1 **Title:** Psilocybin facilitates fear extinction: importance of dose, context, and serotonin receptors

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ABSTRACT

A variety of classic psychedelics and MDMA have been shown to enhance fear extinction in rodent models. This has translational significance because a standard treatment for posttraumatic stress disorder (PTSD) is prolonged exposure therapy. However, few studies have investigated psilocybin's potential effect in fear learning paradigms. More specifically, the extents to which dose, timing of administration, and serotonin receptors may influence psilocybin's effect on fear extinction are not understood. In this study, we used an auditory delay fear conditioning paradigm to determine the effects of psilocybin on fear extinction, extinction retention, and fear renewal in male and female mice. Psilocybin robustly enhances fear extinction when given acutely prior to testing for all doses tested. Psilocybin exerts long-term effects to elevate extinction retention and suppress fear renewal in a novel context, though these changes were sensitive to dose. Administration of psilocybin prior to fear learning or immediately after extinction yielded no change in behavior, indicating that concurrent extinction experience is necessary for the drug's effects. Co-treatment with a 5-HT$_{2A}$ receptor antagonist blocked psilocybin's effects for extinction, extinction retention and fear renewal, whereas 5-HT$_{1A}$ receptor antagonism attenuated only the effect on fear renewal. Collectively, these results highlight dose, context, and serotonin receptors as crucial factors in psilocybin's ability to facilitate fear extinction. The study provides preclinical evidence to support investigating psilocybin as a pharmacological adjunct for extinction-based therapy for PTSD.

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating condition in which a traumatic experience causes difficulty in distinguishing safe and unsafe contexts, resulting in exaggerated responses to stimuli reminiscent of the initial trauma[1]. A standard treatment for PTSD is prolonged exposure therapy, wherein patients learn to extinguish fearful responses through repeated presentations of triggering stimuli in a safe setting[2]. While generally effective, many individuals discontinue the therapy due to the challenging emotional reactions upon re-exposure[3]. Further, extinction learning is often bound to the context in which it was learned[4–6]. In other words, patients may respond well in the clinic but continue to experience PTSD symptoms in their daily lives[2]. To overcome these limitations, a considerable amount of research has focused on developing pharmacotherapies that can promote sustained and context-independent extinction[7–9].

Classic psychedelics and related psychoactive compounds may have therapeutic potential for mental illnesses including PTSD[10–12]. The promise is highlighted by the Phase 3 clinical trials of 3,4-methylenedioxymethamphetamine (MDMA), where patients reported an enduring decrease in PTSD symptoms following MDMA-assisted psychotherapy[13,14]. Consistently, in preclinical mouse models involving auditory cued fear conditioning, a single dose of MDMA reduced conditioned freezing 30 minutes after administration[15]. Importantly, this behavioral change lasted 10 days later and persisted when mice were tested in an unfamiliar context. The enhancing effect of MDMA on extinction was moderated by dose[15] and required 5-HT$_{2A}$ receptors[16]. Recently, classic psychedelics have been evaluated using preclinical mouse models for fear learning. For instance, mice treated with 2,5-dimethoxy-4-iodoamphetamine (DOI) showed less cue-conditioned freezing during fear extinction compared to controls 30 minutes after administration[17]. However, these effects were lost when the mice were tested again 24 hours later, suggesting that the effect of DOI on extinction was not retained. Similar acute, immediate effects on cued fear extinction have been demonstrated for N,N-dimethyltryptamine (DMT) in rats[18,19], 4-hydroxy-diisopropyltryptamine (4-OH-DiPT) in mice[20], and TCB-2 in mice[21]. However, the studies so far with classic psychedelics typically have limited scope: testing a single dose, focusing on only acute effects, or omitting fear renewal, making it unclear if the classic psychedelics can have enduring impact that is retained outside the initial extinction context.

