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

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### ► **To cite this version:**

Pierre-louis Raux, Guillaume Drutel, Jean-michel Revest, Monique Vallée. New perspectives on the role of the neurosteroid pregnenolone as an endogenous regulator of type-1 cannabinoid receptor (CB1R) activity and function. *Journal of Neuroendocrinology*, 2022, Papers from the Virtual International Meeting STEROIDS and NERVOUS SYSTEM, TORINO, ITALY - February 2021, 34 (2), pp.e13034. <10.1111/jne.13034>. <hal-03424066>

**HAL Id: hal-03424066**

**<https://hal.science/hal-03424066v1>**

Submitted on 20 Oct 2022

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1 New perspectives on the role of the neurosteroid pregnenolone  
2 as an endogenous regulator of type-1 cannabinoid receptor  
3 (CB1R) activity and function  
4

5 Pregnenolone : endogenous allosteric signaling specific  
6 inhibitor of CB1R  
7

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15 **Acknowledgments**

16 We acknowledge support from INSERM, Aquitaine Regional Council and University of  
17 Bordeaux, France. Figures were created with Biorender.com.

18 **Abstract**

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

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

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

34

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

## 37 **Introduction**

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

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

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

65 It has been suggested that steroids act as neuromodulators to regulate brain-related  
66 functions<sup>17,18</sup>. Further challenges lie ahead in proving these regulations, such as detecting

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

## 76 **Pregnenolone: a neurosteroid with unique characteristics**

### 77 Neurosteroids synthesis: an overview

#### 78 *Mitochondrial production of pregnenolone from cholesterol*

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

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

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

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

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

#### 119 *Neurosteroidogenesis enzymes*

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

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

138 pregnenolone into 3 $\beta$ -sulfated derivative by sulfotransferase enzymes in the rodent and human  
139 brain<sup>38</sup>. However, the presence of sulfated pregnenolone, which has been reported in  
140 postmortem human brain tissue, is a matter of debate in the rodent brain<sup>39</sup>. It is unlikely that  
141 the sulfated form of pregnenolone crosses the blood-brain barrier (BBB), which would require  
142 the presence of transporter proteins. Moreover, although pregnenolone sulfate could be  
143 uptaken across the BBB in rodents, it would be rapidly metabolized in favor of free  
144 pregnenolone<sup>40</sup>, which is concordant with the low levels of pregnenolone sulfate measured in  
145 rodents brain with highly sensitive mass spectrometry methods<sup>41–44</sup>. The 3 $\beta$ -hydroxysteroid  
146 dehydrogenase (3 $\beta$ -HSD) enzyme required for the conversion of pregnenolone to  
147 progesterone is expressed in the rat and human brain<sup>45–48</sup>. Two enzymes, 5 $\alpha$ -reductase and  
148 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD) or 3 $\alpha$ -hydroxysteroid oxido-reductase (3 $\alpha$ -  
149 HSOR) that convert progesterone to its active metabolite, allopregnanolone were then  
150 localized in principal glutamatergic output neurons in the cortex, hippocampus, and amygdala  
151 of mice, and in the human brain<sup>29,49</sup>. The conversion in the brain of pregnenolone into DHEA  
152 by 17-hydroxylase/17,20 lyase (P450c17) is challenging since no expression of P450c17 has  
153 been detected in rat brain glia<sup>50</sup>, but adult rat brain, and neurons and astrocytes in culture have  
154 been shown to convert pregnenolone to DHEA (for review<sup>39</sup>). In the human brain, postmortem  
155 studies report that P450c17 was not expressed in the temporal lobe and limbic system of the  
156 adult human<sup>51</sup>, but was present in the developing human fetal nervous system, suggesting that  
157 DHEA may be synthesized locally<sup>52</sup>. Finally, DHEA can be converted, in the rat and human  
158 brain, into androstenedione and then into testosterone, which can be metabolized into estradiol  
159 by aromatase<sup>49,53,54</sup>. Similarly, evidence has shown that estradiol can be synthesized from  
160 pregnenolone in the rat hippocampus<sup>55</sup>.

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

## 165 Local measurement of neurosteroids and pregnenolone

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

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

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

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

#### 204 Cellular and molecular targets of pregnenolone

205 Pregnenolone is inactive in modulating the ionotropic receptors, GABA<sub>A</sub> and NMDA receptors  
206 that are the classical targets of neurosteroids, such as allopregnanolone and the sulfated forms  
207 of pregnenolone and DHEA<sup>81-85</sup> (Table 1).

