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Pregnenolone Can Protect the Brain from Cannabis Intoxication

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Further, our findings indicate that the role of TN-GnRH3 may be closely associated with female preference for a visually recognized individual. Individual recognition is central to many types of cooperative interactions (*23*), social decision-making in pair-bonding (*4*), kin recognition (*1*, *24*), and social hierarchy (*25*). The present findings shed new light on the importance of TN-GnRH3 neurons for female preference, as well as for individual recognition, for social neuroscience.

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Pregnenolone Can Protect the Brain from Cannabis Intoxication

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Pregnenolone is considered the inactive precursor of all steroid hormones, and its potential functional effects have been largely uninvestigated. The administration of the main active principle of *Cannabis sativa* (marijuana), Δ^9-tetrahydrocannabinol (THC), substantially increases the synthesis of pregnenolone in the brain via activation of the type-1 cannabinoid (CB_1) receptor. Pregnenolone then, acting as a signaling-specific inhibitor of the CB_1 receptor, reduces several effects of THC. This negative feedback mediated by pregnenolone reveals a previously unknown paracrine/autocrine loop protecting the brain from CB_1 receptor overactivation that could open an unforeseen approach for the treatment of cannabis intoxication and addiction.

Steroid hormones are important modulators of brain activity and behavior (*1*–*4*). Steroids play crucial roles in regulating physiological activities such as food intake, wakening, reproduction, and sexual behavior and participate in the regulation of mood and memory. Steroids also facilitate coping with stress and have been implicated in stress-related pathologies (*1*–*4*).

Although the most-studied steroids are produced in the periphery, some of them, named neurosteroids, are also synthesized directly in the brain (*5*, *6*) from the putatively inactive precursor pregnenolone (3β-hydroxypregn-5-en-20-one) (*5*). Active neurosteroids, such as pregnenolone sulfate (20-oxo-5-pregnen-3β-yl sulfate), allopregnanolone (3α-hydroxy-5α-pregnan-20-one), and DHEA (3ß-hydroxyandrost-5-en-17-one), have been implicated in the regulation of mood and cognitive activities, and their decline has been associated with aging-related impairments (*5*, *7*).

We investigated the involvement of neurosteroids in addiction by studying the effects of the major classes of drugs of abuse on their production in the brains of rats and mice. Concentrations of brain steroids were analyzed using gas chromatography coupled to mass spectrometry (*8*, *9*), which allows measuring, in the same sample, pregnenolone, DHEA, testosterone (17β-hydroxyandrost-4-en-3-one) and its metabolite DHT (dihydrotestosterone; 17β-hydroxy-5α-androstan-3-one), and the three stereoisomers pregnanolone (3α-hydroxy-5β-pregnan-20-one), allopregnanolone, and epiallopregnanolone (3β-hydroxy-5α-pregnan-20-one). As shown for the ventral striatum (the nucleus accumbens, NAc), in the brain of Wistar rats, basal levels were approximately 1 ng per gram of tissue (ng/g) for pregnenolone and testosterone, around 0.4 ng/g for allopregnanolone and DHT, and only traces of epiallopregnanolone (<0.2 ng/g) were found (Fig.1A). In C57BL/6N mice, the highest concentrations were found for pregnenolone and epiallopregnanolone, and the lowest concentrations were observed for testosterone. DHT was undetectable (fig. S1A). In both rat and mice brains, DHEA and pregnanolone were undetectable under basal conditions and after the administration of drugs.

Representative compounds of the major classes of drugs of abuse were injected into Wistar rats at doses corresponding approximately to the median effective dose (ED_{50}) for most of their unconditional behavioral effects: cocaine [20 mg per kilogram of body weight (mg/kg)], morphine (2 mg/kg), nicotine (0.4 mg/kg), alcohol (1 g/kg), and the main active principle of marijuana (*Cannabis sativa*), Δ^9-tetrahydrocannabinol (THC) (3 mg/kg) (*10*). The increase in pregnenolone (Fig. 1B and table S1) induced by THC (around 1500%) was several times higher and longer-lasting (2 hours) than the one induced by the other drugs (around 300% and 30 min). Dose-response studies showed a maximal THC-induced increase in pregnenolone of approximately 3000% in both Wistar rats (Fig. 1C) and C57BL/6N mice (fig. S1).

