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Repeated low doses of psilocybin increase resilience to stress, lower compulsive actions, and strengthen cortical connections to the paraventricular thalamic nucleus in rats

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One Sentence Summary: Repeated low doses of psilocybin increase resilience to stress, lowers compulsive actions, and increases synaptic density in the paraventricular nucleus of the thalamus, thus showing therapeutic potential in mental health.

Abstract: Psilocybin (a classic serotonergic psychedelic drug) has received appraisal for use in psychedelic-assisted therapy of several psychiatric disorders. A less explored topic concerns the use of repeated low doses of psychedelics, at a dose that is well below the psychedelic dose used in psychedelic-assisted therapy and often referred to as microdosing. Psilocybin microdose users frequently report increases in mental health, yet such reports are often highly biased and vulnerable to placebo effects. Here we establish and validate a psilocybin microdose-like regimen in rats with repeated low doses of psilocybin administration at a dose derived from occupancy at rat brain 5-HT_{2A} receptors *in vivo*. The rats tolerated the repeated low doses of psilocybin well and did not manifest signs of anhedonia, anxiety, or altered locomotor activity. There were no deficits in pre-pulse inhibition of the startle reflex, nor did the treatment downregulate or desensitize the 5-HT_{2A} receptors. However, the repeated low doses of psilocybin imparted resilience against the stress of multiple subcutaneous injections, and reduced the frequency of self-grooming, a proxy for human compulsive actions, while also increasing 5-HT_7 receptor expression and synaptic density in the paraventricular nucleus of the thalamus. These results establish a well-validated regimen for further experiments probing the effects of repeated low doses of psilocybin. Results further substantiate anecdotal reports of the benefits of psilocybin microdosing as a therapeutic intervention, while pointing to a possible physiological mechanism.

INTRODUCTION

The serotonergic psychedelic prodrug psilocybin ([3-(2-dimethylaminoethyl)-1H-indol-4-yl] dihydrogen phosphate) has received increasing attention in recent years for the treatment of depression, end-of-life anxiety, and substance use disorders. The efficacy of psilocybin to treat these conditions has been established for acute high doses in combination with psychotherapy *(1–4)*. An experimental alternative to high dosing, known as "microdosing", refers to the repeated intake of serotonergic psychedelic substances (like psilocybin) at a low dose without overt psychedelic effects *(5–7)*.Although there are no randomized control trials attesting to the therapeutic efficacy of microdosing of psychedelics, recent population-based or self-reported studies in healthy individuals point to benefits of repeated low doses of psilocybin on mood, well-being, and substance misuse, as well as heightened perception and mental clarity *(6, 7)*. Despite the lack of evidence-based findings on the efficacy and safety of chronic psychedelic microdosing, increasing numbers of people are testing this approach for psychotherapeutic purposes *(8)*, especially for depression, anxiety, and stress *(6)*. The abundance of anecdotal claims on psychedelic microdosing makes likely the possibility of a placebo effect contributing to perceived benefits in such studies *(9, 10)*. These ambivalent findings drew our interest to study the scientific foundation underlying repeated low doses of psilocybin.

In popular consensus, a psilocybin microdose regimen refers to repeated dosing in the range of 0.8-3 mg synthetic psilocybin, which is around 10% of the commonly applied psychoactive dose of 25 mg *(11)*, followed by one or more days without further exposure to the drug *(6)*. Psilocybin is a prodrug that yields the psychoactive metabolite psilocin *(12)*, which exerts its psychedelic

action through partial agonism at the 5-HT_{2A} receptor *(11, 13)*, while also acting as a partial agonist at 5-HT_{2C}, 5-HT_{1A}, and 5-HT_{7} receptors *(14, 15)*. A more scientific definition of a microdose is based upon the dose-occupancy relationship at 5-HT_{2A} receptors *in vivo* and self-report of psychedelic intensity, as recently described by Madsen et al. *(11)*. In that study, 20% occupancy at 5-HT_{2A} receptors, as evoked by psilocybin at doses in the range of 0.8-1.6 mg in an 80 kg person, represented a threshold for inducing overt psychedelic effects. This stands in agreement to the generally accepted microdose-range in public use *(6)*. Throughout this manuscript we shall refer to psilocybin microdosing as repeated low doses of psilocybin, having shown that such doses indeed have behavioral and pharmacological effects, therefore not being microdoses as defined in pharmaceutical terms.

Given the lack of objective evidence, we set out to establish a psilocybin-dosing regimen in rats that mimics the anecdotal practice of psilocybin microdosing. We then tested common behavioral phenotypes as reported following repeated high doses of psilocybin, namely increased anxiety and deficits in pre-pulse inhibition of the startle reflex. Furthermore, we sought to explore other behavioral effects related to anecdotal reports in humans, namely resilience to stress, mood changes and compulsive-like behaviors. After 21 days of repeated treatment with low doses of psilocybin, we analyzed neuroreceptor and synaptic density using autoradiography. We carried out the study in phases by first establishing a dose-response relationship for 5-HT_{2A} receptor occupancy in rat brain *in vivo*, with correlation to a behavioral index of 5-HT_{2A} receptor activation. We next measured the *in vitro* binding affinity of psilocin for 5-HT_{2A}, 5-HT_{2C}, 5-HT_{1A}, and 5-HT_{7} receptors in rat brain sections, as well as the *in vitro* agonist efficacy of psilocin towards the four

target receptors, thereby establishing which receptors are potentially activated by low doses of psilocybin. In the next phase of the study, we focused on assessing outcomes for anhedonia in the sucrose preference test and anxiety in open-field and elevated plus-maze tests.

Taken together, we established an objective psilocybin dosing regimen for assessing the validity of the microdosing concept in the rat with translational implications for human trials, which are currently lacking evidence of biological mechanisms. We tested the hypothesis that repeated low doses of psilocybin alter rat behaviors related to mood, anxiety, and compulsive-like actions, in combination with altered neuroreceptor expression and synaptic density.

MATERIALS AND METHODS

Animals

Long Evans rats (n=78, M, Janvier Labs) used in experiment 1 were housed in pairs at the animal facility at Rigshospitalet. Rats in experiment 2 (Long Evans, n=18, M, Janvier Labs) were housed four to a cage at the Biomedical Laboratory at University of Southern Denmark. All animals were kept at a 12-hour light/dark (light 6-18) cycle. Cages were equipped with bedding, nesting material, a wooden stick or ball, and a red plastic shelter. All experiments were carried out during the light phase beginning at approximately 10:00 hours. Animals used for receptor binding studies were allowed *ad libitum* access to food pellets and water, but rats used in experiment 1 were maintained on a restrictive diet of 6 pellets a day per rats, in order to keep them motivated, and without excessive weight gain for the startle experiments. Animals in experiment 2 that were fed *ad libitum*

did however undergo brief fasting intervals in relation to the sucrose preference test. The weight of all animals was measured regularly. We conducted all procedures in accordance with FELASA guidelines and with approval from The Danish Animal Experiments Inspectorate (2017-15-0201-01283 or 2016−15−0201−01031).

Radiochemical production of [^{18}F]MHMZ

[^{18}F]MHMZ was prepared at the Cyclotron Unit in Department of Clinical Physiology, Nuclear Medicine and PET (Rigshospitalet) by alkylating MDL105725 with 2-[^{18}F]fluoroethyl tosylate ([^{18}F]FEtTos) in an automated synthesis module *(49, 50)*. Radiochemical purity of [^{18}F]MHMZ was >97% and molar radioactivity was 15-30 GBq/μmol at end of synthesis.

Serotonin 5-HT$_{2A}$ Receptor Occupancy

Four groups of Long Evans rats (n=18) first received s.c. injections of psilocybin (vehicle, 0.05 mg/kg, 0.20 mg/kg and 1.00 mg/kg in saline, s.c.). The rats were anesthetized 40 min later with isoflurane (2–2.5% in oxygen) and placed in a homemade four-rat insert in a Siemens HRRT (High Resolution Research Tomograph) scanner *(51)*. A 45-min dynamic emission scan was started immediately after i.v. injection of approximately 10 MBq [^{18}F]MHMZ and followed by a 6-min rotating point source ^{137}Cs transmission scan. The rats were kept warm using an infrared lamp and monitored for respiration throughout the scan. The list-mode emission data were dynamically reconstructed with the standard 3D-OP-OSEM- algorithm with point-spread function modelling *(52)* (16 subsets, 10 iterations and no post-reconstruction filtering) using a MAP-TR attenuation

map with human head prior and scatter correction. The reconstructed PET image data were first cropped manually to a head-only image in PMOD v3.7, followed by motion-correction with the PFUSEIT tool and summation for the purpose of co-registration. The SUV images were corrected for body weight and injected dose. Automatic co-registration with a tracer-specific PET template as target was executed in FSL 5.0.11 with the *flirt* command using the normalized correlation cost function with 12 degrees of freedom, and the transformation was applied to the dynamic images. A modified Logan reference tissue model with the cerebellum as reference region was used to calculate the non-displaceable binding potential (BP_{ND}) in the frontal cortex *(53)*. Percent occupancy was calculated from the difference in BP_{ND} at a given dose of psilocybin in relation to vehicle administration.

