Biomedicine & Pharmacotherapy 154 (2022) 113612

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Serotonin 5-HT$_{2A}$, 5-HT$_{2c}$ and 5-HT$_{1A}$ receptor involvement in the acute effects of psilocybin in mice. *In vitro* pharmacological profile and modulation of thermoregulation and head-twich response

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ARTICLE INFO

Keywords:
Psilocybin
5-HT$_{1A}$ receptors
5-HT$_{2A}$ receptors
5-HT$_{2C}$ receptors
Head-twitch response
Body temperature

ABSTRACT

The psychedelic 5-HT$_{2A}$ receptor (5HT2AR) agonist psilocybin (or the active metabolite psilocin) has emerged as potential useful drug for various neuropsychiatric diseases, with a rapid onset of therapeutic activity. However, the mechanisms responsible for such effects remain incompletely characterized. We aimed to study *in vitro* pharmacological profile and *in vivo* acute mechanism of psilocin/psilocybin. Competition binding studies with psilocin were performed in brain and cell cultures. The role of 5HT2AR, 5-HT$_{2C}$ receptors (5HT2CR) and 5-HT$_{1A}$ receptors (5HT1AR) on the psychosis–like head-twitch response (HTR) and on body temperature in mice after psilocybin administration were evaluated. Psilocin showed similar affinities for 5HT2AR (K_i: 120–173 nM), 5HT2CR (K_i: 79–311 nM) and 5-HT1AR (K_i: 152–146 nM) in human and mice brain. Psilocybin induced a dose-dependent HTR (maximal effect 17.07 ± 1.31 at 1 mg/kg i.p.) that was completely suppressed by the 5HT2AR antagonist MDL11939 (1 mg/kg). Higher doses of psilocybin (3 mg/kg) induced lower HTR (9.00 ± 0.53). The 5HT2CR antagonist SB242084 (0.1 mg/kg) increased HTR exerted by psilocybin (3 mg/kg). Psilocybin significantly raised core body temperature at low dose (0.125 mg/kg) (E_{max}=0.67 ± 0.15 °C), whereas a significant decrease was induced by doses over 1 mg/kg (E_{max} = −1.31 ± 0.16 °C). Pre-treatment with the 5HT1AR antagonist WAY100635 reversed the decrease of body temperature after psilocybin (1 mg/kg), causing hyperthermia (E_{max} = 0.94 ± 0.26 °C). The present work provides key findings on the 5HT2AR, 5-HT2CR and 5HT1AR involvement in the acute central effects of psilocybin. The results may be relevant for understanding the mechanism of action underlying the therapeutic effects and side effects of this psychedelic drug.

1. Introduction

Psilocybin [3-[2-(dimethylamino)-ethyl]− 1 H-indol-4-ol dihydrogen phosphate] is a naturally occurring alkaloid found in a variety of mushrooms within the *Psilocybe* and other fungal genera. *In vivo*, psilocybin is rapidly dephosphorylated to psilocin (4-hydroxy-N,N-dimethyltriptamine) [1]. The latter drug is considered a psychedelic which profoundly alters perception, mood and a host of cognitive processes [2]. During the 1960s and 1970s, psilocybin, among other psychedelics, was widely used in experimental research for the study of the etiopathogenesis of various mental disorders [3]. Along with other psychedelic substances, including D-lysergic acid diethylamide (LSD) and mescaline, psilocybin became a popular recreational drug, and was classed as Schedule I by the Convention on Psychotropic Substances of 1971, which prohibited their manufacture, use and distribution, causing all research to be discontinued. Nonetheless, the interest in human experimental research with psilocybin has regrown since the 1990s [4], and it is currently one of the most used psychedelics in human studies [5,6]. In the last years, multiple clinical trials have concluded that single or double exposure to psilocybin produces long-lasting positive changes in attitudes, mood and behaviour on healthy individuals [7,8] and significant improvements in the symptomatology of a variety of psychiatric disorders including depression, anxiety and addiction [8–14].

Despite the growing interest in the therapeutic properties of

Abbreviations: 5HT1AR, 5-HT$_{1A}$ receptors; 5HT2AR, 5-HT$_{2A}$ receptors; 5HT2CR, 5-HT$_{2C}$ receptors; HTR, head-twitch response; LSD, D-lysergic acid diethylamide; DOI, 1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine; AUC, area under curve.

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https://doi.org/10.1016/j.biopha.2022.113612
Received 24 June 2022; Received in revised form 12 August 2022; Accepted 24 August 2022
Available online 30 August 2022

psilocybin, there are scarce dose-response studies that allow the in-depth assessment of the mechanism of action of this substance. Acute pharmacological effects associated to psilocybin/psilocin administration have traditionally been attributed to its binding properties to 5-HT_{2A} receptors (5HT2AR) [15]. As shown by *in vitro* data of binding performed on human receptor-expressing cells, psilocin binds with high affinity to 5HT2AR (K_i: 25–107 nM, human cloned receptor using [^{3}H] ketanserin) [16–18]. *In vivo* human data also demonstrates an important mechanistic implication of 5HT2AR in the acute effects of psilocin, according to cerebral 5HT2AR occupancy, psilocin plasma levels and psychedelic experience correlation [19,20]. Likewise, subjective effects of psilocybin are blocked by pre-treatment with ketanserin, a 5HT2AR/5-HT_{2C} receptor (5HT2CR) antagonist [21,22]. Despite this evidence, the pharmacology of psilocin is more complex than originally described. Psilocybin, at clinically used doses, has been proven to interact with a wide variety of serotonergic receptors, particularly 5-HT_{1A} receptors (5HT1AR) and 5HT2CR [23]. *In vitro* data indicate that psilocin binds with high affinity to 5HT1AR (Ki 49–567 nM, human cloned receptor, using [^{3}H]8-OH-DPAT) and 5HT2CR (Ki 10–97 nM, human cloned receptor using [^{3}H]mesulgerine and [^{3}H]mianserin) [16–18]. Although the acute hallucinogenic mechanism of psilocybin/psilocin is strongly associated to 5HT2AR activation, it is not well understood whether the binding profile of psilocin to the 5HT1AR and 5HT2CR is involved in the acute and/or long-lasting effects of this drug.

Given the subjective nature of the effects of psychedelics on humans, a variety of preclinical assays have been used to study hallucinogen mechanisms *in vivo*. Amongst them, the head-twitch response (HTR) is the most translational behavioural assay to characterize the acute effects of hallucinogens in rodents [24]. There is a high correlation between the potency of psychedelic-induced HTR in rodents and their behavioural subjective effects in humans [25]. It is described as a paroxysmal rotational movement of the head induced by hallucinogens in rodents, and it constitutes an easily quantifiable response with low within-subject and between-subject variability [26]. Mechanistic studies have shown that specific activation of 5HT2AR in frontal cortex pyramidal neurons is responsible for HTR [27]. However, dose-response curves derived from HTR present a biphasic appearance [28]. Thus, other receptors may also play an important role in the hallucinogenic effect induced by psychedelics to give rise to this biphasic nature. Special interest arises from the 5HT2CR and 5HT1AR, given their alleged modulatory role on the behavioural effects of psychedelics [26,29,30].

Aside from the HTR, several assays have been commonly used to assess the mechanism of action of serotonergic hallucinogens, such as changes in core body temperature. Systemic administration of psychedelics has been shown to produce changes in body temperature in humans [20,31] and rodents (for review see 32). The involvement of 5HT2AR in thermoregulatory reactions *in vivo* has been previously described. Evidence supports that 5HT2AR activation in the hypothalamus leads to a rise in body temperature [33–35]. However, different serotonergic mechanisms have also been identified as players in the control of body temperature [32].

In spite of the indisputable role of 5HT2AR on the *in vivo* acute effects of psilocybin, the role of different serotonergic receptors should also be considered. Recent studies report that single psilocybin administration exerts an increase in neural plasticity in rodent frontal cortex [36] and hippocampus [37] that correlates with antidepressant effects [37,38]. Preclinical data from such studies suggests that 5HT2AR activation may not be necessary for an antidepressant response to psilocybin. Thus, off-target activities illustrate the need for better functional characterization *in vivo*.

To the best of our knowledge, there are no comparative studies of psilocin's pharmacological profile on human and rodent native brain tissue. In the present study, we have characterized *in vitro* pharmacological properties of psilocin and performed an *in vivo* dose-response study for psilocybin in mice. We have characterized the specific role of 5HT2AR, 5HT2CR and 5HT1AR on psilocybin-induced effects through the evaluation of HTR and core temperature, two physiological and behavioural paradigms known to be sensitive to the effects of hallucinogens. For future *in vivo* studies in order to select the optimal dose of psilocybin, it is essential to determine the implication of different serotonergic receptors in one particular psilocybin-induced effect.