The effects of THC on pregnenolone-derived neurosteroids were not statistically significant in mice (fig. S1). In rats (Fig. 1C), a statistically sig-

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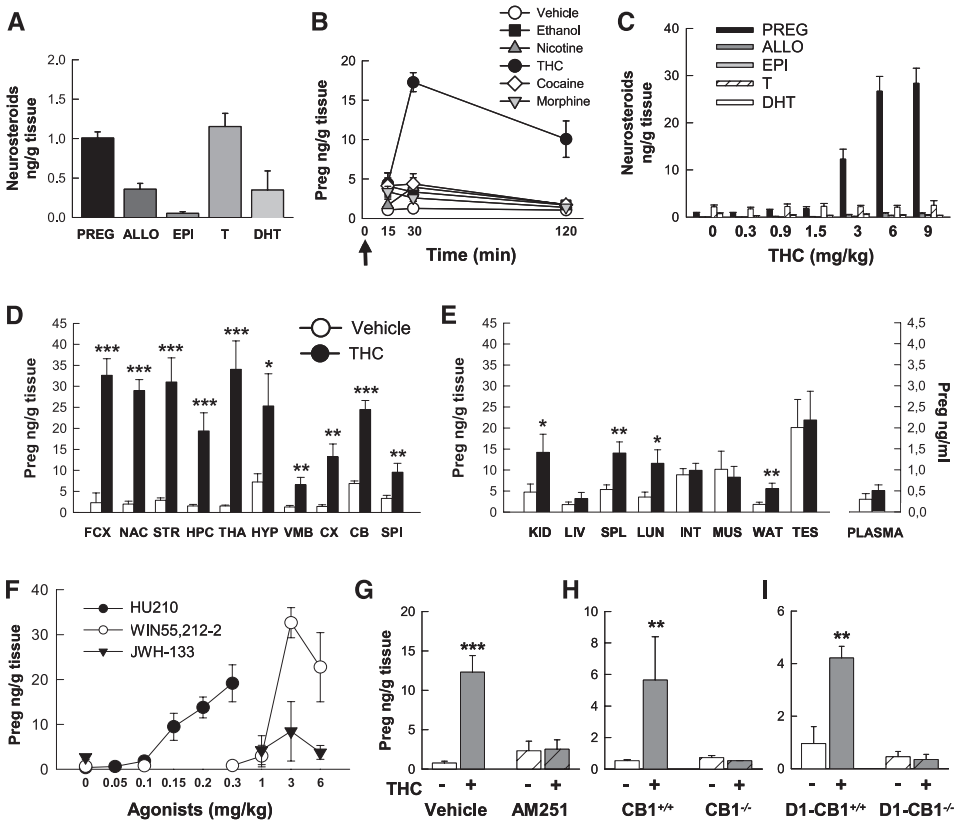

Fig. 1. THC increases pregnenolone levels by activating the CB_1 receptor. (**A**) Basal levels of pregnenolone (PREG), allopregnanolone (ALLO), epiallopregnanolone (EPI), testosterone (T), and dihydrotestosterone (DHT) in the NAc. (**B**) Compared to the major classes of drugs of abuse, cocaine [20 mg/kg, administered intraperitonally (ip)], morphine (2 mg/kg, ip), nicotine (0.4 mg/kg, ip), and ethanol (1 g/kg, ip), THC (3 mg/kg, ip) induced the highest increase in pregnenolone concentrations in the NAc. The arrow indicates the time of drug injection. (**C**) THC dose-dependently increased [$F(6,30) = 17.2$, $P < 0.001$] pregnenolone concentrations in the NAc, with minor effects on pregnenolone-derived downstream steroids. (**D** and **E**) THC at 9 mg/kg differently increased pregnenolone concentrations in brain structures and peripheral tissues: the prefrontal cortex (FCX), NAc, dorsal striatum (STR), hippocampus (HPC), thalamus (THA), hypothalamus (HYP), ventral midbrain (VMB), sensory motor cortex (CX), cerebellum (CB), spinal cord (SPI), kidney (KID), liver (LIV), spleen (SPL), lung (LUN), intestine (INT), muscle (MUS), white adipose tissue (WAT), testis (TES), and plasma. (**F**) In the NAc, the ip injection of the CB_1 agonists HU210 and WIN 55,212-2 dose-dependently increased pregnenolone levels [analysis of variance (ANOVA), $P < 0.001$ in all cases]. The CB_2 agonist JWH-133 had non–statistically significant effects. (**G**) The increase in pregnenolone concentrations induced by THC (3 mg/kg, ip) in the NAc was abolished by the CB_1 antagonist AM251 (8 mg/kg, ip) injected 30 min before THC. THC (12 mg/kg, ip) induced an increase in pregnenolone levels in the NAc of wild-type mice but not in KO mice with a (**H**) complete ($CB_1^{-/-}$) or (**I**) neuron-specific (D_1-$CB_1^{-/-}$) deletion of the CB_1 receptor. Data are expressed as mean ± SEM (n = 6 to 12 animals per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to animals that did not receive THC. All experiments except (H) and (I) were performed in Wistar rats.