Wet back shake – dose-response

Long Evans rats (n=22) were given saline or psilocybin at a range of doses (0.05, 0.1, 0.25, 1.0, 3.0, 6.0 mg/kg in saline, s.c.). They were then placed in a 20 × 20 cm arena with 30 cm high walls including one clear Plexiglas wall to allow for video recording for one hour. Wet back shakes were manually scored as a rapid rotational movement of the head and neck or/and body that visually resembled a wet dog shake. The arena was cleaned and disinfected between trials.

Psilocin competition binding assays

Psilocin competition binding assays were performed using *in vitro* autoradiography, with the addition of a range of psilocin concentrations to the incubation buffer depending on the target

receptor. Psilocin concentrations were 0-100 μM for 5-HT_{1A} receptor binding studies with [^{3}H]WAY100365 (7.5 nM), with hippocampus as the target region for analysis. Psilocin concentrations ranged from 0-100 μM for 5-HT_7 receptor studies with [^{3}H]SB269970 (1 nM) in the presence of 20 nM WAY100635 to block the 5-HT_{1A} receptor binding component, with thalamus as the target region for analysis. In the case of the 5-HT_4 receptor labeled with [^{3}H]SB207145 (0.58 nM), psilocin concentrations ranged between 250 nM-100 μM, with dorsal striatum as the target region for analysis. In the case of 5-$HT_{2A/C}$ receptors labelled with the agonist [^{3}H]Cimbi-36 (0.5 nM), psilocin concentrations ranged between 1-500 nM, with the frontal cortex as the target region for 5-HT_{2A} analysis and the choroid plexus as the target region for 5-HT_{2C} analysis. ImageJ (version 1.53k) was used to extract pixel values and Graphpad Prism (v9) to calculate IC_{50} values, and corresponding K_i values were calculated using the Cheng-Prusoff equation: $IC_{50}/(1+([Ligand]/K_D))$.

Functional properties of psilocin

The functional properties of serotonin and psilocin were characterized at stably expressed 5-HT_{2A}, 5-HT_{2C}, 5-$HT_{1A,}$ and 5-HT_7 receptors in HEK293 cell lines. 5-HT_{2A} and 5-HT_{2C} functional activity were characterized in the Ca^{2+}/Fluo-4 assay essentially as previously described *(54)*. In short, the 5-HT_{2A}- and 5-HT_{2C}-HEK293 cells *(55)* were cultured in a humidified atmosphere at 37 ºC and 5% CO_2. The day before the assay, the cells were split into 96-well plates with a clear bottom (6 x 10^4 cells/well). On an experimental day, the culture medium was aspirated, and the cells were incubated in 50 μL assay buffer supplemented with 6 mM Fluo–4/AM at 37 °C for 1 h. Then the

buffer was removed by aspiration, the cells were washed once with 100 µL assay buffer, and a further 100 µL assay buffer was added to the cells. The plate was then assayed in a FLEXStation^3 (Molecular Devices).

5-HT_{1A} and 5-HT_7 functional activity were evaluated in the cAMP production assay. In short, HEK293 cells expressing 5-HT_{1A} or 5-HT_7 receptors were cultured in 25 cm^2 flasks, grown to 90% confluence, washed twice with PBS prewarmed to 37°C, and centrifuged for 5 min (160×g). The cell pellet was resuspended in a stimulation buffer (1×HBSS, 5 mM HEPES, 0.5 mM IBMX, and 0.1% BSA). The total cAMP production was measured using the LANCE Ultra cAMP detection kit (PerkinElmer), according to the manufacturer's directions.

Repeated low doses of psilocybin as a microdosing regimen

Aliquots of psilocybin prepared by dissolving psilocybin in distilled water to a concentration of 1 mg/ml were stored at -80 °C. Before use, 1 mg/mL titers were diluted with 0.9% saline solution to a concentration of 0.05 mg/mL, which was administered based on body weight. Long Evans rats (male, 2 cohorts of n=18) were habituated to the environment for 12 days after arrival. Following habituation, the rats were divided into treatment and control groups. Two rats in a home cage were assigned to one or the other groups, thus avoiding biases related to animal dominance and secondary exposure to drugs through feces or urine. The treatment group received 0.05 mg/kg psilocybin s.c. every second day for 21 days, while the control group received injections of isotonic saline water. All animals were weighed at intervals during the treatment period, and behavioral

tests were done on the day following the last treatment (Fig. 2A). Two experiments were made; the first tested behavioral outcomes in a habituated open-field test and pre-pulse inhibition of the startle reflex, as well as *postmortem* receptor expression, while the second tested behavioral outcomes in a novel open-field followed by the elevated plus maze test. Experiment 1 was made in two cohorts with subtle differences: Cohort 1 experiments, which were carried out by an experimenter with male pheromones, were exposed to an unsuccessful sucrose preference test (not reported) as well as receiving a high dose of psilocybin after the last behavioral test. Cohort 2 experiments, which were carried out by an experimenter with female pheromones, did not undergo sucrose preference tests, nor did they get the single high dose of psilocybin at the end of the experiments. The experimenters in experiment 1 were not blinded to treatment. Behavioral data from cohorts 1 and 2 of the first experiment were pooled in the main manuscript. The second experiment was carried out be an experimenter with male pheromones, who was blinded to treatment. After the euthanasia of rats by decapitation, brains were removed, rapidly frozen on dry ice, and retained at -80 °C for *postmortem* analysis.

Acoustic startle reflex and open-field behavior

This set of behavioral experiments were carried out as previously described in *(24)*. In short, startle tests were conducted in a sound-attenuated, lighted, and ventilated chamber (StartFear Combined system, Panlab Harvard Apparatus, Barcelona, Spain). The startle reflex of the rats was assessed on days before the open-field and PPI tests. After an exploration period of ten min with a constant background white noise of 65 dB for acclimation, we administered 55 startle stimuli of 20 ms

duration (70-120 dB white noise (w.n.), each stimulus increased in 5 dB intervals, with 5 stimuli given at each volume) were presented in a pseudo-randomized order, with variable pseudo-randomized inter-trial intervals (ITI; range 6-24 s, mean 15 s) to avoid predictability of the next stimulation. The startle reflex corresponding to each pulse was plotted for each rat to establish the mean maximum pulse intensity.

On the day of the PPI and open-field test, rats were brought to the laboratory at least 60 minutes before testing. One test session consisted of a baseline recording of open-field behavior for 40 minutes followed immediately by assessment of PPI for 37 minutes, for a test session lasting 80 minutes in total. The rats were placed in the center of an 80.5 x 80.5 cm square field with walls 40.5 cm in height (no drugs or injections were given on the experimental day). The open-field test was recorded with a Basler ACE (ACA1300-60) camera while the handler had left the room. Spontaneous activity such as distance traveled, rearing, and grooming, as well as arena activity such as time spent in the center or border zone of the field were scored automatically using the Ethovision® XT 11.5 (Noldus Information Technologies) software.

After locomotion recordings, rats were immediately transferred to the startle box for sensorimotor gating assessment in sessions consisting of four blocks. Block I was a five min exploration period with only background noise (65 dB w.n.). Block II was a short-term habituation (STH) test with ten auditory pulses (110 dB w.n., 30 ms each at intra test intervals (ITI) of 10 s). Block III was the startle and pre-pulse inhibition (PPI) test, consisting of seven different trials; a) no stimuli (65 dB w.n.); b) pulse alone (110 dB w.n., 30 ms); c) pre-pulse alone (81 dB w.n., 20 ms); d-g) pulse (110

dB w.n., 30 ms) preceded 100 ms by a 20 ms w.n. pre-pulse at 69 dB (d), 73 dB (e), 77 dB (f) or 81 dB (g). Each trial type was presented 15 times in a pseudo-randomized order with a variable ITI ranging from 6-24 s (15 s mean). Block IV was an additional STH test, conducted just as in Block II. The percentage of PPI for each pre-pulse intensity was expressed as: % PPI = [(mean startle amplitude on pulse alone trial – mean startle amplitude on pre-pulse trial)/mean startle amplitude on pulse alone trial] *100.

