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ORIGINAL ARTICLE

Pregnenolone blocks cannabinoid-induced acute psychotic-like states in mice

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Cannabis-induced acute psychotic-like states (CIAPS) represent a growing health issue, but their underlying neurobiological mechanisms are poorly understood. The use of antipsychotics and benzodiazepines against CIAPS is limited by side effects and/or by their ability to tackle only certain aspects of psychosis. Thus, safer wide-spectrum treatments are currently needed. Although the blockade of cannabinoid type-1 receptor (CB1) had been suggested as a therapeutical means against CIAPS, the use of orthosteric CB1 receptor full antagonists is strongly limited by undesired side effects and low efficacy. The neurosteroid pregnenolone has been recently shown to act as a potent endogenous allosteric signal-specific inhibitor of CB1 receptors. Thus, we tested in mice the potential therapeutic use of pregnenolone against acute psychotic-like effects of Δ^9-tetrahydrocannabinol (THC), the main psychoactive component of cannabis. We found that pregnenolone blocks a wide spectrum of THC-induced endophenotypes typically associated with psychotic-like states, including impairments in cognitive functions, somatosensory gating and social interaction. In order to capture THC-induced positive psychotic-like symptoms (e.g. perceptual delusions), we adapted a behavioral paradigm based on associations between different sensory modalities and selective devaluation, allowing the measurement of mental sensory representations in mice. Acting at hippocampal CB1 receptors, THC impaired the correct processing of mental sensory representations (reality testing) in an antipsychotic- and pregnenolone-sensitive manner. Overall, this work reveals that signal-specific inhibitors mimicking pregnenolone effects can be considered as promising new therapeutic tools to treat CIAPS.

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INTRODUCTION

After tobacco, alcohol and caffeine, cannabis is the most widely used psychotropic drug, with an estimated 125–227 million consumers worldwide.[1] A link between cannabis intoxication and the development of psychosis has long been recognized[2–6] and psychotic-like states have been documented in numerous case reports and estimated to occur at least once in about 20–50% of individuals who use cannabis.[3,7] Cannabinoid agonists such as the main psychoactive component of the *Cannabis sativa* plant, Δ^9-tetrahydrocannabinol (THC), have been shown to produce a full range of positive and negative psychotic-like symptoms in humans, such as hallucinations, delusions, disorganized speech, emotional withdrawal and decreased social interaction, and other endophenotypes typically associated with psychosis, such as cognitive (memory impairments) and somatosensory gating alterations (reduction of pre-pulse inhibition, PPI).[5,6,8–12] Owing to the high prevalence of cannabis use, it is urgent to better understand cannabis-induced acute psychotic-like states (CIAPS) and to develop novel treatments.

Antipsychotic drugs and benzodiazepines currently used against CIAPS have serious limitations and side effects.[13] Typical antipsychotics are linked to extrapyramidal side effects such as tremors, spasticity and tardive dyskinesia,[14,15] whereas atypical antipsychotics can produce sedation and weight gain[16,17] and benzodiazepines induce sedation and potentially addiction.[18] Moreover, these drugs can only tackle certain psychotic symptoms (e.g. positive ones), but not others (e.g. cognitive endophenotypes).[13,19] Given these limits of currently used drugs, researchers are now investigating the role of new neurobiological substrates in the pathophysiology of psychotic-like states.

The endocannabinoid system has been considered as an emerging target for the development of antipsychotic treatments.[20,21] Some years ago, the blockade of CB1 receptors had been suggested as a therapeutical means against psychoses.[22–24] However, the use of orthosteric cannabinoid antagonists is strongly limited by undesired side effects[25,26] and contradictory results have been obtained in animal models,[5,20] possibly due to the ability of these drugs to block all cellular functions of CB1 receptors, hence causing opposite effects. Indeed, the clinical use of CB1 antagonists has been stopped as an antipsychotic treatment in humans, due to lack of proven efficacy.[21]

Recently, the neurosteroid pregnenolone has been shown to act as a potent endogenous signal-specific inhibitor of CB1 receptor. By binding a specific allosteric CB1 site, pregnenolone blocks THC-induced activation of extracellular-regulated kinases and reduction of mitochondrial activity, but not other signaling pathways induced by activation of CB1 receptors.[27] This is a major difference as compared with orthosteric antagonists that block all cellular effects of THC, thus explaining the lack of undesired behavioral effects of pregnenolone.[27,28]

In this study, we analyzed whether pregnenolone was able to block the THC-induced endophenotypes resembling acute

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psychotic-like states such as cognitive impairment, alteration of somatosensory gating (i.e. decreased PPI) and reduction of social interaction in mice. Moreover, we adapted a behavioral approach recently proposed in rodents[29–31] to study alterations in mental sensory representations which are hallmarks of positive psychotic-like states. The results show that pregnenolone can block the full range of acute psychotic-like symptoms and related endophenotypes induced by THC, thereby suggesting that drugs mimicking pregnenolone activity could be used to treat CIAPS.

MATERIAL AND METHODS

Mice

All experimental procedures were approved by the Committee on Animal Health and Care of INSERM and the French Ministry of Agriculture and Forestry (authorization number, A501350). Male C57BL/6-N mice purchased from Janvier (Genest St Isle, France) were used in this study. The age of the animals at the beginning of all the behavioral experiments was 9–10 weeks. Except for the spontaneous alternation and social interaction tasks (see below), all the behavioral approaches were performed with independent groups of mice. All experiments were performed during the first part of the light phase (0900 to 1400 hours). Experimenters were always blind to treatments.

Drugs

Rimonabant, purchased from Cayman Chemical (Ann Arbor, MI, USA), was dissolved in a mixture of 4% ethanol, 4% Cremophor-EL and 92% of saline (NaCl 0.9%). Risperidone and lithium chloride (LiCl), obtained from Sigma-Aldrich (St Quentin Fallavier, France), were dissolved in saline. Amphetamine, obtained from Calaire Chimie (Calais, France), was dissolved in saline. THC was purchased from THC-Pharm (Frankfurt, Germany) and dissolved in 4% ethanol, 4% Cremophor-EL and 92% saline. Pregnenolone was dissolved in Tween 80 (1 drop/3 ml) and dimethyl sulfoxide (2.5%) diluted in saline as previously described.[27] Rimonabant, risperidone, LiCl, amphetamine and THC were all administered intraperitoneally (i.p.) whereas pregnenolone was injected subcutaneously (s.c.). All drugs were injected in a volume of 10 ml kg^{-1}. MK801 was purchased from Tocris (Bristol, UK) and dissolved in saline. It was administered subchronically i.p. during 7 days in 3-week-old mice.

Chemical compounds

The solutions used in the aversion tasks were presented in 50 ml drinking tubes in the home cage with either banana (0.05%, isoamyl acetate) or almond (0.01%, benzaldehyde) for odors, and sucrose (5%) or maltodextrin (5%) for tastes. All compounds were obtained from Sigma-Aldrich.

