The phytocannabinoid, Δ^9-tetrahydrocannabivarin, can act through 5-HT_{1A} receptors to produce anti-psychotic effects

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BACKGROUND AND PURPOSE

To address the questions of whether Δ^9-tetrahydrocannabivarin (THCV) can (a) enhance activation of 5-HT_{1A} receptors *in vitro* and (b) induce any apparent 5-HT_{1A} receptor-mediated anti-psychotic effects *in vivo*.

EXPERIMENTAL APPROACH

In vitro studies investigated the effect of THCV on targeting by 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) of 5-HT_{1A} receptors in membranes obtained from rat brainstem or human 5-HT_{1A} CHO cells, using [^{35}S]GTPγS and 8-[^{3}H]-OH-DPAT binding assays. *In vivo* studies investigated whether THCV induces signs of 5-HT_{1A} receptor-mediated anti-psychotic effects in rats.

KEY RESULTS

We found that THCV (a) potently, albeit partially, displaced 8-[^{3}H]-OH-DPAT from specific binding sites in rat brainstem membranes, (b) at 100 nM, significantly enhanced 8-OH-DPAT-induced activation of receptors in these membranes, (c) produced concentration-related increases in 8-[^{3}H]-OH-DPAT binding to specific sites in membranes of human 5-HT_{1A} receptor-transfected CHO cells, and (d) at 100 nM, significantly enhanced 8-OH-DPAT-induced activation of these human 5-HT_{1A} receptors. In phencyclidine-treated rats, THCV, like clozapine, (a) reduced stereotyped behavior, (b) decreased time spent immobile in the forced swim test, and (c) normalized hyperlocomotor activity, social behaviour and cognitive performance. Some of these effects were counteracted by the 5-HT_{1A} receptor antagonist, WAY100635, or could be produced by the CB_1 antagonist, AM251.

CONCLUSIONS AND IMPLICATIONS

Our findings suggest that THCV can enhance 5-HT_{1A} receptor activation, and that some of its apparent anti-psychotic effects may depend on this enhancement. We conclude that THCV has therapeutic potential for ameliorating some of the negative, cognitive and positive symptoms of schizophrenia.

Abbreviations

5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; CBD, cannabidiol; CBDA, cannabidiolic acid; CHO, Chinese hamster ovary; CLZ, clozapine; FST, forced swim test; NMDA, *N*-methyl-D-aspartate; NOR, novel object

recognition test; PCP, phencyclidine; THC, Δ^9-tetrahydrocannabinol; THCV, Δ^9-tetrahydrocannabivarin

Introduction

In 2005, Russo *et al.* (2005) showed that one of the main components of *Cannabis sativa*, cannabidiol (CBD), in the micromolar range, binds to and functionally activates serotoninergic 5-HT_{1A} receptors. More recently we have reported that, at concentrations in the nanomolar range, CBD as well as its immediate precursor cannabidiolic acid (CBDA) can enhance the ability of the selective 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), to stimulate [^{35}S]GTPγS binding to rat brainstem membranes (Rock *et al.*, 2012; Bolognini *et al.*, 2013). Cannabigerol, another phytocannabinoid, has been reported by our group to behave as a potent apparent competitive antagonist of the 5-HT_{1A} receptor (Cascio *et al.*, 2010; Rock *et al.*, 2011).

The research described in this paper focused on the phytocannabinoid, Δ^9-tetrahydrocannabivarin (THCV) (Figure 1), a *propyl*-analogue of Δ^9-tetrahydrocannabinol (THC), and on the 5-HT_{1A} receptor. So far, it has been shown that this constituent of *Cannabis* can behave in both *in vitro* and *in vivo* experiments as a CB_1 receptor antagonist (Thomas *et al.*, 2005; Pertwee *et al.*, 2007; Dennis *et al.*, 2008; Ma *et al.*, 2008), and a CB_2 receptor partial agonist (Bolognini *et al.*, 2010). In addition, THCV has been reported to activate or block certain transient receptor potential (TRP) cation channels and to target GPR55 receptors (Anavi-Goffer *et al.*, 2012; De Petrocellis *et al.*, 2011; 2012). However, the ability of THCV to interact with 5-HT_{1A} receptors has not yet been investigated.

Here, for the first time, we present evidence that THCV (a) shares the ability of CBD to enhance 8-OH-DPAT-induced activation of 5-HT_{1A} receptors *in vitro* in pharmacological assays performed with membranes obtained from rat brainstem or from CHO cells stably transfected with the human 5-HT_{1A} and (b) produces, in rat models of schizophrenia-like symptoms, apparent anti-psychotic effects that are, at least in part, 5-HT_{1A} receptor-mediated.

Methods

Receptor nomenclature

The nomenclature of all the receptors mentioned in this paper conforms to BJP's Concise Guide to Pharmacology, Alexander *et al.*, 2013.

Animals

For *in vitro* experiments, brainstem tissues were obtained from 6 adult male Sprague Dawley rats maintained on a 12/12 h light/dark cycle with free access to food and water. These animals were purchased from Harlan UK Ltd (Blackthorn, UK). Before the removal of the brainstem, rats were killed by exposure to CO_2 followed by cervical dislocation. All animal care and experimental procedures complied with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines for the use of experimental animals. For *in vivo* experiments, male Sprague-Dawley rats (280-300g at the time of arrival) were purchased from Charles River (Calco, Italy) and randomly housed in groups of 4, on a 12/12 h light-dark cycle (lights on 08:00h) and in a temperature (24 ± 2°C) and humidity controlled environment (50 ± 10%), with a plastic tube for environmental enrichment. All animals had free access to food and water. We used a total of 204 rats that were randomly allocated to the experimental groups as follows: 18 control and 84 treated animals (6 rats for each experimental group) were tested for acute PCP experiments and 18 control and 84 treated animals (6 rats for each experimental group) were submitted to sub-chronic PCP experiments. All *in vivo* experiments were carried out during the light phase and performed in accordance with the guidelines released by the Italian Ministry of Health (D.L.116/92) and (D.L.111/94-B), and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Drugs and materials

THCV, extracted from *Cannabis sativa*, was provided by GW Pharmaceuticals (Salisbury, UK). 8-OH-DPAT was supplied by Tocris (Bristol, UK). [^{35}S]GTPγS (1250 Ci mmol^{-1}) and 8-[^{3}H]-OH-DPAT (135.2 Ci mmol^{-1}) were purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA), GTPγS and adenosine deaminase from Roche Diagnostic (Indianapolis, IN, USA), and GDP, DMSO and phencyclidine hydrochloride (PCP) from Sigma-Aldrich UK. Clozapine (CLZ), WAY100635 and AM251 were obtained from Tocris Bioscience (Italy).

In vitro procedures

CHO cells

Chinese hamster ovary cells stably transfected with cDNA encoding human serotoninergic 5-HT_{1A} receptors (a generous gift from Dr Keith Parker) were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM supplemented with 2

mM L-glutamine, 10% foetal bovine serum, 0.6% penicillin streptomycin and G418 (600 mg·mL-1).