Sucrose preference test

In the second set of experiments, animals were exposed to 5 rounds of the sucrose preference test with approximately one week in between, as outlined in figure 4b. The first two tests were baseline recordings, the next two were made during the psilocybin microdosing regimen, and the final test was conducted on day two following the last saline/psilocybin treatment. Each test was carried out as follows: a 1% w/w sucrose solution in freshwater was made from a 10% stock solution. Water bottles (450 mL) with a metal cap and spout were used. Before the first test, all animals had a habituation session, during which all water bottles in primary housings were replaced for 24 h by water bottles with 1% sucrose. The test consisted of 2 phases across 2 days; on day-1, animals were fasted (with free access to water) for an 8 h period in light (10-18). Immediately after fasting each animal was isolated for 12 h in the dark (18-06) in a cage closely resembling their home cage, with access to two water bottles containing premeasured 1% sucrose solution or regular water. Following this, all animals were weighed and returned to their original home cage with ad libitum access to food and regular water. Consumed water and sucrose solution were measured. The 16 animals were run in batches of 8 on separate days.

Open-field and elevated plus maze.

Following a minimum 2 h rest period after the last sucrose preference test, each animal was exposed to an open-field arena for 15 min, essentially as described above. Following the end of the 8 trials (approximately 3 h total), the animals were introduced to the elevated plus maze. The maze (Panlab) consisted of a black 20x20 cm central platform, with gray 40-cm-long arms extending from each side of the central platform. Making up a plus-shaped platform raised 50 cm above the floor. Two opposite standing arms were fitted with 40-cm-tall gray walls, starting from the central platform. The two remaining arms were fitted with, 5-cm-tall clear walls. An animal was placed in into the central platform and recorded for 15 min with a similar setting as used in the open field test. Upon conclusion of the 15 min recording, the animals were returned to their home cage. The maze was then cleared of droppings and cleaned with a cloth followed by a disinfectant wipe, in preparation for the next subject. For analysis (Ethovision® XT 11.5, Noldus Information Technologies) the maze was segmented into 5 zones: center, open arms and closed arms. Variables measured were movement, distance & time, zones, and behavior. Rearing and grooming were scored manually using the video recordings. Animals were naïve, being unhabituated to the open-field and the elevated plus maze, thereby increasing possible differences in anxiety. The experimenter performing the tests was blinded to the treatment regimen.

Determination of 5-HT receptor and synaptic vesicle protein 2A levels *postmortem*

Protein levels were quantified postmortem using autoradiography. Rats were euthanized by decapitation without anesthesia. The brains were carefully extracted, rinsed with cold isotonic

saline water, placed in cryomold boxes containing Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Europe B.V), and frozen on dry ice. Frozen brains were stored at -80 °C until further use. For cryosectioning, the brains were cut at -20 °C with a thickness of 20 µm, either horizontally (experiment 1, cohort 1) between the approximate stereotaxic coordinates extending -3 to -7 mm dorsal to ventral, or coronally (experiment 2, cohort 2) at intra-aural 5.70 mm. The brain sections were thaw-mounted on Superfrost microscope slides (Thermo Scientific), air dried, and stored at -80 °C until the autoradiographic studies.

Autoradiography studies of 5-HT receptors were performed as described above, using only one concentration of each radioligand; 5-$HT_{1A,}$ [^{3}H]WAY100635 (20 nM), 5-HT_{2A} and 5-$HT_{2C,}$ [^{3}H]Cimbi-36 (2 nM), and 5-HT_7, [^{3}H]SB269970 (5 nM). Autoradiography of the synaptic vesicle protein 2A (SV_2A: a proposed marker for synaptic density) was performed using 60 nM [^{3}H]UCBJ, otherwise similarly to the 5-HT receptors. For each radioligand, we used three sections from each of the 18 animals in experiment 1. We calculated the specific binding ratio relative to a reference region, namely the cerebellum for horizontally cut sections (cohort 1) and the corpus callosum (white matter) for coronally cut sections (cohort 2). The cortex region in cohort 1 refers to the medial prefrontal cortex, while the cortex region in cohort 2 refers to the cingulate.

Statistical analysis

Data are reported as mean ± standard deviation unless otherwise noted. A significant result is indicated with * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. Group-wise comparisons (Fig. 1) were done by one-way ANOVA without repeated measures. Individual columns were compared with

baseline, and a Dunnett's multiple comparison corrections were applied. Cohort and treatment effects on behavior in the open-field (Fig. 2) were tested by two-way ANOVA without repeated measures. Comparisons were done across treatments with a Šídák correction for multiple comparisons. In analyzing SR (Fig. 3), the reflex to different doses was included as a repeated measure in a two-way ANOVA with Šídák correction for multiple comparisons. We did not correct for multiple comparisons across experiments or different measurements, as these measures were defined prior to the experiment and such corrections would increase type-2 errors with no change in observed differences.

RESULTS

Psilocybin induces a dose-dependent occupancy of the 5-HT_{2A} receptor *in vivo*

To determine a "microdose" of psilocybin occupying <20% of brain 5-HT_{2A} receptors, and without inducing overt behavior effects suggestive of psychedelic action, we undertook a dose-occupancy experiment using the 5-HT_{2A}-selective positron emission tomography (PET) tracer [^{18}F]MHMZ (Fig. 1A). As expected, psilocybin induced dose-dependent occupancy of 5-HT_{2A} receptors *in vivo* (One-way ANOVA, $F(3,14)=8.52$ $P=0.002$) at doses ranging from 0.05-1 mg/kg (Fig. 1B+C). The non-displaceable binding potential (BP_{ND}) of [^{18}F]MHMZ in frontal cortex was 2.01 ± 0.13 in control animals, 1.64 ± 0.13 in animals treated with 0.05 mg/kg (not significant; 19 ± 9% occupancy), 1.55 ± 0.17 in animals treated with 0.2 mg/kg ($P=0.028$; 21 ± 16% occupancy), and 1.26 ± 0.35 ($P<0.001$; 41 ± 19% occupancy) in animals treated with 1.0 mg/kg psilocybin ($P<0.001$) (Fig. 1B+C). Psilocybin also induced a classic inverted U-shaped behavioral response of wet-back shakes, which is taken to be indicative of 5-HT_{2A} receptor activation *(16)* (One-way

ANOVA, $F(6,17)=2.89$, $P=0.04$) (Fig. 1D). As expected, psilocybin at low doses did not induce any significant change in wet-back shakes, whereas 1 mg/kg evoked a significant increase ($P=0.01$, 8.5 ± 5.8 per 10 min) compared to the control group. The wet-back shakes declined for doses above 1 mg/kg, mainly due to the sedative effects of high doses of psilocybin (Fig. 1D). Based on these results, we defined our microdose as 0.05 mg/kg, this being a dose inducing <20% occupancy at 5-HT_{2A} receptors and inducing no overt behavioral response.

Psilocin activates 5-HT_{2A} and 5-HT_{2C} receptors upon low psilocybin-induced occupancy of the 5-HT_{2A} receptor

To understand the effects of psilocybin at low doses, we assessed which serotonergic receptors were likely activated at a dose obtaining 20% 5-HT_{2A} receptor occupancy in rats. We used *in vitro* autoradiography to establish the affinity (K_D) of radioligands chosen for their selectivity at 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_7 receptors (Table 1), and then measured the inhibition constant (K_i) of psilocin against those same serotonin receptors, in brain regions where the highest signal-to-background was observed for the given receptor. Psilocin showed moderate *in vitro* affinity for all four receptors (Fig. 1E); $K_i = 80 \pm 28$ nM for 5-HT_{1A} v.s. [^{3}H]WAY100635 (Fig. 1G), $K_i = 15 \pm 2$ nM for 5-HT_{2A} v.s. [^{3}H]Cimbi-36 (Fig. 1H), $K_i = 12 \pm 8$ nM for 5-HT_{2C} v.s. [^{3}H]Cimbi-36 (Fig. 1I), and $K_i = 524 \pm 272$ nM for 5-HT_7 v.s. [^{3}H]SB269970 (Fig. 1J). We then characterized psilocin for its functional activity (EC_{50}) at stably expressed 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_7 receptors in HEK293 cell lines in Ca^{2+}/Fluo-4 and cAMP assays (Table 1, Fig. 1F). The EC_{50} values of psilocin were 727 ± 45 nM for 5-HT_{1A} (Fig. 1K), 36 ± 8 nM for 5-HT_{2A} (Fig. 1L), 11 ± 0.3 nM for 5-HT_{2C} (Fig. 1M), and 45 ± 59 nM for 5-HT_7 (Fig. 1N). Psilocin proved to be a partial agonist at

all four receptors, with efficacy (R_{max}) ranging from 13-71% of full activation (Table 1). These results predict that psilocybin at a dose evoking 20% occupancy at 5-HT_{2A} receptors would likely also obtain significant occupancy and functional activation at 5-HT_{2C} receptors (up to 35%), some activation at 5-HT_7 receptors (up to 13%), but no activation expected at 5-HT_{1A} receptors.