Surgery

Stereotaxical surgeries, performed as previously described,[32] were aimed at implanting guide cannulas (Plastics One, Roanoke, VA, USA) targeting the hippocampus with the following coordinates: AP, −1.8; ML, +/−1; DV, −1.3.[33] Drugs or vehicles were injected using a peristaltic pump (PHD 22/2000 Syringe Pump Infusion; Harvard Apparatus, Holliston, MA, USA, flow rate: 0.5 µl min^{-1}). The correct placement of the hippocampal cannulas was verified *post hoc* by injection of 2% pontamine sky blue solution in 0.5 M sodium acetate.

Behavioral procedures

Spontaneous alternation. Spontaneous alternation was assessed in a Y-maze (42 cm long, 8 cm wide, 120° between arms) in a room bearing a ceiling-mounted camera. Mice were placed in the maze for 8 min during which each arm entry was scored (with all four paws in one arm, defined by a black line in the arm entrance). A spontaneous alternation refers to a triplet of consecutive arm explorations with all three arms being different. Accordingly, we counted the number of correct triplets (exploration of three different consecutive arms) to calculate the percentage of alternation using the following formula:

$$\% \text{ alternation} = \frac{\text{Number of correct triplets}}{\text{Total number of triplets}} \times 100$$

Mice were treated with an injection of vehicle or THC (0.3, 1 and 3 mg kg^{-1}) 120 min before the task. Pregnenolone (6 mg kg^{-1}, s.c.) was administered either 10 min before or 30 min after THC. We controlled the total locomotion by assessing that there was no difference in total number of alternations between the THC groups and the vehicle group. The dose and timing of THC administration were chosen on the basis of preliminary experiments. Whereas a dose of 3 mg kg^{-1} injected 60 min before the test induced a clear reduction in total alternations (Supplementary Figure S1A), the same dose administered 120 min before did not show effects on locomotion (Supplementary Figure S1B).

Morris water maze

The hippocampal-dependent delayed matching-to-place version of the Morris water maze paradigm was performed using a white circular pool as previously described.[34] After a day of habituation to the pool and the platform, mice started training sessions with visual clues placed on the wall. Each training session (one per day) consisted in four trials where the mouse was initially placed in the water facing the wall until it reached the hidden platform (cut off at 90 s), whose location was changed every day. The interval between trials was of 30 s. The starting location was identical for the first and the fourth trials, but different for the other ones. The cognitive performance was assessed by the calculation of the following saving ratio formulas:

$$\text{saving ratio latency} = \frac{\text{latency trial 1} - \text{latency trial 4}}{\text{latency trial 1} + \text{latency trial 4}}$$

$$\text{saving ratio distance} = \frac{\text{distance trial 1} - \text{distance trial 4}}{\text{distance trial 1} + \text{distance trial 4}}$$

During the training phase, animals were discarded if within three consecutive days they did not reach the platform in any of the four trials before the cutoff. Fifteen to 20% of the animals had to be discarded due to this issue. Starting from day 5 of training, all animals received a vehicle injection 30 min before session. The training lasted until the average latency times and distances to reach the platform between trial 2, 3 and 4 were decreased, as compared with trial 1, for three consecutive days (generally 12 days). The average of these three consecutive days (with the vehicle treatment) was calculated as vehicle condition. The day after, all the animals were treated with THC (5 mg kg^{-1}, i.p.) 30 min before the test session. On this day, mice received also an injection of pregnenolone (6 mg kg^{-1}, s.c.) or vehicle 10 min before the THC injection as previously described.[27] The dose and timing of THC administration were chosen in the basis of previous findings.[34]

Pre-pulse inhibition

PPI was measured in a startle chamber (SR-Lab San Diego Instruments, San Diego, CA, USA). Mice were placed in the startle chamber and a 70-dB background noise was presented during a 5-min acclimation period. The PPI session consisted of randomly presented 100 trials: a 120-dB noise trial presented alone (Startle, S), no stimulus trial, prepulse 73-dB trial, prepulse 76-dB trial, prepulse 82-dB trial, prepulse 73-dB + pulse 120-dB, prepulse 76-dB+pulse 120-dB, prepulse 82-dB+pulse 120-dB. The intervals between single trials were randomized between 10 and 30 s. The 100 miliseconds response after the presentation of the 120-dB pulse was analyzed by the PPI setting and we used the maximal response peak to calculate the PPI (% PPI = 100 × (*S* − PPiS)/*S*) and the startle response as a control index for the mouse reaction to the startle pulse. THC administration (1, 5 and 10 mg kg^{-1}, i.p.) was performed 60 min before the PPI experiment and the pre-treatment with pregnenolone (6 mg kg^{-1}, s.c.) was achieved 10 min before THC. A dose response experiment was performed to determine the dose of THC exerting no effects on the startle response (Supplementary Figure S2A).

Social interaction

Mice were tested in an open field (35 × 35 cm) with two plastic containers (plastic cylinders of 8 cm diameter with holes for odor interaction) in two opposite corners, one of them hosting a mouse (8- to 10-week-old adult male C57BL/6-N), while the other container remained empty. In each corner we defined the 'social' and 'non-social' zones as an 8-cm area surrounding the containers. For each experimental group, the position of the container with the mouse was counterbalanced. Mice were put in the middle of the open field whose bottom was divided into squares of 6 cm for 5 min. A ceiling-mounted camera recorded animals' movements. This behavioral test was performed immediately after the spontaneous alternation task, that is, animals had received THC (0.3, 1 and 3 mg kg^{-1})

or its vehicle 130 min earlier. Pregnenolone (6 mg kg^{-1}, s.c.) was injected 10 min before THC. A social interaction index was calculated as:

$$\text{Social interaction index} = \frac{\text{Time spent in "social" zone}}{\text{Total time in both zones}}$$

The total interaction time of the two zones and the total number of crosses were used to control locomotor exploration activity. The dose and timing of THC administration were chosen on the basis of pilot experiments revealing THC effects on social interaction but not on locomotion (Supplementary Figures S3A–C).

Locomotion

Mice were placed in a plexiglas box (35 × 45 cm) whose bottom is divided into squares (6 cm). Locomotion was assessed by the number of crossed squares, this number being manually counted during 5 min by the experimenter in the same room. Mice were treated with THC (0.3, 1 and 5 mg kg^{-1}) or vehicle 45 min before the test. Pregnenolone (6 mg kg^{-1}, i.p.) was injected 10 min before THC administration. Besides, amphetamine (2.5 mg kg^{-1}, i.p.) was injected 45 min before the test as to compare its effect with that of THC administration. The systemic pre-treatment with risperidone (0.3 mg kg^{-1}, s.c.) was performed 10 min before amphetamine. In the MK801 mouse model, we performed the locomotion test in adult mice (9–10 weeks old).