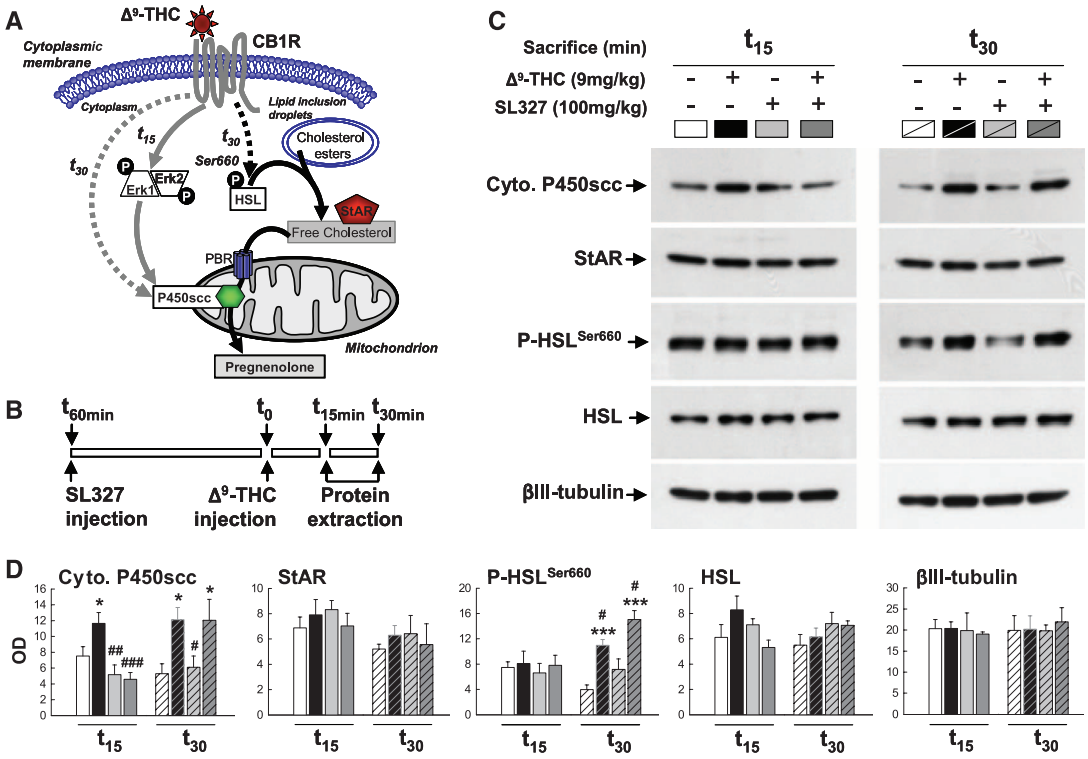

Fig. 2. THC can increase pregnenolone synthesis through proteins involved in neurosteroidogenesis. Schematic representation of (**A**) the proposed molecular mechanism and (**B**) the protocol used. (**C**) Representative Western blots and (**D**) densitometric quantification of NAc expression of cytochrome P450scc, StAR, P-HSL^{Ser660}, HSL, and βIII-tubulin proteins, in Wistar rats ip injected with THC (9 mg/kg) after treatment with SL327 or vehicle. 15 min after THC administration, the levels of cytochrome P450scc increased via an Erk1/2^{MAPK}-dependent mechanism. 30 min after THC administration, with an Erk1/2^{MAPK}-independent mechanism, cytochrome P450scc was still increased and HSL was activated by phosphorylation. THC administration did not modify the levels of StAR proteins. Data are expressed as mean ± SEM (n = 5 to 7 animals per group). OD, optical density. *$P < 0.05$, ***$P < 0.001$ in comparison to vehicle-treated rats (white and white-striped bars). #$P < 0.05$, ##$P < 0.005$, ###$P < 0.001$ in comparison to THC-treated rats (black and black-striped bars). Fisher's protected least significant difference post hoc test was used after ANOVA.