Radioligand displacement assay

Membranes from Sprague Dawley rat brainstem were prepared as described by Bolognini *et al.* (2013). Each assay was carried out with 0.7 nM [^{3}H]-8-OH-DPAT, rat brainstem membranes (50 µg per well) or human 5-HT_{1A} CHO cell membranes (50 µg per well) using Tris-binding buffer (50 mM Tris-HCl, 50 mM Tris-base, 0.1% BSA, pH 7.4), total assay volume 500 µl. All assays were performed at 37ºC for 60 min before termination by the addition of ice-cold Tris-binding buffer and vacuum filtration described previously by Ross *et al.* (1999b). Specific binding was defined by the presence and absence of 1 µM unlabeled 8-OH-DPAT.

[^{35}S]GTPγS binding assay

Each assay was carried out with rat brainstem membranes (10 µg protein per well) or human 5-HT_{1A} CHO cell membranes (50 µg protein per well), GTPγS-binding buffer (50 mM Tris-HCl; 50 mM Tris-Base; 5 mM $MgCl_2$; 1 mM EDTA; 100 mM NaCl; 1mM dithiothreitol; and 0.1% BSA), 0.1 nM [^{35}S]GTPγS and 30 µM GDP, in a final volume of 500 µl (Cascio *et al.*, 2010). Membranes from rat brainstem were pre-incubated for 30 min at 30ºC with 0.5 U ml^{-1} adenosine deaminase (200 U ml^{-1}) to remove any endogenous adenosine. Non-specific binding was measured in the presence of 30 µM GTPγS. Assays were performed at 30ºC for 60 min (Cascio *et al.*, 2010).

Dissociation Kinetics

Dissociation kinetic assays were performed with the 5-HT_{1A} receptor agonist 8-[^{3}H]-OH-DPAT (0.7 nM), human $5HT_{1A}$ CHO cells (50 µg protein per well) and Tris-binding buffer, total assay volume 500 µl (Price *et al.*, 2005). 8-[^{3}H]-OH-DPAT was incubated with human $5HT_{1A}$ CHO cells for 60 min at 25ºC. Dissociation was initiated by the addition of 1 µM unlabeled ligand in the presence or absence of test compounds. Dissociation times of 0.5 to 120 min at 25°C were used. Non specific binding was determined in the presence of a 1 µM concentration of the unlabeled ligand. Binding was terminated by addition of ice-cold wash buffer (50 mM Tris-HCl, 50 mM Tris-base, and 0.1% BSA) followed by vacuum filtration.

In Vitro Data Analysis

Values have been expressed as means and variability as SEM or as 95% confidence limits. Values for IC_{50}, EC_{50}, maximal effect (E_{max}) and SEM or 95% confidence limits of these

values have been calculated by nonlinear regression analysis using the equation for a sigmoid concentration-response curve (GraphPad Prism). The dissociation rate constant for 8-[^{3}H]-OH-DPAT was calculated using a one phase exponential decay equation (GraphPad Prism). P values <0.05 were considered significant.

In vivo procedures

Drug administration

PCP was dissolved in saline and administered at a dose of 5 mg kg^{-1} i.p. (volume of injection 1 ml kg^{-1}). THCV was dissolved in ethanol, cremophor and saline (1:1:18) and administered at a dose of 2 mg kg^{-1} i.p. (volume of injection 5 ml kg^{-1}), 30 min before the test. CLZ was dissolved in 0.2% acetic acid and saline and pH was adjusted to 6.5 using 10M NaOH. It was administered at a dose 2.5 mg kg^{-1} i.p. (volume of injection 5 ml kg^{-1}), 30 min prior to testing. WAY100635 was dissolved in saline and administered at a dose of 1 mg kg^{-1} i.p. (volume of injection 1 ml kg^{-1}), 45 min before the test sessions. AM251 was dissolved in DMSO, Tween-80 and saline (1:1:8) and administered at a dose of 0.5 mg kg^{-1} i.p. (volume of injection 5 ml kg^{-1}), 45 min before testing.

Acute PCP administration

Acute inhibition of the NMDA receptor induces positive-like symptoms of schizophrenia in rodents, such as hyperlocomotion and stereotypies, and this is a model often used to predict the effect of substances with potential antipsychotic properties (Bubenikova-Valesova *et al*., 2008a; Large, 2007).

At post-natal day 75, the effects of drug treatments on the stereotyped behaviour and increases in locomotor activity induced by acute PCP administration (5 mg kg^{-1} i.p.) were assessed according to the treatment schedule shown in 'Results'.

Sub-chronic PCP schedule

A sub-chronic treatment regime with PCP followed by a washout period, with animals tested in the drug-free state, gives lasting cognitive deficits and negative-like signs with reasonable similarity to the neuropathological and behavioural disturbances of the disorder and is currently considered a useful model for testing the efficacy of novel antipsychotics against affective components and cognitive impairments of psychotic disorders (Neill *et al*., 2010, 2014). Animals were treated with either saline or PCP once a day for 7 days, according to a slightly modified version of the treatment schedule described by Seillier *et al.* (2010) and

shown in 'Results'. After 7 days of withdrawal, rats were tested in the novel object recognition (NOR) test, social interaction test and forced swim test (FST).

Behavioural tests

Spontaneous Locomotor Activity

Rats were placed in a computer-controlled infra-red activity monitor arena. The arena consisted of a clear acrylic box, 43×43×32 cm (Ugo Basile, Varese, Italy) placed in a sound-attenuating room. The cage was fitted with two parallel infrared beams, located 2 and 6 cm from the floor and cumulative horizontal and vertical movement counts were recorded for 50 min. During this period, stereotyped behaviours were scored by two observers blind to the treatment groups according to the rating scale described by Sams-Dodd (1998). Horizontal locomotor activity and stereotypies were calculated in 10-min blocks. The total scores of the whole 50 min test session (i.e. the sum of the scores recorded for each 10 min block) were calculated and converted to area under the curve (AUC) values using GraphPad Prism 5.0 software.

Novel Object Recognition Test (Classic and Spatial)

The experimental apparatus used for the object recognition test was an open-field box (43 x 43 x 32 cm) made of Plexiglas, placed in a dimly illuminated room. Animals performed each test individually. The experiment was performed and analyzed as previously described by Zamberletti *et al.* (2012). Briefly, each animal was placed in the arena and allowed to explore two identical previously unseen objects for 5 min (familiarization phase). After an inter-trial interval of 3 min one of the two familiar objects was replaced by a novel, previously unseen object and rats were returned to the arena for the 5-min test phase. During the test phase the time spent exploring the familiar object (Ef) and the new object (En) was videotaped and recorded separately by two observers blind to the treatment groups and the discrimination index was calculated as follows: [(En-Ef)/(En+Ef)] x 100.

Social Interaction Test

This test was carried out in a room illuminated with a dim overhead light. On the day of testing, each animal was habituated for 10 min in the test arena (60 x 60 x 60 cm), an open-field box made of Plexiglas. During the test session, each animal was allowed to explore freely an unfamiliar congener in the arena for 10 min. The arena was cleaned with 0.1% acetic acid and dried after each trial. Social behaviors were defined as sniffing, following, grooming, mounting and nosing. Aggressive behaviours were defined as attacking, biting, tail

rattling and aggressive grooming. The whole testing phase was videotaped, analyzed by two observers blind to the treatment groups; we also recorded the time spent in social behaviors and the number of aggressive behaviors.

