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Jérôme Jeanblanc, Romain Bordy, Grégory Fouquet, Virginie Jeanblanc, Mickaël Naassila. Psilocybin reduces alcohol self-administration via selective left nucleus accumbens activation in rats. *Brain - A Journal of Neurology*, 2024, 147 (11), pp.3780-3788. 10.1093/brain/awae136 . hal-04662033

**HAL Id: hal-04662033**

**<https://u-picardie.hal.science/hal-04662033v1>**

Submitted on 25 Jul 2024

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# Psilocybin reduces alcohol self-administration via selective left nucleus accumbens activation in rats

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<sup>†</sup>These authors contributed equally to this work.

## Abstract

The use of psilocybin to treat alcohol use disorder is very promising, but the mechanisms of action remain poorly understood. We combined behavioral, pharmacological and gene expression analyses to decipher the mechanisms of action of psilocybin, for the first time injected into the brain.

Male Long Evans rats underwent chronic operant ethanol self-administration before testing the effect of intraperitoneal psilocybin or directly within the nucleus accumbens core or the ventral tegmental area. Transcripts from the dopaminergic system were quantified in the nucleus accumbens and prefrontal cortex.

Psilocybin significantly reduced (50%) ethanol self-administration when injected 4 hours before the session either intraperitoneally (1mg/kg) or directly within the left nucleus accumbens (0.15µg) but not the right nucleus accumbens or the left ventral tegmental area. The effect of intraperitoneal injection of psilocybin was prevented by intra left nucleus accumbens injection of 0.3µg of the 5-HT<sub>2A</sub>R antagonist ketanserin. In rats that self-administered ethanol but not in those self-administering saccharin, dopamine D<sub>2</sub> receptor mRNA were increased in both the nucleus accumbens and the prefrontal cortex by psilocybin, while D<sub>1</sub>R mRNA was increased only in the prefrontal cortex.

As in humans, psilocybin reduced ethanol self-administration in rats through the 5-HT<sub>2A</sub>R within the left nucleus accumbens possibly through increased D<sub>2</sub>R expression. Our results open unexpected perspectives regarding the hemispheric lateralization of psychedelic effects.

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**Running title:** Reduced alcohol drinking by psilocybin

**Keywords:** addiction; alcoholism; animals; drinking; psychedelics; serotonin

**Abbreviations:** AUD = alcohol use disorder; CSF = cerebrospinal fluid; DOI = 2,5-dimethoxy-4-iodoamphetamine; LSD = lysergic acid diethylamide; NAcc = nucleus accumbens; PFC = Prefrontal cortex; VTA = ventral tegmental area

## Introduction

Alcohol use disorder (AUD) is a chronic disease characterized by a continuum of severity and is a leading cause of disability-adjusted life year and mortality worldwide. <sup>1</sup> Among the new promising AUD treatments, psychedelics and in particular psilocybin (4-phosphoryloxy-N,N-dimethyltryptamine) and Lysergic acid diethylamide (LSD) are showing a very strong revival of interest. <sup>2</sup>

A double-blind randomized controlled trial with 93 participants with AUD, demonstrated a rapid and long-lasting effect of psilocybin-assisted psychotherapy with an increase rate of abstinence and a robust and sustained decrease days of heavy drinking, 33–36 weeks following the first of two psilocybin dosages administered 4 weeks apart. <sup>3</sup>

Only very few studies have investigated the effect of psilocybin on ethanol drinking in rodents. A single dose (0.5 or 1.0 or 2.0 mg/kg) reduced ethanol drinking in a two-bottle choice paradigm in male C57BL/6J mice. <sup>4</sup> Psilocybin (1 mg/kg) also decreased the alcohol deprivation effect in the rat model of alcohol relapse. <sup>5</sup> We recently demonstrated in the ethanol post-dependent state model of AUD in rats that 1 mg/kg psilocybin reduced ethanol reinstatement during acute withdrawal and that this effect may involve restoration of *Grm2* expression in the NAcc. <sup>6</sup>

The underlying neurobiological effect of psilocybin is still poorly understood. <sup>7</sup> Psilocyn, the psychoactive metabolite of psilocybin, binds on several serotonin receptors but in humans, ketanserin, the antagonist of 5-HT<sub>2A</sub>R and the 5-HT<sub>1A</sub> agonists, reduced the effects of psilocybin, thus suggesting a main role of these two receptors. <sup>7</sup> These receptors are expressed within the reward circuit at both pre-and post-synaptic levels. <sup>8</sup> The D<sub>2</sub>R and 5-HT<sub>2A</sub>R are co-distributed within the NAcc and the D<sub>2L</sub>R/5-HT<sub>2A</sub>R heteromers display receptor-receptor allosteric interactions that can lead to an inhibition of the D<sub>2L</sub>R functioning. <sup>9</sup> This allosteric mechanism has been suggested to underly the ability of the hallucinogens DOI and LSD to increase the density of the D<sub>2</sub>-like receptors in the NAcc core, an action blocked by ketanserin. <sup>10</sup> D<sub>2</sub>R isoforms display specific *in vivo* functions, whereby D<sub>2L</sub>R primarily acts at postsynaptic and D<sub>2S</sub>R in presynaptic dopaminergic transmissions. <sup>11</sup> Dopamine D<sub>2</sub>R in the NAcc has an impact on PFC functioning with a reduction in the prefrontal glutamate drive via the

mediodorsal thalamic nucleus.<sup>9</sup> All of these data support the hypothesis of a role of D<sub>2</sub>R, and particularly of the D<sub>2L</sub>R, in the effect of psychedelics in both the NAcc and PFC.

We hypothesized that the psilocybin-induced reduction in ethanol self-administration in rats chronically consuming ethanol may be due specifically to an effect on 5-HT<sub>2A</sub>R within the NAcc. We also tested whether the effect of psilocybin may involve D<sub>2</sub>R (especially D<sub>2L</sub>R), another main target controlling ethanol self-administration and that is forming heterodimers with 5-HT<sub>2A</sub>R.<sup>12</sup>

## Materials and methods

### Animals and drugs

Sixty-six male adult Long-Evans rats (452–605 g at the beginning of the injections) were obtained from Janvier Labs (Le Genest Saint Isle, France). The sample sizes were chosen based on our previous experience.<sup>13</sup> Animals were individually housed under a light/dark cycle of 12 h (lights on at 7:00 a.m.) with food and water available *ad libitum*. Experiments were carried out in accordance to the guidelines for Care and Use of Laboratory Animals (National Institutes of Health) and the European community regulations for animal use in research (2010/63/UE) and were approved by the local research ethics committee (CREMEAP; N°2145-201510051547534V2). The Rat Grimace Scale was used to control for the welfare of the rats. None of the animals were excluded for reaching welfare endpoints. For drugs, see supplementary materials.