67   Among classic psychedelics, psilocybin has advanced the most in clinical trials. Recent work
68   indicates that psilocybin administration with psychological support may be an effective treatment
69   option for major depression and treatment-resistant depression[22–25]. Excitingly, some clinical
70   reports show reductions in symptom severity months after treatment[26,27], suggesting that
71   psilocybin-based interventions promote long-term behavioral changes. It has been suggested
72   that psilocybin may have therapeutic potential for PTSD as well[28,29]. In a small open-label study,
73   PTSD severity declined in long-term AIDS survivors following psilocybin-assisted group
74   psychotherapy[30]. This early result is now followed by ongoing clinical trials of psilocybin in
75   people with trauma-related disorders from frontline clinical duty (NCT05163496), experience in
76   adulthood (NCT05312151), and combat for veterans (NCT05554094).
77
78   Less is known about the effects of psilocybin in rodent models of fear learning. In one report,
79   mice given low dose (0.1 - 0.5 mg/kg) psilocybin prior to fear conditioning extinguished
80   conditioned freezing more quickly than controls[31]. However, this dosing strategy makes it
81   difficult to discern whether psilocybin's effect was due to a change in extinction learning or in the
82   consolidation and retrieval of the initial fear memory. More recent studies suggest that
83   psilocybin (1 - 2 mg/kg) reduces conditioned freezing when administered 30 minutes prior to
84   extinction[32,33], and that this effect can persist 24 hours later[32], but used contextual and trace fear
85   conditioning paradigms. Thus, despite tantalizing hints from these recent studies, much remains
86   unknown regarding psilocybin's effect on cued fear extinction. Crucially, the extent to which
87   psilocybin's effects may depend on essential parameters such as dose, timing of administration,
88   and serotonin receptors are not well understood.
89
90   In this study, we used auditory delay fear conditioning and determined the effects of psilocybin
91   on fear extinction, extinction retention, and fear renewal with a total of 112 male and female
92   mice. We report that a single 1 mg/kg dose of psilocybin enhances fear extinction and
93   suppresses fear renewal in a novel context up to 8 days after drug administration. We found that
94   the persisting action of psilocybin is sensitive to dose, despite its acute impact being observed
95   at all doses tested. We varied the timing of administration to show that psilocybin was ineffective
96   at altering the acquisition of conditioned fear or consolidation of fear extinction. We tested the
97   role of 5-HT_{1A} and 5-HT_{2A} receptors in supporting psilocybin's effects on fear extinction and
98   renewal. Collectively, these results demonstrate that psilocybin facilities fear extinction in mice
99   and delineate the parameters under which its behavioral effects occur.
100

MATERIALS AND METHODS

Animals. Male and female C57BL/6 mice were purchased from Jackson Laboratory (Stock No. 000664) and given 14 days to acclimate to the vivarium at Cornell University. Within the vivarium, animals were kept in a climate-controlled room and were housed in groups of 4 mice per cage with *ad libitum* access to food and water. Mice were kept on a 12-hour light/dark cycle (lights on between 08:00 and 16:00 hours), with behavioral testing occurring between 08:30 to 13:00 hours. All mice were randomly assigned to different experimental groups prior to testing. Animals were 7 - 8 weeks old at the start of behavioral studies. Animal care and experimental procedures were approved by the Institutional Animal Care & Use Committee (IACUC) at Cornell University.

Drugs. Psilocybin was obtained from the Usona Institute's Investigational Drug & Material Supply Program. The chemical composition of psilocybin was confirmed by high performance liquid chromatography at Usona Institute. A stock 4 mg/mL solution was prepared by dissolving solid in 0.9% saline, then diluted into working solutions of 0.05, 0.1, or 0.2 mg/mL the day before injection. The $5\text{-}HT_{1A}$ receptor antagonist WAY100635 (4380, Tocris Biosciences) was diluted in 0.9% saline for a 5 mg/mL stock solution. The $5\text{-}HT_{2A}$ receptor antagonist MDL100907 (4173, Tocris Biosciences) was dissolved in a 0.9% saline solution containing 0.3% Tween 80 (P1754, Sigma-Aldrich) for a 1 mg/mL stock solution. The day before injection, aliquots of WAY100635 or MDL100907 were diluted in 0.9% saline to produce working solutions of 0.2 mg/mL and 0.1 mg/mL respectively. For injections of two drugs, cocktails of psilocybin (0.1 mg/mL) and either WAY100635 (0.2 mg/mL) or MDL100907 (0.1 mg/mL) were prepared using the same stock solutions and diluting with 0.9% saline. This was done so that both drugs could be administered as a single injection, mitigating stress induced by performing multiple injections. Injections, including vehicle controls, were performed intraperitoneally (i.p.) at a volume of 10 mL/kg. Methods for drug preparation and doses of psilocybin (0.5, 1, or 2 mg/kg), WAY100635 (2 mg/kg), and MDL100907 (1 mg/kg) were chosen based on prior work[33–35].