Forced Swim Test

Animals were tested in a modified version of the FST that included only a single session of swimming (Realini *et al.*, 2011; Zamberletti *et al.*, 2012), since our objective was to measure any changes in a pre-existing behavioral deficit induced by PCP. Briefly, rats were forced to swim for 15 min inside a clear 50 cm tall, 20 cm diameter glass cylinder filled to 30 cm with 25°C water. The session was videotaped for later analysis of the following parameters: immobility (time spent by the animal floating in the water making only those movements necessary to keep its head above the water), swimming (active swimming movements to the centre of the cylinder), and climbing (forceful thrashing movements with forelimbs against the walls of the cylinder). The time spent in each of these behaviors was measured by an experimenter blind to the treatment groups.

In Vivo Data Analysis

Behavioural data were expressed as mean values ± SEM of 6 animals per group and analysed by three-way ANOVA with PCP, THCV and WAY100635 as independent variables, or by two-way ANOVA with PCP and THCV/CLZ/AM251 as independent variables followed by Bonferroni's post hoc test to examine group differences. The level of statistical significance was set at $P < 0.05$.

Results

In vitro experiments

First, we investigated whether THCV shares the ability of CBD to enhance the activation of 5-HT_{1A} receptors in rat brainstem membranes. Interestingly, we found that, unlike CBD, THCV (100 nM) induced a statistically significant increase (240.9-fold) in the potency (EC_{50}), but not in the efficacy (E_{max}), with which 8-OH-DPAT stimulates [^{35}S]GTPγS binding to these membranes (Figure 2A and Table 2). When tested alone, THCV (1 nM to 10 μM) did not affect [^{35}S]GTPγS binding to the same membranes (Figure 2B). Furthermore, we found that in these membranes, THCV potently, but only partially, displaced 8-[^{3}H]-OH-DPAT from specific binding sites (Figure 2C and Table 1).

Next, we performed experiments with membranes obtained from human 5-HT_{1A}-transfected CHO cells that, in contrast to brain tissue, do not express other types of receptor.

We found that, in these membranes, THCV (100 nM) induced a significant increase in the efficacy (E_{max}), but not in the potency (EC_{50}), with which 8-OH-DPAT activates human 5-HT_{1A} receptors (Figure 2D and Table 2). When THCV was tested alone in the [^{35}S]GTPγS binding assay, it did not induce any detectable effect at 1 nM to 10 µM (Figure 2E). Also, in the same membranes, we found that THCV significantly increased the binding of 8-[^{3}H]-OH-DPAT to specific binding sites (Figure 2F), while the binding of this tritium-labelled compound was completely prevented by 8-OH-DPAT (Figure 2F and Table 1).

Since there is evidence that the 5-HT_{1A} receptor possesses an allosteric binding site (Barrondo *et al.*, 2009), we also investigated the ability of the 8-OH-DPAT-potentiating concentration of THCV (100 nM) to alter the rate at which 8-[^{3}H]-OH-DPAT dissociates from specific binding sites in membranes obtained from human 5-HT_{1A} CHO cells (n=4). Our experiments showed that this concentration of THCV did not alter this dissociation rate (data not shown).

Effect of THCV administration on PCP-induced behavioural alterations

Figure 4 shows the effect of THCV administration (2 mg kg^{-1} i.p.) on PCP-induced schizophrenia-like symptoms in rats in comparison with the atypical antipsychotic, CLZ (2.5 mg kg^{-1} i.p.). Two different paradigms of PCP administration were chosen: acute PCP to mimic the positive-like signs of schizophrenia (Panel A) and sub-chronic PCP treatment to produce the apparent cognitive deficits (Panel B) and negative-like symptoms (Panels C and D).

As expected, acute PCP injection (5 mg kg^{-1} i.p.) induced marked hyperlocomotion paralleled by the appearance of stereotyped behaviours during the 50-min test session ($F_{1,20}$=17.56, P=0.0005). THCV treatment *per se* did not affect locomotor activity in control animals but its administration completely normalized PCP-induced hyperlocomotion and significantly reduced stereotyped behaviours (THCV: $F_{1,20}$=4.452, P=0.0477; PCPxTHCV interaction: $F_{1,20}$=5.891, P=0.0248). Similar results were obtained with the atypical antipsychotic, CLZ (2.5 mg kg^{-1} i.p.) (two-way ANOVA for PCP: $F_{1,20}$=14.85, P=0.0010; CLZ: $F_{1,20}$=2.390, P=0.1378; PCPxCLZ interaction: $F_{1,20}$=5.133, P=0.0347) (Figure 4A).

Figure 4, Panel B depicts the effect of THCV on the cognitive impairment induced by sub-chronic PCP pretreatment in the novel object recognition (NOR) test. Sub-chronic PCP significantly impaired recognition memory, as indicated by a significant reduction in the discrimination index of about 50 % compared to controls ($F_{1,20}$=5.266, P=0.0327). It induced even greater apparent cognitive impairment in the spatial version of the test, the

discrimination index being reduced by about 90 % ($F_{1,20}=38.64$, $p<0.0001$). THCV administration completely restored recognition memory in PCP-pretreated rats both in the classic (THCV: $F_{1,20}=14.46$, $P=0.0010$; PCPxTHCV interaction: $F_{1,20}=14.46$, $P=0.0011$) and in the spatial (THCV: $F_{1,20}=11.93$, $P=0.0025$; PCPxTHCV interaction: $F_{1,20}=27.30$, $p<0.0001$) variants of the NOR test, without having any effect in control animals. The recovery induced by THCV was very similar to that observed after CLZ administration (Classic NOR: two-way ANOVA for PCP: $F_{1,20}=19.27$, $P=0.0003$; CLZ: $F_{1,20}=16.47$, $P=0.0006$; PCPxCLZ interaction: $F_{1,20}=24.09$, $p<0.0001$; Spatial NOR: PCP: $F_{1,20}=53.18$, $p<0.0001$; CLZ: $F_{1,20}=39.67$, $p<0.0001$; PCPxCLZ interaction: $F_{1,20}=43.37$, $p<0.0001$). Neither the time spent exploring the two identical objects during the familiarization phase nor locomotor activity were altered in any of the groups analyzed (data not shown).

Figure 4, Panel C shows the effect of THCV administration in the social interaction test. Sub-chronic PCP pretreatment significantly reduced the amount of time spent in active social behaviours in the 10-min test session by about 60 % when compared to vehicle-treated rats ($F_{1,20}=154.9$, $p<0.0001$). THCV administration restored the normal social behaviour in PCP-pretreated rats (THCV: $F_{1,20}=84.40$, $p<0.0001$; PCPxTHCV interaction: $F_{1,20}=4.641$, $P=0.0436$). Similar results were obtained with CLZ (PCP: $F_{1,20}=44.19$, $p<0.0001$; CLZ: $F_{1,20}=9.225$, $P=0.0065$; PCPxCLZ interaction: $F_{1,20}=6.465$, $P=0.0194$). Aggressive behaviours were not observed in any of the groups under investigation. In the FST, sub-chronic PCP pretreatment induced a significant increase of about 80 % in the time spent in immobility compared to controls ($F_{1,20}=11.34$, $P=0.0031$). This was paralleled by a simultaneous reduction in swimming activity. THCV administration to PCP-pretreated rats completely normalized the time spent in immobility during the test session (THCV: $F_{1,20}=19.62$, $P=0.0003$; PCPxTHCV interaction: $F_{1,20}=11.42$, $P=0.0030$), the effect being very similar to that observed following CLZ injection (PCP: $F_{1,20}=17.60$, $P=0.0004$; CLZ: $F_{1,20}=20.89$, $P=0.0002$; PCPxCLZ interaction: $F_{1,20}=8.430$, $P=0.0088$). The rescue of this parameter was accompanied by the normalization of the amount of time spent in swimming activity.