Repeated low doses of psilocybin do not induce anxiety in a familiar environment

In the first set of experiments, we tested if psilocybin microdosing induced anxiety or schizophrenia endophenotypes in rats, as previously reported for the chronic high-dose LSD animal model of schizophrenia *(17)*. We also tested if psilocybin microdosing would induce changes in open-field behaviors like, locomotion, rearing, and self-grooming, as well as preference for sucrose *(18)*. Following the end of the behavioral experiments, we tested if microdosing with psilocybin had induced behavioral desensitization to a high dose of psilocybin, i.e., reduced wet-back shakes, and reduced levels of 5-HT_{2A} receptors *postmortem*. Based on the occupancy and behavioral data reported above, we established a microdosing regimen with 0.05 mg/kg psilocybin (s.c.) every second day for 24 days (Fig. 2B). Sucrose preference in cohort-1 was inconclusive due to suboptimal conditions in the SPT (data not reported). Following the end of the regimen, we tested several behavioral measures in a 40-minute familiar open-field test. Repeated low doses of psilocybin did not change locomotion (Fig. 2C-F), center frequency, or time spent in the center, confirming that psilocybin at these doses does not induce anxiety behavior in a familiar open-field arena, as often seen in animal models of repeated high doses of psilocybin. Neither did psilocybin treatment change unsupported (Fig. 2J-M) or supported rearings (Fig. 2N-Q).

Repeated low doses of psilocybin reduces the frequency of compulsive self-grooming in a familiar environment

Self-grooming is a compulsive and stereotypic behavior in rodents *(23)*, which is directly linked to activation of the cortico-striatal-thalamo-cortical (CSTC) circuit *(24),* and is highly sensitive to stress *(25)*. Animals in the first experiment were habituated to the open-field in the days prior to the test to reduce anxiety and effects of stress on all behavioral outcomes. While exploratory behaviors decreased, self-grooming increased over time in the open-field arena (Fig. 2C, F, H, R, T). Psilocybin microdosing reduced the overall self-grooming frequency by 14% in the microdosing compared to control animals (unpaired, two-tailed t-test, P=0.016) (Fig. 2U), without changing other measurements in the open-field test. Here, grooming frequency refers to an uninterrupted session of grooming and can include one or more grooming bouts. These results point towards an effect of psilocybin microdosing in reducing self-grooming in rats.

Repeated low doses of psilocybin do not induce schizophrenic endophenotypes

Exposure to high doses of psilocybin or other 5-HT_{2A} receptor agonists induce deficits in pre-pulse inhibition (PPI) of the startle reflex *(19–21)*, which is a widely accepted endophenotype of schizophrenia. Indeed, repeated psilocybin dosing (at high dose) has been used to model that aspect of schizophrenia *(21)*. Therefore, we tested whether repeated low doses of psilocybin altered PPI or startle habituation. We first tested basal acoustic startle reflex (SR) to different intensity sound pulses (Fig. 2K). SR was clearly intensity-dependent but plateaued at 110 dB. The SR as measured

at 110 dB did not differ between control (45 ± 12%) and microdosing groups (45 ± 9%). We subsequently chose 110 dB as the pulse intensity for testing SR with or without pre-pulses of different intensity. There were no group differences in SR to pulse (P) and pre-pulse + pulse (PP+P) (Fig. 3A+B). As expected, there was a dose-dependent decrease in SR with increasing pre-pulse intensities in both groups (Fig. 3B). There was no group difference in latency from the startle pulse to maximum peak of startle (Fig. 3C) or in %PPI (Fig. 3D+G). Animals had similar weight gain (Fig. 3E) over the course of the regimen. While control animals presented the expected short-term habituation (STH) to SR over the startle sessions (P=0.007), psilocybin-treated animals showed more resistance to this habituation (-24 ± 29 % in controls and -1 ± 41 % in psilocybin-treated animals) largely due to relatively high standard deviation in the psilocybin-treated animals (Fig. 3F). No effect of treatment could be detected on either the initial startle or post-session startle reflex.

Microdosing of psilocybin increase resistance to stress induced anhedonia

Anhedonia is one of the core symptoms of major depression disorder. In experiment 2 we tested if repeated low doses of psilocybin would change the preference for sucrose in the sucrose preference test, an established model for anhedonic behavior in rats. All rats received two baseline sucrose preference tests and had 81 ± 0.3 % preference for the 1% sucrose solution on the second baseline test (Fig. 4D). Repeated treatments s.c. are bound to induce some stress in experimental animals and control-injected animals indeed showed declining preference for sucrose doing the treatment period to a mean of 50 ± 27 %) (mixed effects model, F(1,64, 11,08)=4.94, P=0.03, as required because one of the individual measurements from one rat had to be excluded), regaining normal

preference at the end of the test period (Fig. 4E). However, psilocybin-treated animals did not show declining sucrose preference, but rather an increase to 92.7 ± 5.2 % on their first within-treatment test. The preference for sucrose by psilocybin-treated animals also returned to baseline following the end of treatment (Fig. 4F). This points towards an increased resistance to stress-induced anhedonia during the psilocybin treatment period, but not following cessation of the treatment. Whether this result is due to central stress inhibition or analgesia effects remains to be established.

Repeated low doses of psilocybin does not change anxiety or exploration in novel environments

We tested the effects of repeated low doses of psilocybin in two test sessions, first in a short exposure to a novel open-field environment and second in the elevated plus maze. There were no changes in center entries or time spent in the center of the open-field, as seen in experiment 1 (Fig. 4G-N). Furthermore, we observed no change in preference for open or closed arms in the elevated plus maze (Fig. 4O-V). These results confirmed our previous findings in a known environment, that repeated low doses of psilocybin do not induce anxiety. Interestingly, the frequency of self-grooming was reduced by 48% in the elevated plus maze, without changes in locomotion or rearing, further confirming our previous findings.

Repeated low doses of psilocybin does not induce behavioral or receptor desensitization towards 5-HT_{2A} receptor agonists

Behavioral desensitization to 5-HT_{2A} agonists has been reported in humans and in experimental animals following repetitive administration of high doses of psilocybin *(22)*; such a desensitization would lower the effect of the drug over time and increase the dose needed for a comparable effect. Therefore, we assessed wet-back shakes following an acute challenge with 1 mg/kg psilocybin following the end of treatment regimen and upon completion of the behavioral studies. There were no differences in the behavioral response to the acute challenge between animals subjected to repeated low doses of psilocybin compared and control animals, or in comparison to naïve rats from the initial study (Fig 5). We further assessed *postmortem* 5-HT_{2A} receptor levels (Fig. 6A-C) in both prefrontal cortex and striatum (Fig. 6A+B), as well as 5-HT_{2C} receptor levels in the choroid plexus (Fig. 6C), finding that psilocybin treatment did not alter the expression of these receptors. Together, these results point towards a well-established model for a microdosing-like treatment regimen with repeated low doses of psilocybin in rats, without evoking behavioral desensitization or conspicuous 5-$HT_{2A/2C}$ receptor changes.