Fear conditioning and extinction paradigms. The timeline for delay fear conditioning and extinction tests was inspired by prior literature[15,17,18]. All behavioral testing was conducted in a near-infrared video fear conditioning system (MED-VFC2-SCT-M, Med Associates Inc). Prior to each session, mice were brought into a side room separated from the fear conditioning system where they habituated for 30 minutes. The near-infrared video camera was calibrated to record

135   with the manufacturer's recommended average intensity of 130 a.u. so that conditions for

136   motion detection were held constant across all experimental days. On day 1 of testing (auditory

137   cued fear conditioning), the mouse was individually placed in the conditioning chamber, which

138   was prepared in "context A" (blank straight walls, metal grid floor, cleaned with 70% ethanol

139   before each session). Each conditioning trial began with a 3-minute habituation period, after

140   which the mouse was given 5 presentations of an auditory tone as the conditioned stimulus (CS;

141   4 kHz, 80 dB, 30 s duration). Each CS co-terminated with a foot shock unconditioned stimulus

142   (US; 0.8 mA, 2 s duration). A 90 s intertrial interval separated the CS + US pairings. On day 2,

143   mice were left undisturbed in their home cages. On day 3 (fear extinction), the mouse received

144   an i.p. injection of vehicle or drug solution and then were returned to their home cages for 30

145   minutes. After this, the mouse was placed in the conditioning chamber prepared in "context B"

146   (black A-frame, white plastic floor, cleaned with 1% acetic acid before each session). A 3-minute

147   habituation period was given, followed by 15 presentations of the CS without a US, each of

148   which was separated by a 15 s intertrial interval. On day 4 (extinction retention), the mouse

149   underwent the same procedure as day 3, except with no injection administered beforehand.

150   Mice were then left undisturbed until day 11 (fear renewal), at which point the mouse was

151   placed in the conditioning chamber prepared in "context C" (striped curved white wall, striped

152   plastic floor, cleaned with Peroxigard^{TM} before each session) and subjected to the same

153   habituation and CS-only schedule used on days 3 and 4. On each experimental day, mice were

154   returned to their housing location immediately after testing. Variations of this general paradigm

155   were performed to determine the behavioral mechanisms supporting psilocybin's effects. In one

156   set of these experiments (**Fig. 3A**), the mouse was administered 0.9% saline (10 mL/kg, i.p.) or

157   psilocybin (1 mg/kg, i.p.) then returned to their home cage for 30 minutes, after which they

158   underwent fear conditioning followed by extinction 48 hours later as described above. In another

159   set of experiments (**Fig. 3D**), the mouse underwent fear conditioning on day 1, then fear

160   extinction on day 3 with 0.9% saline (10 mL/kg, i.p.) or psilocybin (1 mg/kg, i.p.) administered

161   immediately after fear extinction, then tested for extinction retention 24 hours later.

162

163   **Analysis.** Conditioned freezing was used as the primary readout for behavior, and was defined

164   as a lack of detectable movement other than breathing for ≥ 1 s. The videos captured by the

165   near-infrared camera were analyzed with automated procedures using the VideoFreeze^{TM}

166   software from Med Associates Inc (motion threshold: 18 au, detection method: linear, minimum

167   freeze duration: 30 frames = 1 s). For extinction, extinction retention, and fear renewal, we

168   binned the data to calculate the freezing responses, where bin "Hab" corresponds to the 3-

169   minute habituation period with no tone, bin 1 corresponds to CS period during tones 1-3, bin 2

170   corresponds to CS period during tones 4-6, bin 3 corresponds to CS period during tones 7-9,

171   bin 4 corresponds to CS period during tones 10-12, and bin 5 corresponds to CS period during

172   tones 13-15.

173

174   **Statistics.** Statistical testing was conducted in SPSS. Conditioned freezing data were analyzed

175   with generalized linear mixed effects modeling. Fixed factors are listed in the corresponding

176   figure legends. Random factors of subject, cage, and sex were used to account for individual

177   differences in the overall model, and a heterogeneous autoregressive 1 (ARH1) covariance

178   structure was used in accounting for repeated measures. A sequential Bonferroni correction

179   was applied to between-groups comparisons.

180

181   **RESULTS**

182

183   **Psilocybin enhances extinction and attenuates renewal of conditioned fear**

184   Motivated by studies reporting the effects of MDMA on fear extinction[15,16], we implemented a

185   delay fear conditioning protocol to test the effects of psilocybin on fear extinction (**Fig. 1A**; see

186   **Materials and Methods**). Briefly, on day 1, we trained a C57BL/6J mouse to associate an

187   auditory tone (conditioned stimulus; CS) with a noxious foot shock (unconditioned stimulus; US)

188   in context A. On day 3, the mouse was given psilocybin (0.5, 1, or 2 mg/kg, i.p.) or saline

189   vehicle. The animal was then placed back in its home cage for 30 minutes before being moved

190   to context B for fear extinction, which consisted of 15 CS presentations without the US pairing.