2. Material and methods

2.1. Animals

Adult male C57BL/6 J mice (8 weeks old) were purchased from Envigo (Barcelona, Spain), and housed under standard laboratory conditions on a 12 h light/dark cycle, at room temperature (22–24 °C), with food and water available *ad libitum*. Mice weighed 22–26 g, and were housed in groups of 6 (cage size: 22 × 22 × 14.5 cm) in compliance with ARRIVE guidelines [39] throughout the entire experimental procedure. The estimated total number of animals used for this study was 302, and group sizes per experiment were 4–17. Animal care and experimental protocols were carried out in accordance with the principles of animal care established by the EU Directive 2010/63/EU and in agreement with Spanish legislation (Royal Decree 53/2013), and were approved by the UPV/EHU Ethical Board of Animal Welfare (CEEA; reference M20_2020_014).

2.2. Drugs and radioligands

Psilocybin [3-[2-(dimethylamino)-ethyl]− 1 H-indol-4-ol dihydrogen phosphate] was obtained from THC Pharm (Frankfurt, Germany) and psilocin [4-hydroxy-N,N-dimethyltriptamine] from Lipomed AG (Arlesheim, Switzerland). The selective 5HT2AR antagonist MDL11939 [α-phenyl-1-(2-phenylethyl)− 4-piperidinemethanol] and the 5HT2R antagonist methysergide [(4 R,7 R)-N-[(2 S)− 1-hydroxybutan-2-yl]− 6,11-dimethyl-6,11-diazatetracyclo[7.6.1.0^{2,7}.0^{12,16}]hexadeca-1(16),2,9,12,14-pentaene-4-carboxamide] were purchased from Sigma Aldrich (St. Louis, MO, USA). The selective 5HT1AR antagonist WAY100635 [*N*-[2-[4-(2-methoxyphenyl)− 1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate] and the selective 5HT2CR antagonist SB242084 [6-chloro-2,3-dihydro-5-methyl-*N*-[6-[(2-methyl-3-pyridinyl)oxy]− 3-pyridinyl]− 1*H*-indole-1-carboxyamide dihydrochloride] were obtained from Tocris (Bristol, UK). Peripheral 5HT2R antagonist xylamidine tosylate [2-(3-methoxyphenoxy)propyl-[2-(3-methylphenyl)ethanimidoyl]azanium;4-methylbenzenesulfonate] (Kemprotec Limited, Cumbria, UK). [^{3}H]ketanserin [[^{3}H]3-[2-[4-(4-fluorobenzoyl)piperidin-1-yl]ethyl]− 1 H-quinazoline-2,4-dione] (specific activity 22.8 Ci/mmol) was obtained from PerkinElmer (Madrid, Spain) and stored at − 20 °C. [^{3}H]8-OH-DPAT [[^{3}H]− 7-(dipropylamino)− 5,6,7,8-tetrahydronaphthalen-1-ol] (specific activity 187.4 Ci/mmol) was supplied by PerkinElmer (Madrid, Spain) and stored at − 20 °C. [^{3}H]mesulergine [[^{3}H]- (6aR,9S,10aR)− 9-(dimethylsulfamoylamino)− 4,7-dimethyl-6,6a,8,9,10,10a-hexahydroindolo[4,3-fg]quinoline] (specific activity 76.8 Ci/mmol) was obtained from PerkinElmer (Madrid, Spain) and stored at − 80 °C. All other chemicals were obtained from standard sources.

2.3. Postmortem human brain, mice brain and human receptor expressing cells

Human brain samples were obtained from the brain collection facility of the UPV/EHU (#35 at https://biobancos.isciii.es/ListadoColecciones.aspx). Autopsies were performed in the Basque Institute of Legal Medicine, Bilbao, in compliance with policies of research and ethical boards for postmortem brain studies [40]. Six subjects were chosen among the collected brains on the basis of the absence of neuropsychiatric disorders or drug abuse, and an appropriate postmortem interval (time between death and tissue dissection/freezing). Specimens of dorsolateral prefrontal cortex were dissected following

standard procedures [41] and stored at − 80 ºC until membrane preparation. Experimental protocol with postmortem human samples was approved by the UPV/EHU Ethical Board for human studies (CEISH; reference 119/2019). Mice brain samples were obtained from six animals after animal euthanasia by cervical dislocation. Bilateral brain cortices were dissected for 5HT2AR and 5HT1AR binding studies. Whole brain tissue (except cortex and cerebellum) was used for 5HT2CR experiments. Tissue was frozen at − 80 °C, or immediately processed for radioligand binding assays. Cells selectively expressing human 5HT2AR (CHO-K1. Product number: ES-313-M400UA) or 5HT2CR (CHO-K1. Product number: ES-315-M400UA) were purchased from PerkinElmer (Madrid, Spain).

2.4. Membrane preparation

Brain samples and cells were processed to obtain membrane-enriched homogenates as previously described [42]. Briefly, samples were homogenized using an Ultra-Turrax T8 homogenizer in 1 mL of homogenization buffer (5 mM Tris–HCl; 0.25 M sucrose, pH 7.4). The crude homogenate was centrifuged at 3000 *g* for 10 min at 4 ºC, and the supernatant was re-centrifuged at 40,000 *g* for 10 min at 4 ºC. The resultant pellet (P_2 fraction) was washed twice in incubation buffer (50 mM Tris-HCl; pH 7.5) and re-centrifuged in similar conditions. Aliquots were stored at − 80 ºC until assay.

2.5. Binding competition assays

Radioligand binding studies were performed in 96-well microtiter plates in a final total volume of 250 μl incubation buffer (50 mM Tris-HCl, pH 7.5) as previously described [42]. Three independent experimental replication were performed to generate individual data points. For 5HT2AR, competition experiments were carried out in the presence of 2 nM of [^{3}H]ketanserin with 50 μg membrane protein for tissue preparations and 4 μg membrane protein for human 5HT2AR expressing cells. In 5HT2CR studies, 5 nM of [^{3}H]mesulergine with 70 μg membrane protein for rodent tissue preparations and 5 μg membrane protein for human 5HT2CR expressing cells was used. 50 μg membrane protein in the presence of [^{3}H]8-OH-DPAT (1 nM) was utilized for 5HT1AR studies. Competition experiments were performed in the absence or presence of 13 different concentrations (10^{-10}–10^{-4} M) of psilocin. Besides, for 5HT2CR specific binding calculation purposes, an additional competition experiment of psilocin (10^{-10}-10^{-4} M) in the presence of 5HT2CR antagonist SB242084 (100 nM) was performed. Based on previously published data [43] and competition binding experiments performed (Supplementary Fig. 1), SB242084 100 nM was identified as the most suitable drug concentration for the blockade of 5HT2CR specific binding without any 5HT2AR binding displacement. Subtraction of psilocin competition experiments in the presence and absence of SB242084 (100 nM) was considered 5HT2CR specific curve. Non-specific binding was determined in the presence of MDL11939 (10 μM) for 5HT2AR, methysergide (10 μM) for 5HT2CR and WAY100635 (10 μM) for 5HT1AR. Reactions were incubated for 60 min at 37 °C for 5HT2AR and 5HT2CR and 45 min at 37 ºC for 5HT1AR competition assays.

2.6. Pharmacological treatments

Psilocybin was dissolved to 0.025–0.2 mg/mL in saline solution (0.9 % NaCl). MDL11939 was dissolved to 0.1 mg/mL in saline solution with a minimal amount of glacial acetic acid to facilitate dissolution. WAY100635 was dissolved in saline solution to 0.1 mg/mL. SB242084 was dissolved in vehicle (0.9 % NaCl, 8 % β-cyclodextrine, 25 mM citric acid), to 0.01–0.1 mg/mL. Xylamidine tosylate was dissolved in saline solution to 0.2 mg/mL. Antagonists or vehicle were administered 30 min prior to psilocybin or saline. All drugs were administered by intraperitoneal injection. Doses of the different antagonists used as well as the dosing schedule were selected on the basis of preliminary experiments and previously published works [26,28–30].

2.7. Head-twitch response assessment

HTR is described as side-to-side movement of the head. This behaviour occurs naturally in rodents and the frequency of manifestations is strongly enhanced after psychedelic administration [24–26]. HTR increase is not observed with other psychoactive drugs such as cocaine, phencyclidine or amphetamine [16]. In the present study, HTR was individually assessed in an Open Field chamber (43 × 43 × 43 cm), and recorded with a camera device (ASUS Zenfone 3, Taipei, Taiwan) immediately after psilocybin injection for 25 min. Four mice were simultaneously tested for the characteristic rotational head movement, and a trained observer counted the number of these responses recorded between minutes 5 and 25. The time period for HTR evaluation was chosen after an initial analysis of HTR throughout time in 5 min-intervals, for 40 min. The open fields were thoroughly cleaned with 70 % ethanol and were left to fully dry between test sessions. Light intensity was established and fixed at 60 lux for all behavioural experiments.

