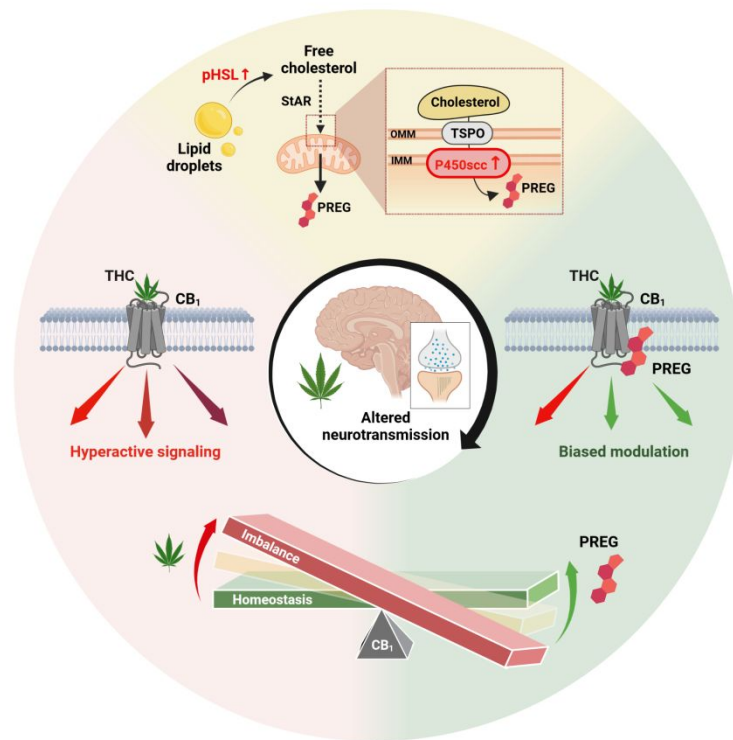
312 Our findings also support a novel role for pregnenolone via its action through the CB1R
313 GPCR. It is more likely that pregnenolone operates as either an autocrine or paracrine
314 neuromodulator than an endocrine one. Indeed, pregnenolone is produced within mitochondria
315 in neurons and astrocytes⁸⁶; yet, the mitochondrial CB1R (mtCB1R) has recently focused all
316 attention with first the discovery of its presence on the outer membrane of mouse brain
317 neuronal mitochondria⁸⁷ described as a G protein-coupled receptor containing the first 22
318 amino acids of the CB1 protein⁸⁸. In addition, mtCB1Rs have been involved in neuronal energy
319 metabolism and CB1-mediated synaptic plasticity such as DSI⁸⁷, as well as in the regulation
320 of memory⁸⁸. Finally, mtCB1Rs in astrocytes are involved in glucose metabolism and THC-
321 induced deficits in social behavior⁸⁹. Further attention is indicated to demonstrate a direct
322 action of pregnenolone on mtCB1. We first assessed the action of pregnenolone on *in vitro*
323 mitochondrial respiration using a human CB1R (hCB1R) mutated at the PREG binding site and
324 preventing its binding to the CB1R. In doing so, we found that in cells transfected with intact
325 hCB1R, PREG blocked the THC-induced decrease in cellular and mitochondrial respiration,
326 whereas this effect was abolished in cells transfected with mutated hCB1R⁶². Ongoing *in vivo*
327 experiments translating this genetic strategy into mice are exploring the effects of endogenous
328 pregnenolone on CB1-mediated behavior.

329 In terms of homeostasis gain and evolutionary purpose, the body has set up an ingenious
330 device such that the disruption of ECS signaling by exogenous THC is attenuated by an
331 endogenous inhibitory modulator produced locally in the brain. The fact that CB1Rs localized
332 in D1 striatal GABAergic neurons have been shown to mediate THC-induced pregnenolone
333 production⁶² suggests that pregnenolone serves as a neuroprotective messenger mediated by
334 GABAergic CB1Rs. This has relevance with the regulatory action of GABAergic CB1Rs during
335 excessive ECS stimulation, as provided by the biphasic function of ECS mediated by the
336 balance between glutamatergic and GABAergic CB1Rs in response to low and high doses of
337 THC, respectively^{90–93}. Thus, pregnenolone can serve as an endogenous "brake" during
338 uncontrolled ECS activation⁹⁴; however, it is not a brake *stricto sensu*, like orthosteric CB1
339 antagonist operates (i.e. by completely shutting down the activity of ECS), but rather a "slowing
340 down mechanism", which is more likely to be recruited after high degrees of CB1 activation.

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

349 recreational cannabis use and CUD while at the same time the perceived risk of cannabis
350 exposure is decreasing^{96,97}.

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



363

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

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