191   On day 4, retention of the extinction memory was tested by exposing the mouse to another 15

192   CS presentations in context B. Finally, on day 11, the mouse was exposed to another 15 CS

193   presentations in context C to determine if its conditioned fear would be renewed in an unfamiliar

194   environment distinct from the initial extinction context. For each drug condition, we tested 4

195   male and 4 female animals. The results are shown in **Figs. 1B-J**.

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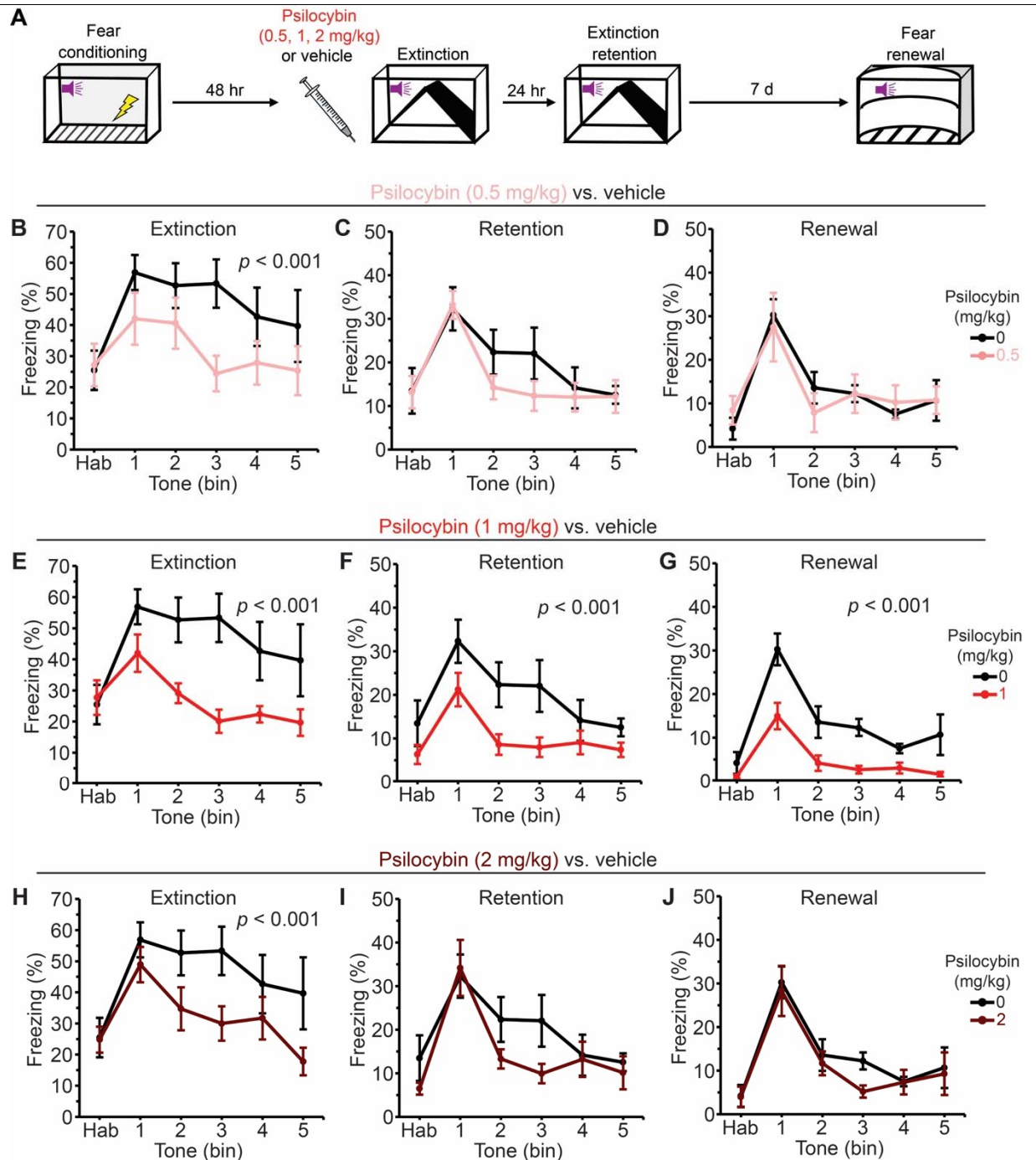
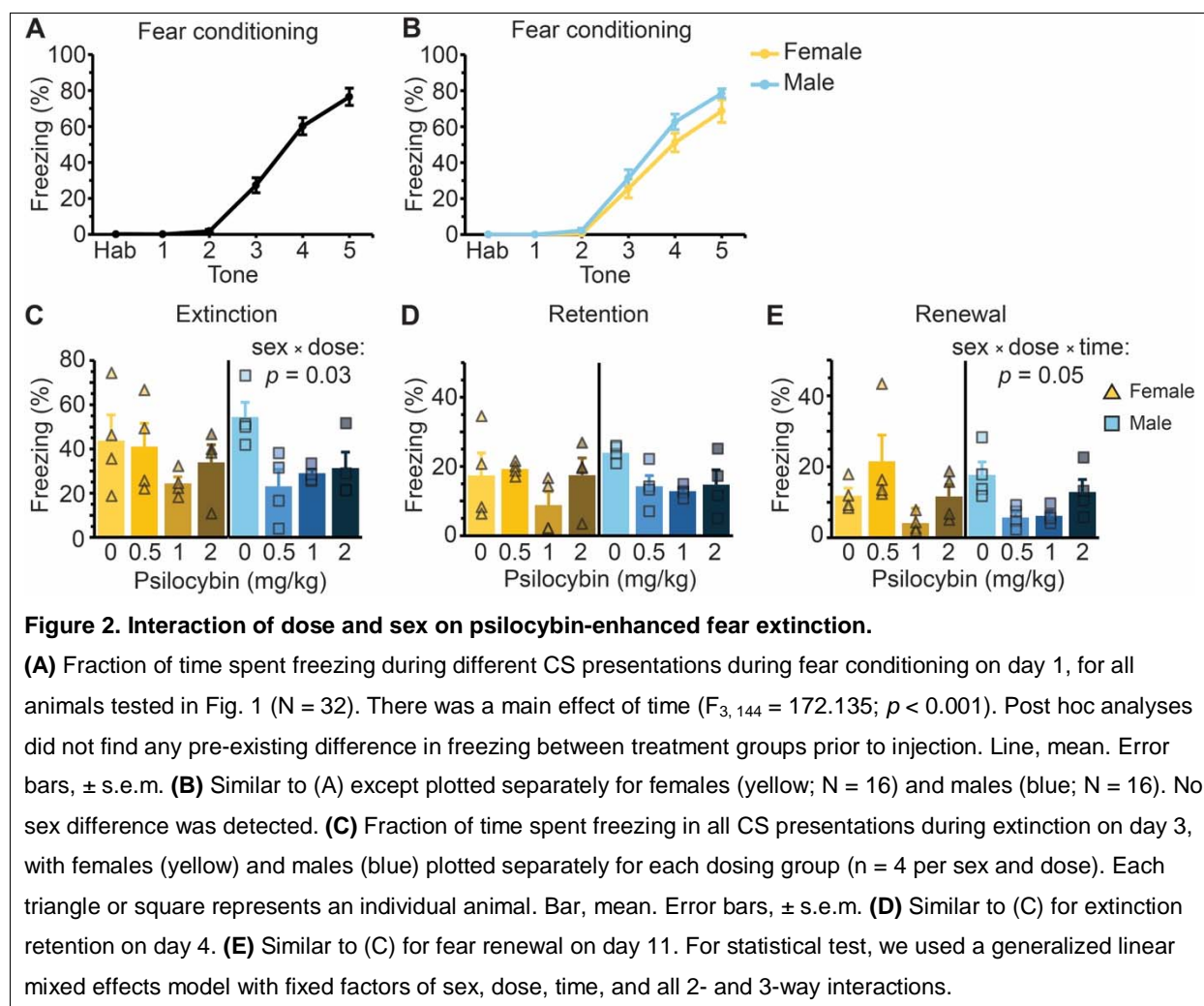**Figure 1. Psilocybin drives persisting enhancement of fear extinction and suppression of fear renewal.**
(A) Experimental timeline. **(B)** Fraction of time spent freezing during habituation (Hab) and different binned CS periods during extinction on day 3. Black, vehicle. Red, 0.5 mg/kg psilocybin. Line, mean. Error bars, ± s.e.m. Shown is the p-value (if <0.05) for between-group post hoc analysis between vehicle and 0.5 mg/kg psilocybin performed after generalized linear mixed effects model with fixed factors of dose, time, and 2-way interactions. **(C)** Similar to (B) for extinction retention on day 4. **(D)** Similar to (B) for fear renewal on day 11. **(E – G)** Similar to (B) – (D) for 1 mg/kg psilocybin. The data for vehicle is repeated from (B) – (D) for purpose of comparison. **(H – J)**