2.8. Rectal temperature measurement

Rectal temperature of mice was measured 30 min prior to psilocybin injection as baseline (right before antagonist administration), immediately before psilocybin administration, and every 20 min post-psilocybin administration for 120 min. A thermocouple probe (RET-3 Rectal Probe for Mice, BiosebLab, Salon de Provence, France) connected to a digital thermometer (Bioseb 8851 K.J.T. Type, BiosebLab, Salon de Provence, France) was inserted 2 cm into the rectum and a steady temperature readout was obtained within 10 s of the insertion. Animals were placed in the testing room 1 h prior to any recording and room temperature was maintained stable at 24 ± 1 °C. All temperature experiments were performed in the early morning (starting at 8:30 AM) in order to avoid baseline temperature differences caused by circadian rhythms.

2.9. Statistical analysis

Data obtained from radioligand binding displacement curves in human or mouse brain and cell membranes were analysed by non-linear analysis using GraphPad Prism™ software version 9.0 (GraphPad Software Inc. CA, USA). Specific binding data obtained from radioligand competition was transformed to percentage of basal binding value (binding in the absence of any exogenous drug) for each radioligand used. In each experimental condition, the selection between models of competition curves by psilocin against different radioligands binding was made by the extra-sum-of-squares (*F* test). Following the nonlinear curve fitting, K_i values for 5HT2AR, 5HT2CR and 5HT1AR were calculated from the corresponding IC_{50} values. K_i values were normalized to –log K_i (pK_i) to perform statistical analyses. Non parametric Mann-Whitney test was used to evaluate differences between pK_i estimations.

For the behavioural assessment, data were tested initially for normality (Shapiro-Wilk) and further analysed using one-way or two-way ANOVA followed by Bonferroni *post hoc* test after statistically significant interaction between factors. Factors were identified as F_{ag} (agonist), F_{ant} (antagonist) and F_i (interaction). For the time-course temperature measurements, data were analysed using repeated measures two-way ANOVA, followed by Bonferroni *post hoc* test after statistically significant interaction. Factors were identified as F_{drug} (drug), F_t (time) and F_i (interaction). The area under curve (AUC) of temperature timelines were analysed by using one-way or two-way ANOVA, followed by Bonferroni *post hoc* test after significant interaction. Factors were defined as F_{ag} (agonist), F_{ant} (antagonist) and F_i (interaction). All results are shown as mean±SEM. In all cases, statistical significance was considered when $p < 0.05$. Data were analysed using GraphPad Prism 9.0.

3. Results

3.1. 5HT2AR, 5HT2CR and 5HT1AR competition assays

We first examined the pharmacological parameters of psilocin displacing [^{3}H]ketanserin binding in mouse brain cortex, in human frontal cortex and human 5HT2AR expressing cells. As shown in Fig. 1A, we found that psilocin displaced [^{3}H]ketanserin binding in a monophasic manner in all the tissues. Similar K_i values were obtained in the different experimental conditions evaluated. pK_i values were 6.741 ± 0.186 (K_i = 173 nM), 6.855 ± 0.051 (K_i = 120 nM) and 6.256 ± 0.244 (K_i = 478 nM) in mouse brain cortex, human brain cortex and human 5HT2AR expressing cells, respectively. Statistical comparison of psilocin pK_i values obtained in cerebral cortex, human frontal cortex and human cells expressing 5HT2AR did not reveal significant differences between the different tissues.

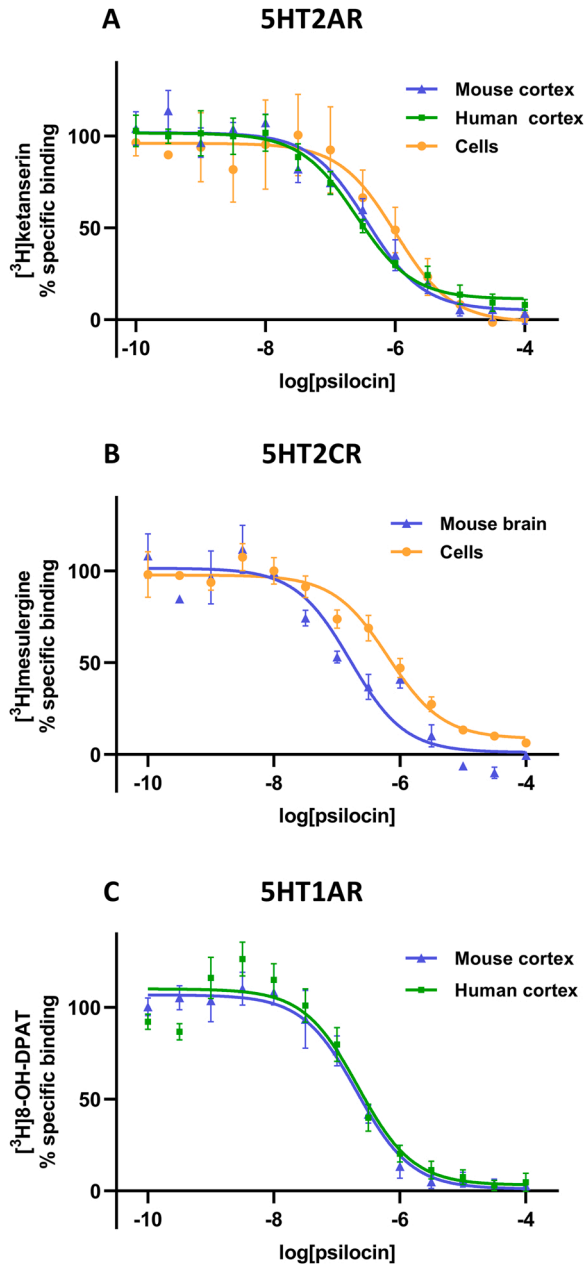

Fig. 1. [^{3}H]Ketanserin (2 nM) (**A**), [^{3}H]mesulergine (5 nM) (**B**) or [^{3}H]8-OH-DPAT (1 nM) (**C**) specific binding displacement curves by psilocin in mouse cortex/whole mouse brain, human brain cortex, or human 5HT2AR or 5HT2CR expressing cells. All values represent means±SEM of six different assays.

For the evaluation of 5HT2CR binding properties of psilocin, we first studied SB242084 displacing [^{3}H]mesulergine binding in mouse brain. SB242084 has been shown as the most selective 5HT2CR ligand over 5HT2AR [43]. As shown in Supplementary Fig. 1, we found that SB242084 displaced [^{3}H]mesulergine binding in a biphasic manner, showing that the fraction of high-affinity binding sites (presumably 5HT2CR binding sites) was completely inhibited at 100 nM of SB242084. For this reason, we examined the pharmacological parameters of psilocin displacing [^{3}H]mesulergine binding in mouse brain in the presence or absence of SB242084 100 nM (Supplementary Fig. 2). We observed that [^{3}H]mesulergine specific binding displacement curve with psilocin was biphasic. Interestingly, in the presence of SB242084 100 nM, [^{3}H]mesulergine specific basal binding decreased and psilocin displaced [^{3}H]mesulergine specific binding in a monophasic manner. The subtraction of both curves shows the specific 5HT2CR binding (Fig. 1B). Since cells selectively express the 5HT2CR, it was not required to perform psilocin competition assays in the presence of SB242084, and psilocin displacing [^{3}H]mesulergine binding was performed directly. pK_i values were 7.019 ± 0.363 (K_i = 79 nM) and 6.497 ± 0.133 (K_i = 311 nM) for mouse brain and human 5HT2CR expressing cells, respectively. No statistical differences were observed between psilocin pKi values obtained.

Finally, competition assays were performed for the evaluation of 5HT1AR binding properties of psilocin. [^{3}H]8-OH-DPAT specific binding displacement curve was monophasic and showed similar K_i values for mouse (pK_i = 6.875 ± 0.104; K_i = 146 nM) and human brain cortex (pK_i = 6.818 ± 0.069; K_i = 152 nM), respectively (Fig. 1C). Again, no statistical differences were observed between psilocin pKi values.

3.2. Temporal evaluation of HTR after psilocybin administration

Psilocybin-induced HTR was assessed throughout time for a total period of time of 25 min post psilocybin (1 mg/kg, i.p.) or saline (5 mL/kg, i.p.) administration. According to the preliminary observations, a 20-minute period was chosen for data analysis, starting 5 min after drug administration, since the number of HTR counted during the first 5 min was negligible. The maximal effect on HTR was reached at the 5–10 min interval post-administration (8.75 ± 0.64 HTR) (Supplementary Fig. 3). Two-way repeated measures ANOVA analysis revealed significant differences between saline and psilocybin-treated groups throughout time intervals ($F_{drug}(1,22)$= 120.30, $p < 0.0001$; $F_{time}(1.75,38.56)$= 73.99, $p < 0.0001$; $F_i(3,66)$= 58.35, $p < 0.0001$). *Post hoc* analysis revealed differences in all four time-periods analysed ($t = 11.75$, $p < 0.0001$ for minutes 5–10; $t = 9.99$, $p < 0.0001$ for minutes 10–15; $t = 5.02$, $p < 0.001$ for minutes 15–20; $t = 5.27$, $p < 0.001$ for minutes 20–25).