Repeated low doses of psilocybin increases 5-HT_7 and SV_2A levels in the paraventricular thalamic nucleus

We wanted to know if repeated low doses of psilocybin were associated with changes in serotonergic receptor density and synaptic connections in the brain, given that high single doses of the drug increase synaptic plasticity *(22, 26)*. Besides the 5-HT_{2A} and 5-HT_{2C} receptor expressions reported above, we also measured 5-HT_{1A} and 5-HT_7 receptor levels using *in vitro* autoradiography with [^{3}H]WAY100635 (Fig 6F+J) and [^{3}H]SB269970 (Fig. 6D+H) respectively. In cohort 1 animals from experiment 1, there was an overall treatment effect of increased 5-HT_{1A}

receptor expression (Fig. 6G) (One-way ANOVA, F (1, 73) = 4,645 P=0.03), yet changes in individual regions were not significant following multiple comparison corrections in septum (P=0.074) and hippocampus (P=0.074) (Fig. 6G). We also observed increased 5-HT_7 receptor expression in the paraventricular nucleus of the thalamus (PVT) (P=0.02), but no such changes in the hippocampus (Fig. 6E) in psilocybin-microdosed animals compared to controls. Since the single high dose of psilocybin used to evoke wet-back shakes in cohort 1 might have affected these expression levels, we repeated the assessments of 5-HT_{1A} (Fig. 6G+K) and 5-HT_7 (Fig. 6E+I) receptor expression in coronal brain sections from cohort 2. This confirmed a 16 % increase in 5-HT_7 receptor expression specific to the PVT (P=0.01), with no increase in the medial amygdala (Fig. 5I). However, the effects on 5-HT_{1A} receptor expression were not replicated, perhaps reflecting an effect of the prior single high dose psilocybin-treatment.

We next assessed levels of synaptic vesicle protein 2A (SV_2A: a proposed marker for synaptic density) using *in vitro* [^{3}H]UCBJ autoradiography in adjacent brain sections from cohort 2 (Fig. 6L). This analysis revealed a 60% increase in the specific binding ratio of [^{3}H]UCBJ in PVT (P<0.001) of psilocybin-treated animals compared with controls, with no change in the hippocampus or cingulate cortex (Fig. 6M). As 5-HT_7 receptors and SV_2A are both located presynaptically, the combined results suggested increased synaptic inputs to the PVT following repeated low doses of psilocybin.

DISCUSSION

Microdosing of psychedelic compounds presents a novel concept for neuropsychiatric treatment that remains largely underexplored. Most such studies in humans have been open-labeled observational and retrospective studies, as recently reviewed by Polito et al. *(6)*. Results of the few prospective placebo-controlled studies to date have been casting doubt on their efficacy *(9, 27)*. Major issues in those studies were the confounding effects of expectation and the lack of effective blinding. Animal experiments can eliminate biases from expectations and subject blinding, thereby providing an objective understanding of drug effects. Furthermore, many of the human observational studies have not considered the pharmacology and pharmacodynamics of different psychedelic serotonin agonists. It should be noted that only male rats were used in this study; as such, we present no information on sex differences.

Based upon human drug-occupancy data *(11)* and reported dosing regimens *(6, 7)*, we designed and validated a 3-week psilocybin treatment regimen in rats. Our choice of 0.05 mg/kg psilocybin (s.c.) every second day was based on our initial finding of approximately 20% occupancy of 5-HT_{2A} receptors *in vivo*, absence of wet-back shakes, and no receptor desensitization at the end of the treatment regimen. We further showed that 20% occupancy of 5-HT_{2A} receptors with psilocin is likely associated with substantial 5-HT_{2C} receptor activation, which may even exceed 5-HT_{2A} activation. A small partial agonism at 5-HT_7 receptors might also occur, but we can exclude important effects at 5-HT_{1A} receptors. While not assessed in our study, previous findings for 5-HT_{2B} receptors suggest lower activation by psilocin compared to 5-HT_{2A} and 5-HT_{2C} sites *(14)*.

Our behavioral studies found several interesting findings. Importantly, we did not detect effects of repeated low doses of psilocybin on anxiety or related behaviors (open-field center and elevated plus maze). To the best of our knowledge, only one prior study has tested for anxiolytic effects of repeated low doses of psilocin in rats *(31)*. In that study, Horsley et al. found increased anxiety in rats treated with 3 intermittent doses of 0.05 or 0.075 mg/kg psilocin every second day, with a two-day window before testing the animals in the elevated plus maze. Another study found that low doses of dimethyltryptamine (DMT, another serotonergic psychedelic compound) did not alter trait anxiety as indexed by the elevated plus maze or the novelty-induced locomotion tests *(32)*; while they did not establish the 5-HT_{2A} receptor occupancy by DMT, the rats in that study did not show any overt behavioral signs of psychedelic effects. The previously reported findings support the lack of effects on trait anxiety with repeated low doses of psilocybin. Effects of repeated low doses of psilocybin on anxiety in humans are more mixed, with either decreased or increased anxiety in healthy people *(28, 30, 33)*, but such studies are vulnerable to bias regarding anxiety that may arise from the use of controlled substances. Yet, the mixed findings in humans generally concur with present findings, where psilocybin-treated rats did not exhibit improved center entry in the open-field test nor open-arm explorations in the elevated plus maze.

Self-grooming is a stereotypic behavioral phenotype that manifests in animal models of obsessive-compulsive disorder (OCD) *(23, 34)*. We found a reduction in grooming frequency with no change in total grooming time in animals treated with repeated low doses of psilocybin and replicated that finding in the second experiment where we assessed grooming during the elevated plus maze test. While we did not analyze the microstructure of their grooming sequences in detail, the present

results suggest that the reduced grooming frequency with no change in grooming time results from longer but less frequent grooming sequences with fewer distractions. A case study on the use of low doses of psilocybin in an individual with OCD showed remarkable long-term amelioration of compulsive symptoms *(35)*, and a clinical study with low to high doses of psilocybin in nine OCD patients found a reduction in their compulsive symptoms *(36)* with no difference in efficacy of increasing dose. While more studies are needed to better understand the effects of repeated low doses of psilocybin on compulsive and perseverative behavior, these few clinical studies do support our findings.

High and repeated doses of serotonergic psychedelics are known to modulate pre-pulse inhibition of the acoustic startle reflex and have been used to model this schizophrenia endophenotype *(19, 21, 37)*. We found no effects of the psilocybin treatment regimen on pre-pulse inhibition or startle amplitude, and no effect on short-term habituation to the startle reflex. Short-term habituation is attributed to focus and attention, and its disruption can lead to increased distraction from incoming stimuli (as in schizophrenia). Several clinical studies have reported a reduction in mind-wandering and increased focus in groups of healthy people who have microdosed *(6, 28)*. Our findings show that repeated low doses of psilocybin did not induce deficits in pre-pulse inhibition, startle reflex or short-term habituation, contrary to what other reportsith high doses *(19, 37)*.

Several studies report elevated mood in healthy, non-depressed humans who self-medicated with serotonergic psychedelics at non-hallucinogenic doses *(6, 28–30)*. The present sucrose preference test showed that the psilocybin-treated rats were more resilient to the stress of repeated s.c.

injections compared to controls, and that an immediate increase in sucrose preference was present following the start of psilocybin treatment. Since both behavioral changes normalized after the end of the psilocybin treatment regimen, this may be an acute drug effect, without long-term benefit. Sucrose preference is only a single measure of the anhedonic phenotype present in depression, which may not manifest to the same degree in healthy rats. As such, measurements of effects on depression should be validated in animal models of depression, such as chronic mild stress or corticosteroid injection.

Although high doses of psilocybin evoke 5-HT_{2A} receptor down-regulation *(22)*, we did not find any changes in 5-HT_{2A} or 5-HT_{2C} receptor expression following the microdose regimen. We initially set out to analyze the low-affinity targets reported for psilocin *in vitro*. Despite findings predicting little or no engagement of 5-HT_{1A} and 5-HT_7 receptors at our dose, we carried out post-mortem receptor quantification of these targets. While 5-HT_{1A} receptor density was unaffected, we found regioselective increases in 5-HT_7 receptor levels in the paraventricular nucleus of the thalamus (PVT) in both experiments with psilocybin-treated rats. The 5-HT_7 receptor is mostly located presynaptically on the axon terminals of glutamatergic pyramidal cells *(38)*, and it could therefore be considered a proxy for increased corticothalamic glutamatergic synaptic density in the PVT. Our observation of increased SV_2A expression levels specifically in the PVT supports the conjecture of increased glutamate synaptic density following repeated low doses of psilocybin.

The 5-HT_7 receptor is a target for second-generation antipsychotic and antidepressant medications *(39, 40)* and is involved in circadian rhythm *(41)*. Online surveys in humans who microdose

psychedelics have reported changes in sleep patterns *(6)*, which might conceivably be mediated by increased 5-HT_7 receptor levels in the PVT, as seen in the psilocybin-treated rats. The PVT is innervated by serotonergic neurons from the dorsal raphé nucleus *(42)*, and by descending axons arising from pyramidal neurons in the frontal cortex in humans *(43)* and rats *(44)*. Increased input from these structures might lead to locally increased synaptic density through cortico-thalamic long-term potentiation *(45)*. The PVT projects mainly to the extended striatum, whereby it plays a major role in motivational behavior *(46)* and approach-avoidance behaviors where reward and avoidance are in conflict *(44, 47)*. Humans who microdose psilocybin often report positive effects on motivational behaviors such as social behaviors, substance use, and compulsive behaviors. Motivation is frequently cited as a precondition for obtaining beneficial effects from psychedelic microdosing *(48),* especially in the contexts of smoking cessation or reducing compulsivity, behaviors where the conflict between approach and avoidance is a key aspect *(28, 29)*; So, to say, you know it is bad for you, but you still want it.