Effect of 5-HT_{1A} receptor blockade on THCV-induced recovery of schizophrenia-like symptoms

Next, we investigated whether the "beneficial" effects exerted by THCV on PCP-induced schizophrenia-like traits were mediated by 5-HT_{1A} receptors. To do this, a selective 5-HT_{1A} antagonist, WAY100635 (1 mg kg^{-1} i.p.), was administered prior to THCV according

to the treatment protocol shown in Figure 3, and animals were then submitted to behavioural testing. WAY100635 administration *per se* did not affect any of the behavioural responses under investigation in control animals. Moreover, treatment with WAY100635 had no effect on PCP-induced positive-like (Figure 5A) or negative-like signs (Figures 5C and D). In contrast, WAY100635 administration did partially prevent PCP-induced cognitive deficits in both variants of the NOR test (Classic NOR: two-way ANOVA for PCP: $F_{1,20}=62.82$, $p<0.0001$; WAY100635: $F_{1,20}=4.617$, $P=0.0441$; PCPxWAY100635 interaction: $F_{1,20}=11.27$, $P=0.0031$; Spatial NOR: PCP: $F_{1,20}=44.90$, $p<0.0001$; WAY100635: $F_{1,20}=8.892$, $P=0.0074$; PCPxWAY100635 interaction: $F_{1,20}=16.22$, $P=0.0007$) (Figure 5B).

Importantly, WAY100635 did prevent the beneficial effect exerted by THCV on PCP-induced stereotypies and social withdrawal. Thus, three-way ANOVA indicated both a significant PCPxTHCVxWAY100635 interaction ($F_{1,30}=5.58$, $P=0.0053$), and a significant THCVxWAY100635 interaction ($F_{1,30}=7.23$, $P=0.0095$) on stereotypies, the data we obtained indicating that WAY100635 pretreatment completely prevented THCV from reducing PCP-induced stereotypies (Figure 5A). A similar statistically significant effect was observed in the social interaction test (PCPxTHCVxWAY100635 interaction: $F_{1,30}=5.046$, $P=0.0129$; THCVxWAY100635 interaction: $F_{1,30}=8.998$, $P=0.0071$), in which WAY100635 pretreatment abolished entirely the ability of THCV to prevent PCP-induced social withdrawal (Figure 5C).

In addition, WAY100635 also partially antagonized the recovery induced by THCV on PCP-induced cognitive deficits in both the classic (PCPxTHCVxWAY100635 interaction: $F_{1,30}=11.80$, $P=0.0002$; THCVxWAY100635 interaction: $F_{1,30}=11.76$, $P=0.0027$) and the spatial (PCPxTHCVxWAY100635 interaction: $F_{1,30}=7.690$, $P=0.0020$; THCVxWAY100635 interaction: $F_{1,30}=5.129$, $P=0.0348$) variants of the NOR test (Figure 5B).

Effect of AM251 administration on PCP-induced behavioural alterations

Acute administration of AM251 (0.5 mg kg^{-1} i.p.) did not affect PCP-induced hyperlocomotion or stereotyped behaviours (Figure 6, Panel A). In contrast, its administration completely prevented PCP-induced cognitive impairments in the classic (two-way ANOVA for PCP: $F_{1,14}=13.69$, $P=0.0024$; AM251: $F_{1,14}=6.476$, $P=0.0234$; PCPxAM251 interaction: $F_{1,14}=9.297$, $P=0.0087$) and spatial (two-way ANOVA for PCP: $F_{1,14}=46.72$, $p<0.0001$; AM251: $F_{1,14}=15.56$, $P=0.0013$; PCPxAM251 interaction: $F_{1,14}=22.17$, $P=0.0003$) variants of the NOR test (Figure 6, Panel B). Acute AM251 administration also significantly reduced PCP-induced negative-like symptoms. Thus, AM251 completely abolished PCP-induced

social withdrawal in the social interaction test (two-way ANOVA for PCP: F1,14=4.290, P=0.0560; AM251: F1,14=4.696, P=0.0467; PCPxAM251 interaction: F1,14=7.676, P=0.0143) (Figure 6, Panel C) and opposed the increased immobility induced by PCP in the FST (two-way ANOVA for PCP: F1,14=35.59, p<0.0001; AM251: F1,14=14.93, P=0.0017; PCPxAM251 interaction: F1,14=5.560, P=0.0334) by significantly increasing the time spent in climbing activity (F1,14=21,91, P=0.0001) (Figure 6, Panel D).

Discussion

The results from our investigation clearly show that THCV possesses an ability to interact with serotoninergic 5-HT_{1A} receptors both *in vitro* and *in vivo*. Turning first to our *in vitro* data, these showed that THCV, at 100 nM, significantly increased the potency (EC_{50}) but not the efficacy (E_{max}), with which DPAT activates 5-HT_{1A} receptors in rat brainstem membranes, thus behaving as a potential positive allosteric modulator of the 5-HT_{1A} receptor. Also, we found that, like 8-OH-DPAT, THCV potently displaced 8-[^{3}H]-OH-DPAT from specific binding sites in these membranes, although the percentage of the maximum displacement induced by 100 nM THCV was significantly lower than that induced by the same concentration of 8-OH-DPAT. These results raised the possibility that THCV does not bind directly to orthosteric sites on these receptors. Moreover, it remains possible too that THCV enhances DPAT-induced 5-HT_{1A} receptor activation through an indirect mechanism that involves the targeting by THCV of another kind of receptor.

To investigate this hypothesis, we performed experiments with human 5-HT_{1A}-transfected CHO cell membranes that, in contrast to brain membranes, do not express other types of receptors. We found that at 100 nM, THCV did significantly increase the binding of 8-[^{3}H]-OH-DPAT to specific binding sites in these CHO cell membranes. However, in contrast to the results we obtained with rat brainstem membranes, THCV induced a significant increase in the efficacy (E_{max}), rather than the potency (EC_{50}), with which 8-OH-DPAT activates the human 5-HT_{1A} receptor. In spite of the different manner in which THCV enhanced the effect of 8-OH-DPAT in brain and CHO cell membranes, these results support the hypothesis that THCV might behave as a positive allosteric modulator at 5-HT_{1A} receptors. However, in the same cells, we found that 100 nM THCV did not alter the rate of dissociation of 8-[^{3}H]-OH-DPAT from specific binding sites.