### Operant Ethanol self-administration

The procedure of ethanol operant self-administration was used as previously described and see supplementary materials.<sup>14</sup> Briefly, rats were allowed to consume 20% ethanol solution for 4 weeks in a 2-bottle-choice intermittent access and then trained to self-administer 20% ethanol solution in Med Associates operant cages (VT, USA) during 15 minutes sessions, 5 days a week. At the end of the training, 3 presses will induce the delivery of 0.1 mL of the 20% ethanol solution associated to a light and a sound stimulus during the 3 seconds of delivery. The timeline of the experiments is depicted in Fig. 1. Outcome measures were the number of lever presses, and ethanol deliveries and the time that were recorded using MED-PC IV software (Med Associates Inc., VT, USA).

### Stereotaxic surgery and cannulae implantation

Of the 10 rats assigned to the 2<sup>nd</sup> experiment and of the 24 rats assigned to the 3<sup>rd</sup> experiment, moderate and high drinker rats (press on the active lever > 30 per session) were included ( $n = 10$  and  $n=16$  respectively) in the interventional group. Rats were anesthetized with inhaled 5% isoflurane and fixed in a stereotaxic frame. Stainless steel guide cannulae (26G) were implanted bilaterally for the 10 rats of the 2<sup>nd</sup> experiments (anteroposterior = +1.6 mm from bregma; lateral =  $\pm 1.6$  mm from the medial suture, vertical =  $-5.2$  mm from the skull). Rats of the 3<sup>rd</sup> experiments were implanted unilaterally on the left core of the NAcc (same coordinates) and on the left VTA (anteroposterior =  $-6.0$  mm from bregma; lateral =  $0.6$  mm from the medial suture, vertical =  $-6.0$  mm from the skull).<sup>15</sup> See supplementary materials for further details.

## Experiments

### Experiment 1: Modification of gene expression after intraperitoneal injection of psilocybin

Psilocybin produces asymmetrical activation of brain regions in humans and in rodents.<sup>16–20</sup> We thus decided to investigate the modulation of selected genes (for details see supplementary materials) within the right and left NAcc 4 hrs after an i.p. injection of psilocybin at 1mg/kg (2mL/kg) in 5 naïve rats.

### Experiment 2: Microinjection of psilocybin into the left or right NAcc

Rats trained to self-administer alcohol ( $n=10$ ) were microinfused with either artificial cerebrospinal fluid (aCSF, Harvard Apparatus, MA, USA) or psilocybin (1mM in aCSF) at the rate of  $0.3\mu\text{L}/\text{min}$  for a total volume of  $0.6\mu\text{L}$ . Four hours after the infusion rats were placed in the operant self-administration chamber for a regular FR3-15 session. A First round of 2 infusions included counterbalanced injections of aCSF or psilocybin within the right NAcc and a second round included counterbalanced infusions of aCSF or psilocybin within the left NAcc. Each infusion was delayed from the previous one of at least 3 days.

### Experiment 3: Effects of systemic injections of psilocybin

Rats were randomly divided into two groups, saline ( $n=8$ ) and psilocybin (1mg/kg) ( $n = 8$ ). The next week, for the second injection, both groups were inverted. One week without injection was left to ensure that there is no after-effect of psilocybin on ethanol consumption.

#### **Experiment 4: Effects of intracerebral injections of psilocybin**

When Experiment 3 was finished, the same animals were submitted to experiment 4 which started with psilocybin (1mM) injection within the left NAcc (0.6  $\mu$ L, 0.3  $\mu$ L/min) or the VTA (0.3 $\mu$ L, 0.3  $\mu$ L/min). Rats were pseudo-randomly distributed in a group of 8 rats that will receive the vehicle (aCSF) and a group of 8 rats that will receive psilocybin. The randomization was based on the basal levels of ethanol self-administration. A 25  $\mu$ L Hamilton syringe (Gastight #1702), attached to a pump was connected with polyethylene tubing to 34G infusion cannulae. The cannulae protruded 2 mm beyond the guide cannulae pedestals when inserted. Psilocybin ( $n = 8$ ) or aCSF ( $n = 8$ ) was injected. To allow drug diffusion, the infusion cannulae were left in place 30 seconds following the injection. Injections in NAcc and VTA were spaced one week apart to ensure that there is no after-effect of psilocybin.

#### **Experiment 5: Implication of serotonergic pathway of NAcc In the effects of psilocybin**

When experiment 2 was finished, the same animals from the experiment 2 were submitted to experiment 3 which started with an injection of 0.6  $\mu$ L (0.5 $\mu$ g/ $\mu$ L) of ketanserin ( $n=16$ ) or aCSF ( $n = 16$ ) in the NAcc at a perfusion rate of 0.3  $\mu$ L/min 5 minutes prior to the psilocybin i.p. injection.

#### **Experiment 6: Genes and protein expression**

Two groups ( $n = 24$  and  $n = 12$ ) were generated in parallel, the first self-administered ethanol similarly to Experiment 1 and the second one was submitted to the same protocol but with the non-caloric rewarding substance saccharin (0.05%), instead of ethanol. This concentration was selected to produce similar active lever presses levels than the ones for the 20% ethanol solution. Four rats from the 1<sup>st</sup> group did not learn the task or reach the 30 presses threshold and thus were excluded. After 2 months of stable baseline of ethanol and saccharin self-administration, rats were i.p. injected with psilocybin (1mg/kg) or saline. Four hours after the injection, rats were euthanized by decapitation under isoflurane anesthesia without behavioral testing. The delay of four hours has been chosen in order to be in line with behavioral testing and our previous study on psilocybin and gene expression and because of protein levels quantification.<sup>6</sup> Brains were rapidly removed and brain tissues were collected on ice and frozen immediately until further process. Details of RNA and proteins extraction, purification and of qPCR were as previously described and see also supplementary materials.<sup>6</sup>

## Statistical Analyses

Data were presented as Mean  $\pm$  SEM and were analyzed using GraphPad Prism software (v. 5.3). Statistical analyses were made using *t*-test or one-way or two-way repeated measures (RM)-ANOVAs followed by a Tukey's *post hoc* test. For the cumulative analysis, the factors were "treatment" and "time bins" for the figures 2H, K and N, and "i.p. treatment", intra-NAcc treatment" and "time bins" for the figure 3C. For the analysis of the effect of ketanserine on psilocybin effect the factors were "i.p. treatment" and "intra-NAcc treatment". For the gene expression, the factors were "Substance consumed" and "treatment".  $P < 0.05$  was considered significant.

## Data availability

All data are available upon reasonable request.

## Results

### Psilocybin differentially regulates gene expression between left and right NAcc

We performed an experiment on gene expression in ethanol-naïve animals that received i.p. injection of 1mg/kg psilocybin, and we found that the genes coding for 5-HT<sub>2A</sub>R and DAT are specifically downregulated in the left NAcc, while BDNF is upregulated in the right NAcc. Additionally, vGluT and mGluR2 genes are similarly upregulated in both the right and left NAcc (Fig 2A and supplementary materials.).

### Left, but not Right, NAcc Microinfusion of Psilocybin Reduces Alcohol Self-Administration

Microinfusion of psilocybin (1mM) within the left NAcc reduced the number of rewards obtained (-38%,  $p < 0.05$ , Fig. 2B) and consequently the total amount of alcohol consumed (-39%,  $p < 0.05$ , Fig 2C). The contralateral infusion, into the right NAcc, did not produce any significant change in the total number of rewards received (-10%,  $p = 0.44$ , Fig. 2D) nor the total amount of alcohol consumed (-11%,  $p=0.43$ , Fig. 2E).