208        Pregnenolone, in contrast to its sulfate derivative, has been described as being inactive or  
209 as having a very low affinity on sigma-1 ( $\sigma_1$ ) receptor<sup>86,87</sup>.  $\sigma_1$  receptors are expressed in many  
210 organs including the nervous system, and located in the specific microdomains of the  
211 endoplasmic reticulum (ER) called mitochondrial associated membranes (MAM)<sup>88</sup>.  $\sigma_1$  receptor  
212 can influence synaptic functions through modulation of NMDA receptor activity and can remove  
213 the negative-regulation of NDMARs by CB1R<sup>89</sup>. Moreover, pregnenolone, along with DHEA,  
214 can interact with the microtubule associated proteins (MAPs), in particular MAP2 and CLIP-  
215 170<sup>90,91</sup>. Pregnenolone or DHEA are able to bind, with affinity in the nanomolar range, with the  
216 amino-terminal region of MAP2 protein, which stimulates MAP2-driven microtubule assembly.  
217 Finally, a novel mechanism of action of pregnenolone involving the GPCR, CB1R, has recently  
218 been demonstrated<sup>72</sup>. In cell models expressing human CB1R (hCB1R), pregnenolone (from  
219 10 nM to 1  $\mu$ M) inhibited the increase in P-Erk1/2<sup>MAPK</sup> and the decrease in cellular and  
220 mitochondrial respiration induced by the CB1 agonist  $\Delta^9$ -THC. By using the Forced-Biased  
221 Metropolis Monte Carlo simulated annealing program (MMC)<sup>92</sup>, a potential binding pocket for  
222 pregnenolone was found in the lipid facing TMH1/TMH7/Hx8 region of the CB1R. This binding  
223 pocket was confirmed, since pregnenolone lost its inhibitory effects on THC-induced decrease  
224 in cellular respiration in cells transfected with a mutant hCB1R that contained a point mutation  
225 that forbid the binding of the ketone end of pregnenolone to the CB1R<sup>72</sup>.

## 226    **CB1R activity and function**

### 227    Brief description of the endocannabinoid system (ECS)

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

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

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

## 246 CB1R distribution

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

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

260 Besides its systemic and cellular localization, CB1R is also distributed in several subcellular  
261 compartments where it plays distinct roles. Central CB1Rs are mainly detected in presynaptic  
262 terminals where they control the retrograde suppression of neurotransmission and ensure the  
263 excitatory/inhibitory balance within the brain. Briefly, eCBs are synthesized *de novo* in  
264 postsynaptic cells upon intracellular Ca<sup>2+</sup> increase and activate presynaptic CB1Rs leading to  
265 the transient or long-lasting suppression of neurotransmitter release (*i.e.* GABA or Glutamate)  
266 <sup>122</sup>.

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

## 275 Endocannabinoids: endogenous ligands of CB1R

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

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

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

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

295 2-AG is the most abundant eCB in the brain and an intermediate in lipid metabolism<sup>126,134</sup>.  
296 It is primarily involved in the retrograde inhibition of neurotransmission and is synthesized upon  
297 depolarization of postsynaptic neurons. Specifically, phosphatidylinositol (PI) is hydrolyzed by  
298 a phospholipase C (PLC) into diacylglycerol (DAG). The hydrolysis of DAG by diacylglycerol  
299 lipase (DAGL) then leads to the formation of 2-AG<sup>126,135–137</sup>. 2-AG travels to presynaptic  
300 neurons retrogradely and is hydrolyzed by the monoacylglycerol lipase (MAGL). While MAGL  
301 is primarily responsible for the termination of 2AG signaling, the  $\alpha/\beta$ -hydrolase domain 6  
302 (ABHD6) and domain 12 (ABHD12) enzymes can degrade 2AG to avoid its excessive  
303 accumulation in postsynaptic neurons and the extracellular space, respectively<sup>138–140</sup>. As for  
304 AEA, alternative pathways may participate in the inactivation of 2AG<sup>141</sup>.

### 305 CB1R activity

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

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

### 315 *Constitutive activity*

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

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

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

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

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

348 certainly linked to the serious adverse psychiatric effects (anxiety, depression and suicidality)  
349 associated with inverse agonists like rimonabant<sup>154,155</sup>.

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

### 353 *Biased signaling*

354 Most GPCRs have long been thought to couple with multiple G $\alpha$  proteins (e.g., Gai/o, Gas,  
355 G $\alpha$ q, or G $\alpha$ 12/13) and  $\beta$ -arrestins, thereby triggering multiple intracellular signaling pathways  
356 in parallel and/or sequentially through different transduction mechanisms. Accumulated  
357 evidence has now established that distinct GPCR agonists have the potential to selectively  
358 activate one specific signaling cascade over another, a phenomenon termed "biased agonism"  
359 or "functional selectivity," and trigger distinct physiological responses<sup>156</sup>.