These *in vitro* results together with evidence already published that activation of 5-HT_{1A} receptors *in vivo* can ameliorate at least some signs of schizophrenia (Ohno, 2011;

Bantick *et al.*, 2001; Shimizu *et al.*, 2013), prompted us to investigate whether THCV can produce any apparent antipsychotic effects *in vivo*, and if so, whether any of these effects are 5-HT_{1A} receptor-mediated. This we did in a pharmacological model of schizophrenia in which rats are treated acutely or pretreated sub-chronically with PCP. Thus, overall, we found first, that on single administration, THCV was as effective as the established atypical antipsychotic, CLZ, in reverting both positive- and negative-like signs of schizophrenia, and cognitive impairments, and second, that many of the effects of THCV that we observed in these *in vivo* experiments appeared to be mediated, at least in part by the 5-HT_{1A} receptor. In rats pretreated with vehicle instead of PCP, neither THCV nor CLZ produced any effect in any of the behavioural tests that we used.

Our *in vivo* experiments with THCV showed that it prevented hyperlocomotion and stereotypies induced by acute PCP administration, and that in the sub-chronic PCP model, it restored social behaviours and counteracted the increase in the time spent in immobility in the FST. Moreover, THCV administration was able to normalize cognitive performance in PCP-pretreated rats. Importantly, the ability of THCV to reverse behavioural changes induced by acute PCP injections, such as stereotypies and hyperlocomotion, is suggestive of a beneficial effect on positive-like signs, whereas its efficacy in normalizing the behavioural alterations induced by sub-chronic PCP in the other tests, although not specific to schizophrenia, may predict THCV effectiveness for treating negative and cognitive symptoms of this disorder, such as memory impairment and deficits in social interaction.

We also found that pretreatment with the selective 5-HT_{1A} antagonist, WAY100635, prevented many of the apparent beneficial effects of THCV on PCP-induced behavioural alterations without affecting any of these effects of PCP in the absence of THCV. Thus, the ability of THCV to interact *in vivo* with these receptors might represent one of the molecular mechanisms responsible for its antipsychotic-like properties, in line with previous reports indicating that increasing the activation of 5-HT_{1A} receptors could be a promising strategy for antipsychotic therapy (Bantick *et al.*, 2001; Kleven *et al.*, 2005; *Meltzer et al.*, 2012; Newman-Tancredi, 2010). It is noteworthy, however, that although WAY100635 completely blocked the reversal by THCV of PCP-evoked stereotypies, it did not reduce the ability of THCV to reverse hyperlocomotion induced by acute administration of PCP, indicating that this latter effect of THCV was probably not 5-HT_{1A} receptor-mediated. These results suggest that 5-HT_{1A} receptors are not mediating the effect of THCV on hyperlocomotion, whereas their modulation may be involved in its action on stereotyped behaviours. Our results are in line with previous findings obtained with wild-type and 5-HT_{1A} receptor knockout mice that

demonstrated that 5-HT_{1A} receptors are implicated in the control of stereotyped movements, but not hyperlocomotion, induced by the non-competitive NMDA receptor antagonist, MK-801 (Scorza *et al.*, 2010).

Furthermore, our findings that pretreatment with WAY100635 prevented THCV from producing any recovery from PCP-induced social withdrawal are in line with evidence obtained from other preclinical studies that 5-HT_{1A} agonism can improve PCP-induced social behaviour deficits in rodents (Snigdha and Neill, 2008; Depoortere *et al.*, 2007; Bubenikova-Valesova *et al.*, 2008b), and with the concept that optimized stimulation of 5-HT_{1A} receptors is required to maximize treatment benefits with regard to some aspects of social abilities (Depoortere *et al.*, 2007; Bruins Slot *et al.*, 2005; Bubenikova-Valesova *et al.*, 2008b). Despite the evidence that 5-HT_{1A} receptor agonists can produce apparent anti-depressant effects both in traditional and in modified versions of the FST (Lucki *et al.*, 1994; Cryan *et al.*, 1997; De Vry, 1995), the ability of THCV to reverse PCP-induced immobility in the FST was not dependent upon its action at 5-HT_{1A} receptors, as pre-treatment with WAY10063 did not prevent THCV from producing this effect.

Finally, in the NOR test, WAY100635 by itself partially restored recognition memory in PCP-pretreated rats, possibly indicating that 5-HT_{1A} receptors may be involved in the impairment of recognition memory triggered by sub-chronic PCP administration in rats. Since sub-chronic treatment with PCP has been reported to increase cortical 5-HT_{1A} receptor binding (Choi *et al.*, 2009) and 5-HT release (Etou *et al.*, 1998; Martin *et al.*, 1998; Adams and Moghaddam, 2001; Amargós-Bosch *et al.*, 2006), it is possible that 5-HT_{1A} antagonism could counteract PCP-induced enhancement of serotonergic stimulation, thus resulting in the observed improvement of cognitive performance. However, the fact that the reversal by THCV of PCP-induced cognitive impairment in the NOR test was not completely prevented by WAY100635, suggests that other molecular mechanisms may have contributed to the ameliorating effect of THCV in this test.

One such mechanism may be antagonism of the cannabinoid CB_1 receptor by THCV. Thus, at the dose used in the present study, THCV has been reported to produce such antagonism (Pertwee, 2008) and we hypothesize that this action could contribute to the observed recovery of recognition memory induced by THCV since CB_1 receptor antagonism has been extensively proven to have pro-cognitive effects (De Bruins *et al.*, 2010; Black *et al.*, 2011; Vaseghi *et al.*, 2012; Guidali *et al.*, 2011; Seillier *et al.*, 2010). In line with previously published data, here we demonstrated that the established CB_1 receptor antagonist, AM251, was effective in reversing the negative-like symptoms and cognitive impairment induced by

sub-chronic PCP. Interestingly, unlike THCV, AM251 did not counteract acute PCP-evoked hyperlocomotion and stereotypies. This is in line with previous reports that after acute administration, CB_1 receptor antagonists fail to show any activity in models of positive symptoms of schizophrenia (Black *et al.*, 2011; Martin *et al.*, 2003; Thiemann *et al.*, 2008), suggesting that antagonism of the CB_1 receptor may not reduce such symptoms. In this context, its ability to enhance activation of 5-HT_{1A} receptors and block activation of CB_1 receptors simultaneously could make THCV particularly effective as a therapeutic agent for the treatment of schizophrenia, a possibility that merits further exploration, for example by investigating its efficacy in other animal models of schizophrenia.

It is well-established that drugs, like rimonabant, that are able to antagonize cannabinoid CB_1 receptors, may also show depressive-like effects, including suicidality (Beyer et al., 2010). The mechanism(s) by which rimonabant shows these side effects are not yet known, one possibility being that at high doses rimonabant behaves as an inverse agonist, rather than as a 'neutral' antagonist at the CB_1 receptors (Pertwee et al., 2005). On the contrary, THCV, at the dose used in this study (2 mg/kg) lacks of inverse effect, thus behaving as a CB_1 receptor 'neutral' antagonist (Pertwee, 2008). The lack of inverse effect might make THCV a drug safer than rimonabant. Moreover, it has been reported that a major limitation in the use of neuroleptics is the risk of short- and long-term side effects, such as significant weight gain and alterations in glucose metabolism (Shams and Muller, 2014). In contrast, THCV has been reported to exert anti-obesity effects in mouse models (Wargent *et al.*, 2013), suggesting that, unlike current antipsychotics, its administration would not produce unwanted increases in body weight.

