Psilocybin prevents activity-based anorexia in female rats by enhancing cognitive flexibility: contributions from 5-HT1A and 5-HT2A receptor mechanisms.

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Abstract

Psilocybin has shown promise for alleviating symptoms of depression and is currently in clinical trials for the treatment of anorexia nervosa (AN), a condition that is characterised by persistent cognitive inflexibility. Considering that enhanced cognitive flexibility after psilocybin treatment is reported to occur in individuals with depression, it is plausible that psilocybin could improve symptoms of AN by breaking down cognitive inflexibility. A mechanistic understanding of the actions of psilocybin is required to tailor the clinical application of psilocybin to individuals most likely to respond with positive outcomes. This can only be achieved using incisive neurobiological approaches in animal models. Here, we use the activity-based anorexia (ABA) rat model and comprehensively assess aspects of reinforcement learning to show that psilocybin (post-acutely) improves body weight maintenance in female rats and facilitates cognitive flexibility, specifically via improved adaptation to the initial reversal of reward contingencies. Further, we reveal the involvement of signalling through the serotonin (5-HT) 1A and 5-HT2A receptor subtypes in specific aspects of learning, demonstrating that 5-HT1A antagonism negates the cognitive enhancing effects of psilocybin. Moreover, we show that psilocybin elicits a transient increase and decrease in cortical transcription of these receptors (*Htr2a* and *Htr1a*, respectively), and a further reduction in the abundance of *Htr2a* transcripts in rats exposed to the ABA model. Together, these findings support the hypothesis that psilocybin could ameliorate cognitive inflexibility in the context of AN and highlight a need to better understand the therapeutic mechanisms independent of 5-HT2A receptor binding.

Introduction

AN is characterised by pathological weight loss driven by restrictive feeding and excessive exercise behaviours, has the highest mortality rate of any psychiatric disorder [1], and is the leading cause of death in females aged 15-24 [2]. Cognitive inflexibility may be a trait marker of vulnerability to AN, considering that dysfunction arises before the onset of symptoms [3] and persists after weight recovery [4]. Impairments in cognitive flexibility have been consistently seen in AN patients [5-8], and are associated with low quality of life [9], making this symptom a primary target for AN therapeutic intervention. Cognitive flexibility is a fundamental element of executive functioning that allows for behavioural adaptation to a variable environment, and as a consequence, is associated with favourable outcomes throughout the lifespan [10]. This capability is compromised across a range of neuropsychiatric disorders that include but are not limited to; depression and anxiety disorders, substance-use disorders, obsessive-compulsive disorder and anorexia nervosa (AN) [11]. In each of these conditions, psilocybin-assisted therapy [12-15] has either been shown to elicit positive outcomes or is being currently trialled.

Converging evidence from clinical trials and preclinical studies indicates that psilocybin is an effective treatment for symptoms of several psychiatric disorders [16]. However, there is little evidence to date that disentangles its pharmacological efficacy from the clinically-guided psychological intervention that accompanies psilocybin exposure in these trials [17]. Moreover, while the pharmacological actions of psilocybin are now better understood [18, 19], how these actions translate to therapeutic outcomes remains unclear. Based on the proposal that the therapeutic effects of psilocybin relate to the promotion of flexible thinking and relaxation of maladaptive, rigidly held beliefs [12], and the evidence that psilocybin elicits long lasting effects on cognitive and neural flexibility [20], it seems likely that at least some aspects of therapeutic efficacy may be driven by enhanced cognitive flexibility. However, the neurobiological mechanisms through which psilocybin acts to improve cognitive flexibility are unknown, and there are multiple components of learning and cognition that could contribute to enhanced flexible thinking and behaviour after psilocybin treatment that have not been systematically addressed.

There is evidence implicating serotonin (5-HT) dysfunction in AN, with positron emission tomography (PET) imaging studies revealing decreased binding to the 5-HT2A receptor (5-HT2AR) subtype [21] and increased binding to the 5-HT1A receptor (5-HT1AR) subtype [22] in the frontal cortex of patients. Psilocybin is an agonist for both receptor subtypes [23], raising the intriguing possibility that psilocybin could rescue or reverse cognitive inflexibility by re-establishing the balance of 5-HT signalling in those with AN. Whether or not psilocybin has therapeutic effects in individuals with AN will be revealed by ongoing clinical trials (e.g., NCT04052568, NCT04661514, NCT05481736, NCT04505189). However, these trials are not capable of testing the mechanisms through which psilocybin acts to elicit improvements in symptoms; moreover, they have been criticised in recent

years for methodological constraints most notably their inability to blind participants to treatment conditions, which can bias outcomes in line with expectancy effects [24, 25].

Preclinical studies in animal models are critical for advancing the understanding of the behavioural and pharmacological mechanisms underlying the therapeutic effects of psilocybin [26], with evidence converging on increased neuroplasticity as a key driver of beneficial outcomes [27, 28]. Unfortunately, efforts in this space have focused largely on traditional assays of depression-related behaviour in rodents [29], with variable findings of either improvements [30] or no effects [31], dependent on the assay or animal model used [32]. Given the growing appreciation in behavioural neuroscience that these types of behavioural tests (i.e. the forced swim test) do not reliably translate to human depressive syndromes [33] and that reinforcement learning tasks offer key advantages including more relevant clinical links and repeatability [34], these early approaches clearly need to be redressed. Other key methodological details in prior studies need to be considered, particularly the role of multiple dosing (cross-over) designs, antagonising 5-HT2AR with ketanserin (a compound with many known non-serotonergic binding sites [35]), and the measurable motoric side effects of acute psilocybin administration [36].

The investigation of neurochemical or neural circuit substrates of these effects centre around the actions of psilocybin on the serotonin-2 (5-HT2) receptor subtypes [30, 37-41] but the evidence for the role of 5-HT2AR in rodent cognitive flexibility is conflicting, where acute activation either impairs [42] or has no effect on performance [43]. Less attention has been paid to the possibility that actions at other 5-HTRs might mediate cognitive effects of psilocybin, despite 5-HT2A *independent* effects seen for alleviation of depression-like behaviour [30], dendritic spine formation [44], and neuronal synchronicity [45]. It is likely that specific aspects of psilocybin-induced cognitive flexibility involve other 5-HT receptors [46, 47] and their integration with other neuromodulatory systems, most notably dopamine [48-50]. The challenge in identifying the neuronal substrates for improved flexibility after psilocybin is heightened when attempting to understand whether there may be disorder-specific effects in individuals AN [51, 52], who present with disturbed 5-HT function that remains inadequately understood.

In the present study, our objective was to comprehensively investigate how psilocybin, in a 5-HT receptor-dependent manner, may alter some of the core components that underlie cognitive flexibility, such as incentive motivation and task engagement [53], response inhibition [53], and reward efficacy [54]. All animals received psilocybin only once, with or without prior administration of selective 5-HT1A and 5-HT2A receptor antagonists, and learning outcomes were assessed post-acutely, when response profiles are not impacted by motor side effects. In addition, we used the most well-established rodent model of AN, activity-based anorexia (ABA) [55], that elicits rapid body weight loss combined with paradoxical hyperactivity [56] to determine whether psilocybin has differential effects on 5-HTR function in the context of eating pathology. ABA rats exhibit impairments in cognitive flexibility on both reversal learning [57] and attentional-set shifting tasks [58], which is rescued by

74 suppressing cortico-striatal circuitry [59] a key site of psychedelic drug action [60]. Finally, we assessed
75 psilocybin-induced alterations in the abundance of 5-HTR mRNA transcripts in the prefrontal cortex
76 to determine the time-course of effects as well as its impact following the development of the ABA
77 phenotype. Together, these studies reveal specific roles of 5-HT receptor subtypes in enhanced
78 flexible learning after psilocybin and point towards a molecular mechanism that may underpin the
79 efficacy of psilocybin for treating symptoms of AN.

80 Methods

81 Animals and housing

82 All animals were obtained from the Monash Animal Research Platform (MARP; Clayton, VIC,
83 Australia). To assess direct effects of psilocybin on the development of the ABA phenotype, female
84 Sprague-Dawley rats (*n*=35 behaviour; *n*=12 RNAscope) were 6 weeks of age on arrival in the
85 laboratory. Young female rats were used in these studies because they are particularly vulnerable
86 to developing the ABA phenotype, a feature that is incompletely understood but has translational
87 relevance to the increased prevalence of AN in young women. In order to asses cognitive and
88 behavioural phenotypes relevant to AN/ABA, we used separate cohorts of aged matched female
89 Sprague-Dawley rats (total *n*=168) that commenced training at 7 weeks of age (see **Supplementary**
90 **Table 1** for details). To examine the effects of psilocybin on 5-HTR subtype abundance across a
91 time course, an additional cohort (*n*=19) of female Sprague-Dawley rats were used, with
92 administration matched to behavioural cohorts at 8 weeks of age. In all cases, animals were group-
93 housed and acclimated to the 12h light/dark cycle (lights off at 1100h) for 7 days in a temperature
94 (22-24 °C) and humidity (30-50 %) controlled room before experiments commenced. Because the
95 behavioural aspects of ABA (i.e. wheel running and food intake) as well as aspects of reinforcement
96 learning are known to fluctuate with the oestrous cycle in female rats [61, 62], a male rat was individually
97 housed in all experimental rooms at least 7 days prior to experimentation in order to facilitate
98 synchronisation of cycling, known as the Whitten Effect [63]. All experimental procedures were
99 conducted in accordance with the Australian Code for the care and use of animals for scientific
100 purposes and approved by the Monash Animal Resource Platform Ethics Committee (ERM 29143).

101 Pharmacological compounds

102 Psilocybin (USONA Institute Investigational Drug Supply Program; Lot# AMS0167) was dissolved in
103 saline and administered at a dose of 1.5 mg/kg. Ketanserin tartrate (Tocris Biosciences, CAS 83846-
104 83-7; 1.5 mg/kg), MDL100907 (volinanserin; Sigma-Aldrich, CAS 139290-65-6; 0.1 mg/kg),
105 WAY100635 maleate (Tocris Biosciences, CAS 1092679-51-0; 0.5 mg/kg) serotonin receptor
106 subtype antagonists were administered 30 min before psilocybin (or 0.9% NaCl saline control)
107 treatment and all animals only received one combination of psilocybin/saline and one receptor
108 subtype antagonist. Dose selection was based on the literature [44, 64-66]. All drugs were administered
109 intraperitoneally at a 1.0 ml/kg injection volume using a 26-guage needle.