### Psilocybin Reduced ethanol self-administration

Acute intraperitoneal injection of psilocybin (1mg/kg, 4 hours before the test) reduced both total received rewards (-48%;  $P < 0.0001$ ; Fig. 2F) and ethanol intake (-51%;  $P < 0.0001$ ; Fig. 2G) compared with the vehicle. Statistical analysis did not show any difference due to the order of treatment between vehicle and psilocybin: there was a significant effect of the “treatment” ( $F_{(1,14)} = 19.66$ ,  $p < 0.001$ ), no effect of the “order of injections” ( $F_{(1,14)} = 0.73$ ,  $p = 0.725$ ) and no interaction between both factors ( $F_{(1,14)} = 0.65$ ,  $p = 0.44$ ). The cumulative consumption (3min-intervals, Fig. 2H), showed that psilocybin did not alter the initiation of the consummatory episode whereas it reduces its duration by ending it earlier as compared to vehicle ( $P < 0.01$ ).

### **Left NAcc but not left VTA is implicated in the effects of psilocybin**

A direct injection within the left NAcc reduced both total received rewards and ethanol intake compared with control treatment (-54% and -51% respectively;  $P < 0.05$ ; Fig. 2I and 2J). The cumulative consumption showed that psilocybin stopped their ethanol consumption earlier than the vehicle ( $P < 0.05$ ; Fig. 2K). Injection within the VTA had no effect either on total received rewards or on ethanol intake compared with control group, and did not affect the way rats consumed ethanol (Fig. 2L-N).

### **5-HT<sub>2A</sub> pathway within the NAcc is implicated in the effects of psilocybin**

The ketanserin specific 5-HT<sub>2A</sub>R antagonist (0.5μg/μL) was injected within the NAcc 5 min before intraperitoneal injection of psilocybin (1mg/kg, 4 hours before the test) and blunted the effects of psilocybin on total received rewards and on ethanol intake ( $P < 0.01$ ; Fig. 3A and B). Ketanserin inhibited early termination of ethanol self-administration observed in rats after psilocybin treatment, re-establishing a cumulative consumption similar to vehicle ( $P < 0.0001$ ; Fig. 3C). No change in neither total number of received rewards nor ethanol intake was observed after ketanserin alone (Fig. 3A and B). Both number of active and inactive lever presses are presented on supplementary Fig. 1. Latencies for the 1st and the last rewards are presented on Fig. 3D, see supplementary materials for details.

### **Effects of psilocybin on genes expression**

Dopaminergic genes expression in both the NAcc and the PFC was quantified 4h after a 1mg/kg psilocybin or a saline i.p. injection (Fig. 4A and B). Two groups of rats were used, one trained to self-administer ethanol for six months with final levels of rewards obtained of  $19.25 \pm 1.19$  for a consumption of pure ethanol of  $0.62 \pm 0.03$  g/kg ( $n = 24$ ; 12 saline vs. 12 psilocybin), and



a group trained to self-administer saccharin (0.05%) for 6 months with a final average of  $20.83 \pm 3.76$  rewards per session ( $n = 12$ ; six saline vs. six psilocybin).

No modification of genes expression associated to dopamine biosynthesis (Th) or dopamine transporter (Slc6a3) was observed in the NAcc (Fig. 4A) and the PFC (Fig. 4B). However, the expression of Slc6a3 in the PFC seems to be changed after psilocybin treatment in the saccharin group and a Kruskal Wallis test indicated a significant main effect ( $H = 9.167$ ,  $p = 0.0272$ ). However, the Dunn's multiple comparison test indicated a  $p$  value of 0.565 between the groups EtOH treated with either saline or psilocybin, and of 0.096 between the saccharine groups treated with either saline or psilocybin.

The mRNA levels of the dopaminergic receptors ( $D_1R$ ,  $D_2R$  and  $D_3R$ ) in PFC showed a significant increase of 2.1-fold for  $D_1R$  and of 2.5-fold for  $D_2R$  induced by psilocybin in the ethanol group ( $P < 0.05$ ; Fig. 4B). The *Drd2* gene expression was increased by 2-fold in NAcc of psilocybin-treated ethanol group ( $P < 0.01$ ; Fig. 4A). While the increase of *Drd2* gene expression is associated with an increase of both the short and the long isoforms in PFC (Fig. 4B), only the long isoform is increased in the NAcc of the psilocybin-treated ethanol group ( $P < 0.05$ ; Fig. 4A).

Results on  $D_2R$  protein levels in the whole NAcc are presented on (Supplemental Fig. 2). The 2-way ANOVA on protein levels revealed no effect of any of the factors or the interaction between factors (ethanol self-administration:  $F(1,16) = 0.22$  ; psilocybin :  $F(1,16) = 1.10$  ; Interaction :  $F(1,16) = 0.09$ ).

## Discussion

At the beginning of 2024, there were eleven ongoing clinical trials on psilocybin and psilocybin-assisted psychotherapy for the treatment of AUD. Psychedelics are thus the most tested substances in current clinical trials for AUD; however, the neurobiological mechanisms remain poorly understood.<sup>21</sup> We showed similarly to results obtained with the two other 5HT<sub>2A</sub>R agonists, TCB-2<sup>22</sup> and the phenethylamine psychedelic (2,5-dimethoxy-4-iodoamphetamine ; DOI)<sup>23,24</sup>, that psilocybin reduced ethanol self-administration. Psilocybin, at a dose of 1 mg/kg which has already been shown to reduce ethanol drinking, ethanol preference and ethanol relapse<sup>6,25,26</sup>, was very effective (50% decrease) in reducing chronic ethanol self-administration in our study. Previous results have shown a lack of dose response with psilocybin on ethanol self-administration.<sup>6,25</sup>

Since psilocybin reduced ethanol self-administration by shortening the duration of the consummatory episode due to its early termination (no change in the initiation), it can be suggested that the seeking for alcohol is not affected by psilocybin whereas the rewarding properties of alcohol seem to be downregulated. It is important to note that in our longitudinal study, each animal received either two (one in the left NAcc and one in the right NAcc) or three administrations of psilocybin (one i.p. and two intracerebral), and this may have had an impact on our results.

Little is known about the brain structures that are targeted by psilocybin. Here and for the first time, we demonstrate that psilocybin affects differentially both genes expression and behavior in regards with the left of right hemispheres. Indeed, among the selected genes investigated some show similar up-regulation within the right and left NAcc (*Grm2*, *SLC17A7*) while other (*Htr2a*, *Htr2b*, *SLC6A3*) are strikingly down-regulated only within the left NAcc and only *bdnf* is specifically up-regulated by psilocybin within the right NAcc. Thus, when both hemispheres react similarly, we observe an upregulation of the genes, and when it is not symmetrical, genes are downregulated within the left NAcc while *bdnf* is up-regulated in the right NAcc. In addition, only micro-infusion of psilocybin within the left NAcc is able to reduce alcohol operant self-administration. Equally striking and intriguing, blocking 5-HT<sub>2A</sub>R by the unilateral injection of ketanserin into the left NAcc alone is sufficient to block the effect of i.p. psilocybin injection, without compensatory action from the right NAcc. These very striking data should be considered in future studies aimed at determining the mechanism of action of psychedelics. Even though we do not have an explanation yet, it is important to emphasize that brain imaging results on the effects of psychedelics often show lateralized effects when comparing the two brain hemispheres but without discussing the functional implications for such interhemispheric differences.