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

376 Apart from G proteins, arrestin proteins are essential components of multiple GPCR  
377 signaling cascades involving kinases or phosphatases<sup>162</sup>. CB1Rs can thus recruit  $\beta$ -arrestin-1  
378 (Arrestin-2) and  $\beta$ -arrestin-2 (arrestin-3) for signaling, and the coupling of CB1R to  $\beta$ -arrestin  
379 may affect the localization of receptors at the surface, their internalization, recycling, and  
380 degradation<sup>163</sup>.

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

383 cannabinoid receptor- $\beta$ -arrestin interactions is limited, although several mutational studies  
384 have explored the importance of C-terminal residues for binding of  $\beta$ -arrestins to CB1R<sup>164</sup>.

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

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

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

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

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

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

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

432 The property of pregnenolone as an endogenous NAM of the CB1R was further confirmed  
433 through *in vivo* studies. Namely, pregnenolone was able to attenuate a complete spectrum of  
434 cannabinergic effects induced by the CB1 agonist  $\Delta^9$ -THC in mice<sup>72,175</sup>.

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

## 442 CB1R function modulation and therapeutic opportunity

### 443 *Advantages of biased signaling and allosteric modulation*

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

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

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

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

#### 467 *Pregnenolone function and proof of concept studies*

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

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

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

490 ventral tegmental area (VTA), leading to the normalization of DA release<sup>184</sup>. In particular, the  
491 reported alleviating effects on THC-intoxication were specific to pregnenolone and not its  
492 downstream steroids<sup>72</sup>.

493 Together, these data highlight the therapeutic potential for pregnenolone against cannabis  
494 use disorder (CUD), for which no pharmacological treatment was available<sup>185</sup> until the recent  
495 development of C3-17 pregnenolone synthetic analogs, named AEF compounds, by the  
496 biotechnology company *Aelis Farma* (WO2012/160006A1; WO2014/083068A1;  
497 WO2019/162328A1 patents). Specifically, those compounds display a better therapeutic profile  
498 than pregnenolone, with increased half-life, no conversion into steroids and good bioavailability  
499 and administration *per os*. One of the lead AEF compounds is currently in a phase II clinical  
500 trial for CUD indications and shows very promising results (ClinicalTrials.gov; Identifier  
501 NCT03717272; Effect of AEF0117 on Subjective Effects of Cannabis in CUD Subjects).

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

513 Furthermore, pregnenolone administration is associated with an amelioration of psychosis-  
514 related symptoms in humans and murine models of schizophrenia. However, it is not known  
515 whether CB1Rs mediate those effects and whether the high doses of pregnenolone used  
516 induced an increase in downstream steroids, such as allopregnanolone, which is a potent  
517 modulator of GABA<sub>A</sub> receptors<sup>74</sup>.

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

525 pregnenolone normalized the firing properties of dopaminergic neurons, DA release, and  
526 somatosensory gating alterations in PCE animals<sup>188</sup>. Furthermore, the pharmacological  
527 inhibition of the enzyme 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) did not impair the  
528 neuroprotective action of pregnenolone, hence confirming that pregnenolone but not its  
529 metabolites were involved in these effects<sup>188</sup>.

## 530 **Conclusions and perspectives**

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

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

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

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**Table1.** Reported molecular targets for pregnenolone

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

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.