In conclusion, this investigation has shown for the first time that THCV can affect the activation of 5-HT_{1A} receptors both *in vitro* and *in vivo*. Our *in vitro* results, obtained from experiments performed with both rat brainstem and human 5-HT_{1A} CHO cell membranes, strongly support the hypothesis that THCV might modulate the activation of these receptors indirectly, rather than by binding directly to their orthosteric sites. Our *in vivo* experiments with rats yielded data showing that, like the established anti-psychotic drug, CLZ, THCV can potently antagonize stereotyped behaviour, reduce the amount of time spent immobile in the forced swim test, and normalize hyperlocomotor activity, social behaviour and cognitive performance in PCP models of schizophrenia-like symptoms. The 5-HT_{1A} receptor antagonist, WAY100635, abolished the ability of THCV to modify PCP-induced stereotyped and social behaviour, but it had no effect in the FST and only partially reduced the suppressant effect of THCV on PCP-induced cognitive deficiency in the NOR test, thus suggesting that these

apparent beneficial effects of THCV were not mediated only by 5-HT_{1A} receptors. We speculate that one additional action that may underlie these apparent beneficial effects, is the antagonism by THCV of the cannabinoid CB_1 receptor.

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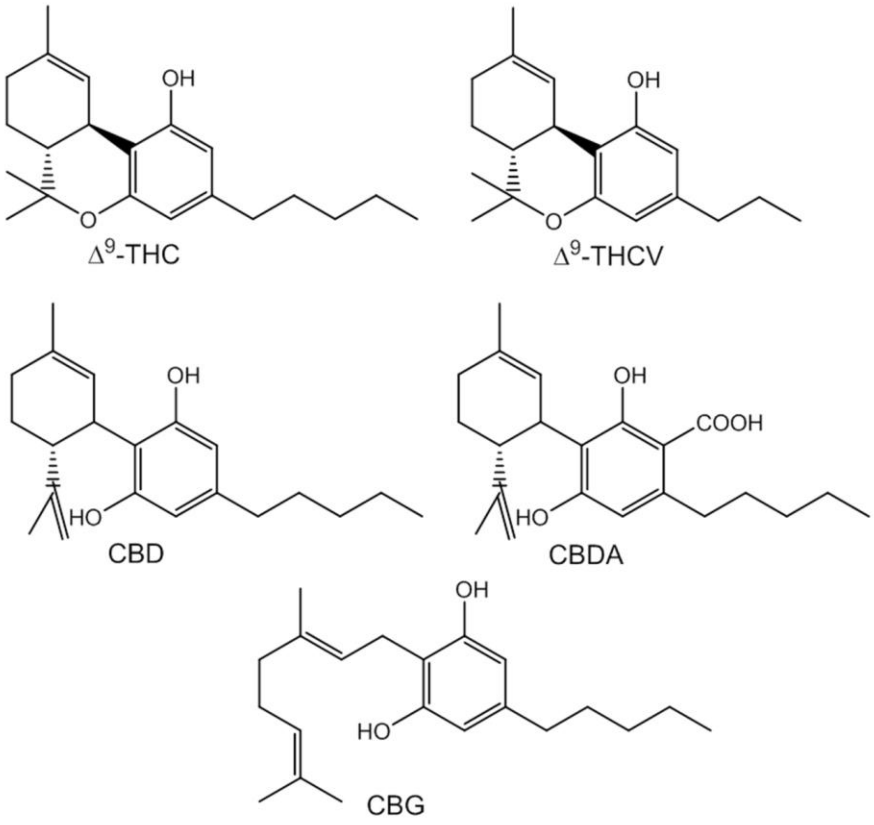
Conflicts of Interest

None



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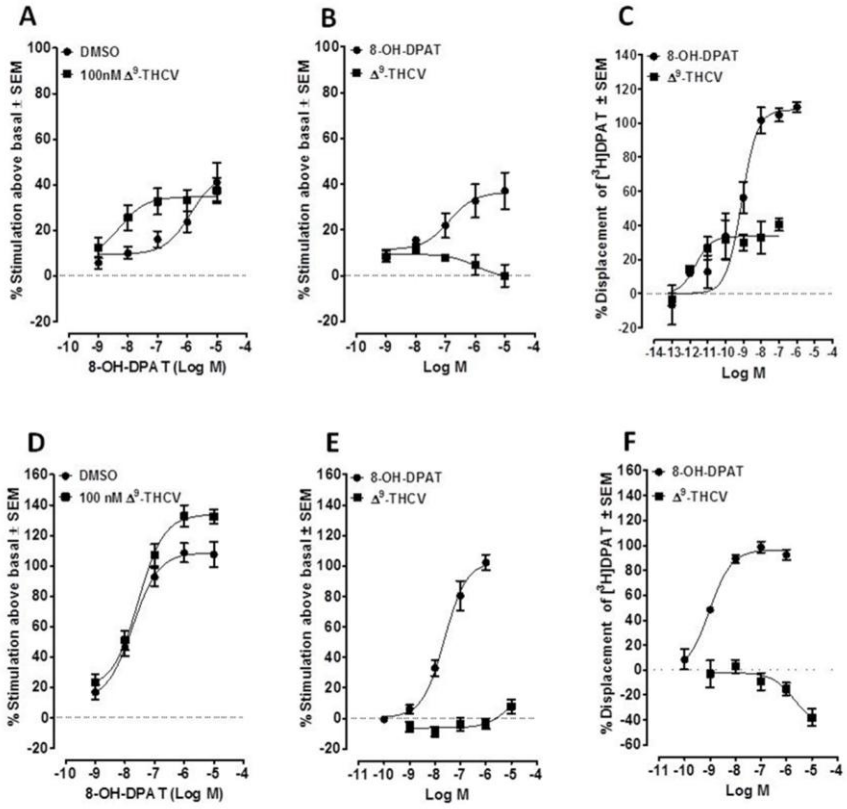

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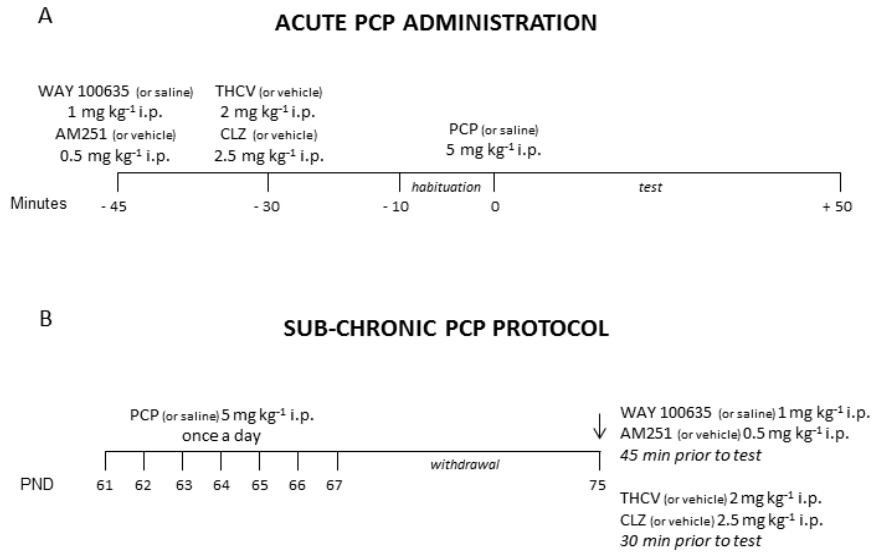