Direct and mediated aversions

Mice were water-deprived in the room where the whole protocol occurred. The basal consumption of each odor and taste was measured in naïve mice and no preference for any stimulus was detected (Supplementary Figures S4A and B). All subjects received 60 min access to water during three consecutive days as *habituation period*. Over the following days, we performed the pre-conditioning phase with different *odor–taste pairings* in which animals were submitted to different schedules of pre-conditioning trainings, consisting of one, three, six or nine odor–taste pairings, respectively (Supplementary Figures S4C–E). Each pairing consisted in 2 days: the first day the subjects received 60 min access to a flavored solution containing a new taste (either 5% sucrose or 5% maltodextrin; Taste 1, T1) and a new odorant (0.05% Banana or 0.01% Almond; Odor 1, O1) mixed with water in order to pair T1 with O1. On day 2, the animals received the taste and the odor that was not provided during the previous day, that is, Taste 2 (T2) and Odor 2 (O2). On the following 6 days, animals entered the *devaluation phase* where O1 (or T1) was devaluated as to become the conditioned stimulus (CS+). On days 1, 3 and 5 of this phase, the subjects have 60 min access to O2 (or T2) followed by an intraperitoneal (i.p.) injection of saline (CS−) whereas on days 2, 4 and 6, they received 60 min access to O1 or T1 immediately followed by an i.p. injection of LiCl (0.3 M, 1% b.w.) (CS+). After this conditioning, the subjects were given a recovery day during which they could only drink water during 60 min. On the next 2 days, mediated and direct aversions were assessed using a 60 min two-choice *test phase*. Mediated aversion was always evaluated on the first test day with a choice between the stimulus T1 (or O1) previously associated with the CS+ (called mediated CS+, mCS+) and the stimulus T2 (or O2) previously associated with the CS− (called mediated CS−, mCS−). On the second test day, we evaluate the direct aversion with a choice between the CS+ and CS− (Supplementary Figures S4D and E). Aversion was revealed by lower consumption of mCS+ over mCS− (mediated aversion) or CS+ over CS− (direct aversion).[32] Data are presented as absolute liquid intakes and as aversion indices, which were calculated using the following formula: [(CS−) − (CS+)]/[(CS+) + (CS−)] for direct aversion or [(mCS−) − (mCS+)]/[(mCS+) + (mCS−)] for mediated aversion, respectively.

'Reality testing' evaluation (mental representation of sensory stimuli)

One odor–taste pairing (short training) during the preconditioning phase is not sufficient to induce mediated aversion, whereas three pairings readily decreased the consumption of mCS+ (see Results), suggesting that with intermediate training there is a similar salience between the CS+ and mCS+ (in other words, CS+ = mCS+ in terms of aversive value). However, after six or nine odor–taste pairings (long training) control animals did not show mediated aversion, suggesting that they are able to separate the values of CS+ and mCS+. Considering that CS+ and mCS+ are separated in reality, this phenomenon is generally called 'reality testing' in the literature,[29–31] and we use this term in the present study. Thus, conditions or treatments that restore mediated aversion are considered as alteration of 'reality testing', in a way that reasonably approximate 'erroneous beliefs that usually involve a misinterpretation of perception or experiences' typically observed in patients experiencing positive psychotic symptoms.[35,36] Thus, using this protocol, we evaluated whether pharmacological treatments applied subchronically during adolescence (MK801, 7 days, 1 mg kg^{-1}, i.p.) or 120 min before the first test (2.5 mg kg^{-1} amphetamine, i.p. or 1 mg kg^{-1} THC, i.p.) impaired the 'reality testing' in the six-pairing protocol, reestablishing a specific aversion to mCS+. THC was also injected 120 min before the test in the nine-pairing protocol to better characterize and confirm its effects on 'reality testing'. A preliminary dose–response experiment was performed to determine the dose of THC not interfering with total liquid intake. The systemic pre-treatments with the antipsychotic risperidone (0.03, 0.1 and 0.3 mg kg^{-1}, i.p.), the CB1 antagonist rimonabant (1 mg kg^{-1}, i.p.) and pregnenolone (6 mg kg^{-1}, s.c.) were performed 10 min before THC or amphetamine (for risperidone only) administration and 120 min before the test of the six-pairing protocol (Supplementary Figures S4D and E). Pregnenolone (6 mg kg^{-1}, s.c.) was also administered 30 min after THC administration, that is, 90 min before the test. Moreover, rimonabant and pregnenolone were also perfused in the hippocampus at a dose of 3 µg/1µl per side 10 min before THC administration (1 mg kg^{-1}, i.p.) and 120 min before the test. Finally, all the systemic treatments (THC, risperidone, rimonabant and pregnenolone) were also given 120 min before the test after three pairings to control their impact on mediated aversion.

Statistical analyses. Two-group comparison were made by *t*-test. Multiple groups comparison were studied through one-way, two- or three-way analysis of variance (ANOVA) with or without repeated values where appropriate. For ANOVA, only when significant interactions between main factors were detected, *post hoc* analyses (Bonferroni's or Fisher's) were performed. In some cases, ANOVA is used and the *P*-value for the main factor (e.g. mCS+ or mCS+) is showed. For detailed statistical analysis see the figure legends.

RESULTS

Action of pregnenolone on the effects of THC on cognitive performance, PPI and social interaction

Pregnenolone blocks THC-induced impairment of cognitive function. Systemic pre-treatment with pregnenolone (6 mg kg^{-1}, s.c.) did not alter behavioral performance *per se* in the spontaneous alternation task[37] and in the hippocampal-dependent delayed matching-to-place version of the Morris water maze.[38] Pregnenolone fully blocked the cannabinoid-induced cognitive impairment in the spontaneous alternation task when injected before or after THC (3 mg kg^{-1}, i.p.) (Figures 1a and b and Supplementary Figures S1A–C). Similarly, pregnenolone pre-treatment abolished the effect of THC at 5 mg kg^{-1} (ref. 34) in the Morris water maze (Figures 1c and d).

Pregnenolone blocks THC-induced decrease of PPI. According to previous results in rats,[39] dose response experiments showed that THC at 10 mg kg^{-1} impairs PPI without any significant effect on the startle response in mice (Figure 2a and Supplementary Figure S2A). Pregnenolone (6 mg kg^{-1}, s.c.) blocked this THC effect on PPI (Figure 2b) without altering the startle response (Supplementary Figure S2B).

Pregnenolone blocks the impairment of social interaction induced by THC. The dose of 3 mg kg^{-1} of THC significantly decreased social interaction in mice (Figure 2c), without affecting possible confounding behaviors, such as locomotion (Supplementary Figures S3A–C). This effect was fully blocked by pregnenolone (6 mg kg^{-1}, s.c.), which have no effect when administered alone (Figure 2d).