Our results suggest that the left NAcc and particularly the 5-HT<sub>2A</sub>R of this specific hemisphere, are responsible for the perception of the rewarding value of alcohol associated with the context and mediates the effects of psilocybin on alcohol consumption. Very interestingly, a recent fMRI pilot study on psilocybin-assisted therapy in patients with AUD showed psilocybin-induced changes in neural reactivity to alcohol and emotional cues that are specific of one brain hemisphere or differential between the two hemispheres.<sup>27</sup> Specifically, across both alcohol and emotional cues, psilocybin induced an increased activity in the left caudate and unique to positive cues, psilocybin increased right hippocampus activity and decreased left hippocampus activity.<sup>27</sup> Other brain imaging studies revealed changes in

specific hemisphere with psilocybin that acutely reduced the response in the right amygdala to negative stimuli, and with LSD that induced hypoconnectivity of the right insula.<sup>28,29</sup>

In other contexts, studies have also shown hemispheric lateralization of behavioral responses. For example, regarding pain, the right and left central amygdala are not directly interconnected and previous study provided demonstration that left and right central amygdala contribute differentially to production of pain affect.<sup>30</sup> Interestingly, similar to our study, it has been shown that the pharmacological blockade of activation the extracellular signal-regulated kinase cascade in the right, but not the left central nucleus of the amygdala, decreases inflammation-induced peripheral hypersensitivity.<sup>31</sup> Thus, suggesting a dominant role of the right amygdala in pain processing.<sup>31</sup>

Regarding the NAcc, unilateral brain structure activation can induce specific behavior and previous study has shown that unilateral virus injection to optogenetically activate the NAcc can induce place preference in mice.<sup>32</sup> Our results suggest that the left NAcc may dominate on the effect of psilocybin on ethanol self-administration relative to the right NAcc, but the exact role of both sides and the potential mutual regulation between NAcc of each side needs further investigation. We cannot conclude about the potential regulation between the two NAcc. The mesolimbic dopamine pathways are known to be ipsilaterally dominated, with less than 10% of fibers from the VTA crossing hemispheres to reach the contralateral NAcc.<sup>33</sup> Interestingly, dopaminergic asymmetry has been shown regarding the influence of the cerebellar dentate nucleus on mesolimbic dopamine pathways.<sup>34</sup> Thus, the stimulation of the cerebellar dentate nucleus can elicit NAcc dopamine release in the contralateral hemisphere, and this dopamine release was significantly greater in the right NAcc relative to the left.<sup>34</sup>

We also unequivocally showed, that the NAcc, but not the VTA, is the critical structure involved in the efficacy of psilocybin and it is important to point out that the amplitude of the effects is similar when psilocybin is injected either intraperitoneally or directly within the NAcc. A previous study in mice showed that impairment of  $\text{Cl}^-$  transport in VTA GABA neurons after ethanol drinking was restored by the 5-HT<sub>2A</sub>R agonist TCB-2 administered intraperitoneally, suggesting a role of 5-HT<sub>2A</sub>R within the VTA.<sup>35</sup> Although the results of the latter study suggested an alteration in the VTA, it did not demonstrate the lack of involvement of other brain structures. Previous study using [<sup>3</sup>H]ketanserin showed that the 5-HT<sub>2A</sub>R are highly expressed in the PFC and the NAcc.<sup>36</sup> We showed that similar to results obtained after other psychedelics such as ayahuasca<sup>37</sup>, the reducing effect of psilocybin on ethanol self-administration is prevented by a pre-treatment with a 5HT<sub>2A</sub>R antagonist. However, we are the first to demonstrate that the effect of the psychedelic is mediated by the 5-HT<sub>2A</sub>R specifically

located within the NAcc. Further studies are needed to identify which specific neuronal pathways targeting the NAcc and expressing the 5-HT<sub>2A</sub>R are involved.

In the post-dependent state model of AUD we demonstrated that psilocybin restored gene expression of *Grm2* in the NAcc, a well-known target whose activation reduces alcohol consumption and that is downregulated in alcohol-dependence.<sup>6</sup> Here, we showed that psilocybin increased *D<sub>2</sub>R* expression in both the NAcc and the PFC and *D<sub>1</sub>R* in the PFC (Fig. 4). First, it is striking that the effectiveness of psilocybin was dependent of the alcohol self-administration exposure since *D<sub>2</sub>R* gene expression was modified only in animals that chronically consumed alcohol and not in the control animals consuming saccharin. Second, *D<sub>2</sub>R* mRNA was increased in the NAcc by psilocybin and this molecular target is a well-known brain target involved in the regulation of ethanol drinking in animals and in AUD in humans.<sup>38,39</sup> High levels of *Drd2* expression reduced ethanol drinking and the risk to develop AUD.<sup>40,41</sup> Increasing the expression of *Drd2* in the NAcc has been shown to decrease ethanol drinking in rodents.<sup>39</sup> All together these results suggest that this increase in *Drd2* gene expression may contribute to the ethanol self-administration decrease by psilocybin.

The 5-HT<sub>2A</sub>R can heterodimerize with the D2LR that are expressed in the post-synaptic neurons. We have shown that D2LR is increased in the NAcc with a significant effect on mRNA levels but only a trend for protein levels (Fig. 4C and supplemental Fig. 2). Within the PFC, both the short and the long forms of the D2R were increased suggesting that both the pre- and the post-synaptic D2R are increased (Fig. 4B). The presynaptic form downregulates DA release and thus could reduce the strength of the DA VTA inputs and this is also corroborated by the trend in the increase of DAT mRNA levels (Fig. 4B) within the PFC. This pathway has been involved in the loss of flexibility and the loss of control over ethanol intake in AUD.

Both *Drd1* and *Drd2* mRNA levels were increased by psilocybin in the PFC (Fig. 4C), whose activation and its role in cognitive function may require optimal balance between activation of dopamine receptors. Since poor cognitive flexibility and decision making is a key factor in AUD, the beneficial effects of psilocybin may involve restoration of cognitive flexibility through change on dopaminergic transmission in the PFC.<sup>6,42,43</sup> Changes in the dopamine receptors balance may be involved in the rewarding value of ethanol and rats stopped drinking either because ethanol is more rewarding (less ethanol needed to feel the same effects) or because ethanol is less rewarding.