nificant effect was observed only for allopregnanolone and epiallopregnanolone. However, even at the highest dose of THC (9 mg/kg), the increase in these pregnenolone metabolites (Fig. 1C) was several times lower than the increase observed in pregnenolone (in ng/g: allopregnanolone = 0.73 ± 0.14, epiallopregnanolone = 0.40 ± 0.08, and pregnenolone = 28.38 ± 3.16).

The highest THC-induced increase in pregnenolone in Wistar rats (Fig. 1D) was observed in the NAc, prefrontal cortex, striatum, and thalamus and the lowest in the spinal cord, ventral midbrain, sensory motor cortex, and peripheral tissues such as the kidney, spleen, lung, and white fat (Fig. 1E). In the liver, gastrointestinal tract, muscle, testis, and plasma, THC had no significant effect.

The effects of THC in the brain are mainly mediated by the type-1 cannabinoid (CB_1) receptor (*11*–*13*). In the NAc, similarly to THC, injection of Wistar rats with the CB_1/CB_2 cannabinoid receptor agonists HU210 or WIN55,212-2 increased pregnenolone levels (Fig. 1F) at doses that are in line with their respective affinities for the CB_1 receptor (*14*). The CB_2-selective agonist JWH133 had no significant effect (Fig. 1F). THC effects on pregnenolone in the NAc were suppressed (i) by the CB_1-selective antagonist AM251 in Wistar rats (Fig. 1G); (ii) in constitutive CB_1 receptor knockout (KO) mice ($CB_1^{-/-}$, Fig. 1H), which lack CB_1 receptors in all cell types; and (iii) in conditional mutant mice (*15*), which lack CB_1 receptors in the majority of striatal γ-aminobutyric acid (GABAergic) spiny neurons [the ones containing the dopamine receptor D_1 (D_1-$CB_1^{-/-}$)] (Fig. 1I) (*9*). The CB1-KO mice were from mice strains in which the C57BL/6N genotype was predominant (six or seven backcrossing generations). These data indicate that THC increases pregnenolone through activation of the CB_1 receptor.

Pregnenolone is synthetized in the mitochondria from cholesterol by cytochrome P450scc (*16*, *17*). When high levels of steroid synthesis are needed (Fig. 2A), new cholesterol is provided first by hydrolysis of cytoplasmic cholesterol esters by the hormone-sensitive lipase (HSL), activated via phosphorylation of serine 660, and then by cholesterol transport into the mitochondria by the steroidogenic acute regulatory protein (StAR) (*16*, *17*).

In Wistar rats, StAR proteins were not modified by THC administration (9 mg/kg) at any time (Fig. 2, C and D) (*9*). In contrast, THC induced a rapid (15 min after THC administration) increase in the levels of P450scc (Fig. 2, C and D) that was dependent on the activity of the extracellular signal–regulated kinases 1/2 mitogen-activated protein kinase ($Erk1/2^{MAPK}$). This rapid increase in P450scc (*9*) was abolished by the selective inhibitor of $Erk1/2^{MAPK}$ phosphorylation SL327 (100 mg/kg; Fig. 2, C and D). Later (30 min after THC administration), an $Erk1/2^{MAPK}$-independent mechanism sustained the increase in P450scc levels and phosphorylated HSL (Fig. 2, C and D). These coordinated modifications can contribute to the increase in pregnenolone induced by THC.

Among the behavioral and somatic CB_1 receptor–dependent effects of THC, the so-called "cannabinoid tetrad" (*9*, *18*) is considered a prototypic signature of cannabinoid intoxication (*14*). The cannabinoid tetrad includes hypolocomotion, hypothermia, catalepsy, and analgesia. The administration of the inhibitor of pregnenolone synthesis aminogluthetimide (AMG, 50 mg/kg) (*18*) to C57BL/6N mice increased all of these behavioral and somatic effects of THC (Fig. 3, A to D). The injection of pregnenolone reversed the effects of AMG and inhibited the effects of THC but had no effect in animals that did not receive THC (Fig. 3, E and H). These data show that THC-induced production of pregnenolone exerts a negative feedback on CB_1 receptor activity.