110 **Activity-based anorexia (ABA)**

111 The ABA paradigm consists of unlimited access to a running wheel and time-restricted food access.
112 At seven weeks of age, rats were individually housed in transparent living chambers with a
113 removable food basket and a running wheel (Lafayette Instruments, IN, USA). Rats were allowed to
114 habituate to the wheel for seven days to determine baseline running wheel activity (RWA). The
115 following day, psilocybin or saline was administered, wheels were locked for 5h and then reopened.
116 Running activity was recorded by the Scurry Activity Wheel Software (Lafayette Instruments, IN,
117 USA). During the ABA period, food access was restricted to 90 min/day at the onset of the dark
118 phase (1100-1230h). Running in the hour before the feeding window (1000-1100h) was considered
119 as food anticipatory activity (FAA). Time-restricted food access persisted for a maximum of 10 days
120 or until rats reached <80 % of baseline body weight (ABA criterion), at which point they were
121 euthanised with 300 mg/kg sodium pentobarbitone (Lethabarb; Virbac, Australia).

122 **Home-cage operant learning paradigms**

123 Open-source Feeding Experimentation Devices (Version 3), known as "FED3" [67], were used for
124 home-cage operant testing, fitted with custom built masks. The task wall consisted of two nose-poke
125 ports situated on either side of a pellet magazine where pellets were delivered with a motorised
126 dispenser. Both operant ports and magazines were fitted with infra-red beams to detect nose-pokes
127 and pellet collection, and were controlled by a commercial microcontroller with data displayed on
128 screen for user feedback. An LED strip underneath the nose-poke ports was used as a light cue.
129 The firmware for FED3 devices were written in the Arduino language, modified from the available
130 Arduino library (https://github.com/KravitzLabDevices/FED3) and flashed in sets of operant training
131 menus (https://github.com/Foldi-Lab/LKM_FED3-tasks).

132 Following light cycle acclimation, rats were individually housed (26cm W x 21cm H 47.5cm D) with
133 *ad libitum* access to water and standard laboratory chow (Barastoc, Australia) throughout. Rats were
134 habituated to sucrose pellet rewards (20 mg, AS5TUT; Test Diet, CA, USA) for two days prior to
135 training. Operant testing was conducted once daily in the home cage for a 3h session between 12:00-
136 15:00 (early dark phase), which began with two days of magazine training on a "free feeding"
137 schedule in which a pellet was dispensed each time one was removed from the magazine.
138 Subsequently, rats were trained to poke for rewards at fixed ratio (FR) schedules (FR1, FR3, FR5)
139 for 2-5 days each until high accuracy (>80% target responding) was achieved. The target side for all
140 experiments was counterbalanced across each cohort to control for any inherent side bias due to in
141 cage FED3 position. Between animal variability in training performance was always balanced
142 between treatment groups and any animals failing to learn the penultimate training step were
143 removed from the experiment before drug administration.

144 *Between-session reversal learning task*

To test the effects of psilocybin on cognitive flexibility, saline or 5-HTR antagonists (pre-treatment) were administered 30 min prior to either saline or psilocybin (treatment), at the completion of the final FR5 training session. The following day (18h post-administration) the reward contingencies of the nose-poke ports were reversed (un-cued), and rats underwent 3 days of testing on the reversed FR5 schedule.

Fixed and variable ratio schedule training and extinction

To test the effects of psilocybin on suppression of learned FR responding, saline or psilocybin was administered immediately following the final FR5 training session. Over the next 3 days rats underwent extinction testing in which the FED3 was provided as usual except no rewards were delivered regardless of animal activity. To test the effects of psilocybin on training under variable reward schedules, and the long-lasting effects on response suppression, rats were trained to nose-poke at FR1 for 4 days FR1 after which saline or psilocybin was administered. The following day rats were trained at variable ratio (VR) schedules of VR5, VR10 and VR20 (two days on each schedule), where the number of target pokes required to deliver a pellet on each trial was randomly selected from 1-5, 6-10 or 11-20, respectively. Subsequently, rats underwent 2 consecutive days of extinction testing, with 24h access to the inactive FED3 device.

Progressive ratio and re-setting task

To test the effects of psilocybin on motivated (effortful) responding, saline or psilocybin was administered at completion of the final FR5 training session and the next day rats underwent a progressive ratio (PR) reinforcement schedule, where the exponential schedule increased according to the formula (5 * e(0.2*n)- 5), where *n* is the trial number, producing response requirements of 1, 2, 4, 6, 9, 12 etc., to the nearest whole number. This was followed by a session at FR5 to reinstate responding and a session during which the PR schedule reset to 1 following any 10-minute period of FED3 inactivity, called a re-setting progressive ratio (R-PR) task.

5-HT receptor subtype abundance

For detection and quantification of 5-HTR subtypes, rats were administered psilocybin or saline and euthanized with sodium pentobarbitone (Lethabarb 150 mg/kg; Virbac, AU) at a time course (6, 12 or 24h) post-administration. ABA rats underwent exposure to the model as described above and were administered psilocybin or saline after they had lost at least 15% baseline body weight (15.1-17.6%). Six hours later they were euthanized as above and all rats were transcardially perfused with 200 mL 0.9% saline followed by 200 mL 4% paraformaldehyde in phosphate buffer. Brains were excised and postfixed in 4% paraformaldehyde in phosphate buffer solution overnight at 4°C, followed by submersion in increasing concentrations of 10%, 20% and 30% sucrose in phosphate buffer solution across 3 to 4 days. Brains were then sectioned at 15µm using a cryostat (CM1860; Leica Biosystems) and the medial prefrontal cortex (mPFC) was collected in a 1:4 series. Two mPFC sections per animal, from the same series (spanning from bregma, anteroposterior: +3.2 mm to +2.2

181 mm), were placed onto SuperFrost Plus slides, and stored at – 20 °C until used. The RNAscope ™
182 Multiplex Fluorescent V2 detection reagent kit (Advanced Cell Diagnostics, USA) was used
183 according to manufacturer's instructions and included specific *in situ hybridisation* probes
184 complementary to the mRNA of the 5-HT1AR (*Rn-Htr1a*; RDS404801) and 5-HT2AR (*Rn-Htr2a*;
185 ADV424551). Detection of mRNA was achieved using Opal™ fluorophore dyes from the 520 (1:500)
186 and 620 (1:750) reagent packs (Akoya Biosciences, USA). Full protocol details are available in
187 **Supplementary Methods**. Micrographs were captured using a fluorescent deconvolution
188 microscope (Thunder Imager 3D, Leica Microsystems, Germany) using a 40x oil immersion
189 objective, with the infralimbic and prelimbic cortices selected as regions of interest. Images were
190 pre-processed and masked using ImageJ (v1.53t [68]) and objects were fed into CellProfiler (v4, [69]) for
191 quantification of nuclear bodies as well as *Htr1a* and *Htr2a* transcripts (See **Supplementary**
192 **Methods**). Selected sections were analysed further using Imaris software (v9.9, Oxford Instruments)
193 to establish the anatomical location of identified differences in transcript abundance across the
194 cortical layers [70]. The DAPI-channel was used as a mask to define individual cells (cell body
195 selection) and the number of *Hrt1a* or *Hrt2a* puncta surrounding DAPI was analysed using the vesicle
196 detection feature.

197 **Statistical analyses**

198 Statistical analyses were performed using GraphPad Prism 9.5.1 (GraphPad Software, San Diego,
199 CA, USA). Statistical significance was set at $p<.05$, with $p<.01$ considered a trend though not
200 significant. Analyses used are two-tailed unpaired t-test, one-way and two-way analysis of variance
201 (ANOVA) with Bonferroni's, Dunnett's or Sidak's post hoc multiple comparisons, and a mixed-effects
202 model, chosen appropriately considering the type of data, number of groups, and comparisons of
203 interest. Full details of all statistical tests performed (including group composition) can be found in
204 the Statistics Tables in **Supplementary Materials**. For RNAscope analyses each individual animal's
205 data point represents an average value from 4 (individual regions) or 8 (combined regions) sections.

206 Results

207 **Psilocybin improves body weight maintenance in ABA rats**

208 In order to assess the influence of a single dose of psilocybin on subsequent adaptation to conditions
209 of ABA, psilocybin was administered 24h prior to the onset of timed food restriction, which facilitated
210 improved body weight maintenance throughout ABA exposure (**Fig 1A**) and increased the proportion
211 of animals resistant to the paradigm (**Fig 1B**). Psilocybin-treated rats spent significantly more days
212 above 85% of their baseline body weight ($p=.0172$; **Fig 1C**) and although the reduction in average
213 daily weight loss after psilocybin treatment did not reach statistical significance ($p=.0638$; **Fig 1D**),
214 psilocybin prevented severe weight loss associated with ABA ($p=.0394$; **Fig 1E**). This ability to better
215 maintain body weight under ABA conditions was not driven by marked alterations to overall wheel
216 running (**Fig 1F**), with psilocybin and saline treated animals running similar amounts during both

217 baseline and ABA phases (baseline p>.9999, ABA p=.3089; **Fig 1G**) and during the food anticipation
218 period (p=.2800; **Fig 1H)**. Similarly, food intake increased over successive days of ABA exposure
219 regardless of treatment (**I**) and psilocybin did not change the average amount of food consumed
220 across the ABA period (p=.3290; **Fig 1J**). When comparing psilocybin treated rats that were
221 susceptible versus resistant to weight loss, it appeared that psilocybin-induced resistance was not
222 qualitatively distinct from previous work [56, 71], but was similarly defined by both reduced food-
223 restriction evoked hyperactivity (**Fig 1K**) that was specific to running during ABA (baseline p=.6279,
224 ABA p<.0001; **Fig 1L**), increased running in anticipation of food (p<.0001; **Fig 1M**) and increased
225 food intake across days (**Fig 1N**) and the overall ABA period (p<.0001; **Fig 1O**). Notably, the only
226 behavioural feature that predicted improved body weight maintenance after psilocybin treatment was
227 wheel running on the day prior to administration (see Baseline Day 7; **Fig 1K**).