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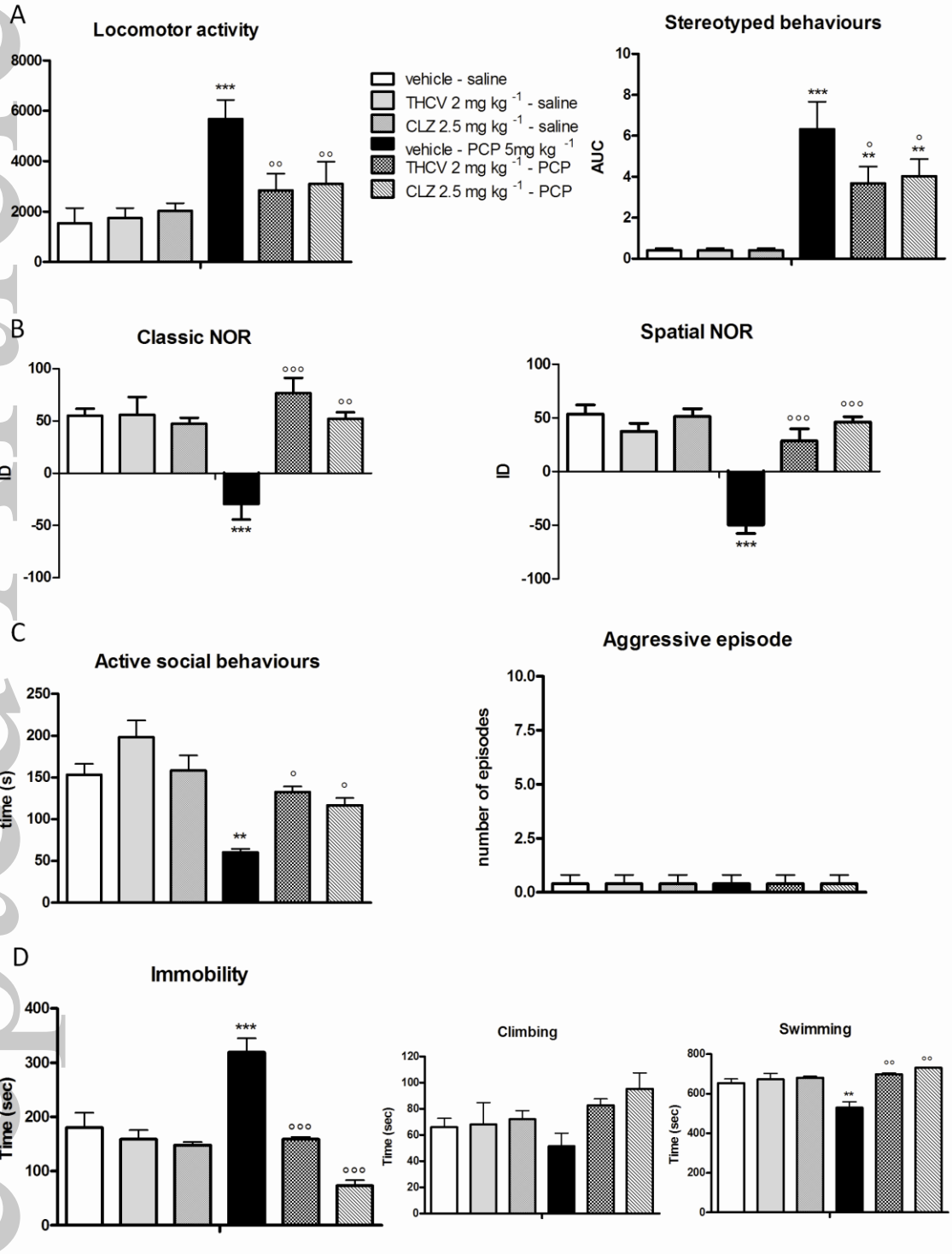

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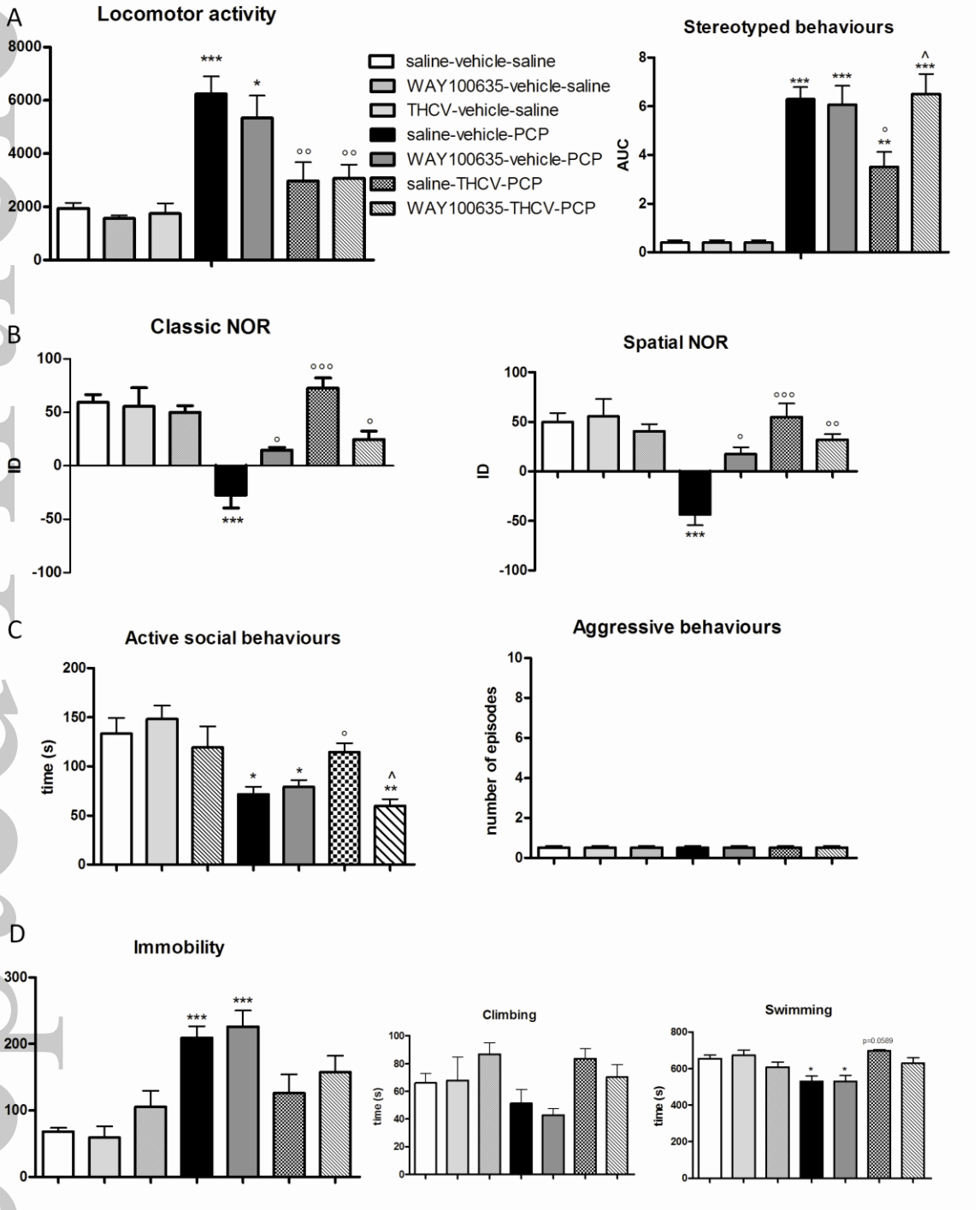