## Conclusions

In summary, we identified the role of the 5-HT<sub>2A</sub>R located in the left NAcc and associated changes in dopaminergic receptors gene expression both in the NAcc and PFC, in the beneficial effects of psilocybin on ethanol self-administration. Our study opens new perspectives and adds further interest to the better understanding of the mechanisms of action of psilocybin with its fast-acting and long-lasting effects reported in patients with AUD. Further studies are warranted to enhance our understanding of the hemispheric lateralization of psilocybin's effects in individuals with AUD.

## Funding

This work was supported by the Agence Nationale de la Recherche (ANR)–funded ERA-NET program: Psi-Alc (FKZ: 01EW1908), the ANR PAPAUD (Psilocybin-assisted psychotherapy in Alcohol use disorder) project and by the IReSP in the framework of the 2021 call for the research against the uses and addictions to psychoactive substances (SPAV1-22-019) and by the INSERM.

## Competing interests

The authors report no biomedical financial interests or potential conflicts of interest.

## Supplementary material

Supplementary material is available at *Brain* online’.

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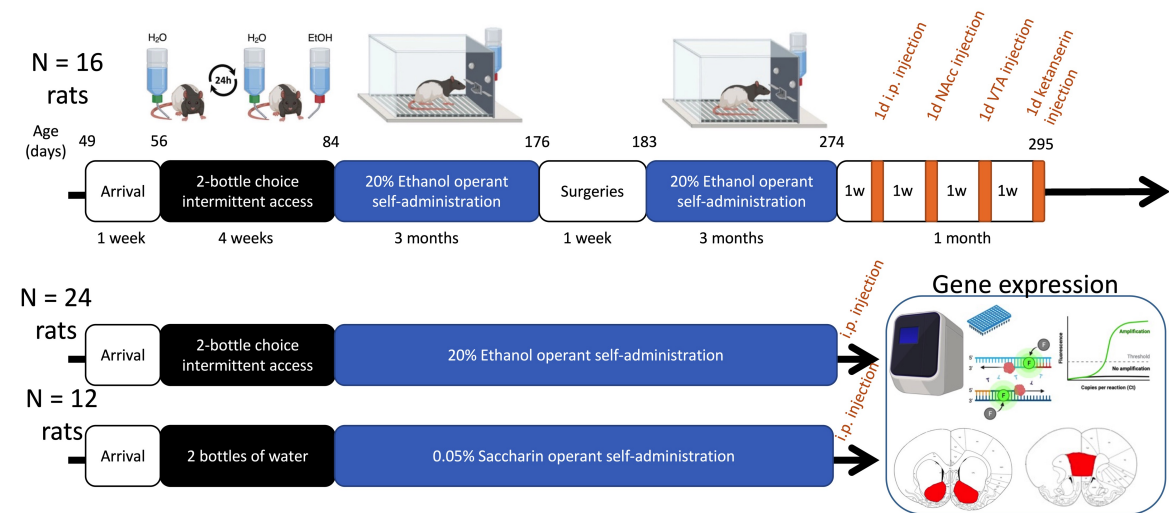
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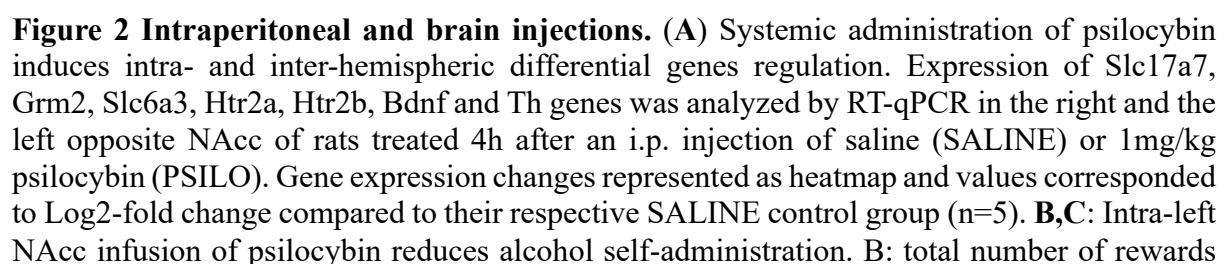
Table 1 Primers used for RTqPCR

Gene	Forward	Reverse
GAPDH	5'-GTTCAACGGCACAGTCAAGG -3'	5'- CGACATACTCAGCACCAGCA -3'
Th	5'- CCCTGAGGGGTACAAAACCC -3'	5'- CACCTCGAAGCGCACAAAAT -3'
Slc6a3	5'- GCTGCTGGTGTCTGGAAGAT -3'	5'- AGGTCCATGGTGAAGGAGGA -3'
Drd1	5'- TCCAGAAGGCGTTCTCAACC -3'	5'- AGTCCTTGAGATGGAGCCT -3'
Drd2	5'- CGTGGGTTGTCTACCTGGAG -3'	5'- CAATGCTGATGGCACACAGG -3'
Drd3	5'- AGTCTGGAATTCAGCCGCA -3'	5'- AGTGAAGTGGCATGACCACC -3'
D2 Long isoform	5'- CCAGTGAACAGGCGGAGAAT -3'	5'- GAGAGTGAGCTGGTGGTGAC -3'
D2 Short isoform	5'- AGCCAACCTGAAGACACCAC -3'	5'- ATCAGGGAGAGTGAGCTGGT -3'