3.3. Dose-response evaluation of the effect of psilocybin administration on HTR

The number of HTR elicited by different doses of intraperitoneal administration of psilocybin was evaluated (Fig. 2). Psilocybin induced a dose-dependent response on HTR: saline solution (5 mL/kg, 2.17 ± 0.37, n = 12), 0.125 mg/kg (3.75 ± 0.73, n = 8), 0.25 mg/kg (5.14 ± 0.55, n = 7), 0.5 mg/kg (16.13 ± 1.65, n = 8), 1 mg/kg (17.07 ± 1.31, n = 14). However, a higher dose of psilocybin (3 mg/kg) induced a lower HTR (9.20 ± 0.44, n = 10) compared to 1 mg/kg. One-way ANOVA revealed a significant effect of psilocybin on elicited HTR ($F(5,53)$= 42.84, $p < 0.0001$). *Post hoc* analysis showed that doses of 0.5 and 1 mg/kg caused a significantly higher response when compared to control group (saline, 5 mL/kg) ($t = 9.55$, $p < 0.0001$; $t = 11.83$, $p < 0.0001$, respectively). Additionally, the effect of 3 mg/kg psilocybin was significantly lower than 1 mg/kg ($t = 5.94$, $p < 0.0001$), confirming an inverted U-shaped effect of psilocybin on HTR (Fig. 2).

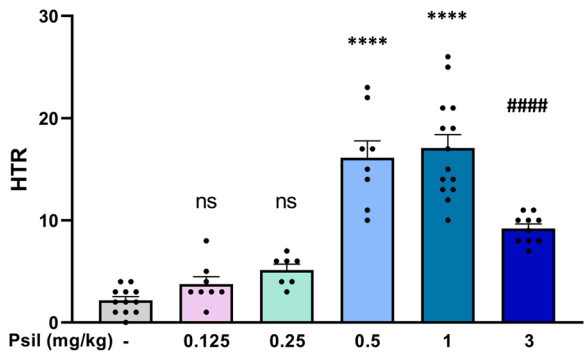

Fig. 2. Effect of intraperitoneal administration of increasing doses of psilocybin (0.125/0.25/0.5/1/3 mg/kg, i.p., n = 8, 7, 8, 14, 10, respectively) or saline (5 mL/kg i.p., n = 12) on HTR. Points represent individual evaluations. Data are presented in bars as mean±SEM. One-way ANOVA followed by Bonferroni *post hoc* test. ****p < 0.0001 psilocybin *vs* saline, $^{\#\#\#\#}$p < 0.0001 psilocybin 1 mg/kg *vs* 3 mg/kg, ns: no statistical significance (p > 0.05).

3.4. Evaluation of the role of 5HT2AR on psilocybin-induced HTR

In order to evaluate the role of 5HT2AR on psilocybin-induced HTR, the 5HT2AR antagonist MDL11939 (1 mg/kg, i.p.) was administered 30 min prior to psilocybin (1 mg/kg, i.p.). On saline pre-treated mice, psilocybin caused 17.83 ± 1.38 HTR (n = 12), whereas MDL11939 (1 mg/kg, i.p.) pre-treated mice did not show HTR after psilocybin administration (n = 12) (Fig. 3A). Likewise, treatment with MDL11939 + saline did not induce any HTR. Two-way ANOVA revealed significant difference between compared groups ($F_{ag}(1,43) = 111.00$, $p < 0.0001$; $F_{ant}(1,43) = 181.70$, $p < 0.0001$; $F_i(1,43) = 115.8$, $p < 0.001$). *Post hoc* analysis revealed a significant difference between vehicle + psilocybin and MDL11939 + psilocybin groups ($t = 16.96$, $p < 0.0001$), confirming that psilocybin-induced HTR is mainly mediated by 5HT2AR.

3.5. Evaluation of the role of 5HT2CR on psilocybin-induced HTR

In order to elucidate the role of 5HT2CR on psilocybin-induced HTR, the 5-HT2CR antagonist SB242084 was selected (Fig. 3B). In a first set of

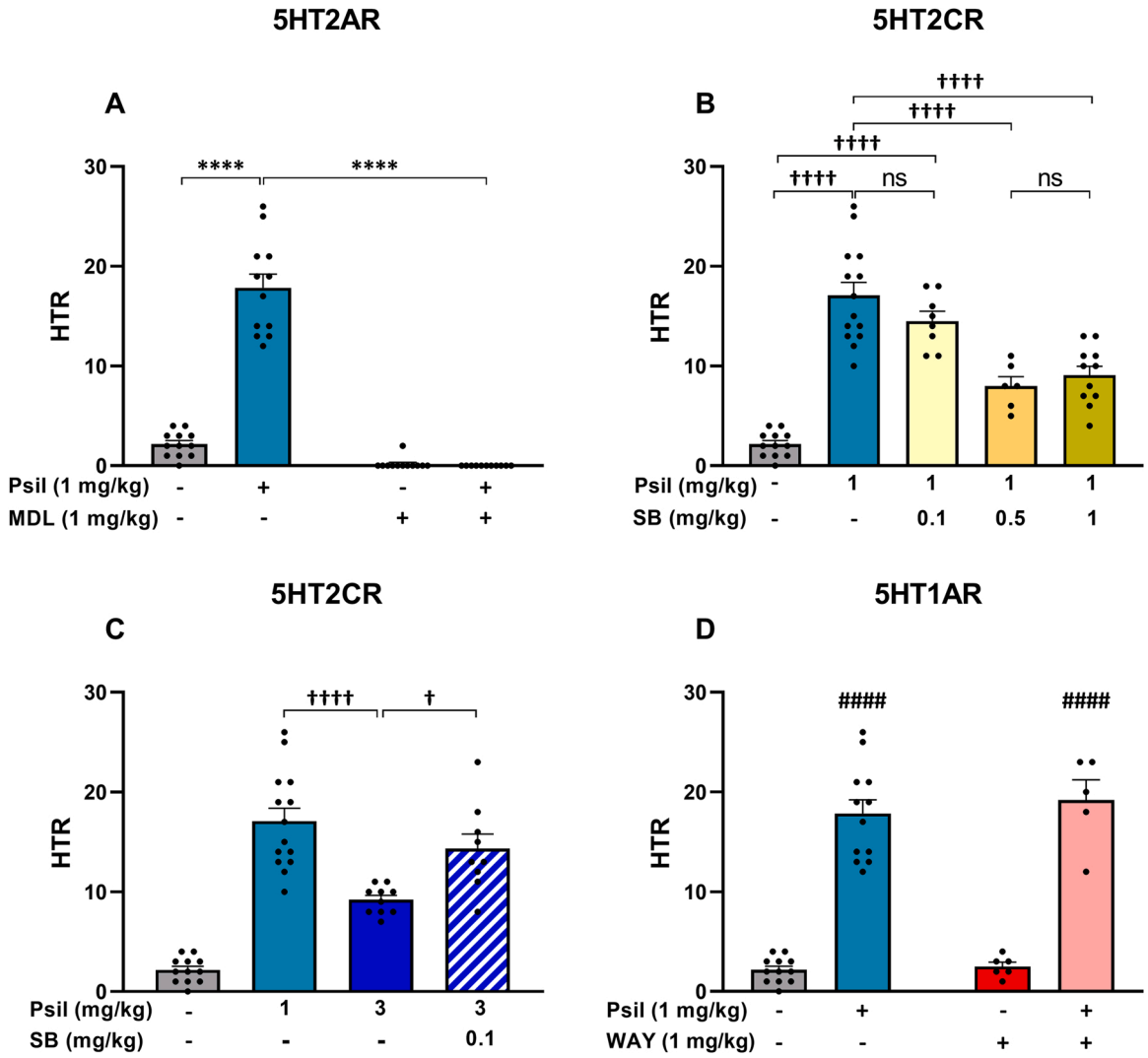

Fig. 3. Evaluation of the role of 5HT2AR (A) 5HT2CR (B, C) and 5HT1AR (D) on psilocybin-induced HTR by pre-treatment with the selective 5HT2AR antagonist MDL11939 (MDL) (1 mg/kg, i.p.), the selective 5HT1AR antagonist WAY100635 (WAY) (1 mg/kg, i.p.) and the selective 5HT2CR antagonist SB242084 (SB). Points represent individual evaluations. Data are presented in bars as mean±SEM. Veh+SS n = 12; veh+psil n = 12–14; MDL+SS n = 12; MDL+psil n = 11; SB (0.1 mg/kg)+psil n = 8; SB (0.5 mg/kg)+psil n = 6; SB (1 mg/kg)+psil n = 11; SS+psil (3 mg/kg) n = 10; SB (0.1 mg/kg)+psil (3 mg/kg) n = 9; WAY+SS n = 6; WAY+psil n = 5. Two-way ANOVA, $^{\#\#\#\#}$p < 0.0001. Two-way ANOVA followed by Bonferroni *post hoc* test, ****p < 0.0001. One-way ANOVA followed by Bonferroni *post hoc* test. †p < 0.05, ††††p < 0.0001, ns: no statistical significance (p > 0.05).