197

198 For day 3, as expected for an extinction session, freezing response decreased as tones were
199 repeatedly presented (main effect of time: $F_{12, 420}$ = 2.568, p = 0.001; generalized linear mixed
200 effects model with fixed factors of dose, time, and 2-way interactions). Psilocybin reduced
201 conditioned freezing (main effect of dose: $F_{3, 420}$ = 6.223, p < 0.001), which was statistically
202 significant for all doses tested compared to vehicle controls (p < 0.001 in all cases; between-
203 group post hoc analyses), with no psilocybin dose significantly outperforming the others (**Figs.**
204 **1B, E, H**). By contrast, for retention on day 4, we detected the effect of psilocybin (main effect of
205 dose: $F_{3, 420}$ = 4.387, p = 0.005; main effect of time: $F_{12, 420}$ = 4.998, p < 0.001), however post
206 hoc analyses revealed only mice previously given 1 mg/kg psilocybin froze less than vehicle
207 controls (p < 0.001), while no significant differences were found between mice given 0.5 or 2
208 mg/kg psilocybin and vehicle (**Figs. 1C, F, I**). For fear renewal on day 11, we observed a drug
209 effect again (main effect of dose: $F_{3, 420}$ = 5.72, p < 0.001; main effect of time: $F_{12, 420}$ = 5.665; p
210 < 0.001), which was driven by mice in the 1 mg/kg psilocybin group, as these mice froze less
211 than their counterparts injected with vehicle (p = 0.001), 0.5 (p = 0.005), or 2 mg/kg psilocybin (p
212 = 0.022) (**Figs. 1D, G, J**). Together, the data indicate that psilocybin robustly enhances fear
213 extinction when given acutely prior to testing. Psilocybin also exerts long-term effects to elevate
214 extinction retention and suppress fear renewal up to 8 days later, though these changes were
215 sensitive to dose and were most reliably elicited by 1 mg/kg of psilocybin.

216

217 To explore potential difference in male versus female animals, we analyzed the dose-response
218 data using fixed factors of sex, dose, time, and all 2- and 3-way interactions. For fear
219 acquisition, we found a main effect of time ($F_{3, 152}$ = 193.417; p < 0.001), but no sex effects were
220 detected (**Fig. 2A, B**). This changed in the extinction task (**Fig. 2C**), which showed a sex × dose
221 interaction ($F_{3, 360}$ = 3.018; p = 0.03) and a main effect of time ($F_{14, 360}$ = 2.965; p < 0.001). For
222 extinction retention, we detected main effects of dose ($F_{3, 360}$ = 5.298; p = 0.001) and time ($F_{14, 360}$ = 4.915; p < 0.001) (**Fig. 2D**). Finally, for fear renewal, there was a significant sex × dose ×
223
224 time interaction ($F_{42, 360}$ = 1.42; p = 0.05) (**Fig. 2E**). These additional analyses suggest that sex
225 may be a factor in psilocybin's dose-dependent effects on fear extinction.

226

Figure 2. Interaction of dose and sex on psilocybin-enhanced fear extinction.

(A) Fraction of time spent freezing during different CS presentations during fear conditioning on day 1, for all animals tested in Fig. 1 (N = 32). There was a main effect of time ($F_{3, 144}$ = 172.135; p < 0.001). Post hoc analyses did not find any pre-existing difference in freezing between treatment groups prior to injection. Line, mean. Error bars, ± s.e.m. **(B)** Similar to (A) except plotted separately for females (yellow; N = 16) and males (blue; N = 16). No sex difference was detected. **(C)** Fraction of time spent freezing in all CS presentations during extinction on day 3, with females (yellow) and males (blue) plotted separately for each dosing group (n = 4 per sex and dose). Each triangle or square represents an individual animal. Bar, mean. Error bars, ± s.e.m. **(D)** Similar to (C) for extinction retention on day 4. **(E)** Similar to (C) for fear renewal on day 11. For statistical test, we used a generalized linear mixed effects model with fixed factors of sex, dose, time, and all 2- and 3-way interactions.