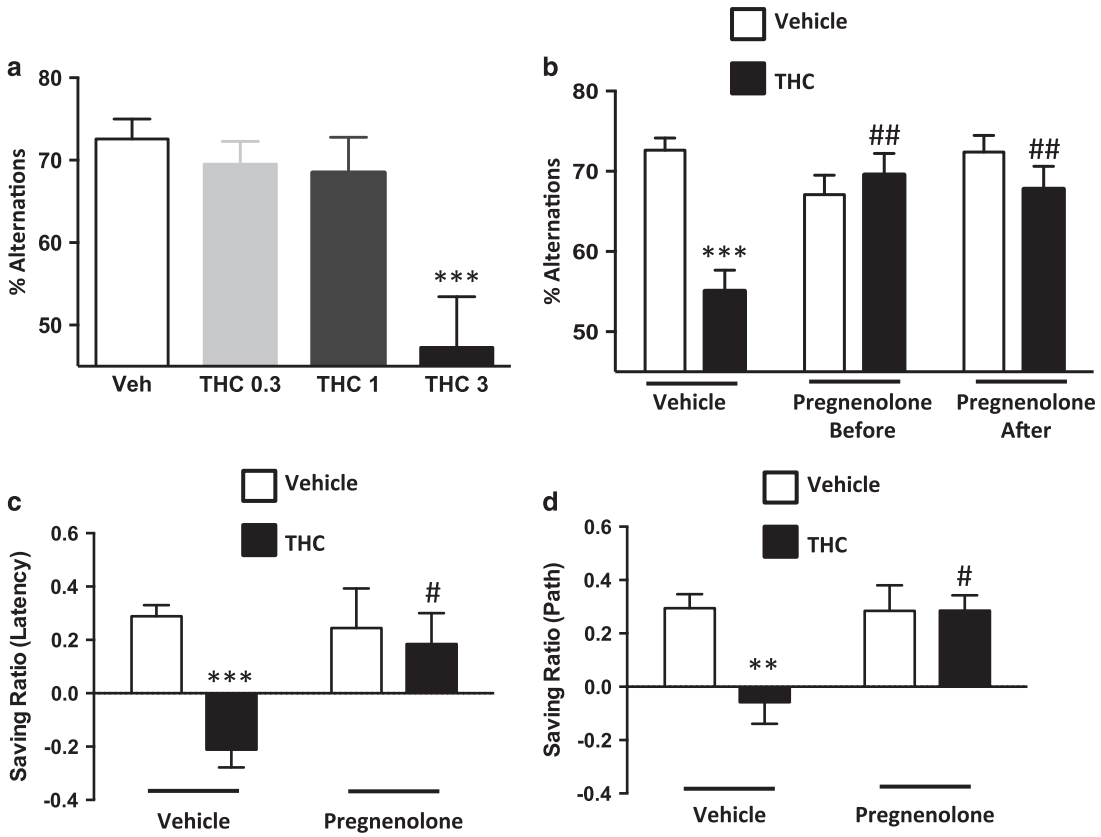

Figure 1. Pregnenolone blocks the cognitive impairment induced by THC. (**a**) Dose–response of THC effects in the spontaneous alternation task (one-way ANOVA, $F(3,32) = 3.8$, $P < 0.001$, $n = 6–12$). (**b**) Effect of pregnenolone (6 mg kg^{-1}, s.c.) 10 min before or 30 min after THC (3 mg kg^{-1}, i.p.) administration on the impairment of spontaneous alternation (*before*: two-way ANOVA, interaction; $F(1,58) = 14.83$, $P < 0.001$; *after*: two-way ANOVA, interaction; $F(1,62) = 6.871$, $P < 0.05$, $n = 10–20$). (**c**,**d**) Effect of pregnenolone (6 mg kg^{-1}, s.c.) on the cognitive impairment induced by THC (5 mg kg^{-1}, i.p.) in the delayed matching-to-place version of the Morris water maze. (**c**) Saving ratio for the latency (two-way ANOVA, interaction, $F(1,64) = 6.48$, $P < 0.05$, $n = 15–20$). (**d**) Saving ratio for the path (two-way ANOVA, interaction, $F(1,64) = 5.09$, $P < 0.05$, $n = 15–20$). $^{**}P < 0.01$; $^{***}P < 0.001$ for the effect of THC. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ for the effect of pregnenolone in THC injected mice. ANOVA, analysis of variance; THC, Δ^9-tetrahydrocannabinol.

Action of pregnenolone on the THC-induced acute positive psychotic-like states

Pregnenolone blocks the hyperlocomotor effects induced by THC. The presence of positive symptoms is a key feature of psychotic-like states[35,36,40] and represents a major challenge to model in rodents.[41–43] Rodent hyperlocomotion induced by human psychotogenic drugs has been long considered an acceptable laboratory approximation of positive symptoms of drug-induced psychotic-like states.[41–43] Cannabinoids are known to exert biphasic effects on locomotion in rats, with high doses inducing sedation and low doses increasing locomotor activity.[44] Whereas the 5 mg kg^{-1} (i.p.) dose of THC strongly decreased locomotion, that of 0.3 mg kg^{-1} induced hyperlocomotion in mice (Figure 3a; see ref. 45 for different results using other mouse strains and experimental settings). Administration of pregnenolone (6 mg kg^{-1}, s.c.) did not alter locomotion in control mice, but it fully blocked the hyperlocomotor effect of 0.3 mg kg^{-1} THC (Figure 3b), suggesting that the neurosteroid can block also acute 'positive' psychotic-like effects of cannabinoids.

Validation of a paradigm to study alterations of mental sensory representation in mice ('reality testing'). Alterations in the mental representation of stimuli leading to mismatches between perception and reality are key features of positive symptoms of psychotic-like states.[35,36,46] For instance, delusions are defined as erroneous beliefs involving a misinterpretation of perception or experiences.[36] Obviously, these alterations cannot be caught by analyzing locomotor activity,[41–43] but recent studies have used procedures to measure 'reality testing', defined as the accuracy of mental representation of reality.[29–31,47] Alterations of 'reality testing' as an impairment of perception might therefore approximate deficits that can lead to positive psychotic-like states such as perceptual delusions or hallucinations.[29–31,47] Thus, we adapted these previous protocols[29–31,47] to design a behavioral paradigm to measure 'reality testing' in mice (see Materials and Methods, Figure 4a, Supplementary Figures S4D and E).