Altogether, we report that repeated low doses of psilocybin in rats imparted resilience to stress-induced anhedonia and lower compulsive actions, together with increased neuroreceptor expression and synaptic density, possibly through long-term activation, in the PVT. The present results substantiate many anecdotal and self-reported claims from psychedelic microdosing in recent years and point to a possible mechanism involving the PVT. Furthermore, these results may indicate a therapeutic potential of repeated low doses of psilocybin in cases where approach and avoidance motivations are in conflict.

1

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Data and materials availability: All data and statistical analysis, can be found at our Open Science Framework website https://osf.io/gy6m9/.

Tables

Table 1: Psilocin pharmacokinetics

					Psilocin			
Receptor	Ligand	Region	K_d	C	IC_{50}	K_i	EC_{50}	R_{max}
5-HT_{1A}	[^{3}H]WAY100635	HIPP	6.04	7.5	179	80 ± 28	727 ± 45	22 ± 9
5-HT_{2A}	[^{3}H]Cimbi-36	PFC	0.23	0.5	51	15 ± 2	36 ± 7.7	68 ± 1
5-HT_{2C}	[^{3}H]Cimbi-36	CP	0.96	0.5	18	12 ± 8	11 ± 0.3	71 ± 1
5-HT_7	[^{3}H]SB269970	THAL	0.78	5.0	3935	524 ± 272	45 ± 59	13 ± 3

Values are in nM (± SD when noted), R_{max} values are given are % of R_{max} for a full agonist/serotonin at the receptor. The data are based on 2-4 individual experiments. HIPP = hippocampus; PFC = prefrontal cortex; CP = choroid plexus; THAL = thalamus

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Figure text

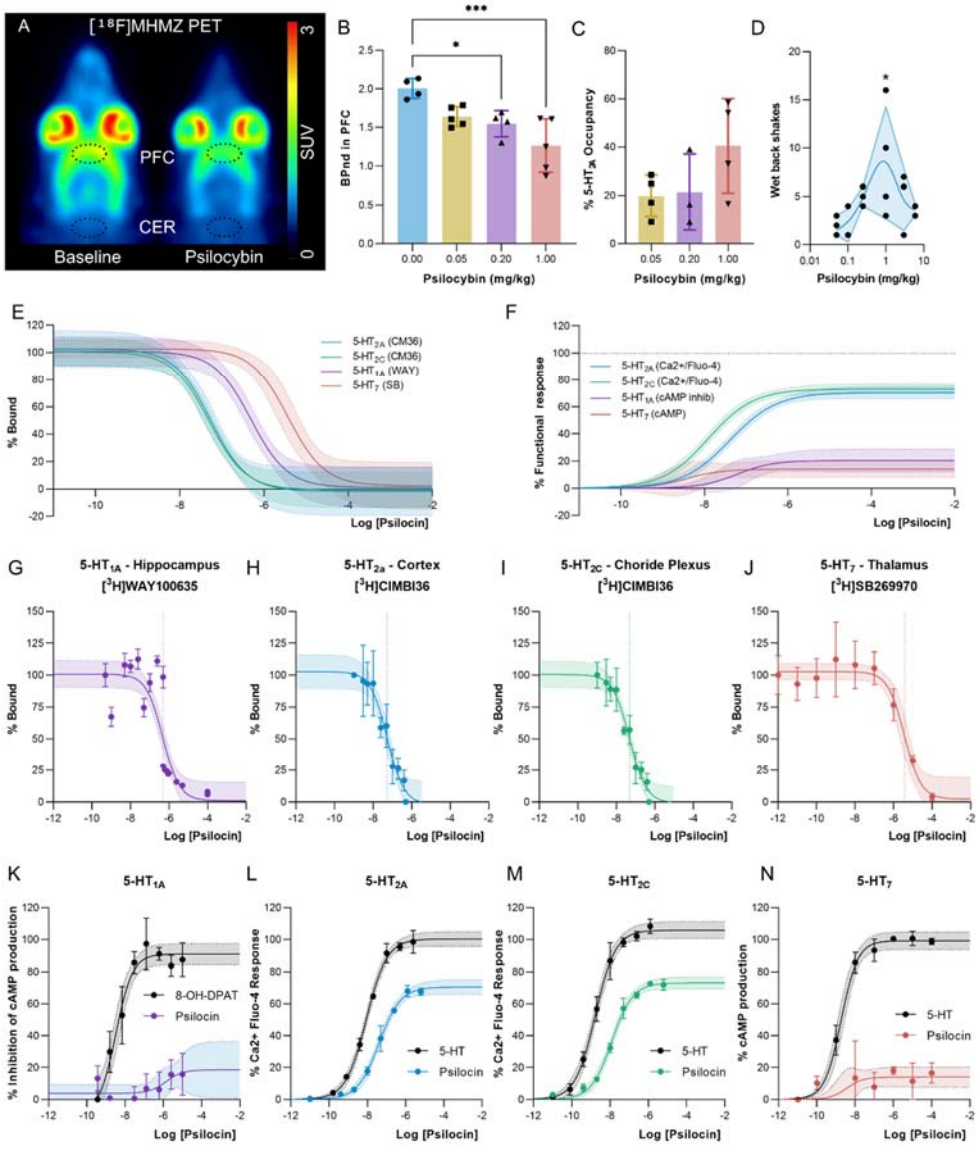

Fig. 1. Pharmacokinetics of low doses of psilocybin. (A) *In vivo* PET imaging of 5-HT_{2A} occupancy by psilocybin (1 mg/kg) measured with [^{18}F]MHMZ, PET images are an average of 20-45 minutes post injection. **(B)** Dose-dependent decline in non-displaceable binding potential (BP_{ND}) in the prefrontal cortex (PFC), a region high in 5-HT_{2A} receptors. **(C)** Dose-dependent occupancy of the 5-HT_{2A} receptor and **(D)** U-shape induction of wetback shakes. Wetback shakes are fitted to an inverted U-shape curve for graphical presentation. **(E+G-J)** Comparable dose-dependent *ex vivo* autoradiographic displacement of radioligands from selected receptor targets, 5-HT_{1A} (against the antagonist [^{3}H]WAY 100635, 5-HT_{2A} and 5-HT_{2C} (in different receptor-specific regions against the 5-HT_{2A} and 5-HT_{2C} agonist [^{3}H]Cimbi36), and 5-HT_7 (against the antagonist [3H]SB269970) in regions with high receptor availability. (**F+K-N**) Comparable *in vitro* dose-dependent efficacy at selected receptor targets, 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_7.