227

Psilocybin is only effective when administered prior to extinction context

229 How can psilocybin facilitate extinction? Classic psychedelics may boost learning through their
230 effects on neural plasticity, which have been demonstrated for psilocybin as it evokes structural
231 rewiring in the medial frontal cortex[34] and induces plasticity-related gene expression[37,38]. Given
232 that learning and memory are linked to structural plasticity[39–42], one possibility here is that
233 learning is enhanced broadly by psilocybin. If true, psilocybin should also improve fear memory
234 acquisition and extinction memory consolidation. An alternative possibility is that learning is
235 enhanced but only for specific contexts. Preclinical studies involving MDMA indicate that the
236 context immediately after drug administration is an important factor in determining the eventual
237 behavioral changes in mice[15], but it is unknown if this applies to psilocybin and extinction.

238

239 To disambiguate the possibilities, we devised two variants of the conditioned fear extinction
240 protocol. For the first variant, mice received 1 mg/kg psilocybin or vehicle 30 minutes prior to

fear conditioning on day 1, then underwent fear extinction on day 3 (**Fig. 3A**). As expected, mice

readily acquired conditioned freezing during fear conditioning (main effect of time: $F_{4, 70} = 105.696$, $p < 0.001$; **Fig. 3B**) and progressively reduced conditioned freezing during extinction

(main effect of time: $F_{14, 210} = 2.641$, $p < 0.001$; **Fig. 3C**). However, no difference was observed

between the psilocybin and vehicle-treated groups.
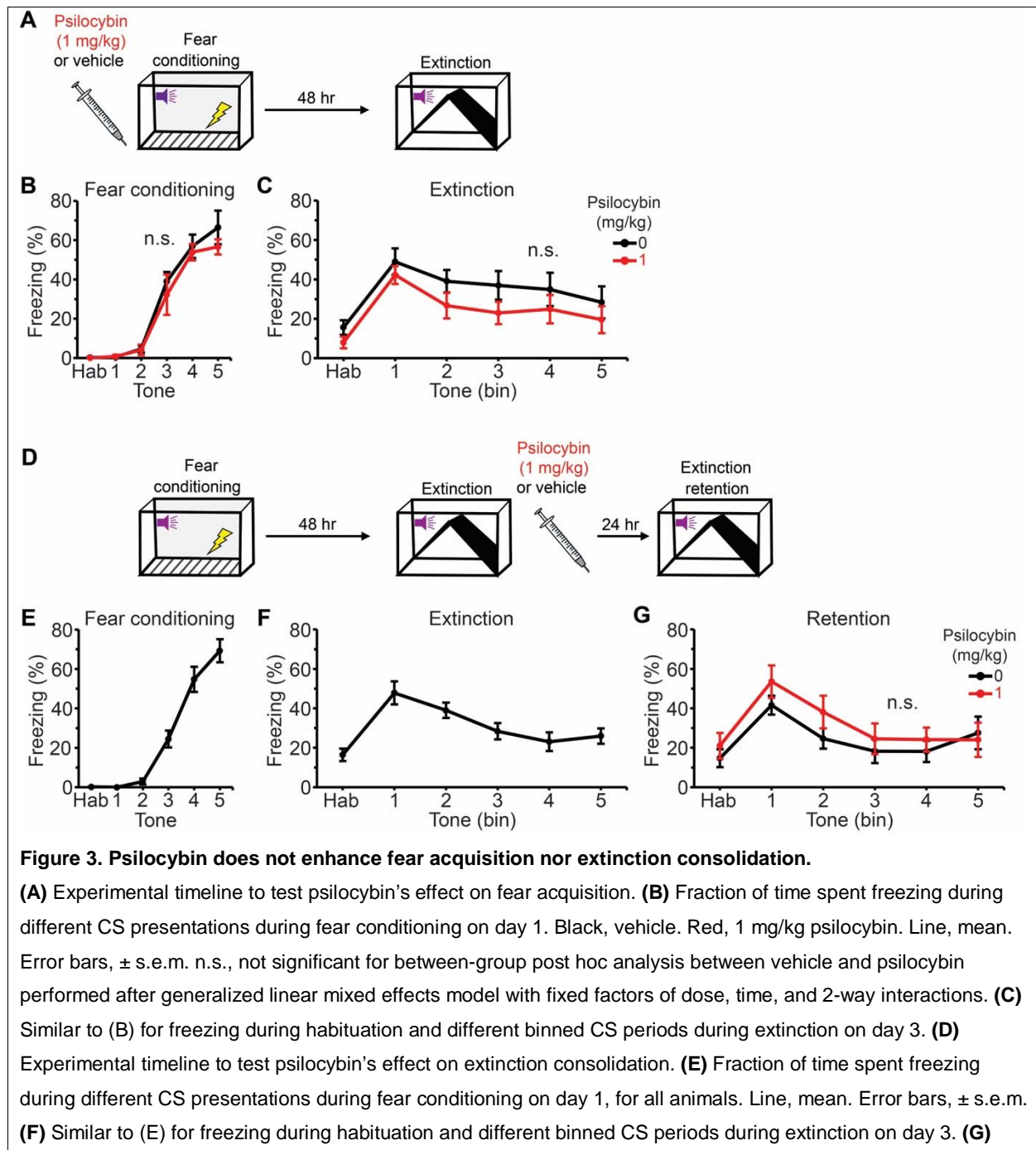
**Figure 3. Psilocybin does not enhance fear acquisition nor extinction consolidation.**