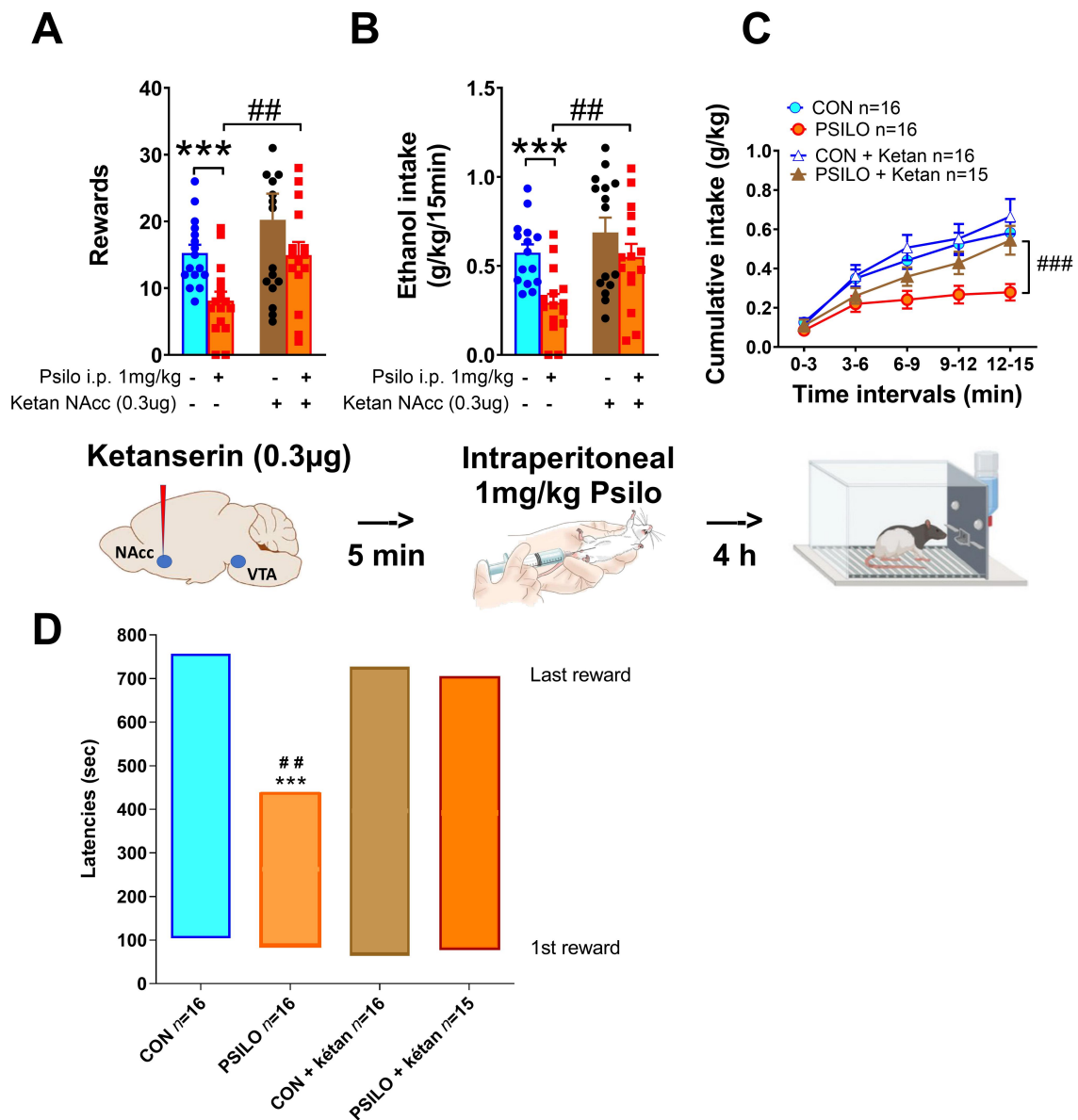
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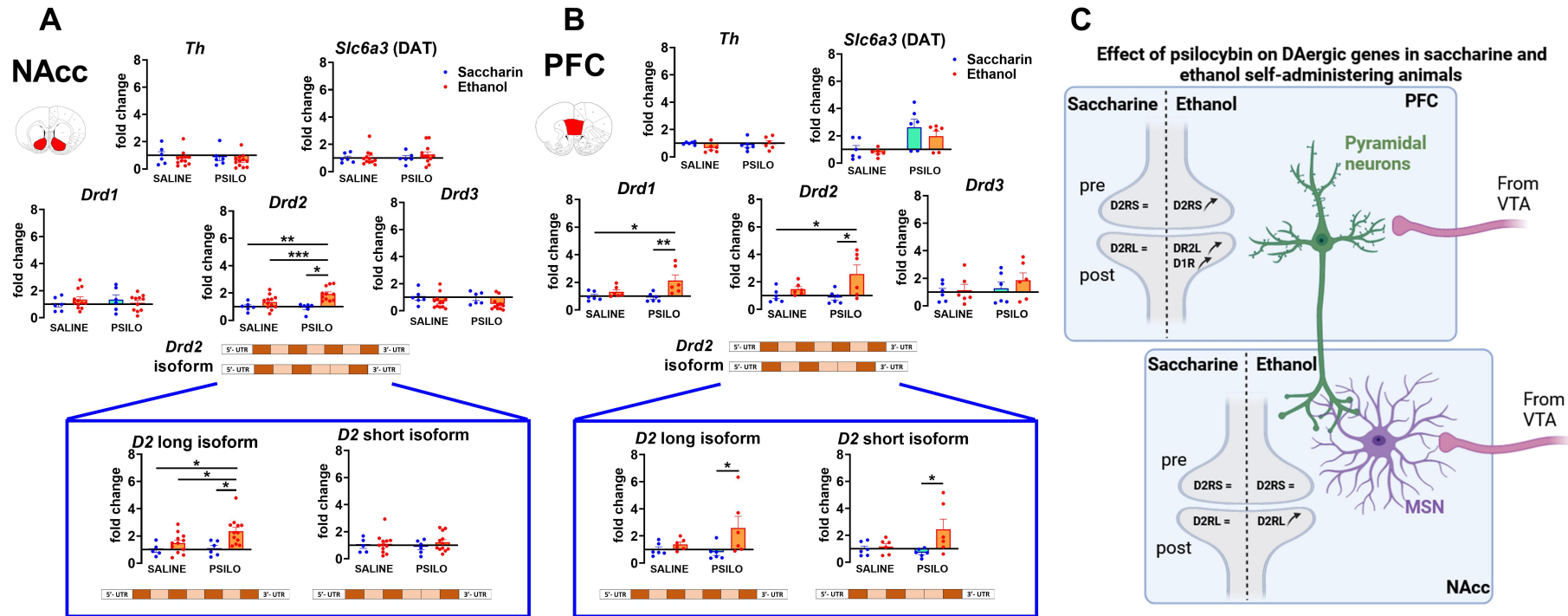
**Figure 1 Time line of the experiments and cohorts of animals.** Three cohorts of rats are shown with the *N* in each cohort. The first one was used for pharmacological testing and the two other ones for gene expression analysis.



are reduced (expressed as Mean  $\pm$  SEM), and the total amount of alcohol consumed (C, expressed as Mean  $\pm$  SEM g/kg). D,E: Intra-right infusion of psilocybin does not alter alcohol self-administration as shown on the number of rewards (D) or total amount of alcohol consumed (E) ( $n=10$ ). F,G,H: Systemic administration of psilocybin (1 mg/kg) 4 hours prior to the test session reduced the number of rewards received (F), the total amount of ethanol consumed during the test session (G) and dampened the rate of ethanol consumption after 6 minutes into the test session (H). I,J,K: Intra-left NAcc micro-infusion of psilocybin (0.15  $\mu$ g) 4 hours prior to the test session decreased the number of rewards received (I), the total amount of ethanol consumed during the test session (J) and dampened the rate of ethanol consumption after 6 minutes into the test session (K). L,M,N: Intra-VTA micro-infusion of psilocybin (0.15  $\mu$ g) did not alter ethanol self-administration. CON = Saline for A, B, C and aCSF for D-I; PSILO = psilocybin. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . A,  $n = 5$ ; B-E,  $n=10$  within design; F-H,  $n = 16$  within design; I-K and L-N,  $n = 8$  between subjects design.



**Figure 3 Antagonism of the 5-HT<sub>2A</sub> receptor within the NAcc blocked the effect of systemic psilocybin administration.** Ketanserin was micro-infused into the NAcc 5 minutes prior to the i.p. injection of psilocybin. The number of rewards (**A**) and the total amount of ethanol consumed (**B**) that were reduced by psilocybin went back to control levels when ketanserin was administered. The rate of consumption was also normalized by ketanserin (**C**). \*\*\*  $P < 0.001$  vs. CON, ##  $P < 0.01$  and ###  $P < 0.001$  between Psilo and Psilo + Ketan.  $N = 16$  within subject design. Average latencies to both the first (lower limit) and the last rewards (upper limit) are depicted in (**D**). The duration of the consummatory episode under each condition is thus depicted as the height of each bar. Significant differences between the control and psilocybin groups (\*\*\*  $P < 0.001$ ), and between the psilocybin and psilocybin + ketanserin groups (##  $P < 0.01$ ) were found for the latencies to obtain the last reward.