Two well-known behavioral disturbances accompany cannabis use in humans: (i) an increase in food palatability and craving that can promote food intake (*19*, *20*) and (ii) a decrease in memory performance (*21*). THC increases food intake in both sated rats and food-deprived C57BL/6N mice (*22*, *23*) and also impairs memory consolidation in an object-recognition task in C57BL/6N mice (*24*). Pregnenolone administration (2 to 6 mg/kg) blocked THC-induced food intake in Wistar rats (Fig. 4A) and in C57BL/6N mice (Fig. 4B) and blunted the memory impairment induced by THC in mice (Fig. 4C), but it did not modify these behaviors per se (Fig. 4, A to C). As previously shown (*24*), THC-induced memory impairments were not due to nonspecific motor effects of THC, because THC did not significantly modify locomotor activity during the object-recognition task.

Cannabinoid drugs modulate brain activity and behavior principally by the activation of presynaptic CB_1 receptors, which inhibit the release of several neurotransmitters and in particular GABA and glutamate (*25*). We assessed the effect of THC on glutamate release (*9*) by measuring excitatory postsynaptic currents (EPSCs) in NAc principal neurons in brain slices obtained from adult Sprague-Dawley rats (Fig. 4, D and E). Bath application of THC (20 μM) reliably inhibited synaptic transmission in control slices (34.3 ± 3.7% of inhibition). The effect of THC was significantly attenuated when slices were pre-treated with pregnenolone 100 nM (15.1 ± 1.8% of inhibition). These effects were probably due to a presynaptic action of pregnenolone. Thus, pregnenolone blocked the increase in paired-pulse ratio (PPR) induced by THC (*9*) but did not modify either the amplitude or the decay time of miniature EPSCs (mEPSCs) (table S2). Changes in PPR and in the mEPSC parameters studied here are indicators of changes in neurotransmitter release and in postsynaptic response, respectively.

To analyze the potential effects of pregnenolone on the development of cannabis abuse and dependence, we first studied dopamine release in the NAc (*9*). Activation of dopaminergic neurons and increase in NAc dopamine extracellular levels are common effects of most drugs of abuse and have been implicated in drug addiction (*26*).

In anesthetized Sprague-Dawley rats, we simultaneously used microdialysis and extracellular unitary recordings (*9*) to estimate dopamine release in the NAc and the firing activity of