experiments, we evaluated the effect of several doses of SB242084 on psilocybin-induced (1 mg/kg) HTR. Psilocybin caused 17.07 ± 1.31 HTR (n = 14) on vehicle pre-treated mice; 14.5 ± 0.98 events (n = 8), 8.00 ± 0.93 (n = 6) and 9.09 ± 0.88 (n = 11) on SB242084 (0.1, 0.5 and 1 mg/kg i.p. respectively) pre-treated mice. One-way ANOVA revealed differences between groups ($F(4,46)= 37.70$, $p < 0.0001$). *Post hoc* analysis showed differences between vehicle + psilocybin and SB242084 (0.5 mg/kg) + psilocybin ($t = 5.66$, $p < 0.0001$) and between vehicle + psilocybin and SB242084 (1 mg/kg) + psilocybin ($t = 6.04$ $p < 0.0001$). However, the lowest dose of SB242084 tested (0.1 mg/kg) did not affect psilocybin-induced HTR ($t = 1.77$, $p = 0.84$). These results reveal that SB242084 at a dose of 0.1 mg/kg presumably behaves as a selective antagonist for 5HT2CR.

In a second set of experiments, SB242084 (0.1 mg/kg) was chosen for pre-treatment to elucidate the role of 5HT2CR on the lowering phase of the inverted U-shaped dose-response profile of psilocybin-induced HTR. Pre-treatment with SB242084 (0.1 mg/kg, i.p.) potentiated HTR (14.33 ± 1.45 n = 9) induced by psilocybin 3 mg/kg i.p. (Fig. 3C). Statistical analysis revealed significant differences between groups (one-way ANOVA, $F(3,41) = 43.25$, $p < 0.0001$). Bonferroni *post hoc* analysis revealed significant differences between saline pre-treated and SB242084 (0.1 mg/kg, i.p.) pre-treated psilocybin groups ($t = 3.20$, $p < 0.05$).

3.6. Evaluation of the role of 5HT1AR on psilocybin-induced HTR

Due to the possible role of 5HT1AR in the HTR, the 5HT1AR antagonist WAY100635 (1 mg/kg, i.p.) was administered 30 min before psilocybin (1 mg/kg, i.p.). WAY100635 (1 mg/kg, i.p.) pre-treated mice showed 19.20 ± 2.04 (n = 5) after psilocybin administration (Fig. 3D). Two-way ANOVA revealed no interaction between variables in the compared groups ($F_{ag}(1,31) = 170.30$, $p < 0.0001$; $F_{ant}(1,31) = 0.47$, $p = 0.50$; $F_i(1,31) = 0.17$, $p = 0.68$). This fact rules out an action mediated by the 5HT1AR on psilocybin-induced HTR.

3.7. Evaluation of the effect of psilocybin administration on core body temperature: dose-response

The effect of different doses of psilocybin in core body temperature was evaluated using a rectal probe (Fig. 4). The lowest psilocybin dose (0.125 mg/kg, i.p., n = 6) caused an increase in temperature, compared to control (n = 12) (E_{max} = 0.75 ± 0.15 °C, time = 25 min post-administration). Two-way repeated measures ANOVA revealed significant differences between compared groups ($F_t(2.62,41.86)= 27.20$, $p < 0.0001$; $F_{drug}(1,16)= 16.05$, $p = 0.001$; $F_i(5,80)= 10.21$, $p < 0.0001$). Two-way repeated measures ANOVA also found significant differences between 0.25 mg/kg psilocybin (n = 8) and control ($F_t(2.32,41.80)= 13.92$, $p < 0.0001$; $F_{drug}(1,18)= 6.58$, $p < 0.05$; $F_i(5,90)= 1.96$, $p = 0.09$). No effects were observed on body temperature after 0.5 mg/kg intraperitoneal administration (n = 7) when compared to control group ($F_t(2.58,43.85)= 11.94$, $p < 0.0001$; $F_{drug}(1,17)= 0.71$, $p = 0.41$; $F_i(5,85)= 0.97$, $p = 0.44$). In contrast, a significant decrease was induced by dose 1 mg/kg psilocybin (n = 14) (E_{max}= −1.31 ± 0.16 °C, time=25 min) as revealed by two-way repeated measures ANOVA ($F_t(2.94,70.57)= 21.67$, $p < 0.0001$; $F_{drug}(1,24)= 13.52$, $p < 0.01$; $F_i(5120)= 12.49$, $p < 0.0001$), as well as by 3 mg/kg psilocybin (n = 8) (E_{max}=−1.69 ± 0.34 °C, time=25 min, ($F_t(1.98,35.67)= 12.93$, $p < 0.0001$; $F_{drug}(1,18)= 12.76$, $p < 0.01$; $F_i(5,90)= 9.59$, $p < 0.0001$) (Fig. 4A).

AUC analysis also revealed significant differences between psilocybin doses (one-way ANOVA, $F(5,50)= 17.57$, $p < 0.0001$). *Post hoc* analysis found differences between control group and 0.125 m/kg psilocybin ($t = 3.58$, $p < 0.05$), 1 mg/kg psilocybin ($t = 3.98$, $p < 0.01$) and psilocybin 3 mg/kg ($t = 4.11$, $p < 0.01$) (Fig. 4B).

3.8. Evaluation of the role of 5HT2AR, 5HT2CR and 5HT1AR on psilocybin-induced hypothermia

In order to assess the role of 5HT2AR, 5HT2CR and 5HT1AR on psilocybin-induced hypothermia, selective antagonists were administered 30 min before psilocybin (1 mg/kg, i.p.) and rectal temperature was measured every 20 min.

After baseline measurement, MDL11939 (1 mg/kg, i.p) or vehicle (10 mL/kg, i.p) was administered 30 min prior to psilocybin (1 mg/kg, i.p.) or saline (5 mL/kg, i.p.). Vehicle pre-treatment + psilocybin caused a decrease in body temperature (E_{max} = 1.16 ± 0.28 °C, time = 25 min, n = 11). 5HT2AR antagonist MDL11939 caused a slight hypothermic effect (E_{max} = −0.56 ± 0.22 °C, time = 0 min, n = 11). In addition, antagonist pre-treatment did not block the effect of psilocybin on the decrease of body temperature (two-way repeated measures ANOVA, $F_t(2.98,59.65) = 17.91$ $p < 0.0001$; $F_{drug}(1,20) = 0.11$, $p = 0.74$; $F_i(6120) = 1.92$, $p = 0.08$) (Figs. 5A and 5B).

Likewise, SB242084 (1 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.) was administered, 30 min prior to psilocybin (1 mg/kg, i.p.) or saline (5 mL/kg, i.p.), Antagonist pre-treatment did not block the effect of psilocybin on core body temperature (two-way repeated measures ANOVA,

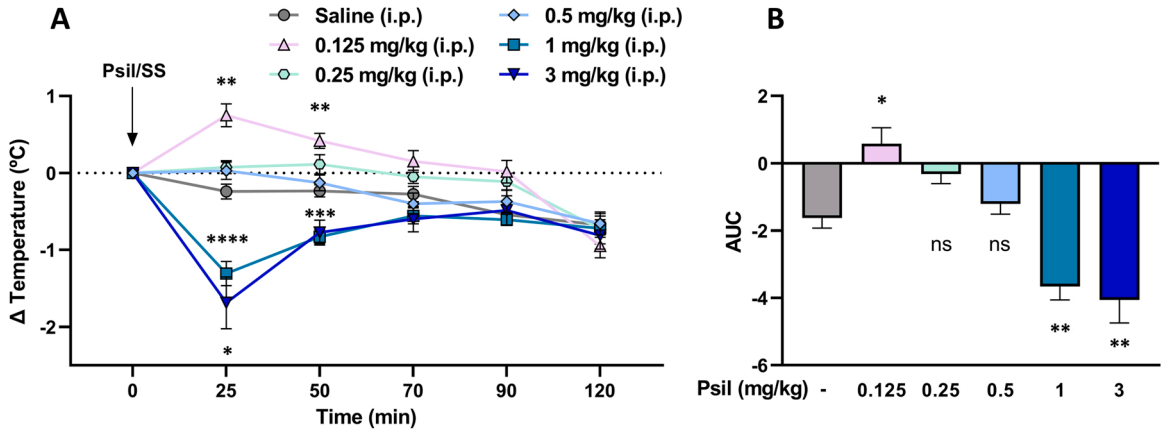

Fig. 4. Effect of systemic administration of different doses of psilocybin (Psil) on body temperature. Data are plotted as mean±SEM. A. Time-course effects of psilocybin (0.125/0.25/0.5/1/3 mg/kg, i.p., n = 8, 8, 8, 14, 8, respectively) or saline solution (5 mL/kg, i.p., n = 12) on rectal temperature. Values are expressed as mean temperature variation±SEM (°C) relative to basal temperature (time 0 min). Two-way repeated measures ANOVA followed by Bonferroni *post hoc* test. *p < 0.05, * *p < 0.01, * **p < 0.001, * ** *p < 0.0001 psilocybin *vs* saline. B. AUC values obtained from rectal temperature variation after psilocybin (0.125/0.25/0.5/1/3 mg/kg, i.p.) or saline (5 mL/kg, i.p.) administration. One-way ANOVA followed by Bonferroni *post hoc* test. *p < 0.05, **p < 0.01, ns: no statistical significance (p > 0.05), psilocybin *vs* saline.