228 **Psilocybin enhances flexible behaviour in a reversal learning task**

229 Considering that the ability to maintain body weight during exposure to ABA in rats has been
230 previously linked to improved cognitive flexibility on a reversal learning task [59] and that exposure to
231 ABA conditions impairs reversal learning [57], we hypothesised that the improvements in ABA after
232 psilocybin were associated with improved flexibility in the present study. Psilocybin was administered
233 18h prior to reversal of reward contingencies (**Fig 2A**), and produced a transient improvement in
234 response accuracy (p=.0312; **Fig 2B**), evidenced by a rapid shift in responding towards the reversed
235 port and an increase in the proportion of rats that reached performance criterion (**Fig 2C**). In order
236 to quantify performance, we used a moving window accuracy (80% accurate, within a 100-trial
237 window) to demonstrate that psilocybin-treated rats required fewer trials to learn the task (**Fig 2D**).
238 Improved performance after psilocybin was not driven by faster acquisition (p=.1474; **Fig 2E**) or
239 altered total (p=.1420; **Fig 2F**) or target responses (p=.5815; **Fig 2G**), but specifically by reduced
240 responding to the non-target (incorrect) port (p=.0260; **Fig 2H**), indicating psilocybin treatment
241 facilitated learning from negative feedback and faster behavioural adaptation, which was also evident
242 in improved reward efficiency (reduced non-target pokes per pellet; see **Supplementary Fig 1F**).
243 While psilocybin did not significantly improve the rate of reward collection (p=.0956; **Fig 2I**), it
244 increased engagement with the reversal task evident in reduced latencies to respond (p=.0332; **Fig**
245 **2J**) and win the first reward (p=.0343; **Fig 2K**). To confirm that this improvement was not related to
246 increased effortful responding or response suppression, we tested separate cohorts of rats on
247 progressive ratio (PR), variable ratio (VR) and extinction tasks. Here, we show that psilocybin
248 administration 18h prior to test did not increase the willingness of rats to expend effort to obtain
249 rewards (p=.4436; **Fig 2L**), the ability to extinguish a previously learned response (p=.5783; **Fig 2M**)
250 or response vigour under uncertain (variable) schedules of reinforcement (p=.2013; **Fig 2N**).
251 Moreover, there was no improvement in response suppression 7 days following psilocybin treatment
252 (p=.6100; **Fig 2O**). See **Supplementary Fig 1** for full session data, including for animals that did not
253 reach performance criterion on the first reversal session.

254 Because classical PR tasks require the test session to be terminated after a 10 min period of
255 inactivity, and yet psilocybin was shown to increase task engagement in the reversal learning task,
256 we were interested to see if psilocybin also acted to restore responding after periods of inactivity.
257 We tested this in two ways; firstly, by allowing animals access to the operant devices for 3h using a
258 standard PR schedule and secondly, by implementing a variation of the PR task in which after any
259 10 min period of inactivity the ratio reset to 1 [re-setting PR (R-PR); see **Supplementary Fig 2A-B**].
260 Breakpoint itself was not different between tasks (all *p's* >.2666; **Supplementary Fig 2C**), however,
261 psilocybin increased task engagement specifically during the R-PR session, when increased
262 engagement is considered economical because the effort required to receive a reward is lower.
263 Moreover, psilocybin-induced task engagement was directed rather than arbitrary, with increases in
264 the number of target but not non-target pokes observed when the ratio reset (PR *p*>.9999, R-PR
265 *p*=.0209; **Supp Supplementary 2D-E**). None of these changes were observed prior to the first re-
266 setting (i.e. first breakpoint; **Supplementary Fig 2I-M**), indicating that experience with the new
267 reward economy was required to elicit increased engagement after psilocybin.

268 **5-HT1AR and 5-HT2AR subtype mechanisms differentially drive psilocybin-induced flexible**
269 **learning**

270 To determine whether psilocybin improved flexibility on the reversal learning task via actions at 5-
271 HT receptor subtypes relevant to anorexia nervosa, selective antagonists to these receptor subtypes
272 were administered 30 min prior to administration of saline (control) or psilocybin and the following
273 day the reward-paired port was reversed (**Fig 3A&K**). For control rats, 5-HT2AR antagonism
274 completely abolished reversal learning capability, with 0% of rats administered the MDL100907
275 compound reaching performance criterion, compared to approximately 53% of rats administered the
276 WAY100635 compound or saline treatment alone (**Fig 3B**). This impairment was driven by all
277 aspects of learning throughout the reversal session, including reduced accuracy (*p*=.0221, **Fig 3C**),
278 rewards obtained (*p*=.0324, **Fig 3D**), target pokes (*p*=.0292, **Fig 3E**) and non-target pokes (*p*=.0048,
279 **Fig 3F**). Importantly, 5-HT2AR antagonism did not cause an impairment in task initiation, since the
280 latency to respond was equivalent across groups (*p*=.4572, **Fig 3G**), although it increased the latency
281 to make a target poke (*p*=.0502, **Fig 3H**), suggesting MDL100907 administration prevented control
282 rats from adapting to the new reward rules. Moreover, the impairment elicited by 5-HT2AR
283 antagonism in control rats was not due to reduced willingness to engage in the task, considering
284 there were no significant changes in the latency to receive the first reward (*p*=.1507, **Fig 3I**) or
285 session duration (*p*=.6048, **Fig 3J**), however, it should be noted that only three MDL100907 treated
286 control rats ever earned rewards. Conversely, 5-HT1A antagonism did not significantly alter most
287 performance measures throughout the test session (all *p*s>.5609; **Fig 3 C-E, G, I, J**) but specifically
288 reduced the number of non-target pokes performed (*p*=.0041, **Fig 3F**) and increased the latency to
289 first target poke *p*=.0244, **Fig 3H**), suggesting administration of WAY100635 allowed rats to learn to

290 the same degree as saline controls with less negative feedback and despite being slower to respond
291 at the initial reversal of reward contingencies.

292 When combined with psilocybin treatment (**Fig 3K**), antagonism of 5-HT1A and 5-HT2A receptors
293 resulted in an opposing pattern of results, with a substantial reduction in WAY100635 treated animals
294 able to learn the task to criterion (15.4%; **Fig 3L**), compared to 55.6% MDL100907 treated and 75%
295 treated with psilocybin alone. This impairment in reversal learning was demonstrated in reduced
296 accuracy (*p*=.0024, **Fig 3M**), rewards obtained (*p*=.0015, **Fig 3N**) and target pokes performed
297 (*p*=.0013, **Fig 3O**), however, 5-HT1A antagonism prior to psilocybin treatment did not alter
298 suppression of responding to the previously rewarded (non-target) side (*p*=.9497, **Fig 3P**) or
299 willingness to initiate a session (*p*=.1636, **Fig 3Q**), although it did increase the latency to poke on
300 the reversed port (*p*=.0212, **Fig 3R**). Compared to psilocybin treatment alone, selective 5-HT2A
301 antagonism reduced the number of non-target pokes (*p*=.0210, **Fig 3P**) but did not significantly alter
302 any other performance measures (all *p*s>.0542; **Fig3 M-T**).

303 **Psilocybin rescues learning impairments induced by 5-HT2AR antagonism potentially via**
304 **preferential actions at 5-HT1AR**

305 This differential impact of 5-HT2A antagonism is highlighted when comparing performance between
306 saline and psilocybin treated animals that all received MDL100907, a large number of which did not
307 reach performance criterion (**Fig 4A**). Whereas selective 5-HT2A antagonism alone (with saline)
308 impaired performance across the board, co-administration of psilocybin rescued impairments in
309 accuracy (*p*=.0079, **Fig 4B**), rewards earned (*p*=.0385, **Fig 4C**) and target responses (*p*=.0436, **Fig**
310 **4D**), potentially via preferential actions at the 5-HT1AR. MDL100907 administration did not cause
311 differential effects on non-target pokes (*p*=.4578, **Fig 4E**), or the latencies to first poke (*p*=.3163, **Fig**
312 **4F**), first target poke (*p*=.6202, **Fig 4G**) or first reward won (*p*=.2428, **Fig 4H**) in psilocybin or saline
313 treated rats, nor was the duration engaged in a session (*p*=.2741, **Fig 4I**) different for psilocybin or
314 saline treated animals administered MDL100907. While 5-HT1A antagonism combined with
315 psilocybin substantially reduced the number of rats able to reach performance criterion (**Fig 4J**), and
316 induced a trend toward reduced accuracy (*p*=.0556, **Fig 4K**), instead of a performance impairment
317 *per se* what seems to be the case is that co-administration of WAY100635 negated psilocybin-
318 induced improvements in reversal learning, with no significant differences in learning measures or
319 response profiles observed between saline and psilocybin treated animals that were administered
320 WAY100635 (all *p*s>.0834; **Fig 4L-R)**. Further supporting a role of 5-HT1A antagonism negating the
321 improvement elicited by psilocybin rather than *impairing* performance is the finding that WAY100635
322 alone facilitated reversal learning compared to saline alone, by reducing non-target responding
323 (**Supplementary Fig 3F**). Intriguingly, co-administration of the mixed antagonist ketanserin impaired
324 performance in saline and psilocybin treated animals to a similar extent, with the notable exception
325 of reducing the session duration for those rats administered saline but not psilocybin (see
326 **Supplementary Fig 4**).

327 **Psilocybin causes a transient shift in the balance of 5-HT1AR and 5-HT2AR mRNA in the**
328 **prefrontal cortex**

329 To examine whether a change in the abundance of 5-HTR subtypes in medial prefrontal cortex
330 (mPFC) was elicited by psilocybin, which could explain the differential effects of psilocybin on
331 reversal learning after pharmacological blockade of the 5-HT1A vs 5-HT2A receptor subtypes, we
332 performed RNAscope on cortical sections (**Fig 5A**) collected 6, 12 and 24h after psilocybin treatment.

333 Psilocybin had no effects on the proportion of cells positive for both *Htr1a* and *Htr2a* transcripts in
334 either the prelimbic (p=.5233, **Fig 5B**) or infralimbic (p=.8637, **Fig 5C**) subregions of the mPFC, but
335 significantly increased the proportion of cells exclusively positive for *Htr1a* in both subregions
336 (prelimbic; p=.0500, **Fig 5D**, infralimbic; p=.0103, **Fig 5E**) at 12h post-administration. This was
337 matched with complementary reductions elicited by psilocybin in the proportion of cells exclusively
338 positive for *Htr2a* in both subregions, although this did not reach statistical significance for the
339 prelimbic cortex (p=.0931, **Fig 5F**) and was evident at both 6h and 12h timepoints for infralimbic
340 cortex (6h; p=.0335, 12h; p=.0129, **Fig 5G**). We further examined the anatomical localisation of *Htr1a*
341 and *Htr2a* positive cells across the layers in infralimbic cortex (**Fig 5H**) at 12h post-administration to
342 show an overall reduction in double positive cells after psilocybin treatment (p=.0026, **Fig 5I$_1$**) that
343 did not reach significance when analysed as an area under the curve (AUC; (p=.0887, **Fig 5I$_2$**). What
344 was clear, however that the respective increase and decrease in the proportion of cells exclusively
345 positive for *Htr1a* or *Htr2a* 12h after psilocybin treatment were specifically localised to cortical Layer
346 V (*Htr1a*; p<.0001, **Fig 5J$_1$,** p=.0211, **Fig 5J$_2$**, *Htr2a*; p<.0001, **Fig 5K$_1$** p=.0212, **Fig 5K$_2$**), which
347 corresponded to 900-1200 µm distance from the midline of Layer I (see **Fig 5H**).