Whereas pre-conditioning with one odor–taste pairing followed by a selective conditioned devaluation of one of the two stimuli did not induce mediated aversion, three pairings induced a reliable mediated aversion (Figures 4b and c), suggesting that the mice formed a unique mental representation of the two previously associated stimuli (odor and taste). However, six pairings before selective conditioned devaluation suppressed this mediated aversion (Figures 4b and c), indicating that the extended training during pre-conditioning induced a mental representation of the stimuli as two separated entities. Considering that the stimuli are separated in reality, one can argue that extended training allows animals acquiring additional information that enables the 'reality testing' of the independent salience of the two stimuli,[29–31] implying a correct mental representation of the values of the stimuli. Importantly, (i) total liquid consumption was similar among the groups (Supplementary Figure S5A), (ii) direct aversion

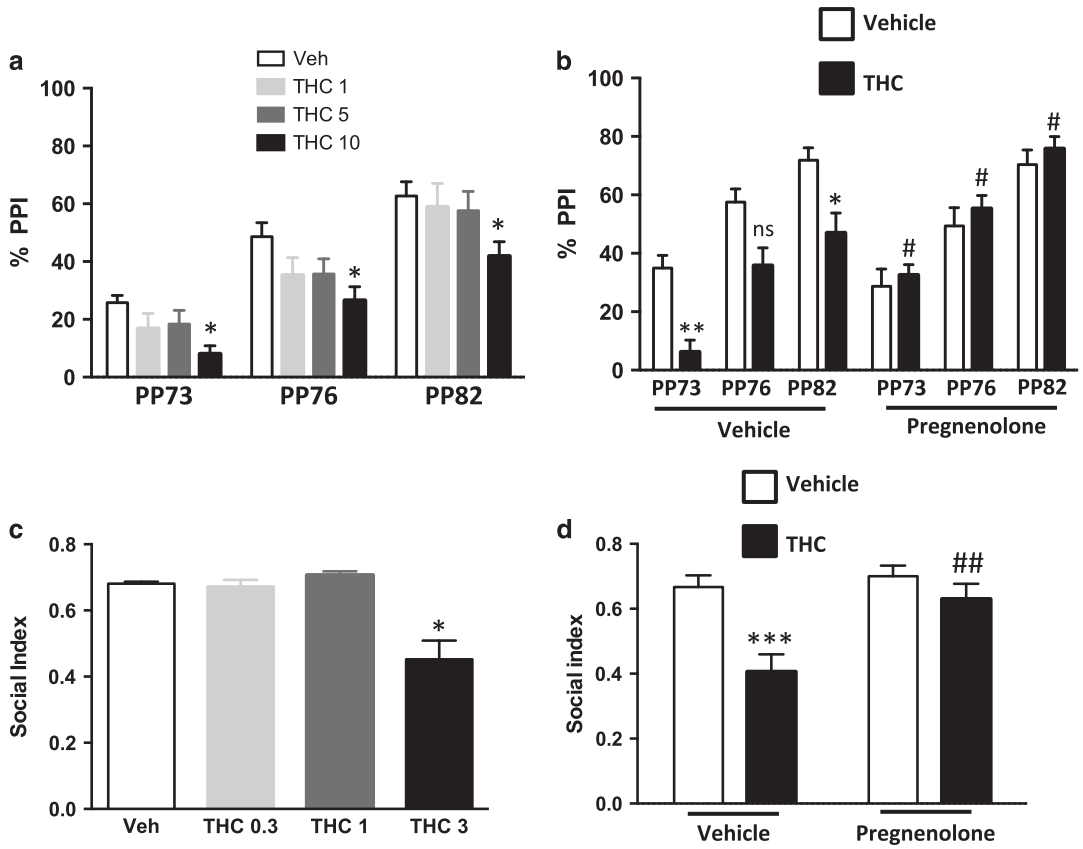

Figure 2. Pregnenolone blocks the effects of THC on PPI and social interaction. (**a**) Dose response of THC effects in the PPI test (one-way ANOVA, pre-pulse 73 db (PP73), $F(3,37) = 0.47$, $P < 0.01$; PP76, $F(3,37) = 0.14$, $P < 0.05$; PP82, $F(3,37) = 0.11$, $P < 0.05$; $n = 7–13$). (**b**) Effect of pregnenolone (6 mg kg^{-1}, s.c.) on the reduction of PPI induced by THC (10 mg kg^{-1}, i.p.) (two-way ANOVA, interaction; PP73 $F(1,35) = 11.93$, $P < 0.01$; PP76 $F(1,35) = 6.31$, $P < 0.05$; PP82 $F(1,35) = 7.63$, $P < 0.01$; $n = 8–12$). (**c**) Dose response analysis of THC effects in the social interaction task (one-way ANOVA, $F(3,33) = 11.23$, $P < 0.01$, $n = 10–12$). (**d**) Effect of pregnenolone on the reduction of social interaction induced by THC (3 mg kg^{-1}, i.p.) (two-way ANOVA, interaction, $F(1,47) = 4.39$, $P < 0.05$, $n = 10–15$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for the effect of THC. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for the effect of pregnenolone in THC-injected mice. ANOVA, analysis of variance; PPI, pre-pulse inhibition; THC, Δ^9-tetrahydrocannabinol.

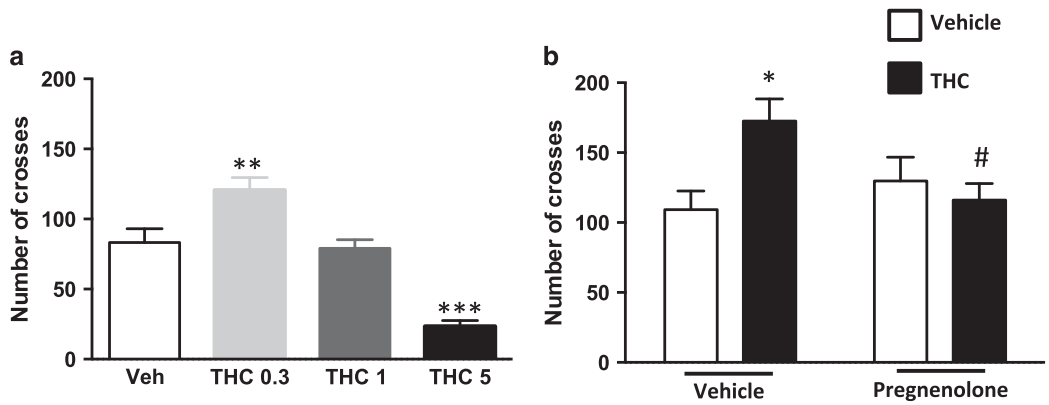

Figure 3. Pregnenolone blocks the hyperlocomotor effect induced by THC. (**a**) Dose response analysis of the effects of THC on spontaneous locomotion (one-way ANOVA, $F(3,28) = 0.7$, $P < 0.001$, $n = 8$). (**b**) Effect of pregnenolone (6 mg kg^{-1}, s.c.) on the hyperlocomotion induced by THC (0.3 mg kg^{-1}, i.p.) (two-way ANOVA, interaction, $F(1,33) = 6.8$, $P < 0.05$, $n = 10–12$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared with vehicle control. $^{\#}P < 0.05$ as compared with THC alone group. ANOVA, analysis of variance; THC, Δ^9-tetrahydrocannabinol.

was present under all pre-conditioning odor–taste pairing conditions (Supplementary Figures S5B–D) and (iii) the loss of mediated aversion was present irrespectively of whether odor or taste were devaluated during conditioning (Supplementary Figures S6A–F). This indicates that the behavioral procedure does not alter motivation to drink or direct aversive memory, and that the effects of different preconditioning schedules on mediated aversion are independent of the sensory modalities used.