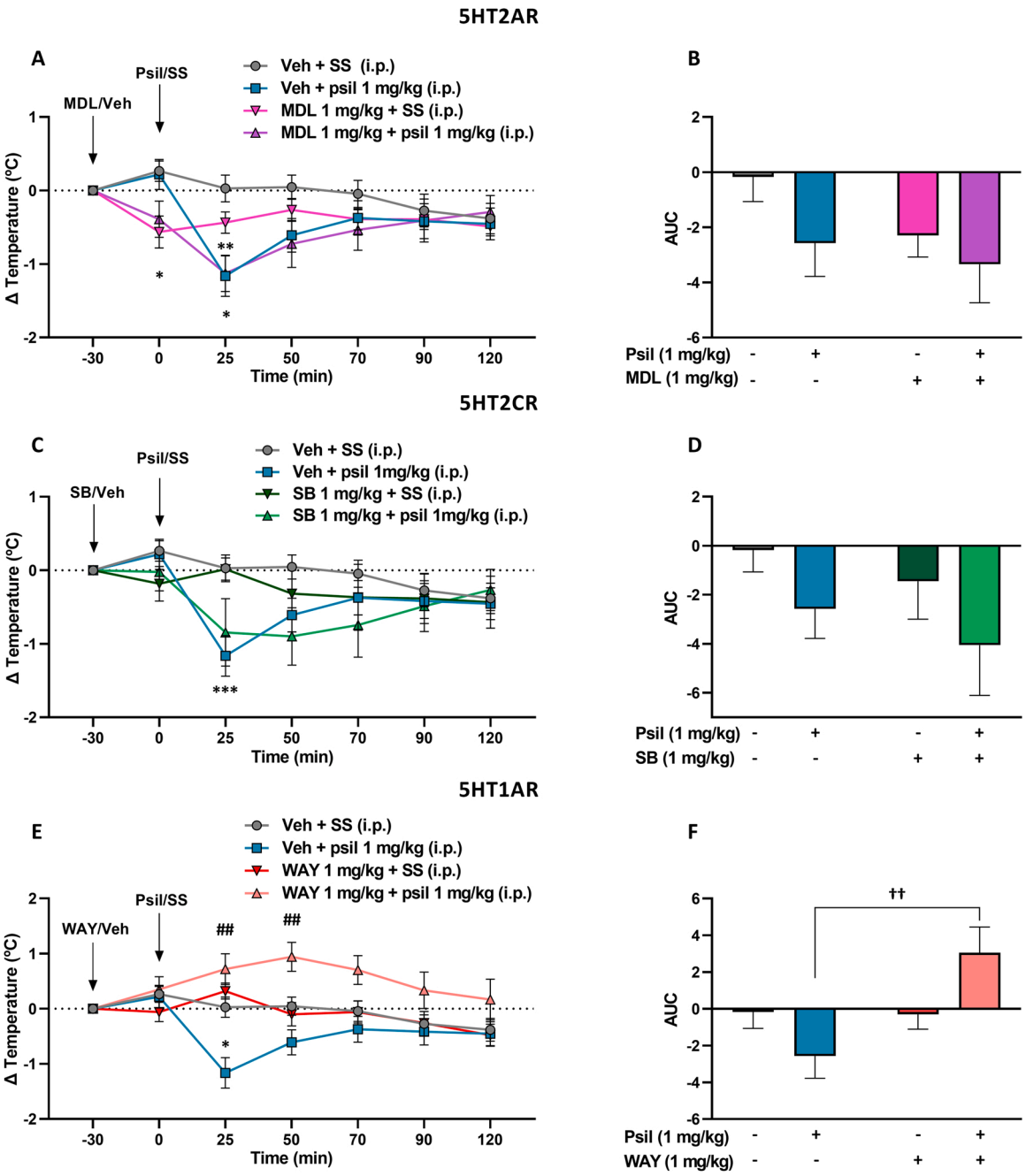

Fig. 5. Evaluation of the role of 5HT2AR, 5HT2CR and 5HT1AR on the modulation of body temperature induced by psilocybin (1 mg/kg) or saline (SS) administration. Effect of pre-treatment with the 5HT2AR antagonist MDL11939 (MDL) (1 mg/kg, i.p.) (A, B), the 5HT2CR antagonist SB242084 (SB) (1 mg/kg, i.p.) (C, D), the 5HT1AR antagonist WAY100635 (WAY) (1 mg/kg, i.p.) (E, F) or vehicle (Veh) (10 mL/kg, i.p.). Data are plotted as mean±SEM (°C) relative to basal temperature (time −30 min) or mean AUC±SEM. Veh+SS n = 11; veh+psil n = 11; MDL+SS n = 11; MDL+psil n = 11; SB+SS n = 6; SB+psil n = 9; WAY+SS n = 10; WAY+psil n = 6. Repeated measures two-way ANOVA followed by Bonferroni *post hoc* test. *p < 0.05, **p < 0.01, * **p < 0.001 treated *vs* control, $^{\#\#}$p < 0.01 vehicle *vs* WAY 100635 pre-treatment on psilocybin-treated groups. Two-way ANOVA followed by Bonferroni *post hoc* test. ††p < 0.01.

$F_t(2.49,44.41) = 13.83$ $p < 0.0001$; $F_{drug}(1,18) = 0.02$, $p = 0.89$; $F_i(6107) = 1.52$, $p = 0.18$) (Figs. 5C and 5D).

In the same way, after baseline temperature measurement, WAY100635 (1 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.) was administered 30 min prior to psilocybin (1 mg/kg, i.p.) or saline (5 mL/kg). Of note, WAY100635 and psilocybin coadministration caused a hyperthermic effect ($E_{max} = 0.94 \pm 0.26$ °C, time = 50 min post-psilocybin, n = 5). Two-way repeated measures ANOVA revealed significant difference between vehicle + psilocybin and WAY100635 + psilocybin groups ($F_t(2.96,43.95) = 3.32$ $p < 0.05$; $F_{drug}(1,15) = 7.82$, $p = 0.014$; $F_i(6,89) = 11.94$, $p < 0.0001$). *Post hoc* analysis revealed significant difference at time points 25- and 50-minutes post-psilocybin administration between studied groups ($t = 4.79$, $p < 0.01$ and $t = 4.44$, $p < 0.01$, respectively) (Fig. 5E). Two-way ANOVA of AUC also revealed significant difference between groups ($F_{ag}(1,34 = 0.19$; $p = 0.66$; $F_{ant}(1,34) = 6.24$, $p = 0.018$; $F_i(1,34) = 6.83$, $p = 0.013$), and *post hoc* analysis showed significant effect of WAY100635 pre-treatment on psilocybin treated groups ($t = 3.37$, $p < 0.01$) (Fig. 5F).

3.9. Evaluation of the role of 5HT2AR on psilocybin-induced hyperthermia

In order to evaluate the role of the 5HT2AR on the hyperthermic effect of psilocybin, a low dose of the drug was selected (0.125 mg/kg, i.p.). Considering the hypothermic effect exerted by MDL11939 (1 mg/kg) (Fig. 5A), first, we selected a dose of MDL11939 that did not have an effect on core body temperature. Thus, a very low dose of MDL11939 (0.1 mg/kg, i.p.) was chosen to be administered 30 min prior to psilocybin (0.125 mg/kg, i.p.). Pre-treatment with the antagonist at such dose blocked the hyperthermic effect of 0.125 mg/kg psilocybin (two-way repeated measures ANOVA: $F_t(2.17,\ 26.04) = 4.34$, $p < 0.05$; $F_{drug}(1,12) = 1.42$, $p = 0.26$; $F_i(5,60) = 4.42$, $p = 0.0017$) (Fig. 6A).