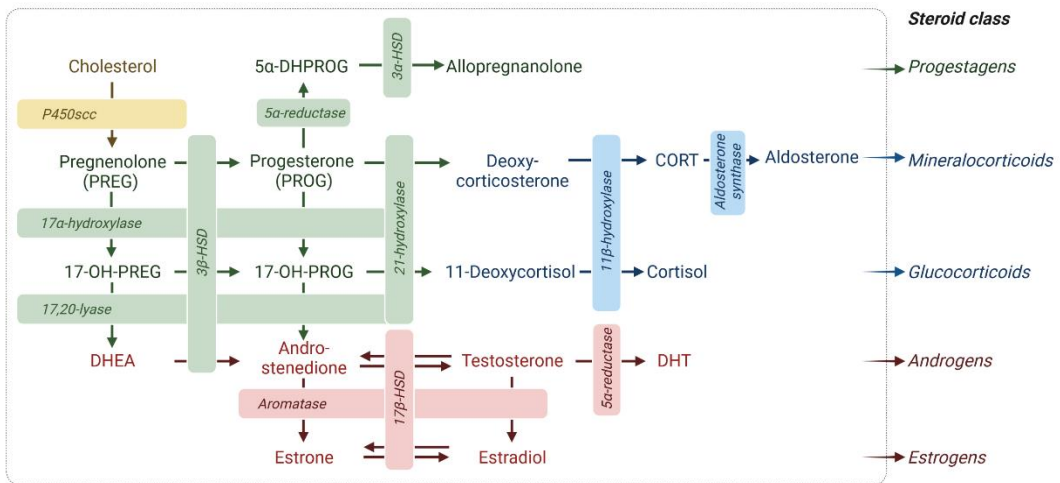
560 **Figure legends**

561 **Figure 1. Main steroid synthesis pathways from pregnenolone.** Cholesterol is converted  
562 into pregnenolone by the cholesterol side-chain cleavage enzyme (P450scc). Pregnenolone  
563 then serves as a precursor for the synthesis of other progestagens and other steroids, including  
564 mineralocorticoids, glucocorticoids, androgens, and estrogens. Abbreviations: 3 $\beta$ -HSD, 3 $\beta$ -  
565 hydroxysteroid dehydrogenase; 5 $\alpha$ -DHPROG, 5 $\alpha$ -dihydroprogesterone; 17 $\beta$ -HSD, 17 $\beta$ -  
566 hydroxysteroid dehydrogenase; CORT, corticosterone; DHEA, dehydroepiandrosterone; DHT,  
567 dihydrotestosterone; PREG, pregnenolone; PROG, progesterone. *Adapted from Hanukoglu,*  
568 *1992.*

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

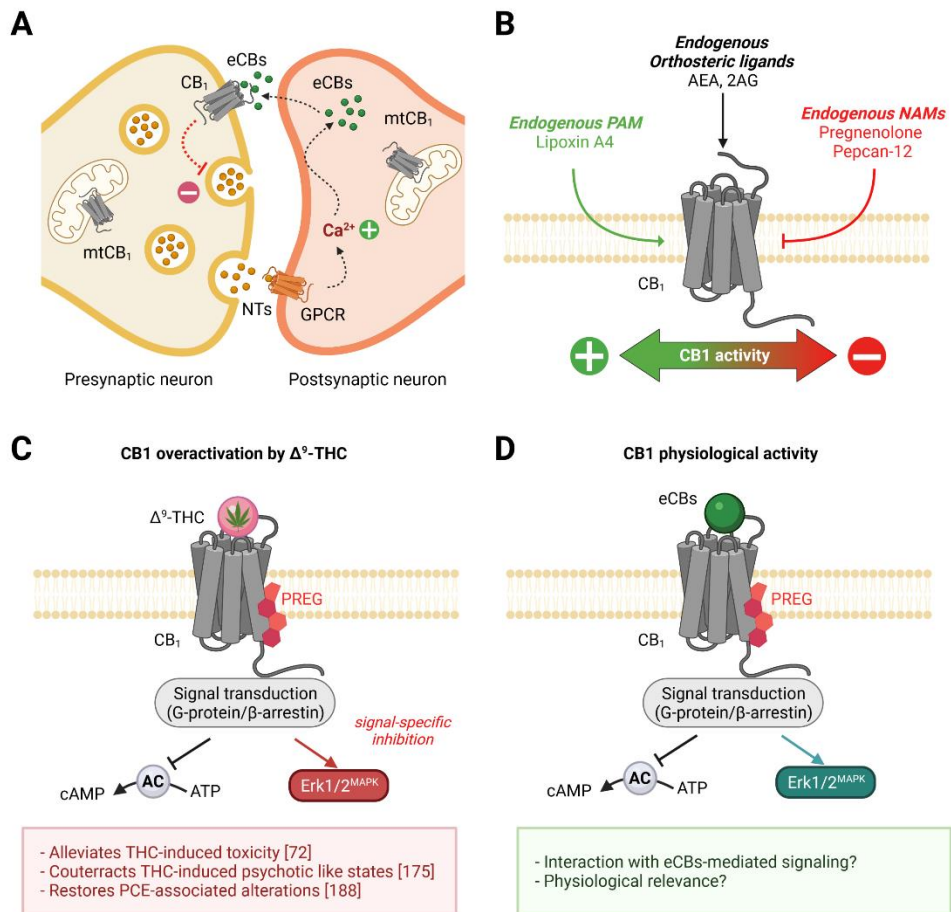
588

589 **Figure 1**



590

591 **Figure 2**



592