Interestingly, previous studies showed that rodent models of psychotic-like behaviors present a 'reality testing' impairment.[29–31] Thus, we tested two psychotogenic pharmacological mouse models in our paradigm. Both subchronic MK801 treatment

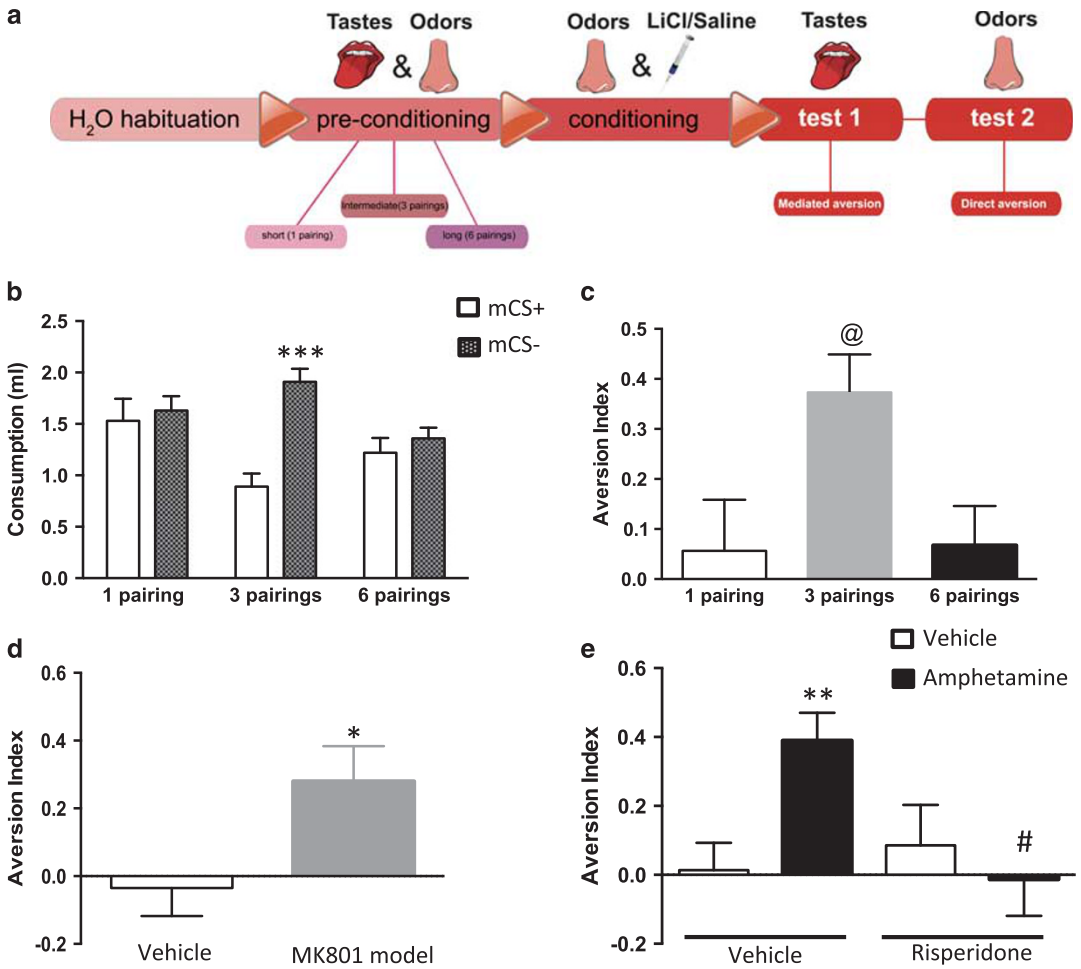

Figure 4. Validation of the mediated aversion 'reality testing' paradigm in mice. (**a**) Schematic representation of the behavioral protocol. For further details see Materials and Methods and Supplementary Figure S4. (**b**,**c**) Effect of the number of odor–taste pairings during preconditioning phase on odor-mediated taste aversion. (**b**) Consumption of tastes (i.e. mediated CS) (two-way ANOVA with repeated measures, interaction, $F(2,27) = 3.98$, $P < 0.05$; $n = 10$). Solid bars indicate consumption of taste paired with devaluated odor (mCS+) and dashed bars indicate consumption of taste paired with undevaluated odor (mCS−, see Materials and Methods). (**c**) Aversive index (one-way ANOVA, $F(2,27) = 0.3$, $P < 0.05$; $n = 10$–12). (**d**,**e**) Effects of psychotogenic treatments on mediated aversion 'reality testing' paradigm (mice receiving six odor/taste pairings during pre-conditioning): (**d**) Aversion index in adult animals subchronically treated with MK801 during adolescence (postnatal days 21–28, 1 mg kg^{-1}, i.p.) (*t*-test, $P < 0.05$, $n = 10$–11). (**e**) Effect of acute pre-test administration of amphetamine (2.5 mg kg^{-1}, i.p.) (two-way ANOVA, interaction, $F(1,56) = 5.9$, $P < 0.05$; $n = 10$–20). $^{*}P < 0.05$; $^{**}P < 0.01$ as compared with vehicle control, $^{***}P < 0.001$ as compared with mCS+; $^{@}P < 0.05$ as compared with one- and six-pairing groups; $^{\#}P < 0.05$ as compared with amphetamine group. ANOVA, analysis of variance; mCS, mediated conditioned stimulus.

during adolescence and acute treatment with amphetamine,[41] which induced hyperlocomotion in adult mice (Supplementary Figures 7A and B), impaired 'reality testing' performance, hence re-establishing mediated aversion in mice pre-conditioned with six odor–taste pairings (Figures 4d, e, Supplementary Figures 8A–E and 9A–F). The fact that amphetamine was acutely administered before the tests excludes possible confounding factors during the pre-conditioning or conditioning associative learning phases. Moreover, both the effects of amphetamine on 'reality testing' and locomotion were blocked by the acute pre-administration of the antipsychotic drug risperidone (0.3 mg kg^{-1}, i.p.) (Figure 4e, Supplementary Figures S7B and S9B–F). These results provide good face validity to this paradigm as a mouse behavioral tool to reflect perceptual alterations, typically associated with positive psychotic-like states in humans.