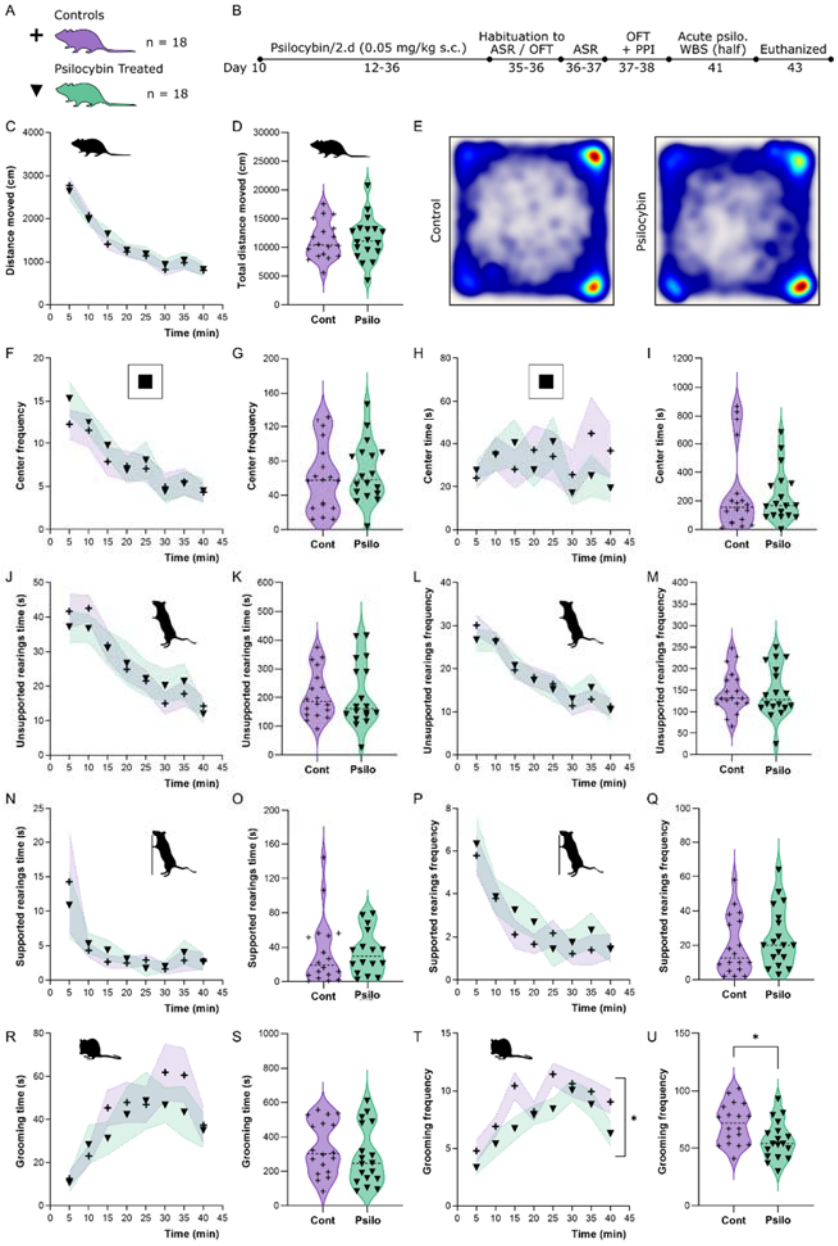

Fig. 2. Repeated low doses of psilocybin reduces compulsive behavior of rats in a familiar open-field environment. (A+B) Experiment setup and timeline. **(C-E)** No differences in locomotion over the timecourse or in totalt. No difference in entries into the center square **(F+G)**, **(H)** time spend in the center (H-I), or rearings, either unsupported **(J-M),** or supported **(N-Q)** following psilocybin microdosing. **(I-J)** Self-grooming increase over time and peak at 15-35 minutes in both psilocybin-treated and control animals, with a lower frequency **(T+U)** in animals treated with repeated low doses of psilocybin. ASR: Acoustic startle response, OFT: Open field test, PPI: Pre-pulse inhibition, WBS: Wet back shakes.

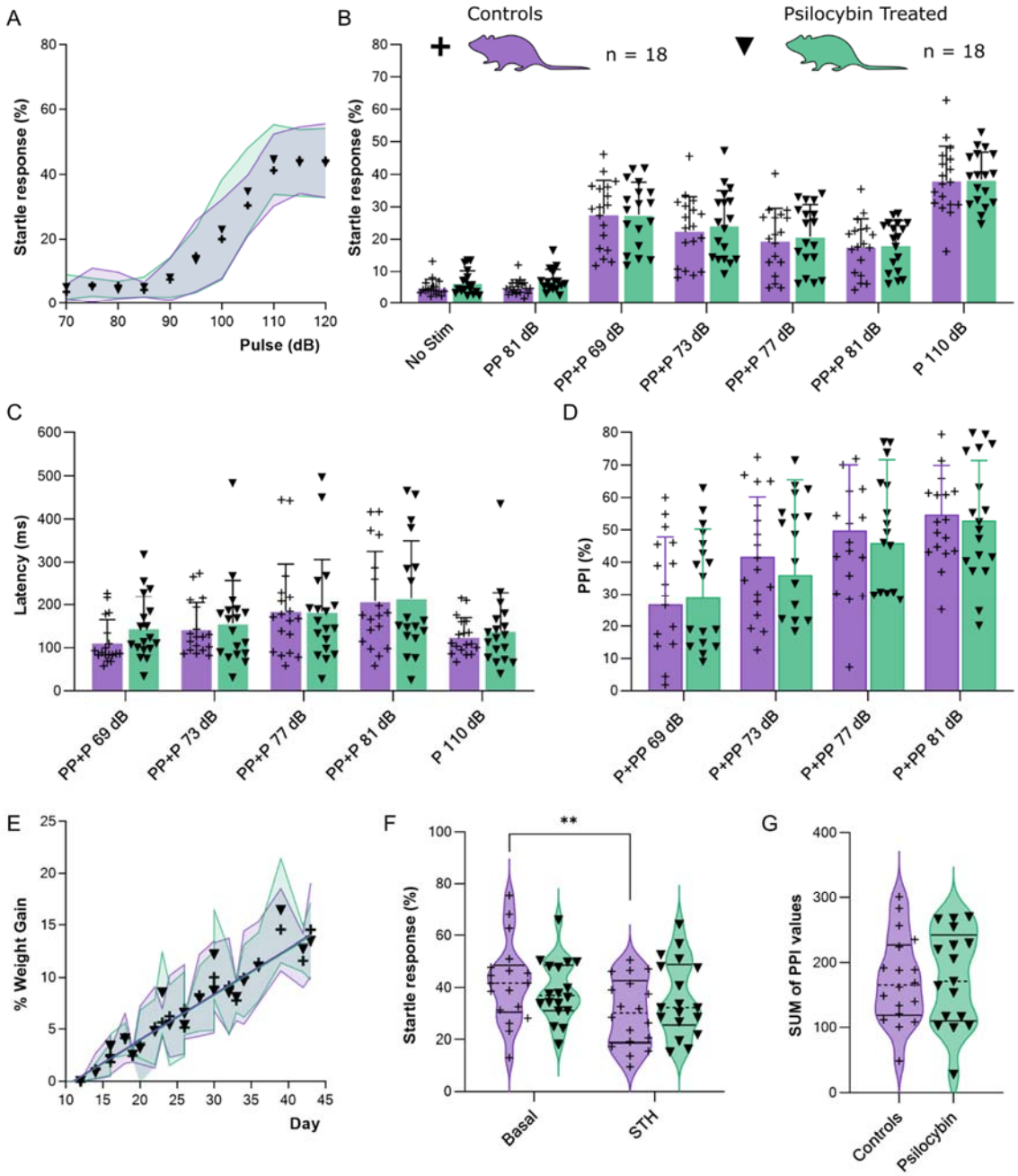

Fig. 3. Repeated low doses of psilocybin does not induce deficits in pre-pulse inhibition (PPI) of the startle reflex in the rat. (A) No change in startle response following increasing intensity sound stimuli, **(B)** following different pre-pulse intensities, **(C)** latency to startle, **(D)** calculated pre-pulse inhibition to individual or SUM of **(G)** PPI values, following psilocybin treatment. **(E)** Animals had similar weight gain yet control animals were more prone to **(F)** short-term habituation compared to the psilocybin-treated animals. P: Pulse, PP: Pre-pulse, STH: Short-term habituation.