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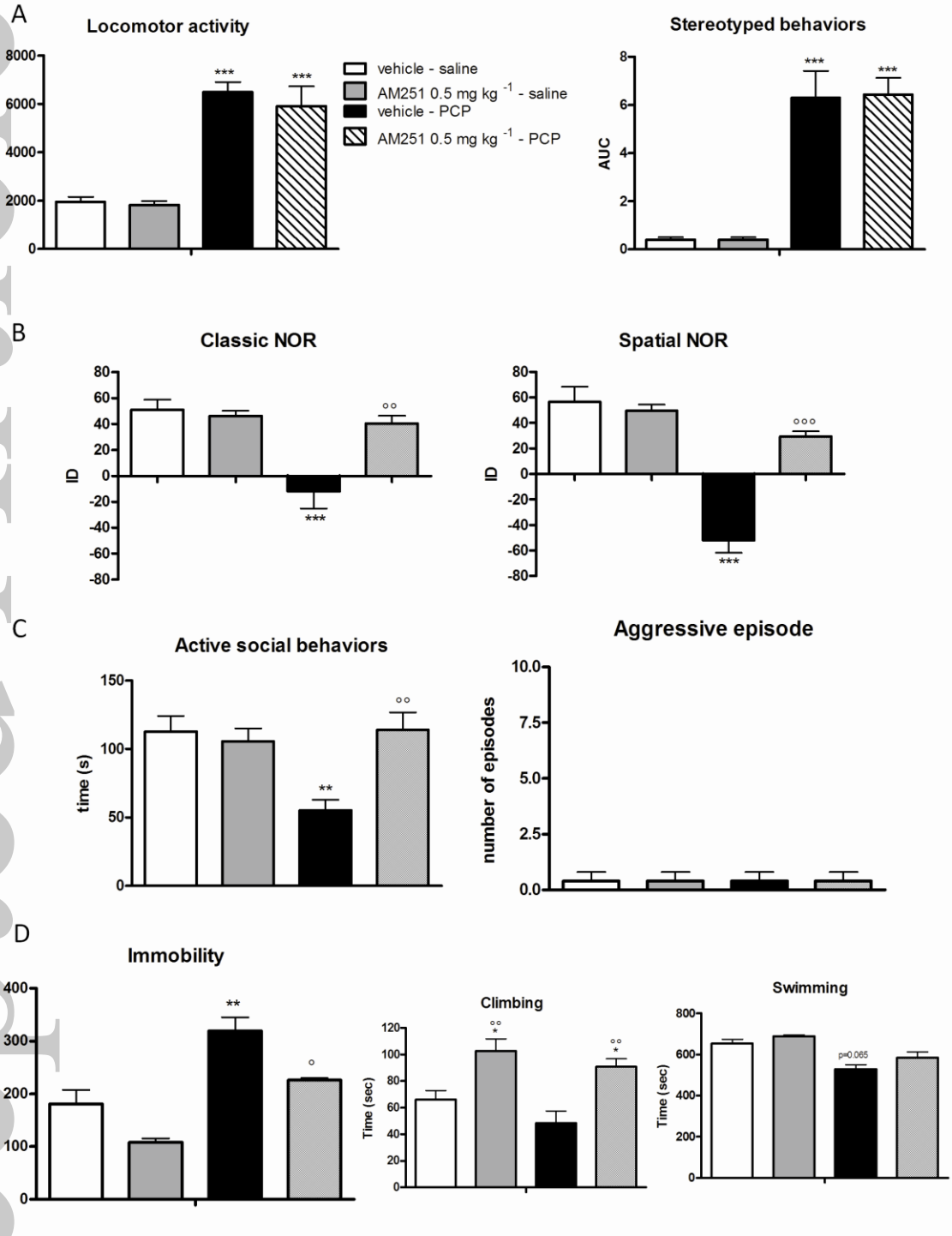

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Table 1: Mean IC_{50} and maximal percentage displacement values for the displacement of 8-[^{3}H]-OH-DPAT from specific binding sites in membranes obtained from Sprague Dawley rat brainstem or human 5-HT_{1A} CHO cells, with 95% confidence intervals (CI) shown in brackets.

Compound	**Tissue**	**IC_{50}, nM (95% CI)**	**Maximal Displacement, % (95% CI)**	**n**
8-OH-DPAT	Rat brainstem	0.8 (0.4 & 1.4)	107.6 (95.4 & 119.8)	4-9
Δ^9-THCV	Rat brainstem	0.002 (0.0002 & 0.002)	33.5* (27.0 & 40.0)	4-9
8-OH-DPAT	human 5-HT_{1A} CHO cells	0.9 (0.5 & 1.7)	96.5 (90.1 & 102.9)	6
Δ^9-THCV	human 5-HT_{1A} CHO cells	2060 (194.6 & 21810)	-45.4* † (-73.5 & -17.4)	10

*The 95% confidence intervals of this mean value do not overlap with those of the mean value in the previous row, indicating it to be significantly lower than the mean value obtained from experiments with vehicle-treated membranes ($P<0.05$). See also Figures 2C and 2F.

†The 95% confidence intervals of this mean maximal displacement value indicate it to be significantly less than zero ($P<0.05$). See also Figure 2F.

Table 2: Effect of 100 nM Δ^9-THCV on the mean EC_{50} and E_{max} values of 8-OH-DPAT for its stimulation of [^{35}S]GTPγS binding to membranes obtained from Sprague Dawley rat brainstem or human 5-HT_{1A} CHO cells, with 95% confidence intervals (CI) shown in brackets.

Pretreatment	**Tissue**	**EC_{50}, nM (95% CI)**	**E_{max}, % (95% CI)**	**n**
Vehicle (DMSO)	Rat brainstem	1301 (234 & 7236)	45.0 (30.0 & 60.0)	11
100 nM THCV	Rat brainstem	5.4* (0.4 & 67.4)	34.7 (27.8 & 41.5)	11
Vehicle (DMSO)	human 5-HT_{1A} CHO cells	18.4 (8.8 & 38.5)	108.5 (99.7 & 117.4)	8
100 nM THCV	human 5-HT_{1A} CHO cells	28.3 (14.9 & 53.7)	133.8* (124.5 & 143.1)	8

*The 95% confidence intervals of this mean value do not overlap with those of the mean value in the previous row, indicating it to be significantly lower than the mean value obtained from experiments with vehicle-treated membranes ($P<0.05$). See also Figures 2A and 2D.