**Figure 4 Effects of psilocybin on dopaminergic genes expression.** Dopaminergic genes expression was analyzed by RT-qPCR in NAcc ( $n = 12$ ) (A) and PFC ( $n = 6$ ) (B) of control and ethanol rats treated 4h before by an i.p. injection of saline (SALINE) or psilocybin (PSILO). Gene expression changes of Th, Slc6a3, Drd1, Drd2, Drd3, D2 long isoform and D2 short isoform represented as fold change in relation to saline-treated control group (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ). Schematic representation of the changes in both the pre and postsynaptic compartments (C).

## Supplemental materials

### Material and methods

#### Drugs

For self-administration experiments, ethanol (VWR, France) was diluted in tap water to a final concentration of 20% (v/v) and saccharin (Sigma-Aldrich, France) to 0.05 % Psilocybin (THC Pharm, Germany) was dissolved in 0.9% saline for intraperitoneal injection at a dose of 1 mg/mL in a volume of 2mL/kg; or in sterile artificial cerebrospinal fluid (aCSF, Phymep, France) for intracerebral injection at a dose of 1 mM. Ketanserin, an antagonist of 5-HT<sub>2A</sub> receptors, (Sigma-Aldrich, France) was dissolved in aCSF at a dose of 0.5 µg/µL.

#### Operant Ethanol Self-Administration

Rats were trained to consume 20% ethanol solution for 4 weeks in a 2-bottle-choice intermittent access to one bottle of 20% ethanol (v/v) and one bottle of tap water for three 24h sessions per week (Mondays, Wednesdays and Fridays). Rats had unlimited access to two bottles of water between ethanol-access periods. Following stable preference for ethanol (among 60% compared to water), rats were assigned to one operant conditioning chamber and were trained to press one of the two levers (the active lever) for 0.1 mL 20% ethanol solution delivery. Rats were trained in two 12h overnight sessions under a fixed ratio 1 (FR1) followed by 60 min daily sessions. After a stable number of presses on active lever, the response requirement was raised to 3 lever presses to get the same quantity of ethanol (FR3), and the length of the session was gradually decreased from 60 min daily sessions, to 30 min daily sessions, and finally to 15 min daily sessions for 2 months before and after the surgery. Self-operant administration sessions were carried out five days per week at the same time (2:00 pm). At the end of the psilocybin treatments, self-administration of sucrose was carried out on rats to verify: (i) the effect of psilocybin cannot be attributed to a locomotor effect; (ii) the generalization to non-addictive reinforcers. In this objective, self-administration sessions were carried out in the same condition as describe above by replacing ethanol by sucrose 0.5%. After few sessions of habituation to obtain a consumption similar to that of ethanol, similar i.p. injections with saline and psilocybin were performed 4 hours before the test session. During the self-administration sessions, the outcome measures were the number of lever presses and the number of ethanol deliveries that were recorded using MED-PC IV software (Med Associates Inc., St. Albans,

VT, USA). Nine animals pressing less than 25 times during 3 FR3-15min sessions were excluded of the study because of their low levels of ethanol consumption.

## **Stereotaxic surgery and cannulae implantation**

Cannulae were fixed to the skull with dental cement (Palavit G<sup>®</sup>, Kulzer, France) and a stainless steel screws (Phymep, France). After surgery, the animals received an analgesic (0.015 mg/kg, i.p; buprenorphine) to prevent post-operative pain for at least 3 days. The endpoints for this part of the study were strong hemorrhage, infection at the site of surgery. None of the animals reached these endpoints.

Rats implanted with cannulae were perfused transcardially with fixative (4% PFA), and 200  $\mu$ m coronal slices were cut and examined for cannula placements. As all cannula placements were appropriately located, no animals were omitted from the study.

## **Western blot experiments of the D2R in the whole NAcc**

Twenty rats were randomly assigned to Saccharin/Saline (n=5) ; Saccharin/Psilo (n=5), Ethanol/Saline (n=5) or Ethanol/Psilo (n = 5). Total proteins of whole isolated nucleus accumbens were extracted with RIPA buffer (SERVA) supplemented with 1mM Na<sub>3</sub>VO<sub>6</sub>, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.1% Triton-X100 and a cocktail of protease inhibitors (Promega). Equal amounts of each protein sample were separated by electrophoresis on SDS-PAGE, transferred onto PVDF Immobilon membranes (Merck Millipore) and blotted with primary antibodies against D2R 1/500 (ABN462, Merck Millipore), and GAPDH 1/2000 (#2118, Cell Signaling Technology) and a HRP-conjugated secondary antibody anti-rabbit 1/3000 (#7074, Cell Signaling Technology). Blots were developed with the Clarity<sup>TM</sup> chemiluminescence system (BioRad) and analyzed using ImageLab software.

## **Experiment 4: Genes expression**

Quantitative PCR was performed according to the SYBRGreen Gene Expression protocol (Applied Biosystems) and the transcription of GAPDH was measured as an endogenous housekeeping control. For quantitative Real Time PCR (RTqPCR), primers are presented in supplemental table 1. Reactions were run on a StepOne Plus thermocycler (Applied Biosystems) and the reaction conditions were as follows: pre-denaturation at 95°C for 10 min, 40 cycles of amplification (denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 30 sec) and final extension at 72°C for 10 min. Data were analyzed on StepOne Software using the  $\Delta\Delta C_t$  method.



## Results

### 5-HT<sub>2A</sub> pathway within the NAcc is implicated in the effects of psilocybin

To identify pathways implicated in the effects of psilocybin in the NAcc, the specific 5-HT<sub>2A</sub>R antagonist, ketanserin (0.5 µg/µL), was injected within the NAcc 5 min before intraperitoneal injection of psilocybin (1mg/kg, 4 hours before the test), see [supplemental Fig. 1](#). Psilocybin injected either intraperitoneally or directly within the NAcc reduced the total number of active lever presses ( $P < 0.05$  and  $P < 0.0001$ , respectively). The effect of psilocybin injected within the NAcc was blocked by the injection of ketanserin directly within the NAcc. No effect was observed after injection of psilocybin directly within the VTA.