Additionally, in order to assess the central action of 5-HT2AR, the effect of 5HT2R antagonist xylamidine, a drug that does not cross the blood brain barrier [44] was evaluated. Peripheral 5HT2AR antagonist xylamidine (1 mg/kg, i.p.) or saline was administered 30 min prior to psilocybin (0.125 mg/kg) or saline, and temperature measurements were performed as described before. As expected, pre-treatment with the drug did not block hyperthermic effects of psilocybin ($F_t(2.41,\ 24.13) = 10.11$ $p < 0.001$; $F_{drug}(1,10) = 0.92$, $p = 0.36$; $F_i(5,50) = 1.04$, $p = 0.41$) (Fig. 6B), confirming that psilocybin-induced hyperthermia is mediated by central action.

4. Discussion

Currently, there is increasing evidence suggesting that serotonergic psychedelics, particularly psilocybin, behave as fast-acting and long-lasting therapeutic agents after single administration [36,37]. Nevertheless, the role of evoked psychedelic experience and 5HT2AR activation in these mechanisms has been subject to debate [36,37,45]. In order to clarify the complex pharmacological profile of psilocybin, we first confirmed similar affinity values of psilocin for 5HT2AR, 5HT2CR and 5HT1AR both in human and mouse brain tissue. We have identified functional interaction between these serotonergic receptors on behavioural and central physiological effects after psilocybin administration at the range of doses commonly used in preclinical studies.

In accordance with previous results, psilocybin produced a dose-dependent increase in HTR [24,46]. Higher doses of the drug (3 mg kg i.p.) decreased the number of HTR elicited, as observed in other studies with different hallucinogens such as 1-(4-iodo-2,5-dimethoxyphenyl) propan-2-amine (DOI) [28], LSD [47] or psilocybin [46], in which inverted U-shape dose-response curves were described. Currently, there is no compelling rationale for the descending segment of the HTR U-shaped curves after psilocybin administration, but different evidence suggest that 5HT2CR or 5HT1AR might be implicated in such effect [24,26,30]. We first confirmed the role of 5HT2AR on maximal psilocybin-induced HTR (1 mg/kg i.p.) by the administration of the 5HT2AR antagonist MDL11939. This antagonist has been proven one of the most selective 5HT2AR antagonists available [43]. In line with the present results, several studies have reported a blockade of drug-elicited HTR by non-selective 5HT2AR/5HT2CR antagonist ketanserin or by selective 5HT2AR MDL100907 administration [48–51].

As opposed to the consensus on the key role of 5HT2AR on drug-elicited HTR, controversy exists regarding the role of 5HT2CR on such effect. Correlations have been established between the hallucinogenic potency of a variety of drugs and their affinity for 5HT2CR. In that sense, some evidence indicates that administration of 5HT2CR agonists causes a dose-dependent increase in HTR in mice [24,52]. However, reports might have overestimated *in vivo* selectivity for the 5HT2CR of the pharmacological tools used. On this matter, pre-treatment with the 5HT2CR antagonist SB242084 has been reported to strongly inhibit DOI-induced HTR [50]. Again, the dose of 5HT2CR antagonist used in this work (3 mg/kg) is probably too high to be considered 5HT2CR-specific. On the contrary, several studies report an inhibitory effect on HTR induced by the activation of 5HT2CR. For instance, pre-treatment with 5HT2CR agonist Ro60–0175 dose-dependently suppressed DOI-induced HTR in mice [28]. Moreover, it has been described that pre-treatment with the 5HT2CR antagonist SB242084 reversed the decrease in HTR produced by high doses of DOI, causing a rightward shift in the descending limb of the dose-response curve of DOI-induced HTR [28].

In this context, we first evaluated the effect of different doses of 5HT2CR antagonist SB242084 at the maximally effective dose of psilocybin (1 mg/kg i.p.). A low dose of SB242084 (0.1 mg/kg) did not alter psilocybin-elicited HTR, but doses of 0.5 and 1.0 mg/kg of SB242084 were able to significantly inhibit such response. We hypothesized that observed inhibition presumably reflects *in vivo* antagonist effects at 5HT2AR at the higher doses of SB242084. Interestingly, pre-treatment with 0.1 mg/kg of SB242084 potentiated the HTR induced by psilocybin 3 mg/kg (Fig. 3C). Hence, doses of SB242084 that significantly potentiated HTR elicited by psilocybin 3.0 mg/kg failed to alter psilocybin-induced HTR at maximal dose (1 mg/kg) of the ascending limb of the psilocybin dose-effect curve. *In vivo* data reported here support the idea of functional interaction between 5HT2AR and 5HT2CR, which exert opposite effects in the induction of HTR. Moreover, these data clearly indicate that doses of SB242084 over 0.1 mg/kg

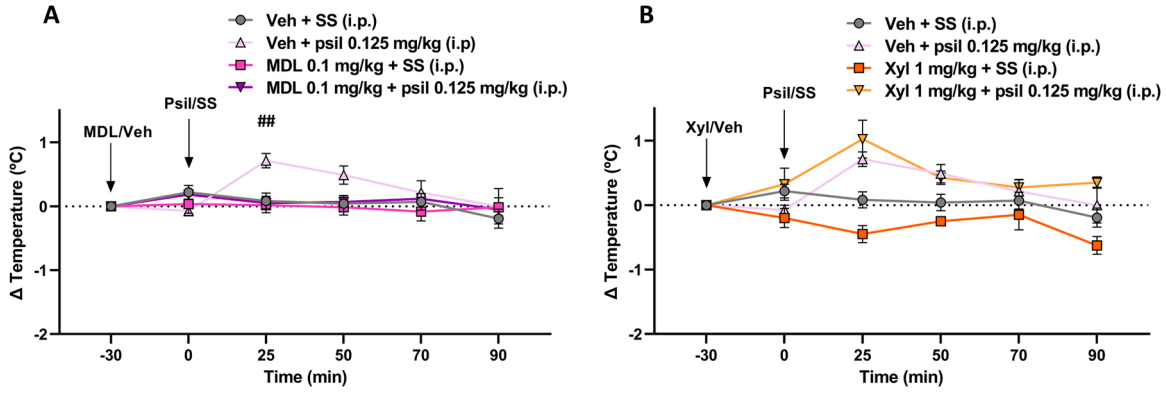

Fig. 6. Evaluation of the role of 5HT2AR on psilocybin-induced hyperthermia. Data are plotted as mean±SEM (°C) relative to basal temperature (time −30 min). A. Time-course effects of MDL11939 (0.1 mg/kg, i.p.) or vehicle (5 mL/kg, i.p.) pre-treatment on psilocybin (0.125 mg/kg, i.p.) or saline (SS, 5 mL/kg, i.p.) on rectal temperature. Veh+SS n = 17; veh+psil n = 8; MDL+SS n = 6; MDL+psil n = 6. Two-way repeated measures ANOVA followed by Bonferroni *post hoc* test. $^{\#\#}p < 0.01$ vehicle pre-treated *vs* MDL11939 (0.1 mg/kg, i.p.) pre-treated psilocybin-treated (0.125 mg/kg, i.p.) mice. B. Time-course effects of xylamidine (1 mg/kg, i.p.) or vehicle (5 mL/kg, i.p.) pre-treatment on psilocybin (0.125 mg/kg, i.p.) or saline (5 mL/kg, i.p.) on rectal temperature. Veh+SS n = 17; veh+psil n = 8; xyl+SS n = 4; xyl+psil n = 4. Two-way repeated measures ANOVA followed by Bonferroni *post hoc* showed no differences between vehicle and xylamidine pre-treated psilocybin groups.

have poor *in vivo* selectivity for 5HT2CR over 5HT2AR. Accordingly, the overall shape of the dose-response curve for psilocybin-elicited HTR may enable some estimation of *in vivo* 5HT2AR selectivity over 5HT2CR. This fact should be considered for future experiments to study the specific role of 5HT2AR or 5HT2CR in a particular effect of psilocybin, specifically in understanding potential psychedelic experience mechanisms (or lack thereof) for the evaluation of long-lasting psilocybin effects.

A more limited number of studies denote possible modulatory roles of 5HT1AR on the HTR induced by hallucinogens. It has been described that pre-treatment with the 5HT1AR agonist 8-OH-DPAT attenuates 5HT2AR-elicited HTR in rats [29]. According to this, selective 5HT1AR antagonism has been suggested to induce 5HT2AR-mediated HTR in an indirect manner [30], although other studies do not corroborate such hypothesis [26]. In the present study, the selective 5HT1AR antagonist WAY100635 [53] failed to modulate (neither enhancing nor inhibiting) psilocybin-elicited HTR. Thus, the involvement of 5HT1AR in the acute psychedelic-like experience induced by psilocybin seems to be discarded.