348 To determine whether psilocybin had similar effects on *Htr1a* and *Htr2a* expression in the context of
349 weight loss and feeding pathology relevant to anorexia nervosa, we compared transcripts from the
350 saline and 6h psilocybin treated rats (non-ABA) to rats that had exhibited substantial weight loss
351 after exposure to ABA conditions and collected 6h post-administration (**Fig 5L**). Main effects of
352 psilocybin identified in non-ABA rats were recapitulated for ABA rats, with no changes in the number
353 of double labelled cells (p=.4620, **Fig 5M**) but complementary increases in *Htr1a* (p=.0011, **Fig 5N**)
354 and decreases in *Htr2a* (p=.0079, **Fig 5O**) positive cells, indicating similar consequences of
355 psilocybin treatment occurred in the ABA brain. Multiple comparisons revealed that the increase in
356 the number of *Htr1a* positive cells elicited by psilocybin was stronger in ABA rats than non-ABA rats
357 (non-ABA; p=.0298, ABA; p=.0206, **Fig 5N**), while the decrease in *Htr2a* positive cells was weaker
358 (non-ABA; p=.0463, ABA; p=.2102, **Fig 5O**), however, additional changes in the abundance of *Htr2a*
359 transcripts (**Fig 5P**) were observed following weight loss under ABA conditions, whereby psilocybin
360 elicited a substantial reduction in the overall number of *Htr2a* transcripts (p=.0005, **Fig 5Q**) and in
361 the number of *Htr2a* transcripts per cell (p=.0007, **Fig 5R**) in the mPFC of ABA rats, that was not
362 evident in non-ABA rats. Further analyses of changes in *Htr1a* and *Htr2a* expression over the 24h
363 time course and effects of exposure to ABA are provided in **Supplementary Fig 5**.

364 # Discussion

365 Clinical trials evaluating the safety and efficacy of psilocybin in people with AN have been ongoing
366 since 2019, with the first pilot study recently reporting that it improves eating disorder symptoms in
367 some individuals [72]. Psilocybin may have transdiagnostic efficacy[16, 29, 38] through several
368 mechanisms relevant to the pathology of AN, including actions on the serotonergic system [37] and
369 cognitive flexibility [20]. However, the details of how such mechanisms are altered by psilocybin in the
370 context of AN remains unknown. Here, we show that psilocybin improves body weight maintenance
371 in the ABA rat model and enhances cognitive flexibility in a reversal learning task by both reducing
372 perseverative responding and promoting task engagement when reward contingencies are initially
373 reversed. That psilocybin did not elicit changes in motivated responding (PR) or response
374 suppression (extinction) following the same training and drug administration protocol suggests a
375 selective improvement in adaptive cognition in the face of changing rules.

376 Further, we demonstrate that psilocybin-induced improvements in reversal learning performance
377 were not dependent on binding to the 5-HT2AR, because co-administration of the selective 5-HT2AR
378 antagonist (MDL100907) did not significantly alter performance measures. Instead, the action of
379 psilocybin at the 5-HT1AR was required for improved cognitive flexibility, whereby improvements in
380 reversal accuracy and engagement were abolished when psilocybin was co-administered with the
381 selective 5-HT1AR antagonist (WAY100635). This finding is complicated by the relatively fast-acting
382 effects of psilocybin observed on *Hrt2a and Hrt1a* transcription in the mPFC, which indicates that
383 psilocybin rapidly and transiently alters the balance of the cellular machinery required to support
384 receptor binding in this region associated with cognitive flexibility [47]. In such a way, the differential
385 effects of 5-HT2A and 5-HT1A antagonism on reversal learning after psilocybin may reflect functional
386 interactions between these two receptor subtypes that depend on serotonin availability during the
387 post-acute (~24h) administration period [73]. These outcomes also call into question reports of the
388 necessity of 5-HT2A binding for “therapeutic” outcomes of psilocybin in animal models, particularly
389 those that use the non-selective antagonist, ketanserin. Not only does ketanserin bind multiple
390 serotoninergic and non-serotonergic receptors but it also only blocks ~30% of 5-HT2AR in the rat
391 cortex [74]. It is plausible, therefore, that partial blockade with ketanserin shifts the binding of psilocybin
392 to other 5-HTR subtypes, including 5-HT1A, which may explain the acute improvement in reversal
393 learning after ketanserin alone previously reported in rats [36].

394 The finding that psilocybin administration specifically prevented severe weight loss in ABA rats is
395 critical in light of the evidence that lower body mass increases the risk for fatal outcomes in AN [75-77].
396 That psilocybin treatment did not have overall effects on feeding or exercise independently is
397 unsurprising considering that psilocybin does not alter feeding or energy balance in mouse models
398 of obesity [78] and supports the proposal that the therapeutic effects of psilocybin for anorexia nervosa
399 are more likely driven by adaptive cognition than through metabolic alterations [51]. In line with this,
400 resistance to weight loss after psilocybin was associated with all aspects of behavioural adaptation

401 to ABA conditions (i.e. increased food intake, reduced compulsive running and increased motivated
402 running), which we have previously shown to be linked with improved cognitive flexibility in ABA rats
403 [57, 59]. While we only observed trend level reductions in overall body weight loss after psilocybin
404 administration, the treatment group is clearly comprised of two distinct subgroups – those that
405 respond to psilocybin with improved weight outcomes and those that fare similar to controls. This
406 divergence in response profiles exists in multiple clinical populations, where between 40-80% of
407 individuals report therapeutic benefits of psilocybin assisted psychotherapy at follow-up, dependent
408 on trial parameters [14, 72, 79]. Response variation was also seen in the pilot study of psilocybin in people
409 with AN, with clinically significant improvements seen in 4/10 participants [72]. Considering the specific
410 effects of psilocybin on perseverative behaviour during reward reversal in this study, perhaps those
411 individuals (humans or rats) who respond to psilocybin with positive body weight outcomes represent
412 a subgroup whose profile is typified by rigid patterns of thought and behaviour. This information could
413 guide the clinical application of psilocybin to those individuals demonstrating high rigidity, however,
414 it is important to recognise the short generation time for the ABA phenotype compared to the often
415 long and protracted pathogenesis of AN. In both cases (human and rat) further research is required
416 to understand how psilocybin might elicit meaningful changes in body weight maintenance over the
417 long term [80].

418 The specific focus in the present study on the involvement of 5-HT2A and 5-HT1A receptor subtypes
419 was based in the evidence from imaging studies that AN is associated with decreased 5-HT2A and
420 increased 5-HT1A binding in cortical regions [21, 22]. The finding that psilocybin has the same main
421 effects on the number of cortical cells positive for the *Htr1a* and *Htr2a* transcripts in animals that had
422 been exposed to ABA conditions is important for the clinical application of psilocybin for AN, and
423 suggests that at least some of the neurobiological effects are unchanged by the development of AN-
424 relevant symptoms. Notably, psilocybin treatment in ABA rats was associated with an augmented
425 increase in the number of cells exclusively positive for *Htr1a* transcripts and an additional reduction
426 in the abundance *Htr2a* transcripts (i.e. number of transcripts per cell) that was not seen after
427 psilocybin treatment in rats that were naïve to ABA. This suggests that in the context of AN-
428 associated symptoms, the actions of psilocybin on cellular activity in the mPFC is more inhibitory in
429 nature, which could indeed be therapeutically relevant in light of the evidence that AN is associated
430 with exaggerated cortical activity [81]. Considering that cognitive inflexibility does not predispose rats
431 to weight loss under ABA conditions [57], but that it is impaired after ABA exposure [57, 58], it is likely that
432 an inflexible phenotype develops coincident with weight loss and would therefore be reflected in the
433 samples analysed here. Perhaps then, it is this additional boost of inhibitory tone elicited by
434 psilocybin in ABA rats that underlies the improvements in adaptation to the experimental conditions
435 of ABA after psilocybin treatment.

436 The overall influence of psilocybin on the number of mPFC cells that exclusively express *Htr1a* and
437 *Htr2a* transcripts is also relevant for understanding the involvement of activity in this brain region for

438 cognitive inflexibility in ABA rats. If one considers the large majority (60-75%) of mPFC Layer V cells
439 (where the effects of psilocybin were localised) that express these mRNAs are pyramidal
440 (glutamatergic) cells, the net effect of psilocybin during this 12h window would be hyperpolarisation
441 of the mPFC, via both increasing the inhibitory 5-HT1AR and decreasing the excitatory 5-HT2AR
442 machinery. This aligns with our previous work, in which chemogenetic suppression of mPFC
443 projection neurons could both prevent weight loss in ABA and improve flexibility on a reversal
444 learning task [59]. However, *Htr1a* and *Htr2a* transcripts are also present on at least two classes of
445 GABAergic interneurons in this cortical region, complicating the interpretation of effects of psilocybin
446 on excitatory output [82, 83]. Moreover, 5-HT1AR are expressed both pre- and post-synaptically, with
447 differential effects of binding on serotonergic transmission [84, 85]. Finally, the alterations observed at
448 a transcriptional level does not preclude other mechanisms such as protein degradation or changes
449 in receptor cycling [86, 87] from being involved in the serotonergic consequences of psilocybin
450 treatment.