Pregnenolone blocks the impairment of 'reality testing' induced by THC. THC (1 mg kg^{-1}, i.p) did not affect liquid intake (Supplementary Figures 10A and B), but it blocked the effect of extended pre-conditioning (six or nine pairings before devaluation), re-establishing the mediated aversion typical of three pairings (Figure 5 and Supplementary Figures S11A–C). Importantly, different doses of the antipsychotic risperidone acutely blocked this effect (Figure 5a, Supplementary Figures S12A, B and S13A–C), suggesting that THC impairs 'reality testing' in mice, most likely triggering a psychotic-like state. The orthosteric CB1 receptor antagonist rimonabant, although slightly reducing the total amount of liquid intake (Supplementary Figures S14A and B), abolished mediated aversion in THC-treated mice (Figure 5b and Supplementary Figures S14A, B). This indicated that activation of CB1 receptors was required for this psychotic-like effect of THC. Importantly, pregnenolone treatment (6 mg kg^{-1}, s.c.) blocked the 'reality testing' impairment when administered before or after THC (Figure 5c, Supplementary Figures S15A–E). Besides, none of these treatments altered direct aversion (Supplementary Figures S12C–E, S13D–F, S14C–E and S15C–E) or mediated aversion

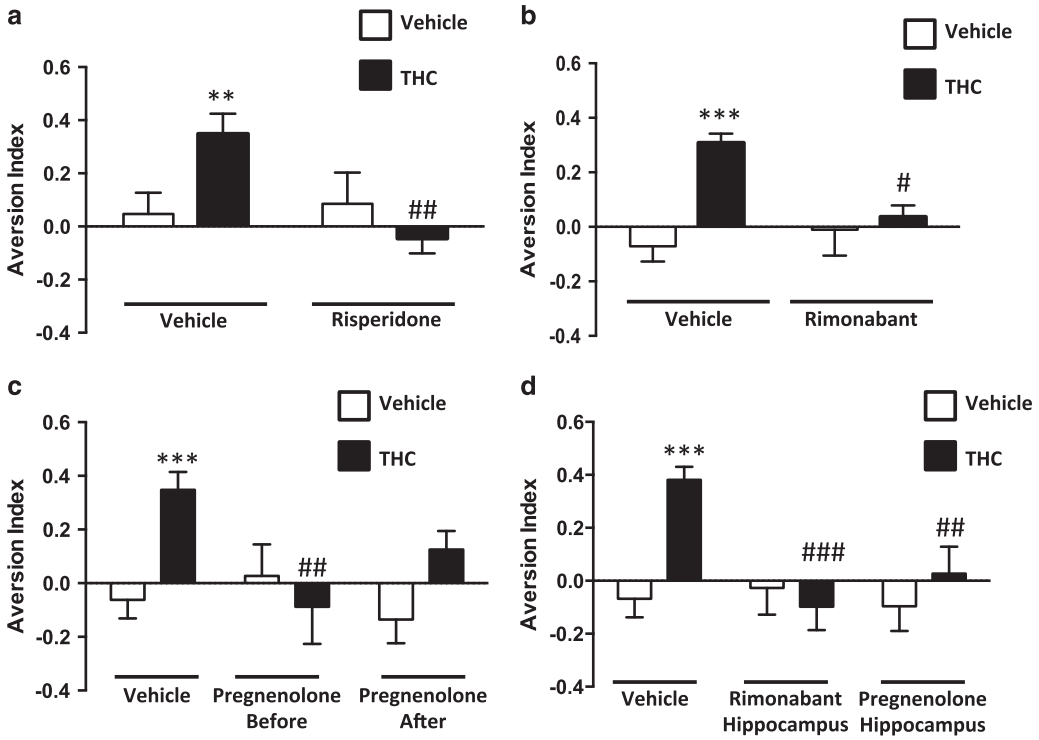

Figure 5. Pregnenolone blocks the THC effects on 'reality testing' in mice. (**a**–**c**) Effect of pre-test THC administration (1 mg kg^{-1}, i.p.) on mediated aversion in mice trained with six odor–taste pairings. (**a**) Risperidone (0.3 mg kg^{-1}, i.p.) effect on the THC induced 'reality testing' impairment (two-way ANOVA, interaction, $F(1,53) = 5.9$, $P < 0.05$; $n = 11$–19). (**b**) Effect of the orthosteric CB1 receptor antagonist rimonabant (1 mg kg^{-1}, i.p.) (two-way ANOVA, interaction, $F(1,39) = 7.1$, $P < 0.05$; $n = 10$–11). (**c**) Effect of the signal-specific allosteric inhibitor of CB1 receptors, pregnenolone (6 mg kg^{-1}, s.c.) before (two-way ANOVA, interaction, $F(1,46) = 9.172$, $P < 0.01$; $n = 14$–18) and after THC administration. (**d**) Effect of hippocampal injections of rimonabant (two-way ANOVA, interaction, $F(1,58) = 12.04$, $P < 0.001$; $n = 11$–21) and pregnenolone (two-way ANOVA, interaction, $F(1,55) = 4.252$, $P < 0.05$; $n = 14$–18) (3 μg μl^{-1}) before THC administration. $^{**}P < 0.01$, $^{***}P < 0.001$ for the effect of THC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.01$ for the effect of pregnenolone in THC injected mice. ANOVA, analysis of variance; THC, Δ^9-tetrahydrocannabinol.

induced by the three-pairings pre-conditioning protocol (Supplementary Figures S16A–D). Notably, hippocampal injections of rimonabant or pregnenolone fully blocked the THC-induced 'reality testing' impairment (Figure 5d, Supplementary Figures S17A, B and S18A–E). Thus, activation of hippocampal CB1 receptors mediated the aforementioned pregnenolone-sensitive psychotic-like effect of THC.

DISCUSSION

Finding a potential drug to tackle the full psychotic-like symptomatology induced by acute cannabis consumption is a challenging issue, mostly because of the difficulty to model these symptoms in laboratory animals. In this study, we show that the signaling-specific CB1 receptor inhibitor pregnenolone reverts a wide range of acute consequences of THC that can resemble acute cannabis intoxication. This included psychotic-like negative states or endophenotypes, such as decreased social interaction, cognitive impairment and inhibition of PPI. The presence of positive symptoms is a key feature to define a psychotic-like state.[35–36] Indeed, our data show that pregnenolone blocks THC-induced acute hyperlocomotion, which is often used as a surrogate for positive psychotic-like phenotypes.[41–43] Considering that hyperlocomotion is an indirect means to assess positive psychotic states, thus we set a behavioral task allowing to investigate the accuracy of internal representation of stimuli ('reality testing'). We found that acute administration of THC, like other psychotogenic treatments, alters 'reality testing' in mice. Importantly, pregnenolone fully reverts this effect, indicating that also THC-induced acute positive psychotic-like symptoms are also counteracted by this neurosteroid. These results are clinically relevant as these perceptual alterations can later lead to persistent delusions and other positive psychotic-like states.[9,10,13] From the pharmacological point of view, it is interesting to note that the different psychotic-like effects of THC are observed in mice at different doses and at different times after administration, resembling the dose-dependence and the time course of cannabis effects in humans.[48–50] Pregnenolone was able to block all effects of THC independently from dose or timing, suggesting that the whole spectrum of psychotic-like symptoms induced by acute cannabis intoxication is a target for this neurosteroid. Our data also show that pregnenolone is able to block at least some psychotic-like effects of THC (e.g. spontaneous alternation or 'reality testing') in a 'real-life' scenario, in which the neurosteroid is administered after THC intoxication. Considering that chronic cannabis intake during vulnerable periods (e.g. adolescence) is often associated to the development of persistent psychoses in adult humans,[51] these results might provide a proof of concept for future studies in order to extend the impact of similar treatment regimens in rodents and the potential therapeutic impact of pregnenolone.