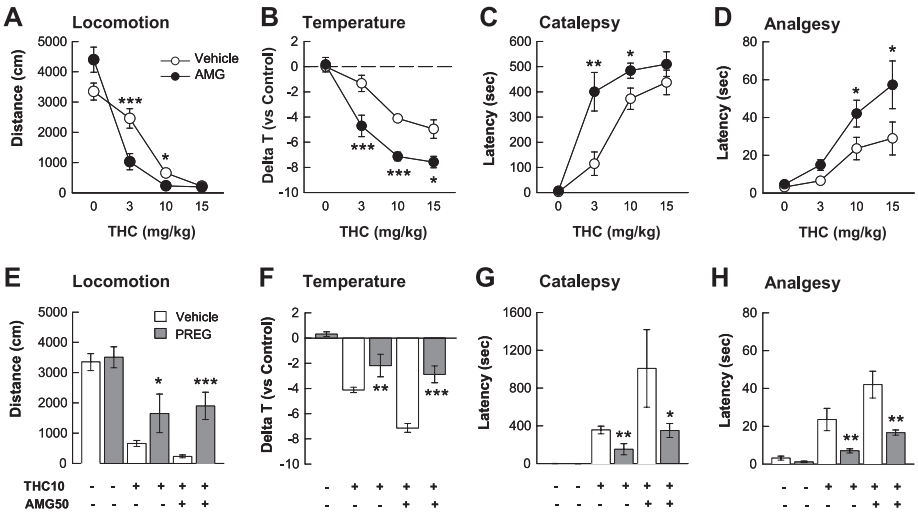

Fig. 3. The cannabinoid tetrad induced by THC was inhibited by pregnenolone. In C57Bl/6N mice, the administration, 30 min before THC, of AMG (50 mg/kg, ip), a P450scc inhibitor that blocks pregnenolone synthesis, increased the effects of THC: (**A**) hypolocomotion [$F(3,98) = 13.8$, $P < 0.001$], (**B**) hypothermia [$F(3,98) = 4.7$, $P < 0.01$], (**C**) catalepsy [$F(3,98) = 2.1$, $P < 0.05$], and (**D**) analgesia [$F(3,98) = 2.2$, $P < 0.05$]. (**E** to **H**) Pregnenolone [6 mg/kg, administered subcutaneously (sc)] administered at the same time as AMG (50 mg/kg, ip) prevented the increase in the responses to THC (10 mg/kg, ip) induced by AMG. Injection of pregnenolone (6 mg/kg, sc) alone 30 min before THC (10 mg/kg, ip) also reduced the behavioral effects of THC. Pregnenolone had no effects in animals that did not receive THC. Data are expressed as mean ± SEM (n = 6 to 12 animals per group). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ as compared to vehicle-treated mice.

dopaminergic neurons in the ventral tegmental area (VTA), respectively. THC, administered intravenously at escalating cumulative doses (0.15, 0.3, 0.6, and 1.2 mg/kg) infused at 1-min intervals (*9*), induced a significant increase in extracellular NAc dopamine levels (Fig. 4G) and in the firing activity of VTA neurons (Fig. 4F). Both effects were blunted by pretreatment with pregnenolone (2 m/kg) (Fig. 4, F and G).

We then analyzed the impact of pregnenolone on the reinforcing effects of cannabinoid drugs, using the intravenous self-administration model (*9*). In this model, CD1 mice were used, because this strain readily learns to produce an operant response (nose-poking into a hole) to obtain an intravenous infusion of CB_1 agonists. Mice readily learned to self-administer the CB_1 agonist WIN55,212-2, showing a clear preference for the device that triggered the infusion of the drug (active hole) in comparison to the inactive device, in which responding had no scheduled consequences (inactive hole) (Fig. 4H). Injections of pregnenolone (2 and 4 mg/kg) before each self-administration session reduced the intake of WIN 55,212-2 (Fig. 4I) and reduced the break point in a progressive ratio schedule (Fig. 4J), which is considered a reliable measure of the motivation for the drug (*9*).

To provide first insights about the mechanism of action through which pregnenolone can modify the behavioral and neurobiological effects of THC, we studied the effects of pregnenolone in cell lines expressing the human CB_1 (hCB_1) receptor (fig. S2). Briefly (*9*), pregnenolone (up to 100 μM) did not modify the equilibrium binding of the radiolabeled CB_1 receptor agonists [^{3}H]CP55,940 and [^{3}H]WIN 55,212-2 (fig. S2A). In contrast, pregnenolone (between 10 nM and 1 μM, depending on the cellular model) inhibited the increase in P-Erk1/2^{MAPK} and the decrease in cellular and mitochondrial respiration induced by THC (*27*) (fig. S2, B to F). This range of pregnenolone concentrations is compatible with the ones (between 10 and 80 ng/g, approximately 30 and 250 nM, respectively) that are observed after THC injections (Fig. 1 and fig. S1) or pregnenolone injections at behaviorally active doses (fig. S3). Pregnenolone up to 1 μM did not decrease the THC-induced reduction of adenosine 3′,5′-monophosphate (cAMP).

These effects suggest that pregnenolone acts as a signaling-specific negative allosteric modulator. Synthetic negative allosteric modulators of CB_1 receptors have been described to display signaling pathway specificity (*28*, *29*). However, these drugs increase agonist binding affinity to the CB_1 receptor, increase agonist-induced Erk1/2^{MAPK} phosphorylation, and inhibit CB_1 agonist–induced inhibition of adenylyl cyclase (*28*, *29*). One possible explanation of these differences is that synthetic antagonists bind to a structural pocket that is devoid of a physiological binding function. In contrast, the endogenous negative allosteric modulator pregnenolone probably binds to a different, evolution-selected, physiologic binding pocket. By using the Forced-Biased Metropolis Monte Carlo (MMC) simulated annealing program (*9*, *30*), we found a potential binding pocket for pregnenolone in the lipid facing the TMH1/TMH7/Hx8 region of the CB_1 receptor (fig. S4A). This binding pocket was validated using a mutant hCB1 receptor (*9*) that contained a point mutation that should forbid the binding of the ketone end of pregnenolone to the CB_1 receptor (fig. S4B). Pregnenolone lost its inhibitory effects on THC-induced decrease in cellular respiration in cells transfected with the mutant hCB1 receptor (fig. S4E).