451 With respect to the specific improvement in reversal learning elicited by psilocybin as a mechanism
452 to explain improved body weight maintenance during ABA, it is notable that the reduced
453 perseverative responding when reward contingencies were reversed was also driven by a
454 subpopulation of "responders". This raises the possibility that individual differences in baseline
455 serotonin signalling may underlie responses to psilocybin treatment, as proposed by the inverted "U-
456 shaped" dose-effect relationships reported for many active compounds and their relation to cognitive
457 function [88]. If adaptive cognition requires an appropriate balance between 5-HT1A and 5-HT2A
458 receptor function [47], our molecular findings suggest that individuals (humans or rats) exhibiting
459 elevated 5-HT1AR function (or for that matter reduced 5-HT2AR function) may not respond positively
460 (since further elevation or reduction elicited by psilocybin would push them into the tail ends of the
461 inverted "U"). It is also important to note, in light of the recent observation that psilocybin,
462 administered acutely, did not facilitate flexibility [36], that there are important methodological
463 differences that may explain this discordance. Specifically, we examined effects of psilocybin post-
464 acutely, using a single administration paradigm, and the reversal learning task used in the present
465 study relied on action-outcome learning rather than Pavlovian cue-outcome learning. Performance
466 on this task is also dependent on the incentive salience of rewards to elicit appropriate responding,
467 with psilocybin-induced improvements observed in reversal task engagement, leading to faster
468 receipt of the first (unexpected) reward. This demonstrates the potential of psilocybin to alter the
469 explore/exploit trade-off common in reinforcement learning, where the subject has the option of
470 maximizing reward based on its current information (exploitation) or by accumulating more evidence
471 (exploration) [89] and may improve the balance between the two for more effective adaptation.

472 One of the most intriguing issues related to the actions of psilocybin in the brain is the means via
473 which it changes neuronal morphology and function to exert its effects. The canonical pathway
474 through which psilocybin is proposed to promote plasticity (and presumably therefore flexible

475 learning) is through binding to the 5-HT2A receptor, an act that elicits a “glutamate surge” through
476 rapid increases in neuronal excitability [27, 28]. The dendritic and synaptic changes that occur
477 downstream may or may not be related to this surge of glutamate since psilocybin induced structural
478 plasticity was still observed in the presence of ketanserin [44]. It is convenient to focus the actions of
479 psilocybin at 5-HT2AR located in the PFC because of their requirement for the subjective
480 (psychedelic) effects [41], however, this view discounts the abundant expression of 5-HT2A in other
481 brain regions relevant to learning and memory, including the hippocampus, claustrum and striatum
482 [90]. The results of the present study suggest that improvements in flexible learning after psilocybin
483 are not mediated by binding to the 5-HT2A receptor, but that selective 5-HT2A antagonism impaired
484 learning in all animals, an effect that was partially restored with co-administration with psilocybin. A
485 possible explanation for these results is that while 5-HT2A receptor function is required for reversal
486 learning, it only partly supports the cognitive enhancing effects of psilocybin. A major challenge is in
487 understanding the role of the 5-HT1A receptor in mediating learning outcomes, especially since firing
488 activity of 5-HT neurons in the dorsal raphe nucleus is controlled by pre-synaptic expression of 5-
489 HT1AR where binding inhibits serotonin release [90]. We show that 5-HT1A antagonism did not affect
490 the ability to reach performance criteria or obtain reward in controls, but preferentially impaired
491 learning improvements elicited by psilocybin. Taken together, this highlights the 5-HT1AR as an
492 important target mediating the effects of psilocybin on cognitive flexibility [90].

493 The key outcomes of this study underline the fact that animal studies are required for understanding
494 the mechanisms that underlie the therapeutic efficacy of psilocybin because they allow detailed
495 interrogation of behaviour and brain function in the absence of effects of expectancy. Future studies
496 should aim to examine how psilocybin influences serotoninergic tone via other (i.e. non-5-HT2A)
497 known 5-HT binding targets. Particularly relevant for understanding the therapeutic mechanisms for
498 AN is the examination of the interaction between serotonergic and dopaminergic mechanisms that
499 could play a role in the way that inflexible patterns of thought and behaviour relate to food reward,
500 aversion, and avoidance [91]. That psilocybin has direct actions on the dopamine system in humans
501 [92] and rats [49] has long been known, but surprisingly paid little attention [93], even though the interaction
502 between serotonin receptor binding and dopamine release is well established [94, 95]. The proposal to
503 study dopaminergic effects of psilocybin is brought into sharper focus by recent evidence of brain-
504 wide activation of the dopamine system by ketamine [96] and that dopamine D2R blockade attenuates
505 the psychedelic-induced head-twitch response [97]. These considerations, in concert with the new data
506 presented here, will provide a better understanding of a mechanistic framework, insight that will
507 provide greater confidence in the potential therapeutic use of psilocybin for conditions such as AN.
508 This is an important and arguably necessary step to include psilocybin in the armoury of tools to treat
509 mental health disorders.

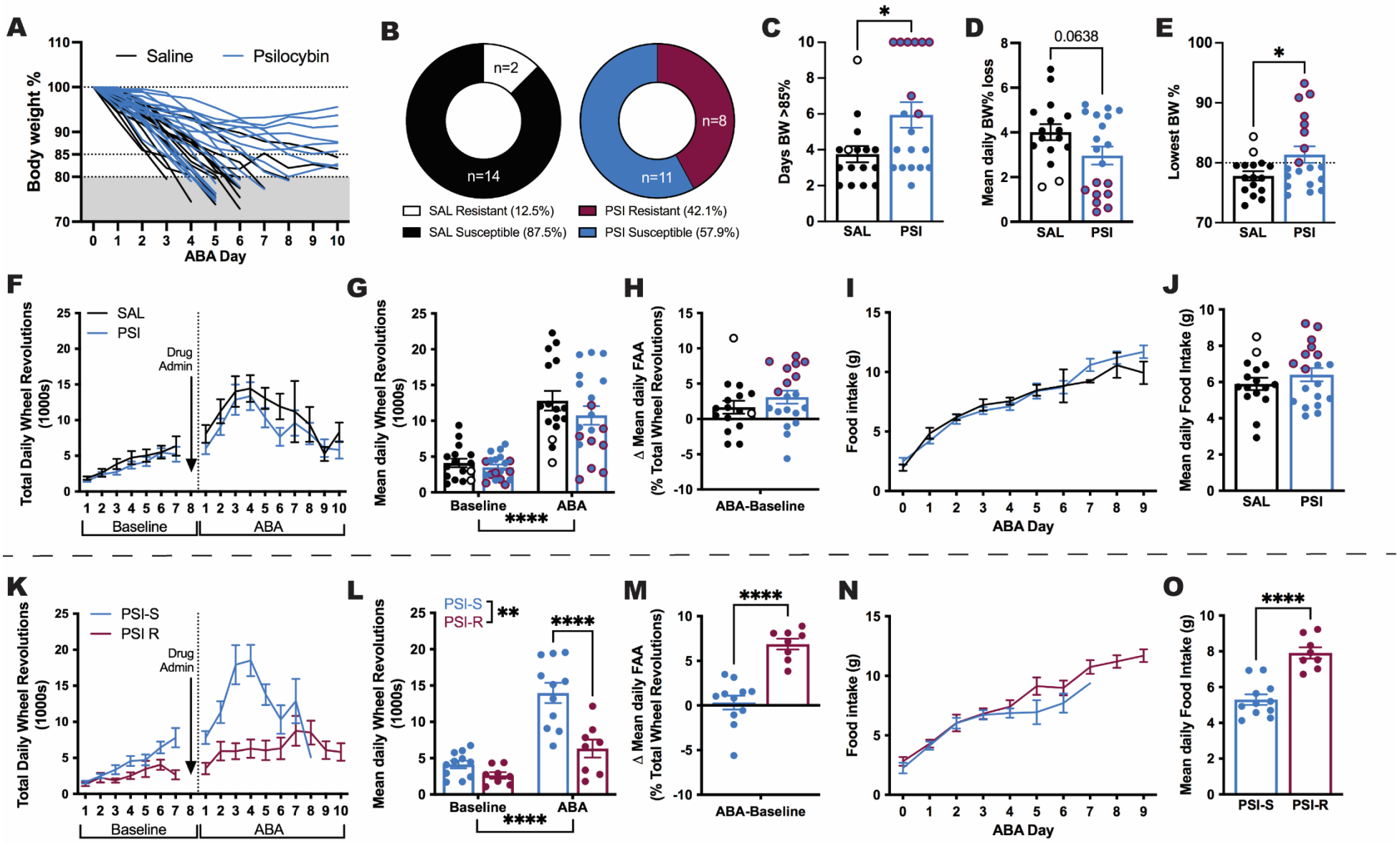


Figure 1. Effects of psilocybin on body weight maintenance in ABA. Weight loss trajectories of individual rats (*n*=16 saline; *n*=19 psilocybin) over the 10-day ABA period (**A**) and proportion resistant to weight loss (**B**). Psilocybin facilitated body weight maintenance over 85% for more days (**C**, *t*(33)=2.508, *p*=.0172), with a trend

toward lower body weight % loss per day (**D**, $t(33)=1.918$, $p=.0638$) that resulted in attenuation of severe weight loss (**E**, $t(33)=2.146$, $p=.0394$). Total daily wheel revolutions (**F**) increased as expected in ABA (**G**, ABA Phase $F(1, 33)=126.5$, $p<.0001$) but were similar between groups (Treatment $F(1, 33)=1.159$, $p=.2985$) across both baseline ($p>.9999$) and ABA ($p=.3089$; Interaction $F(1, 33)=1.033$, $p=.3169$) with no difference in the change in proportional running wheel activity in the penultimate hour before food access (**H**, $t(33)=.1.098$, $p=.2800$). Ninety-minute food intake (**I**) increased similarly across the ABA phase with no difference in mean daily intake (**J**, $t(33)=0.9908$, $p=.3290$). Comparison of only psilocybin treated rats that were susceptible (PSI-S) versus resistant (PSI-R) to ABA highlights the characteristic starvation-induced hyperactivity displayed by PSI-S (**K**) following the onset of ABA conditions (**L**, ABA Phase $F(1, 17)=80.86$, $p<.0001$**;** ABA Outcome $F(1, 17)=13.17$, $p=.0021$; Interaction $F(1, 17)=16.67$, $p=.0008$**;** ABA PSI-S>PSI-R $p<.0001$), in contrast to the selective increase of running in anticipation of food access displayed by PSI-R (**M**, $t(17)=6.203$, $p<.0001$), accompanied by diverging food intake trajectories (**N**) with greater mean daily intake by PSI-R (**O**, $t(17)=5.940$, $p<.0001$). Grouped data show mean ± SEM, with individual data points on bar graphs. *$p<.05$, **$p<.01$, ***$p<.001$, ****$p<.0001$. SAL saline; PSI psilocybin; BW body weight; ABA activity-based anorexia; FAA food anticipatory activity; PSI-S psilocybin treated ABA susceptible; PSI-R psilocybin treated ABA resistant. For full statistical analysis details see **Figure 1 Statistics Table**.