The blockade of CB1 receptors has been previously suggested as a therapeutical means against psychoses,[22–24] but its use is strongly limited by undesired side effects.[25,26] Moreover, clinical studies had to be stopped due to lack of efficacy.[21] Thus, the discovery of more specific, safer and efficient approaches is required for the treatment of CIAPS. Accordingly, it is worth noting that excessive activation of CB1 receptors induces large increases in pregnenolone levels in rodent brain (up to 3–4000%).[27] Pregnenolone, in turn, acts as an endogenous signaling-specific inhibitor of excessive CB1 receptor signaling, which might explain

the absence of side effects as compared with classical orthosteric antagonists.[27–28] Indeed, pregnenolone binds to an allosteric site of the CB1 receptor and inhibits only several of the signaling pathways that are triggered by cannabinoids. Thus, pregnenolone has no apparent effect on CB1 receptor-dependent regulation of cAMP cellular levels, but it antagonizes the activation of extracellular-regulated kinases[27] and the CB1 receptor-dependent reduction of mitochondrial activity recently described in the brain.[27,52] Interestingly, decreased blood levels of endogenous pregnenolone are present in schizophrenic patients and exogenous pregnenolone administration slightly improves the symptomatology in psychotic women.[53–55] Moreover, recent evidence from human post-mortem studies suggests that extracellular-regulated kinases signaling could contribute to the pathogenic events that occur in psychosis.[56,57] The ability of antipsychotics to affect the extracellular-regulated kinases pathway has been also demonstrated *in vitro* and *in vivo*.[58] In parallel, the impact of energy metabolism and mitochondria in the brain is emerging as a promising novel research field in psychopathology.[59–61] For instance, brain-specific alterations of the metabolic profile in the cerebrospinal fluid of first-onset schizophrenic patients have been reported.[62]

Whereas cannabinoid-induced effects on cognition, PPI and sociability have been studied in laboratory animals, psychotic-like positive symptoms are often neglected due to the lack of suitable animal models.[6,41,63] To overcome this methodological issue, we adapted a representation-mediated learning protocol[29–31] in mice as a paradigm to evaluate the mental representation of stimuli and its potential disturbance under psychotic-like states. In this paradigm, extended pre-conditioning training allows animals becoming able to interpret the real situation in the external world and respond consequently. Thus, the term 'reality testing' has been used in rodents to describe this mental process.[29–31] Interestingly, commonly used animal 'models' of psychotic-like states (e.g. ventral hippocampal lesion)[30,64] and known psychotogenic pharmacological treatments (e.g. amphetamine, subchronic MK801, present results) alter 'reality testing'. Moreover, this psychotogenic-like effect was also observed after acute injection of THC both in the six- and nine-pairing pre-conditioning protocol. Human hallucinations and delusions can be viewed as mismatches between external reality and internal mental representations. For instance, the Fifth Edition of the 'Diagnostic and Statistical Manual of Mental Disorders' defines delusions as 'erroneous beliefs that usually involve a misinterpretation of perception or experiences',[36] which implies an erroneous mental representation of stimuli. For this reason, the 'reality testing' impairment produced by the acute administration of THC and psychotogenic drugs before the test of the six-pairing protocol, together with the lack of effect of THC in the three-pairing protocol, seems to reflect a cognitive alteration linked to the perception of the stimuli during retrieval rather than their encoding, resembling human perceptual delusions, which are hallmarks and starting points of positive psychotic-like symptoms.

Importantly for the aims of the present study, the 'reality testing' impairment induced by acute THC or amphetamine treatments is reversed by antipsychotic pre-treatment, supporting the psychotic-like nature of this effect. Moreover, the THC effect is also blocked by the systemic injection of the CB1 antagonist rimonabant, indicating that excessive CB1 receptor activity alters the relationship between sensory 'percept' and mental 'concept'. This denotes a cannabinoid-dependent top-down control of sensory representations in the brain, as we recently proposed in the olfactory system.[65] Notably, this acute impairment of 'reality testing' is blocked by pregnenolone, confirming that pregnenolone-like compounds represent a novel potential therapeutic tool against a wide range of acute psychotic-like states resembling the effects of acute cannabis intoxication in humans. Unfortunately, pregnenolone has a very short half-life, poor bioavailability and low efficacy in clinical studies[53–55] and it is the precursor of many other steroids, making it virtually impossible to use as a human drug.[66] To obviate these limitations, we recently developed a new class of pregnenolone-derivative drugs (Synthetic Signaling Specific inhibitors of the CB1, sCB1-SSi, see ref. 28), one of which will soon enter clinical trials against cannabis use disorders and addiction. Interestingly, more than 40% of psychotic patients regularly consume cannabis, generally leading to worse prognosis of the disease.[4,9] Thus, the present results clearly suggest an additional possible application for sCB1-SSi in psychotic patients consuming cannabis. However, as psychotic symptomatology in humans mostly appear when people consume cannabis chronically during vulnerable periods,[51] future studies in animals and humans will investigate the efficacy of this class of drugs in the treatment of psychotic-like states induced by acute or chronic cannabinoid intoxication.

Finally, hippocampal CB1 receptor blockade by rimonabant and pregnenolone abrogated the THC impairment of 'reality testing', corroborating recent results that implicate this brain region in representation-mediated learning in rats.[47] Accordingly, hippocampal alterations have been demonstrated in different psychotic disorders.[67,68] In particular, imaging studies showed changes in hippocampal activity and altered plasticity mechanisms of the hippocampus in schizophrenic patients.[69,70] Thus, our data support the idea that cannabinoids induce psychotic-like states by altering hippocampal functions. Nevertheless, the potential involvement of other brain regions (e.g. prefrontal cortex) and the role played by different brain cell populations in these effects will require further studies to be assessed.

In summary, we show that pregnenolone fully blocks the wide range of psychotic-like effects and related endophenotypes induced by acute administration of different doses of THC in mice. These results represent a proof of concept indicating a suitable therapeutic profile of signal-specific inhibitors of excessive CB1 receptor signaling. Thus, this work identifies pregnenolone-like drugs as powerful and promising therapeutic means and provides an adapted approach to study altered mental sensory representations that can be used to model the positive symptomatology of a complex human disorder like CIAPS.

CONFLICT OF INTEREST

PVP and GM are founders, stakeholders and consultants for the start-up company Aelis Farma. The remaining authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS

AB-G, GF, P-VP and GM designed research; AB-G, ES-G, BR, YM and FC performed research; AB-G, GF, P-VP and GM supervised research; MV helped with some experiments; AB-G, ES-G, GF and GM analyzed data; and AB-G, GF and GM wrote the manuscript. All authors edited the manuscript.

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