Data obtained from HTR could lead us to consider that the same phenomena might also apply to other psilocybin-mediated effects. In that sense, it has been largely described that psychedelics modulate different physiological processes such as body temperature regulation. Several studies have reported hyperthermic effect induced by hallucinogenic agonists on mice [54], rats [33] and humans [31]. This effect is likely mediated by 5HT2AR activation, as pre-treatment with 5HT2AR antagonists is able to block the raise in body temperature [33,55]. In an attempt to further evaluate the pharmacological profile of psilocybin, core body temperature was also assessed following drug administration.

As identified for HTR, a U-shaped dose-response curve induced by psilocybin administration was also observed when core body temperature was measured. Low doses of psilocybin caused a significant raise in body temperature whereas an intermediate dose did not have any effect. In contrast, at doses that generate a maximum effect on HTR in mice (1 mg/kg), a decrease in core temperature was measured. The differential effects indicate that, at the doses of psilocybin commonly used for preclinical evaluation, multiple serotonergic mechanisms seem to be effectively modulated in combination with the 5HT2AR-mediated effects. In that sense, it is well known that 5HT1AR-mediated mechanisms have an important function on the central regulation of body temperature. For instance, different 5HT1AR agonists have been reported to induce a dose-dependent reduction in rodent body temperature that is efficiently blocked by 5HT1AR antagonists [56]. Such mechanism has also been proven to operate in humans [57].

Based on previous literature and pharmacodynamic properties of psilocybin, we hypothesized that different balance between 5HT2AR and 5HT1AR mechanisms could explain opposite effects observed after drug administration at different doses. Thus, 5HT2AR-mediated mechanism prevails *in vivo* at low-doses. Because of the lack of selectivity of psilocin for 5HT2AR, 5HT1AR-agonist properties of psilocin at moderate to high doses cause an opposite effect on the regulation of body temperature. Confirming the proposed hypothesis, we observed that the hypothermic effect of psilocybin at 1 mg/kg was blocked by pre-treatment with 5HT1AR antagonist WAY100635 (Fig. 5E). Interestingly, blockade of 5HT1AR revealed hyperthermic effect of psilocybin, probably due to 5HT2AR activation. In accordance with the present results, 5HT1AR and 5HT2AR functional interaction has been described in core body temperature control, when the hypothermic effect of 5HT1AR agonist 8-OH-DPAT was attenuated dose dependently by the 5HT2AR/5HT2CR agonist DOI coadministration in rats [29].

In addition, in order to completely discard the role of 5HT2AR and 5HT2CR in the hypothermic effect induced by psilocybin, pre-treatment with the 5HT2AR antagonist MDL11939 (1 mg/kg) or the 5HT2CR antagonist SB242084 (1 mg/kg) before psilocybin administration (1 mg/kg) was also tested. We confirm that 5HT2AR/5HT2CR blockade did not prevent psilocybin-induced hypothermia. Remarkably, antagonist MDL11939 administration exerted a hypothermic effect when administered alone, revealing an active role of 5HT2AR in body temperature maintenance under physiological conditions. This is consistent with evidence, which suggests that 5HT2AR antagonists may cause decreases in body temperature both in humans and rodents. According to clinical data, atypical antipsychotics cause mild to severe hypothermia, in particular regarding olanzapine, risperidone and clozapine [58], and animal studies have suggested this effect to be mainly mediated by blockade of 5HT2AR [59]. In this sense, in an attempt to prevent the blockade of serotonergic tone at the 5HT2AR, a low and inactive dose of MDL11939 was chosen for psilocybin hyperthermic effect evaluation. MDL11939 pre-treatment (0.1 mg/kg) was able to block psilocybin-induced hyperthermic effect at 0.125 mg/kg, confirming the involvement of 5HT2AR activation in such effect.

In recent years, central or peripheral origin for mechanisms underlying hallucinogen-induced hyperthermia have been subject of debate. It has been proposed that the increase of core temperature after psychedelic administration in rodents could be attributed to peripheral 5HT2AR-mediated vasoconstriction, and brown adipose tissue thermogenesis [32,60]. Nonetheless, results from the current study corroborate that central 5HT2AR activation is responsible for the observed hyperthermia, since pre-treatment with peripheral serotonergic antagonist xylamidine did not block psilocybin-induced temperature increase. This is consistent with previous works, which have assessed the effects of serotonergic agonists at the central and peripheral levels after coadministration of antagonists that do not cross blood-brain barrier or by central/peripheral drug administration strategies [32,44,61]. In brief, considering that psilocin affinities for 5HT1AR and 5HT2AR are close, measuring core body temperature might serve as a simple physiological means of studying central 5HT2AR/5HT1AR mechanisms after administration of a certain dose of the drug. The question of the influence of external temperature on the hyperthermic effect caused by the activation of 5HT2AR has been raised [35], and therefore these studies should be carried out under strict control of environmental temperature.

There is an ongoing debate regarding whether subjective effects of psychedelics are necessary components for the achievement of therapeutic efficacy [6,62–64]. Very recent preclinical studies have revealed that single dose psilocybin (1 mg/kg i.p.) elicits rapid antidepressant effects in mice that correlate with increased plasticity in the frontal cortex or hippocampus [36,37]. Here the authors showed that pre-treatment with 5HT2AR/5HT2CR antagonist ketanserin (2 mg/kg i.p.) attenuated (but not abolished) HTR without affecting the antidepressant-like effect on a stress-based model of depression [37]. In addition, they observed that a high dose of psilocybin (10 mg/kg i.p.) was able to decrease the power of CA1 hippocampal oscillations in the delta frequency band of local field potentials (LFPs). Shao *et al.* reported that single dose of psilocybin (1 mg/kg i.p.) induced rapid and persistent growth of dendritic spines in the frontal cortex of mice that was only partially blocked by ketanserin pre-treatment (1 mg/kg i.p.) [36]. Remarkably, ketanserin pre-treatment completely abolished the psilocybin-induced HTR. Therefore, it is not yet fully understood whether the activation of 5HT2AR-dependent signalling and/or multiple serotonergic activation induced by psychedelics must operate to elicit persistent long-term effects.

It is known that psychedelic-induced 5HT2AR canonical signalling pathway activation (Gq/11 protein activation) in the hypothalamus induces hyperthermic effects [35,65]. In contrast, it has been demonstrated that psychedelics activate 5HT2AR non-canonical signalling pathways in cortical pyramidal neurons which are responsible for behavioural (HTR) effects [27,66]. Thus, concomitant evaluation of HTR and body temperature increase induced by 5HT2AR activation may serve as an attractive *in vivo* methodological tool to investigate molecular and signalling mechanisms and to address the relationship between psychedelic experience and 5HT2AR-mediated non-psychedelic effects.

Besides the debate concerning the pharmacological mechanism of action, pharmacokinetic issues related to the coadministration of psychedelics and the 5HT2R antagonist (time interval between drug

administrations) cannot be dismissed, as previously suggested [67]. The results of the present work allow us to establish an accurate posology to study the role of serotonergic receptors, since the pre-administration of the corresponding 5HT2AR, 5HT2CR and 5HT1AR antagonists 30 min before psilocybin administration resulted in a complete suppression of the observed responses.

5. Conclusions

We have confirmed a similar pharmacological profile of psilocin in humans and rodents. In addition, a dose-response study for psilocybin to support dose finding for research and psilocybin-assisted therapy was performed. Our findings have important implications for the theory that not only 5HT2AR but also 5HT2CR and 5HT1AR agonism are important components of the pharmacological actions of psilocybin *in vivo*. Results may provide a novel insight concerning therapeutic targets of the drug for future clinical studies.

Funding

This work was supported by Grant PID2021–123508OB-I00, funded by MCIN/AEI/ 10.13039/501100011033 and by ERDF A way of making Europe, by the Basque Government (IT-1211–19; IT-1512–22), by CIBER -Consorcio Centro de Investigación Biomédica en Red- (CB/07/09/0008), Instituto de Salud Carlos III, and by Fundación Vital Fundazioa (VITAL21/17). IE-S received a predoctoral fellowship from the UPV/EHU.

CRediT authorship contribution statement

Ines Erkizia-Santamaría: investigation, validation, visualization, formal analysis, writing - original draft. **Roser Alles-Pascual**: investigation, formal analysis. **Igor Horrillo**: investigation, formal analysis. **Javier J. Meana**: conceptualization, methodology, resources, writing - review and editing, funding acquisition. **Jorge E. Ortega**: conceptualization, methodology, validation, supervision, writing- reviewing and editing, funding acquisition.

Declaration of Competing Interest

JJM is supported by an unrestricted grant from Janssen. The rest of the authors declare no conflict of interest.

Data availability

The data sets generated for this study are available on request to the corresponding authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113612.

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