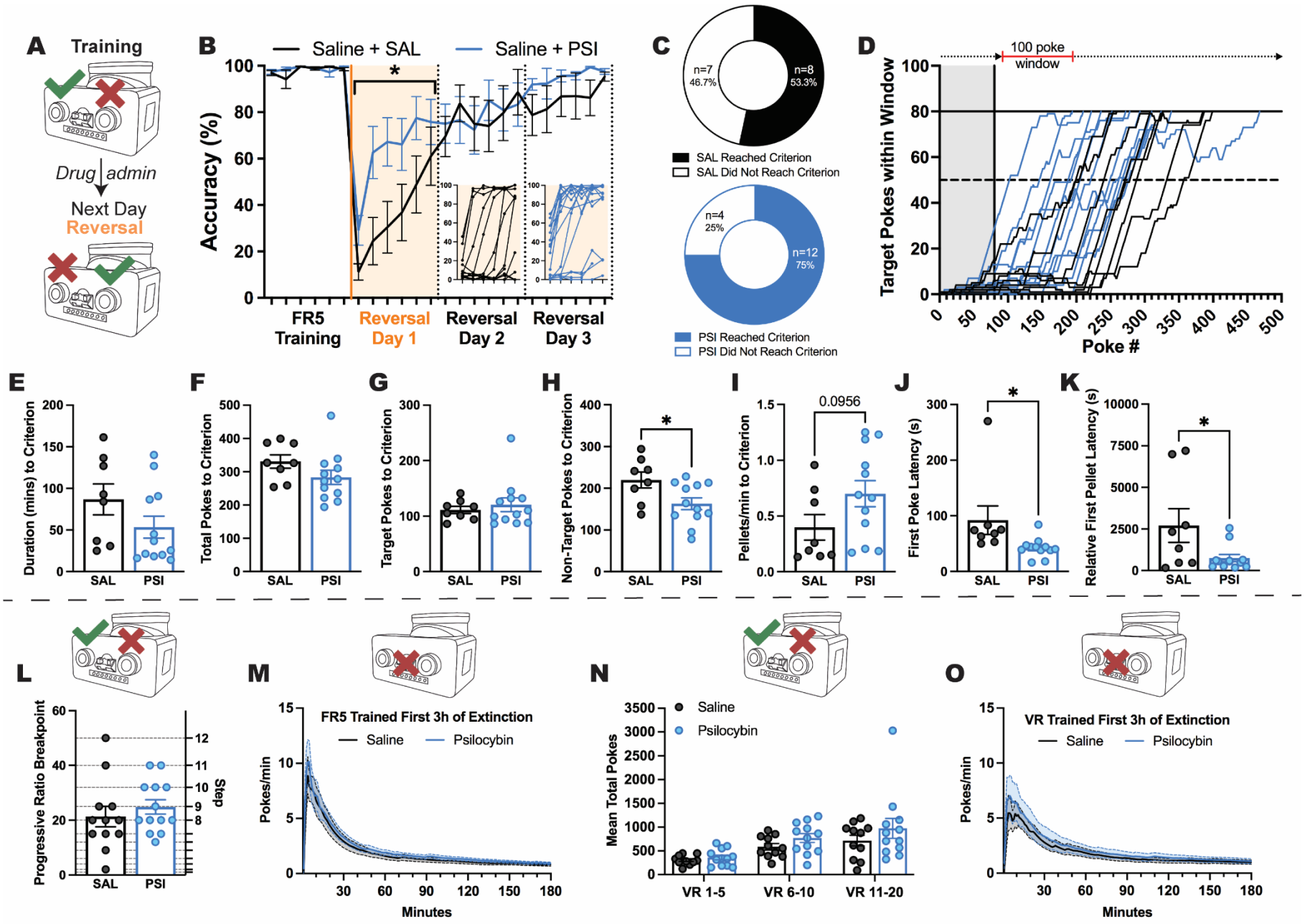

A
Training
Drug admin
Next Day
Reversal
B
Saline + SAL
Saline + PSI
Accuracy (%)
FR5 Training
Reversal Day 1
Reversal Day 2
Reversal Day 3
C
n=7 46.7%
n=8 53.3%
SAL Reached Criterion
SAL Did Not Reach Criterion
n=4 25%
n=12 75%
PSI Reached Criterion
PSI Did Not Reach Criterion
D
100 poke window
Target Pokes within Window
Poke #
E
Duration (mins) to Criterion
F
Total Pokes to Criterion
G
Target Pokes to Criterion
H
Non-Target Pokes to Criterion
I
Pellets/min to Criterion
0.0956
J
First Poke Latency (s)
K
Relative First Pellet Latency (s)
SAL
PSI
L
Progressive Ratio Breakpoint
Step
M
FR5 Trained First 3h of Extinction
Saline
Psilocybin
Pokes/min
Minutes
N
Mean Total Pokes
VR 1-5
VR 6-10
VR 11-20
O
VR Trained First 3h of Extinction

Figure 2. Effects of psilocybin on reversal learning, effortful responding and response suppression. Psilocybin administered after training the day prior to reversal of reward contingencies (**A**) significantly improved accuracy of responding during the initial 3h reversal session (**B**, Treatment $F(1, 29)=5.128$, $p=.0312$; 6 x 30min time bins) and increased the number of rats (**C**) able to reach performance criterion (**D**, 80 target pokes in a 100-poke moving window) on the first day of reversed reward contingencies. While there was no difference in the time (from first poke to poke that achieved criterion; **E**, $t(18)=1.514$, $p=.1474$), total pokes (**F**, $t(18)=1.536$, $p=.1420$) or target pokes (**G**, $t(18)=0.5614$, $p=.5815$) required to reach criterion, psilocybin-treated rats required fewer non-target pokes to reach criterion (**H**, $t(18)=2.425$, $p=.0260$), tended to earn rewards faster (**I**, $t(18)=1.759$, $p=.0956$), and were both faster to first engage with the task (time from device access to first poke; **J**, $t(18)=2.307$, $p=.0332$) and to earn their first reward (time from first poke to earning first pellet; **K**, $t(18)=2.291$, $p=.0343$). Psilocybin treatment had no effect on breakpoint (pokes required to earn final pellet before 10min of inactivity) on a classic progressive ratio task (**L**, $t(23)=0.7795$, $p=.4436$), extinction following fixed ratio training (**M**, Treatment $F(1, 17)=0.3212$, $p=.5783$; Interaction $F(179, 3043)=0.2623$, $p>.9999$), goal directed engagement on increasingly uncertain schedules of reinforcement (**N**, Treatment $F(1, 21)=1.741$, $p=.2013$; Interaction $F(2, 42)=0.7244$, $p=.4906$), or extinction following variable ratio training (**O**, Treatment $F(1, 21)=0.2681$, $p=.6100$; Interaction $F(179, 3759)=0.3509$, $p>.9999$). Grouped data show mean ± SEM, with individual data points on bar graphs. *$p<.05$. SAL saline; PSI psilocybin; FR5 fixed ratio 5; VR variable ratio. For full statistical analysis details see **Figure 2 Statistics Table**.

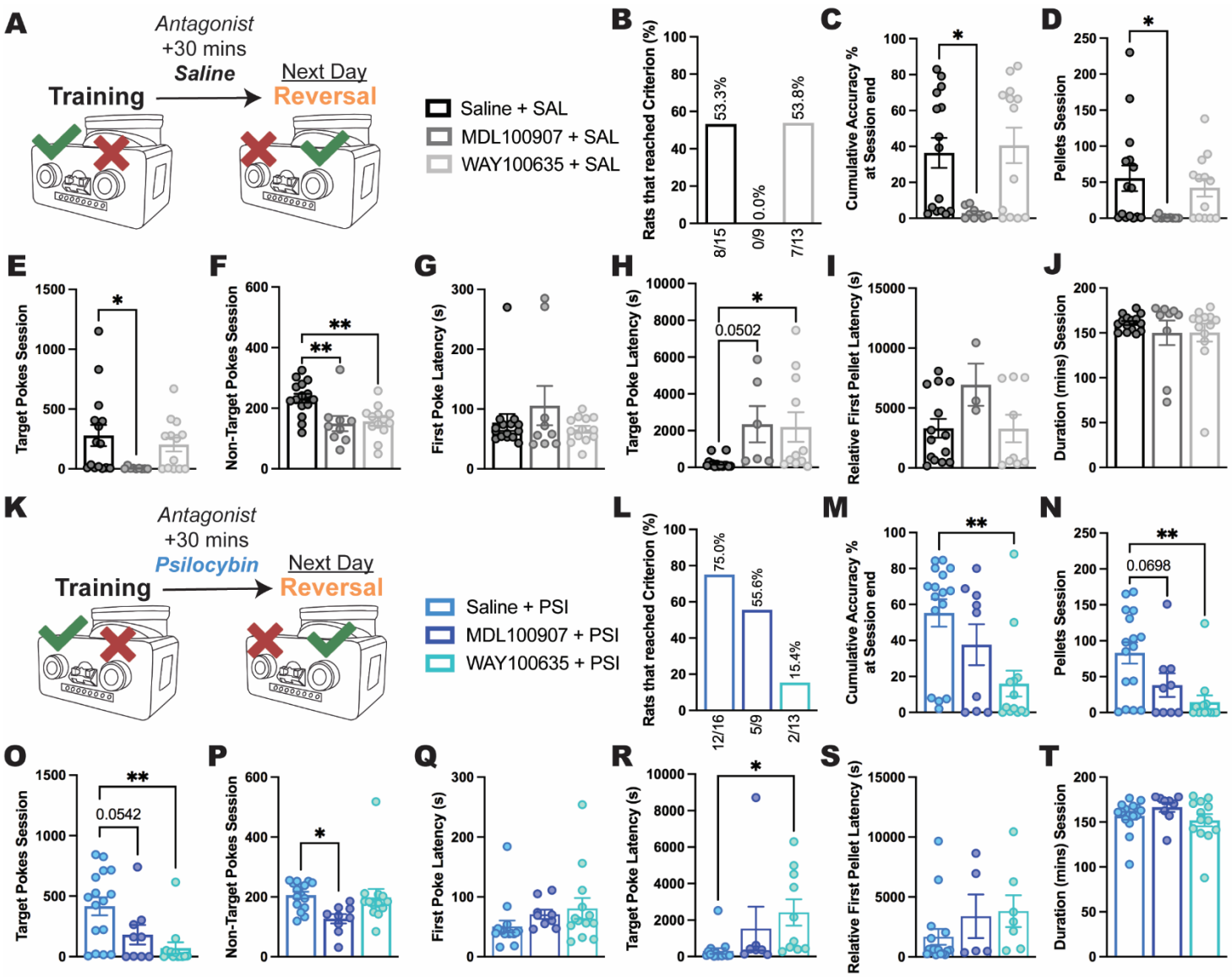


Figure 3. Effects of 5-HT2A and 5-HT1A antagonism on reversal learning in control and psilocybin-treated rats. Following the final training session pre-treatment with either saline (vehicle control), the 5-HT2AR antagonist MDL100907, or the 5-HT1AR antagonist WAY100635, was followed 30min later by treatment with either