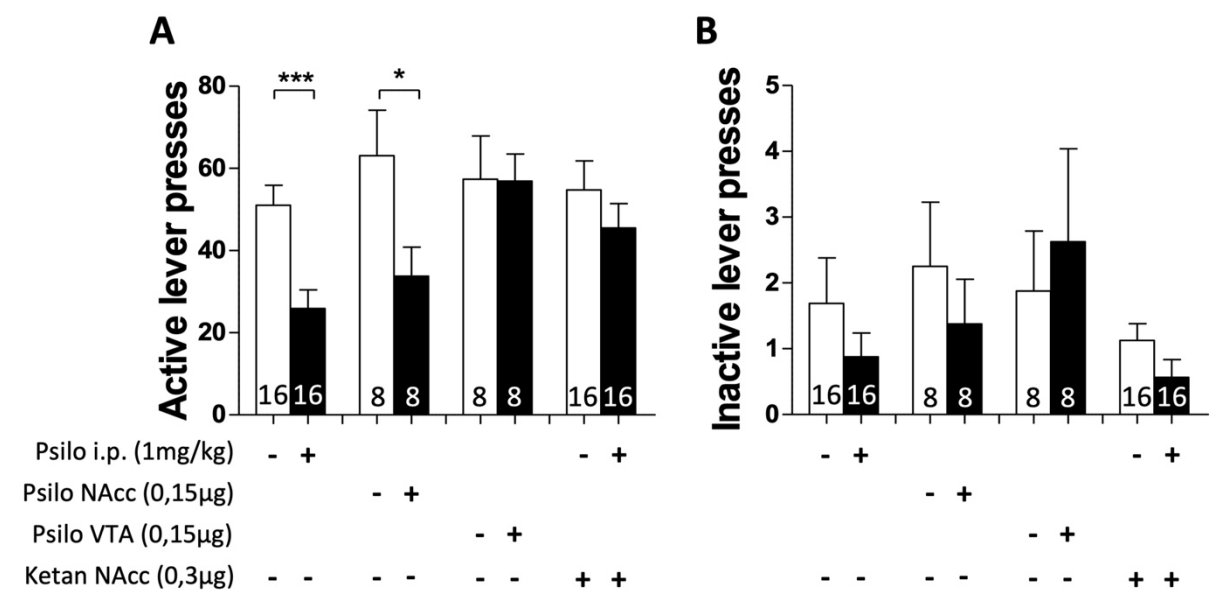
### Latencies for the 1st and the last rewards

Latencies for the 1st and the last rewards are presented on Fig. 3D. The 2-way RM-ANOVA on the 1st reward latencies revealed no effect of any of the factors or the interaction between factors (psilocybin:  $F(1,15) = 0.02$  ; ketanserin :  $F(1,15) = 2.70$  ; Interaction :  $F(1,15) = 1.21$ ). The analysis of the last reward revealed an effect of the factor psilocybin ( $F(1,15) = 11.71$ ,  $P < 0.01$ ), of the factor ketanserin ( $F(1,15) = 8.69$ ,  $p < 0.01$ ), and of the interaction ( $F(1,15) = 4.65$ ,  $P < 0.05$ ). The Tukey *post hoc* test indicated significative differences between the control and psilocybin groups ( $*** P < 0.001$ ), and between the psilocybin and psilocybin + ketanserin groups ( $## P < 0.01$ ).



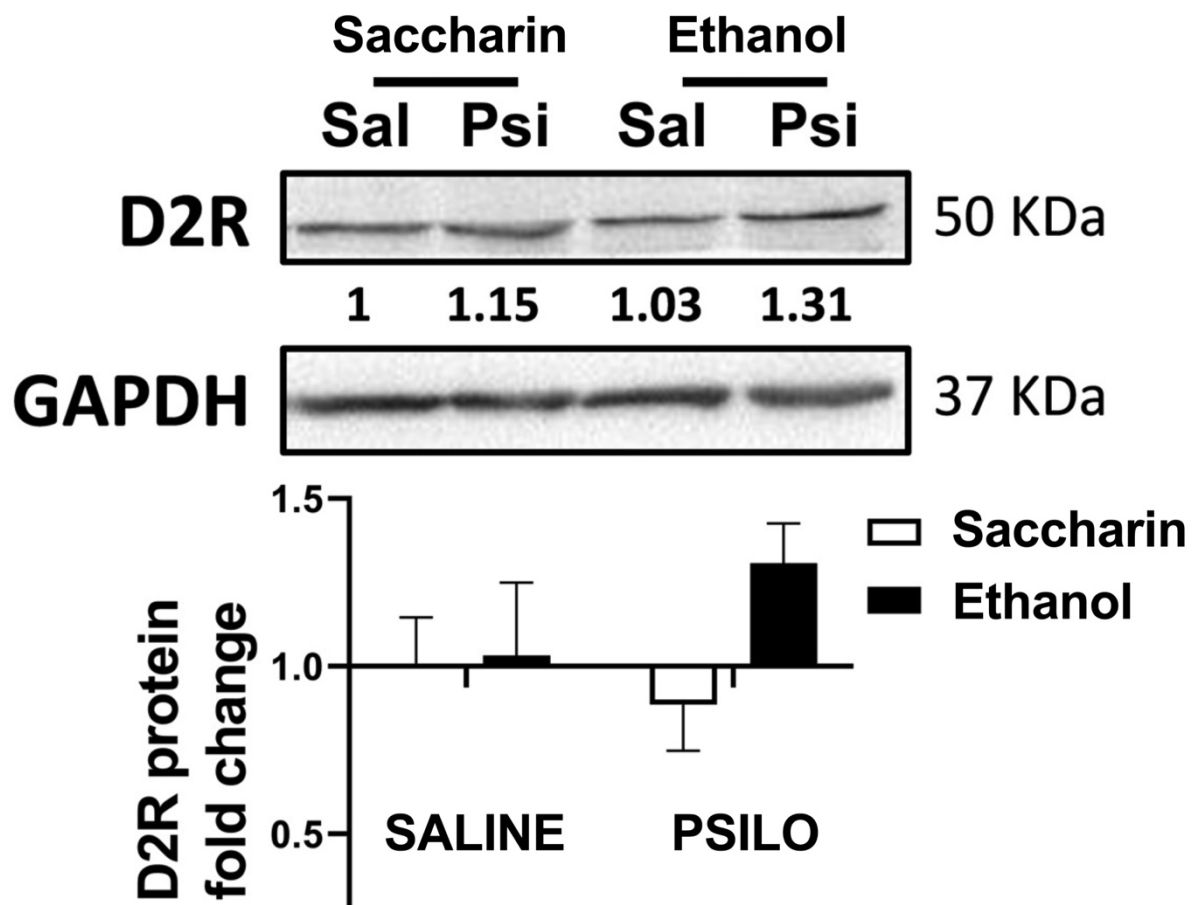
## Legend of the supplementary figure

### Supplementary figure 1



**Effects of psilocybin injected intraperitoneally or the NAcc or the VTA and after, or not, injection of ketanserin into the NAcc. Results of the total number of active (A) and inactive (B) lever presses. Numbers in the bars indicate the number of rats. (-) indicate no injection while (+) indicate injection.**

## Supplementary figure 2



**D2R protein levels in the whole NAcc in either ethanol or saccharin self-administering rats treated with saline or psilocybin.** D2R protein expression was analyzed by western blot in the NAcc of ethanol (Ethanol) or saccharin (Saccharin) self-administering rats treated 4h before by i.p. injection of saline (SALINE) or psilocybin (PSILO). Representative western blot for D2R protein levels in the four different groups. The bar graph represented D2R fold change compared to SALINE/Saccharin group as mean  $\pm$  SEM (n=5).