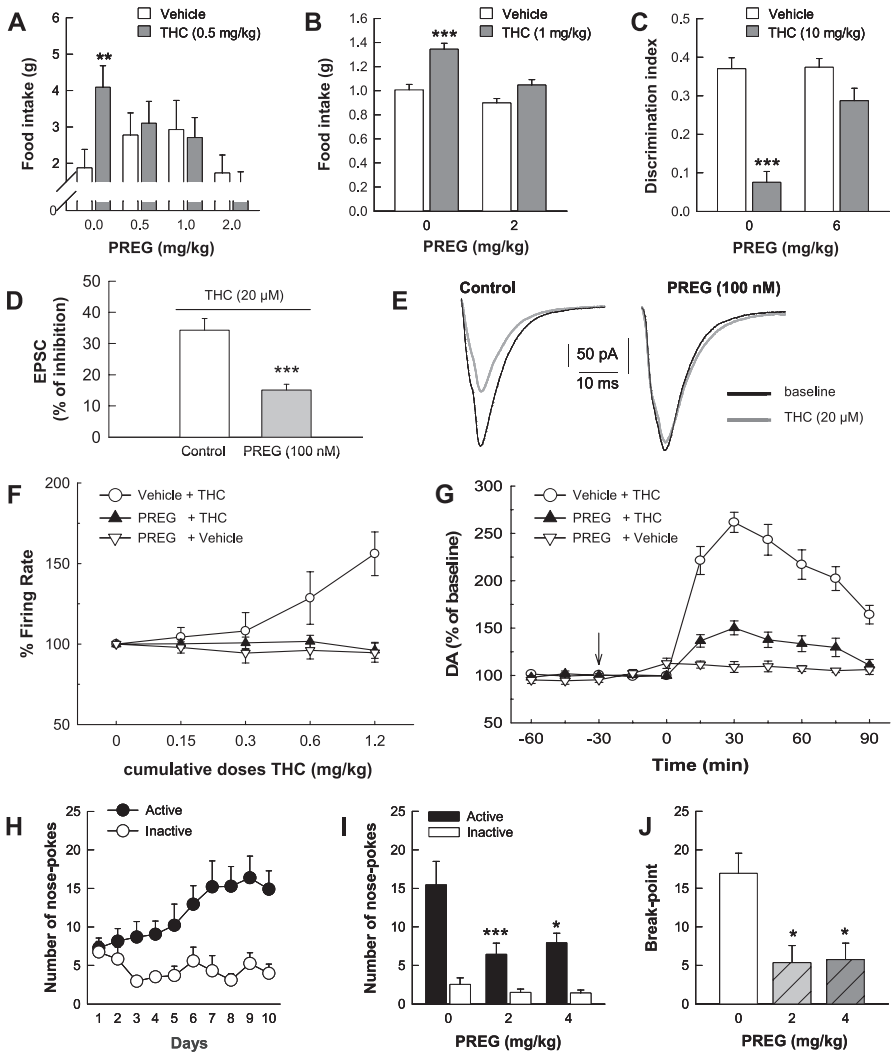

Fig. 4. Pregnenolone inhibits behavioral and neurobiological effects of cannabinoid drugs. Pregnenolone injections inhibited the increase in food intake in (**A**) ad libitum fed Wistar rats [$F(3,94) = 3.65$, $P < 0.02$] and (**B**) 24-hour food-deprived C57Bl/6N mice, as well as (**C**) the memory impairment [$F(3,23) = 24.6$, $P < 0.001$] induced by THC in C57Bl/6N mice. (**D**) Bath application of THC (20 μM) inhibited glutamatergic synaptic transmission in NAc principal neurons in brain slices obtained from adult Sprague-Dawley rats (controls, $n = 8$). This effect was reduced when brain slices were preincubated with pregnenolone 100 nM ($n = 9$). (**E**) Synaptic current traces from representative experiments averaged during baseline and after 40 min of THC exposure. Pregnenolone injections (2 mg/kg, sc, 30 min before THC) in Sprague-Dawley rats decreased the THC-induced increase in (**F**) the firing rate of VTA dopaminergic neurons [$F(4,48) = 8.33$, $P < 0.001$] and in (**G**) the dopamine outflow in the NAc [$F(10,120) = 20.28$, $P < 0.001$]. THC was administered intravenously at escalating cumulative doses (0.15, 0.3, 0.6, and 1.2 mg/kg) infused at 1-min intervals. (**H**) CD1 mice acquired intravenous self-administration of the cannabinoid agonists WIN 55,512-2 (0.0125 mg/kg per infusion) as shown by the higher number of nose pokes in the active device (hole) than in the inactive one [$F(1,18) = 38.3$, $P < 0.001$]. (**I**) After acquisition, the injection of pregnenolone (2 or 4 mg/kg, sc) decreased the number of responses in the active device. (**J**) Pregnenolone also decreased the motivation for WIN 55,512-2, as measured by the reduction in the break point in a progressive ratio schedule. Data are expressed as mean ± SEM. (A) to (C) ($n = 6$ to 12 animals per group), (F) and (G) ($n = 6$ or 7 animals per group), (H) to (J) ($n = 8$ animals per group). The arrow indicates the time of pregnenolone injection, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle-treated controls.