saline (**A**) or psilocybin (**K**) before reversal of reward contingencies the following day. While 53/5% (8/15) of Saline+SAL rats reached reversal day 1 criterion, 0% (0/9) of MDL+SAL treated rats did so (**B**), showing global impairment compared to Saline+SAL across nearly all outcome measures, achieving significantly lower session accuracy (**C**, SAL+>MDL+ p=.0221), earning fewer pellets (**D**, SAL+>MDL+ p=.0324), and making fewer target (**E**, SAL+>MDL+ p=.0292) and non-target (**F**, SAL+>MDL+ p=.0048) pokes in the session. While there was no delay in task engagement (time from device access to first poke; **G**, SAL+ vs MDL+ p=.4572), target poke latency was increased (**H**, SAL+<MDL+ p=.0502) in the 6/9 rats that made a target poke, and only 3/9 rats earned a single pellet (i.e. made at least 5 target pokes; **I**, time from first poke to earning first pellet, SAL+ vs MDL+ p=.1507), even though task engagement duration did not differ (time from first to final poke; **J**, SAL+ vs MDL+ p=.6048). Conversely, WAY+SAL resulted in 53.8% (7/13) of rats reaching criterion, nearly identical to Saline+SAL, with these groups being similar across most measures except WAY+SAL having fewer non-target pokes (**F**, SAL+>WAY+ p=.0041) despite an elongated target poke latency (**H**, SAL+<WAY+ p=.0244). With 75% (12/16) of Saline+PSI rats reaching reversal day 1 criterion, MDL+PSI treatment produced a moderate decrease to 55.6% (5/9) reaching criterion, although only a non-significant decrease in accuracy (**M**, SAL+ vs MDL+ p=.2837), while there was a trend toward fewer pellets (**N**, SAL+>MDL+ p=.0698) and target pokes (**O**, SAL+>MDL+ p=.0542), and a significant reduction in non-target pokes (**P**, SAL+>MDL+ p=.0210) across the session, with no differences in any latency measures (**Q-S**, all SAL+ vs MDL+ ps>.2991) or session duration (**T**, SAL+ vs MDL+ p=.4345). In contrast, WAY+PSI produced severe impairment with only 15.4% (2/13) of rats reaching criterion, with significantly reduced session accuracy (**M**, SAL+>WAY+ p=.0024), pellets earned (**N**, SAL+>WAY+ p=.0015), and target pokes (**O**, SAL+>WAY+ p=.0013), and delayed target poke latency (**R**, SAL+<WAY+ p=.0212, with only 10/13 rats achieving a target poke) compared to Saline+PSI, whilst there were no differences for non-target pokes (**P**, SAL+ vs WAY+ p=.9497), first poke latency (**Q**, SAL+ vs WAY+ p=.1636), relative first pellet latency (**S**, SAL+ vs WAY+ p=.2588, although only 7/13 earned a pellet), nor session duration (**T**, SAL+ vs WAY+ p=.7309). Bar graphs show mean ± SEM with individual data points. *p<.05, **p<.01. SAL saline; PSI psilocybin; SAL+ saline pre-treatment; MDL+ MDL100907 pre-treatment; WAY+ WAY100635 pre-treatment. For main ANOVA results and full statistical analysis details see **Figure 3 Statistics Table**.

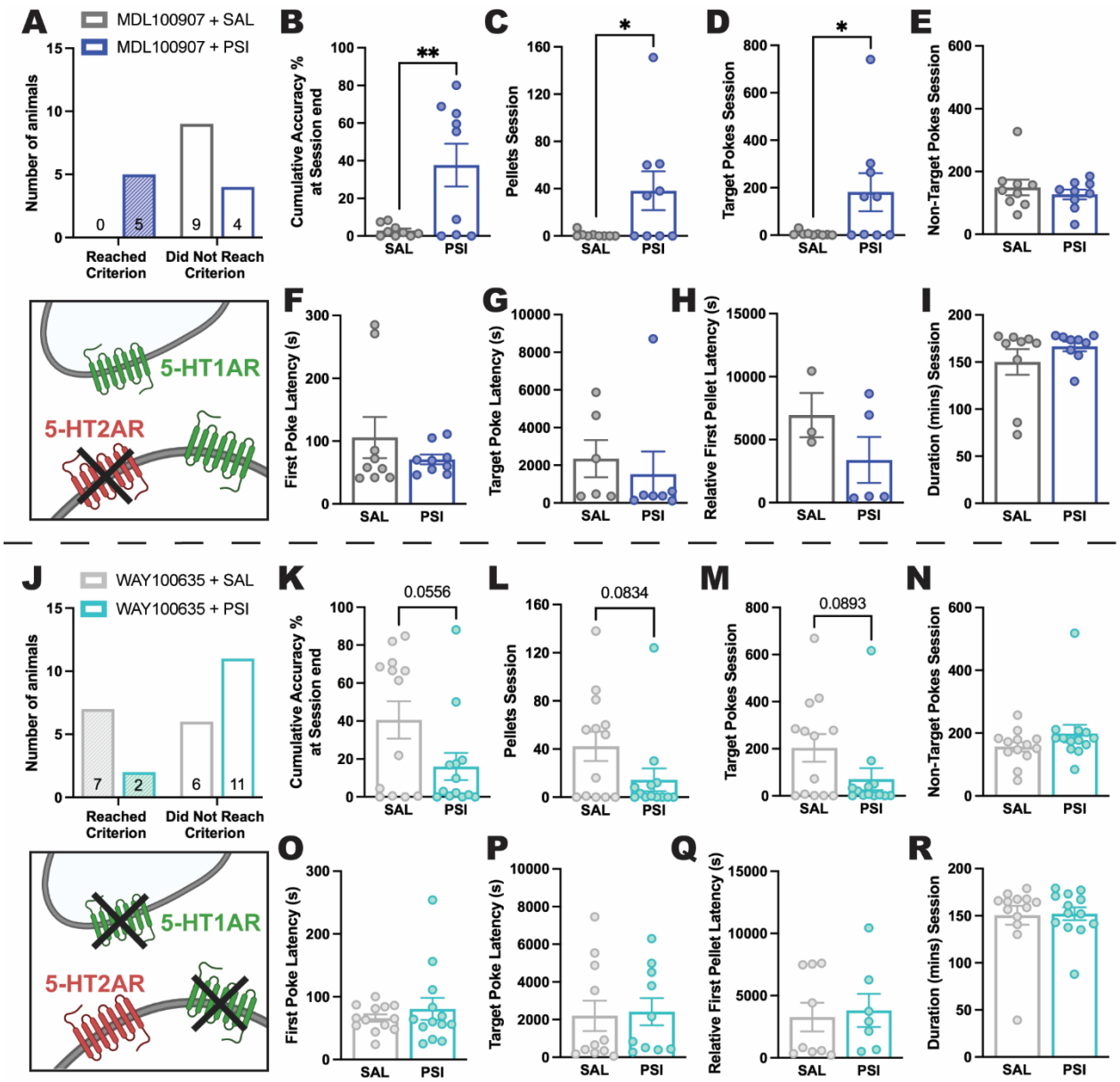
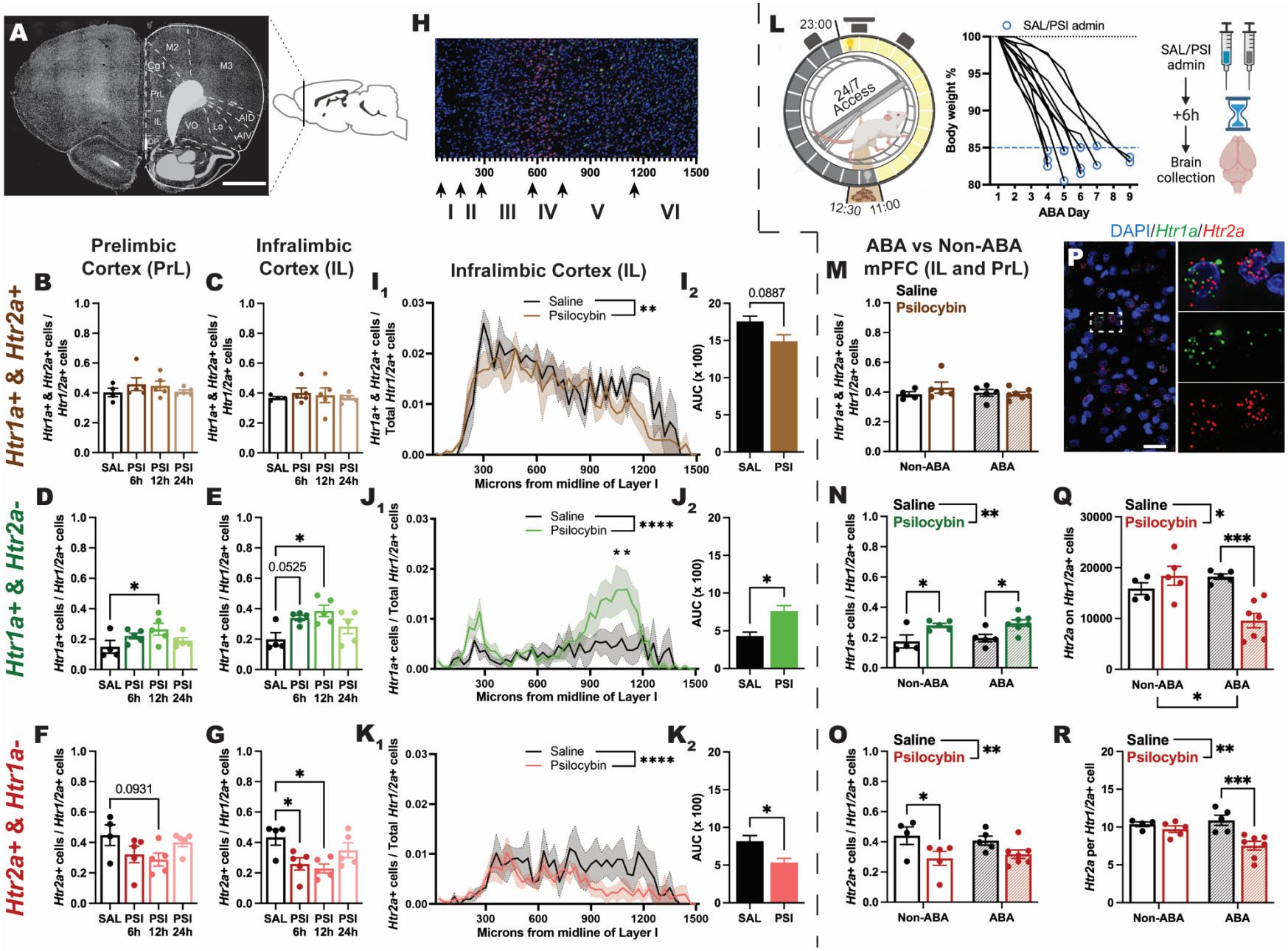