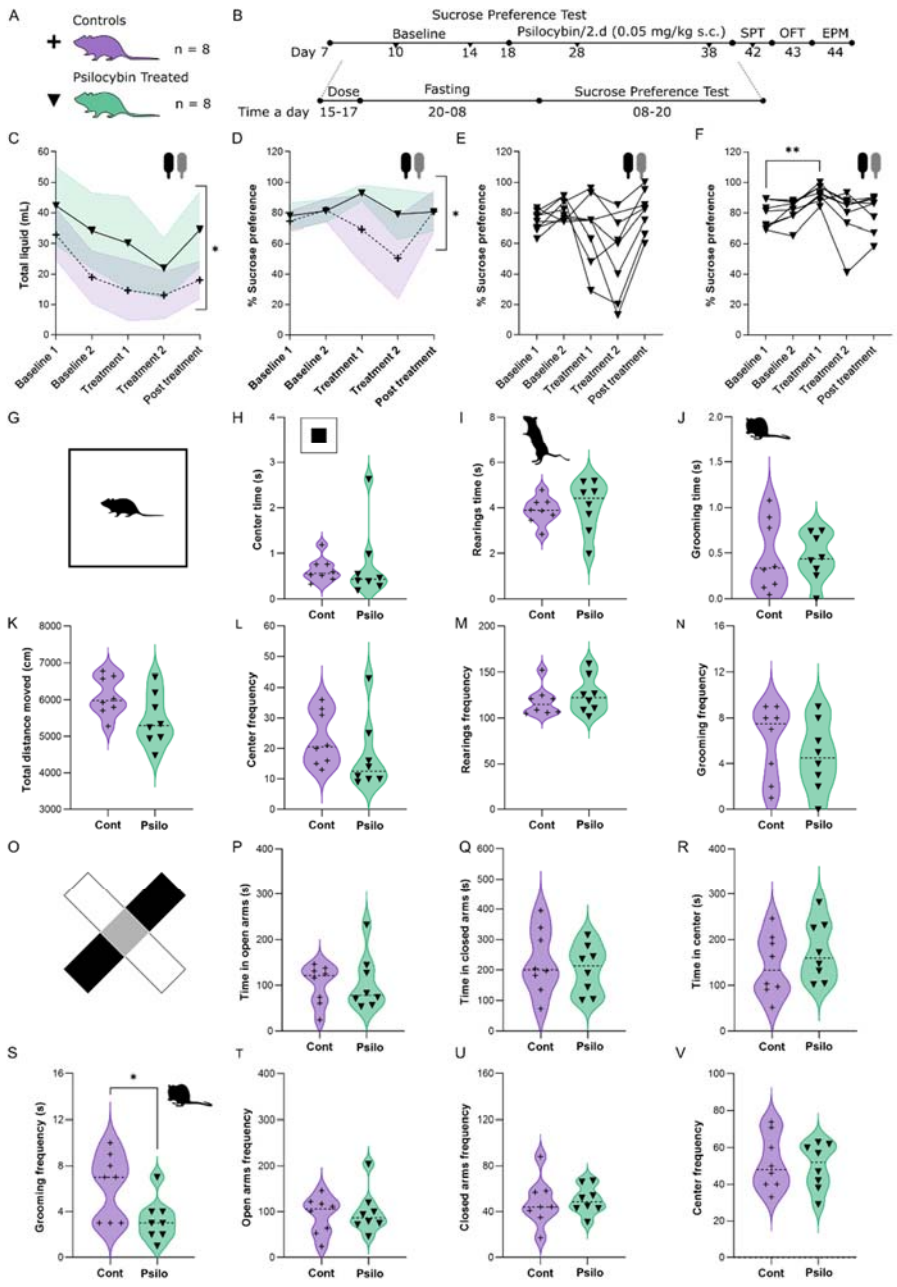

Fig. 4. Repeated low doses of psilocybin increases resiliance to stress-induced anhedonia, but does not change trait anxeity in rats in a novel environments. (A+B) Setup of experiment. **(C-F)** Sucrose preference of rats at baseline during treatment with repeated s.c. injections, and 2 days following the last s.c. treatment. (marked with ▼). **(E)** Animals that did not receive repeated psilocybin were prone to anhedonic responses in the sucrose preference test. **(G-N)** No changes in open-field behavior, or in **(O-R + T-V)** elevated plus maze. **(S)** Self-grooming frequency was reduced in animals who received repeated low doses of psilocybin compared to controls. SPT: Sucrose preference test, OFT: Open field test, EPM: Elevated plus maze.

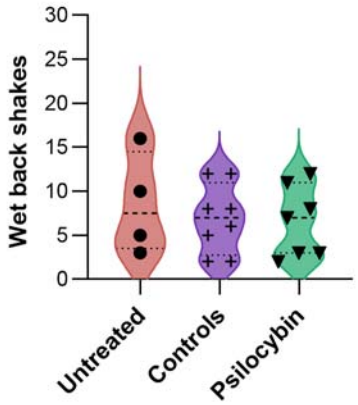

Fig 5: Wet-back shakes in rats after 1 mg/kg psilocybin.

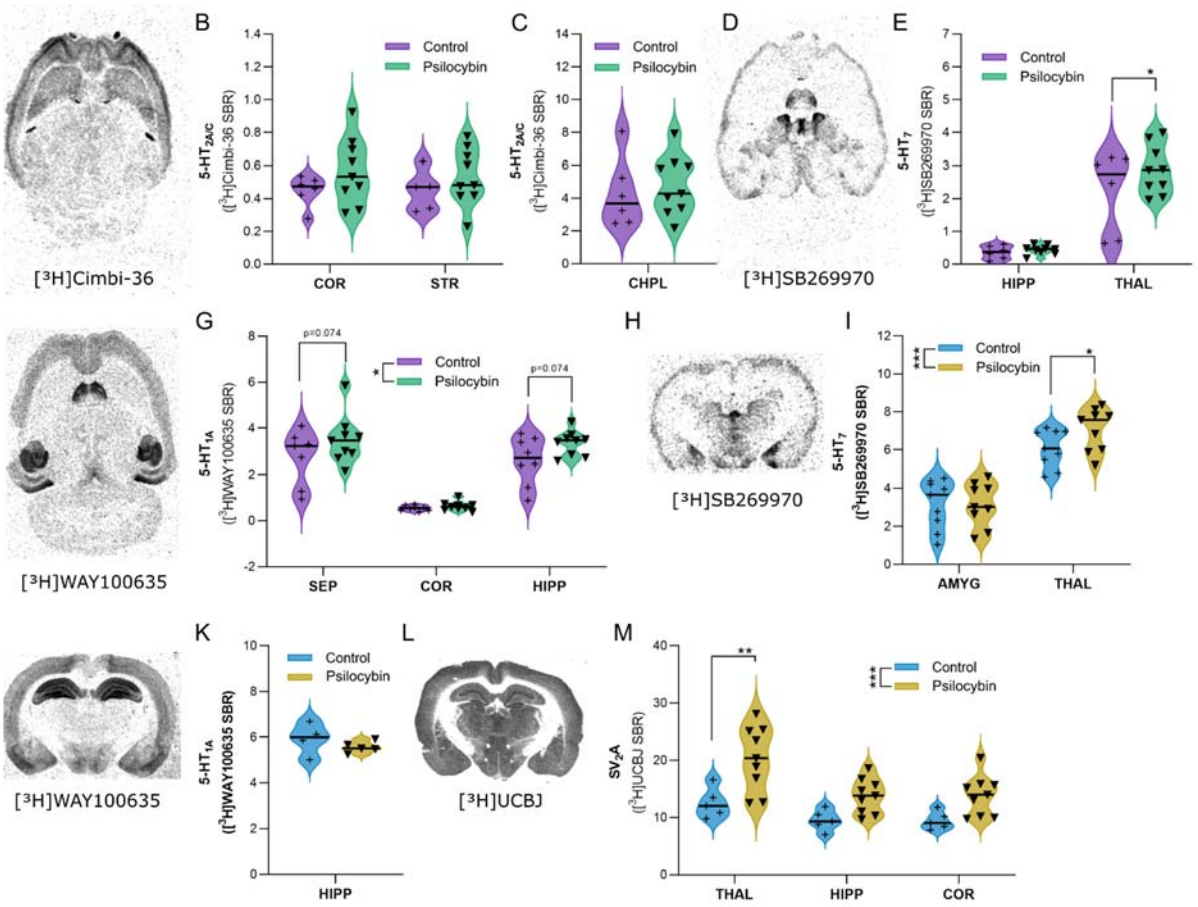

Fig. 6. Repeated low doses of psilocybin increases receptor levels and synaptic density in the paraventricular thalamic nucleus (PVT) of the rat. (**A-G**) Purple and green represents one cohort of animals, while (**H-M**) blue and yellow represents a different cohort of animals, both cohorts are from experiment 1. **(A)** 5-$HT_{2A/C}$ receptor distribution ([^{3}H]CIMBI-36), *postmortem* levels of 5-HT_{2A} **(B)** and 5-HT_{2C} **(C)** does not change following repeated low doses of psilocybin. **(D+H)** 5-HT_7 receptor distribution ([^{3}H]SB269970), **(E+I)** *postmortem* levels of 5-HT_7 are increased in the PVT in psilocybin-treated animals. **(F+J)** 5-HT_{1A} receptor distribution ([^{3}H]WAY 100635), *postmortem* levels of 5-HT_{1A} are increased following a high dose **(G)** but not repeated low doses of psilocybin **(K)**. **(L)** SV_2A distribution, a proxy of synaptic density as measured by [^{3}H]UCBJ autoradiography in rat brain. **(M)** SV_2A levels in the PVT are higher in animals that received repeated low doses of psilocybin compared to control rats.COR: Cortex, STR: Dorsal Striatum, CHPL: Choride Plexus, HIPP: Hippocampus, THAL: Thalamus, SEP: Septum, AMYG: Amygdala.