The results presented here provide an example of an unforeseen paracrine/autocrine loop, through which brain steroids can control the activity of a G protein–coupled receptor (GPCR). Thus, CB_1 receptor stimulation increases brain pregnenolone levels, which in turn exerts a negative feedback on the activity of the CB_1 receptor, antagonizing most of the known behavioral and somatic effects of THC. Pregnenolone probably acts as a signaling-specific negative allosteric modulator binding to a site distinct from that occupied by orthosteric ligands. Pregnenolone, similarly to some of the other previously described allosteric modulators (*31*, *32*), does not modify agonist binding but only agonist efficacy; these effects are compatible with the allosteric two-state model (*31*).

Other drugs of abuse also increased pregnenolone levels, but such an increase was in a much lower range of concentrations than the ones induced by THC, which suggests a different mechanism of action. However, most drugs of abuse also modify the activity of the endocannabinoid system (*33*) and could increase pregnenolone through an indirect activation of the CB_1 receptor. This seemed to be the case for cocaine, whose effects on pregnenolone were blocked by pretreatment with a CB_1 antagonist (fig. S5).

Although pregnenolone has been considered an inactive precursor, our data indicate that pregnenolone, and not its downstream-derived neurosteroids, inhibits the effects of THC that are mediated by the CB_1 receptors. Thus, in mice, the administration of THC or of pregnenolone, in the range of behaviorally active doses (2 to 8 mg/kg), did not modify pregnenolone downstream-active steroids such as allopregnanolone (figs. S1 and S3). In addition, the administration of allopregnanolone did not modify behavioral responses to THC, such as THC-induced food intake (fig. S6).

An increasing number of synthetic allosteric modulators of GPCRs have been described (*28*, *29*, *31*, *32*, *34*, *35*). However, whether endogenous allosteric modulators physiologically regulate the activity of GPCRs has been questioned (*31*). Recently, the lipid lipoxin A4 has been proposed as a positive allosteric modulator of CB_1 receptors, suggesting that endogenous modulation of endocannabinoid signaling is a physiological process (*36*). Our findings confirm and extend this hypothesis, uncovering an endogenous negative allosteric modulator of the CB_1 receptor and revealing one of the possible functions of endogenous negative allosterism: the control of GPCR overactivation.

Allosteric modulators may offer several advantages as therapeutic drugs (*31*, *32*, *34*, *35*). Allosteric modulators do not modify the activity of the receptors per se but enhance or attenuate the effects of endogenous or exogenous ligands. Allosteric drugs can also be signaling-specific, thereby regulating only some of the functions of the receptor. As such, they respect the physiology of the target system, can modify only the signaling pathway involved in the disease, and have a more targeted action than orthosteric compounds (*31*, *32*, *34*, *35*).

In comparison with orthosteric antagonists, drugs with the pharmacological profile of pregnenolone could have supplementary advantages for the treatment of drug dependence. When used at high doses, which effectively block the activity of the target receptor, orthosteric antagonists often induce a profound discomfort that is not well tolerated by patients. Lower doses of orthosteric antagonists are also not practical, because their reversible antagonism can be overcome by taking higher doses of the drug. Signaling pathway–specific allosteric inhibitors, such as pregnenolone, should be better tolerated because they do not produce an inhibition of all CB_1 receptor activities, and their effects cannot be overcome by increasing drug intake. This new understanding of the role of pregnenolone has the potential to generate new therapies for the treatment of cannabis dependence.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S10
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References

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