A
MDL100907 + SAL
MDL100907 + PSI
Number of animals
Reached Criterion
Did Not Reach Criterion
B
Cumulative Accuracy % at Session end
C
Pellets Session
D
Target Pokes Session
E
Non-Target Pokes Session
5-HT1AR
5-HT2AR
F
First Poke Latency (s)
G
Target Poke Latency (s)
H
Relative First Pellet Latency (s)
I
Duration (mins) Session
SAL
PSI
J
WAY100635 + SAL
WAY100635 + PSI
K
0.0556
L
0.0834
M
0.0893
N
O
P
Q
R

Figure 4. Effects of 5-HT2AR and 5-HT1AR antagonism on psilocybin-induced improvements in reversal learning. Reversal learning following 5-HT2AR antagonism via pre-treatment with MDL100907 (**A**) was completely impaired in saline treated animals (0/9 [0%] reached reversal day 1 criterion) whereas psilocybin treatment prevented this impairment (5/9 [55.6%] reached criterion). Psilocybin treatment following MDL-mediated 5-HT2AR antagonism resulted in significantly greater session accuracy (**B**, $t(16)=3.034$, $p=.0079$), pellets earned (**C**, $t(16)=2.255$, $p=0.385$), and target pokes made (**D**, $t(16)=2.191$, $p=.0436$) compared to saline treatment, with no differences for non-target pokes (**E**, $t(16)=0.7609$, $p=.4578$), first poke latency (time from device access to first poke; **F**, $t(16)=1.034$, $p=.3163$), target poke latency (**G**, $t(11)=0.5098$, $p=.6202$), relative first pellet latency (time from first poke to earning first pellet; **H**, $t(6)=1.295$, $p=.2428$), or session duration (time from first to final poke; **I**, $t(16)=1.133$, $p=.2741$). The opposite performance pattern was observed following 5-HT1AR antagonism via WAY100635 pre-treatment (**J**), with 7/13 (53.8%) saline treated rats reaching criterion compared with only 2/13 (15.4%) psilocybin treated rats. Although not significant, psilocybin treatment produced a trend toward lower session accuracy (**K**, $t(24)=2.011$, $p=.0556$), fewer pellets earned (**L**, $t(24)=1.806$, $p=.0834$), and target pokes made (**M**, $t(24)=1.771$, $p=.0893$), whilst there was no difference between groups for non-target pokes (**N**, $t(24)=1.317$, $p=.2001$), first poke (**O**, $t(24)=0.7925$, $p=.4538$), target poke (**P**, $t(19)=0.1996$, $p=.8440$), or relative first pellet (**Q**, $t(14)=0.3062$, $p=.7639$) latency, or session duration (**R**, $t(24)=0.1337$, $p=.8948$). Bar graphs show mean ± SEM with individual data points. *$p<.05$, **$p<.01$. SAL saline; PSI psilocybin. For full statistical analysis details see **Figure 4 Statistics Table**.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Body weight %
ABA Day
SAL/PSI admin
+6h
Brain collection
Prelimbic Cortex (PrL)
Infralimbic Cortex (IL)
Infralimbic Cortex (IL)
ABA vs Non-ABA mPFC (IL and PrL)
DAPI/Htr1a/Htr2a
Htr1a+ & Htr2a+
Htr1a+ & Htr2a-
Htr2a+ & Htr1a-
Htr1a+ & Htr2a+ cells / Htr1/2a+ cells
Htr1a+ cells / Htr1/2a+ cells
Htr2a+ cells / Htr1/2a+ cells
Htr1a+ & Htr2a+ cells / Total Htr1/2a+ cells
Htr1a+ cells / Total Htr1/2a+ cells
Htr2a+ cells / Total Htr1/2a+ cells
AUC (x 100)
Htr2a on Htr1/2a+ cells
Htr2a per Htr1/2a+ cell
SAL PSI PSI PSI
6h 12h 24h
Saline
Psilocybin
Microns from midline of Layer I
Non-ABA
ABA
0.0887
0.0525
0.0931

Figure 5. Effects of psilocybin on the expression of *Htr1a* and *Htr2a* transcripts in the mPFC. Coronal section with brain atlas overlay (AP+3.2mm from bregma; **A**) depicting regions of interest (PrL and IL). The proportion of *Htr1/2a*+ cells that were double labelled with *Htr1a* and *Htr2a* was not changed by psilocybin treatment in either the PrL (**B**, $F(3, 15)=0.7801$, $p=.5233$) or IL (**C**, $F(3, 15)=0.2449$, $p=.8637$). The proportion of *Htr1/2a*+ cells that were exclusively *Htr1a* labelled was increased following psilocybin administration in both the PrL (**D**, $F(3, 15)=2.443$, $p=.1043$, SAL<PSI12h $p=.0500$) and the IL (**E**, $F(3, 15)=4.277$, $p=.0227$, SAL<PSI6h $p=.0525$, SAL<PSI12h $p=.0103$), whilst those exclusively *Htr2a* labelled decreased following psilocybin treatment at a trend level in PrL (**F**, $F(3, 15)=2.192$, $p=.1314$, SAL>PSI12h $p=.0931$) and significantly in IL (**G**, $F(3, 15)=4.426$, $p=.0203$, SAL>PSI6h $p=.0335$, SAL>PSI12h $p=.0129$). The spatial distribution of IL *Htr1/2a*+ cells along the midline from layer 1 (**H**) was significantly different for each uniquely labelled cell population (**I_1** $F(49, 300)=10.34$, $p<.0001$; **J_1** $F(49, 300)=3.549$, $p<.0001$; **K_1** $F(49, 300)=3.436$, $p<.0001$). In each case psilocybin treatment also had a significant effect, producing a significantly reduced overall proportion of double labelled cells (**I_1**, $F(1, 300)=9.214$, $p=.0026$) and exclusively *Htr2a* labelled cells (**K_1**, $F(1, 300)=19.16$, $p<.0001$), but a significantly increased overall proportion of exclusively *Htr1a* labelled cells (**J_1**, $F(1, 300)=22.38$, $p<.0001$) accompanied by a significant Distance by Treatment interaction (**J_1**, $F(49, 300)=1.459$, $p=.0313$). AUC was decreased at a trend level for double labelled cells (**I_2**, $t(6)=2.030$, $p=.0887$), significantly increased for exclusively *Htr1a* labelled cells (**J_2**, $t(6)=3.102$, $p=.0211$) and significantly reduced for exclusively *Htr2a* labelled cells (**K_2**, $t(6)=3.097$, $p=.0212$). A separate cohort of animals underwent ABA induction, were administered either saline or psilocybin when they reached <85% baseline body weight, and culled ~6h later (when bodyweight had dropped to close to ~80% in most cases; **L**). The proportion of mPFC *Htr1/2a*+ cells that expressed both *Htr1a* and *Htr2a* was not effected by psilocybin administration nor ABA exposure (**M**, Treatment $F(1, 17)=0.5663$, $p=.4620$; ABA Exposure $F(1, 17)=0.4685$, $p=.5029$; Interaction $F(1, 17)=1.208$, $p=.2871$), whereas psilocybin significantly increased or significantly decreased the proportion of exclusively *Htr1a* labelled (**N**, Treatment $F(1, 17)=15.50$, $p=.0011$; Non-ABA SAL<PSI $p=.0298$, ABA SAL<PSI $p=.0206$) or *Htr2a* labelled (**O**, Treatment $F(1, 17)=9.038$, $p=.0079$; Non-ABA SAL>PSI $p=.0463$) cells, respectively, in a generally consistent and ABA independent manner (all ABA exposure and interaction ps>.4607). *Htr1a* (green) and *Htr2a* (red) expression on distinct cell populations in the mPFC (**P**) identified through DAPI (blue). The absolute number of *Htr2a* transcripts associated with mPFC *Htr1/2a*+ cells (**Q**) was significantly altered by psilocybin ($F(1, 17)=4.587$, $p=.0470$), ABA exposure ($F(1, 17)=5.098$, $p=.0374$), and their interaction ($F(1, 17)=15.26$, $p=.0011$), such that psilocybin treatment significantly reduced *Htr2a* copy number specifically in the ABA brain (ABA SAL>PSI $p=.0005$). This pattern was mostly replicated by the number of *Htr2a* copies per mPFC *Htr1/2a*+ cell (**R**, Treatment $F(1, 17)=12.12$, $p=.0029$; ABA Exposure $F(1, 17)=2.048$, $p=.1705$; Interaction $F(1, 17)=5.606$, $p=.0300$), with a significant reduction in the density of *Htr2a* specifically following psilocybin treatment after ABA induction (ABA SAL>PSI $p=.0007$). Grouped data show mean ± SEM, with individual data points on bar graphs (except AUC). Values are the average of 4 (PrL and IL) or 8 (mPFC) sections per animal. *$p<.05$, **$p<.01$, ***$p<.001$, ****$p<.0001$. AP anterior-posterior; SAL saline; PSI psilocybin; PrL prelimbic cortex; IL infralimbic cortex; AUC area under the curve; mPFC medial prefrontal cortex (PrL and IL combined); *Htr1/2a*+ cells expressing *Htr1a* and/or *Htr2a*; ABA activity-based anorexia. Scale bars for **A**) 2mm and **P**) 30µm. For full statistical analysis details see **Figure 5 Statistics Table**.

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Conflict of interest

CJF sits on the scientific advisory board for Octarine Bio, Copenhagen, Denmark.

Supplementary materials

Supplementary information is available at MP's website.

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