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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.

References and Notes

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Supplementary Materials
www.sciencemag.org/content/343/6166/91/suppl/DC1
Materials and Methods
Figs. S1 to S19
Movies S1 to S3
References 18–29
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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), δ9-tetrahydrocannabinol (THC), substantially increases the synthesis of pregnenolone in the brain via activation of the type-1 cannabinoid (CB1) receptor. Pregnenolone then, acting as a signaling-specific inhibitor of the CB1 receptor, reduces several effects of THC. This negative feedback mediated by pregnenolone reveals a previously unknown paracrine/autocrine loop protecting the brain from CB1 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, wakefulness, 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-hydroxypreg-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 allopregnanolone ((3β-hydroxy)-5α-pregnan-20-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. 1A). In both rat and mice brains, DHEA and pregnenolone 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 (ED50) for most of their unconditioned 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), δ9-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 30% 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-
Fig. 1. THC increases pregnenolone levels by activating the CB1 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 intraperitoneally (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 \[P_{6.30} = 17.2, P < 0.001\] pregnenolone concentrations in the NAc, with minor effects on pregnenolone-derived downstream steroids. (D and E) THC at 9 mg/kg differentially 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 CB1 agonists HU210 and WIN 55,212-2 dose-dependently increased pregnenolone levels [analysis of variance (ANOVA), \(P < 0.001\) in all cases]. The CB2 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 CB1 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 (CB1–/–) or (I) neuron-specific (D1–CB1–/–) deletion of the CB1 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. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) 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.
significant 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 kidney, spleen, lung, and white fat (Fig. 1E). These data showed that THC-induced production of pregnenolone exerts a negative feedback on CB1 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.

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 showed that THC-induced production of pregnenolone exerts a negative feedback on CB1 receptor activity.

Among the behavioral and somatic CB1 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 aminoglutethimide (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 showed that THC-induced production of pregnenolone exerts a negative feedback on CB1 receptor activity.

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 dopamine neurons.

Cannabinoid drugs modulate brain activity and behavior principally by the activation of presynaptic CB1 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 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.

**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 mg/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 CB1 agonists. Mice readily learned to self-administer the CB1 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 CB1 (hCB1) receptor (fig. S2). Briefly (9), pregnenolone (up to 100 µM) did not modify the equilibrium binding of the radiolabeled CB1 receptor agonists [3H]CP55,940 and [3H]WIN 55,212-2 (Fig. S2A). In contrast, pregnenolone (between 10 nM and 1 µM, depending on the cellular model) increased the preference for CB1 and decreased the motivation for 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 CB1 receptors have been described to display signaling pathway specificity (28, 29). However, these drugs increase agonist binding affinity to the CB1 receptor, increase agonist-induced Erk1/2 MAPK phosphorylation, and inhibit CB1 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 endogeneous 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 CB1 receptor (fig. S4A). This binding pocket was validated using a mutant hCB1 receptor that contained a point mutation in the TMH3 (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) an 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.

www.sciencemag.org SCIENCE VOL 343 3 JANUARY 2014 97
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 CB1 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.

References and Notes