(A) Experimental timeline to test psilocybin's effect on fear acquisition. **(B)** Fraction of time spent freezing during different CS presentations during fear conditioning on day 1. Black, vehicle. Red, 1 mg/kg psilocybin. Line, mean. Error bars, ± s.e.m. n.s., not significant for between-group post hoc analysis between vehicle and psilocybin performed after generalized linear mixed effects model with fixed factors of dose, time, and 2-way interactions. **(C)** Similar to (B) for freezing during habituation and different binned CS periods during extinction on day 3. **(D)** Experimental timeline to test psilocybin's effect on extinction consolidation. **(E)** Fraction of time spent freezing during different CS presentations during fear conditioning on day 1, for all animals. Line, mean. Error bars, ± s.e.m. **(F)** Similar to (E) for freezing during habituation and different binned CS periods during extinction on day 3. **(G)**

Fraction of time spent freezing during different CS presentations during extinction retention on day 4. Black, vehicle. Red, 1 mg/kg psilocybin. Line, mean. Error bars, ± s.e.m. N = 16, including 4 males and 4 females for each of the vehicle and psilocybin conditions for fear acquisition, 4 males and 4 females for each of the vehicle and psilocybin conditions for extinction retention.

247

248 For the second variant, mice underwent fear conditioning on day 1 (**Fig. 3D**). Instead of

249 administering psilocybin prior to extinction on day 3, the paradigm was modified such that 1

250 mg/kg psilocybin or vehicle was administered immediately after extinction. The mice were then

251 tested for extinction retention on day 4. Again, mice readily acquired conditioned freezing during

252 fear conditioning (main effect of time: $F_{3, 70} = 63.946$; $p < 0.001$; **Fig. 3E**) and progressively

253 reduced conditioned freezing during extinction (main effect of time: $F_{14, 210} = 3.275$; $p < 0.001$;

254 **Fig. 3F**), with no pre-existing differences between treatment groups prior to drug administration.

255 The post-extinction administration of 1 mg/kg psilocybin did not impact extinction retention on

256 day 4 compared to vehicle controls (**Fig. 3G**). Collectively, these experiments suggest that the

257 timing of psilocybin administration – shortly prior to the initial extinction experience – is key to its

258 effect on retention of the extinction memory.

259

260 **5-HT$_{2A}$ and 5-HT$_{1A}$ receptors contribute to psilocybin's long-term behavioral effects**

261 Upon entering the body, psilocybin is rapidly converted to its active metabolite, psilocin, which is

262 an agonist at various serotonin receptors[43], including the 5-HT$_{1A}$ and 5-HT$_{2A}$ subtypes which are

263 highly expressed in the neocortex[44]. To test the role of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in

264 mediating psilocybin's effects on fear extinction, we modified the auditory fear conditioning

265 protocol such that the mouse received an i.p. injection of a mixture containing MDL100907 (1

266 mg/kg; 5-HT$_{2A}$ receptor antagonist[45,46]), WAY100635 (2 mg/kg; 5-HT$_{1A}$ receptor antagonist[47]) or

267 vehicle, paired with psilocybin (1 mg/kg) or vehicle (**Fig. 4A**). For each of the 4 conditions in

268 MDL100907/vehicle + psilocybin/vehicle, we tested 4 male and 4 female animals. For each of

269 the 4 conditions in WAY100635/vehicle + psilocybin/vehicle, we tested 4 male and 4 female

270 animals. We did not find any notable difference in the results involving the vehicle +

271 psilocybin/vehicle conditions, therefore combined the data to produce **Figs. 4B-D**. The

272 remainder of the results are shown separately for MDL100907 + psilocybin/vehicle (**Figs. 4E-G**)

273 and WAY100635 + psilocybin/vehicle (**Figs. 4H-J**).

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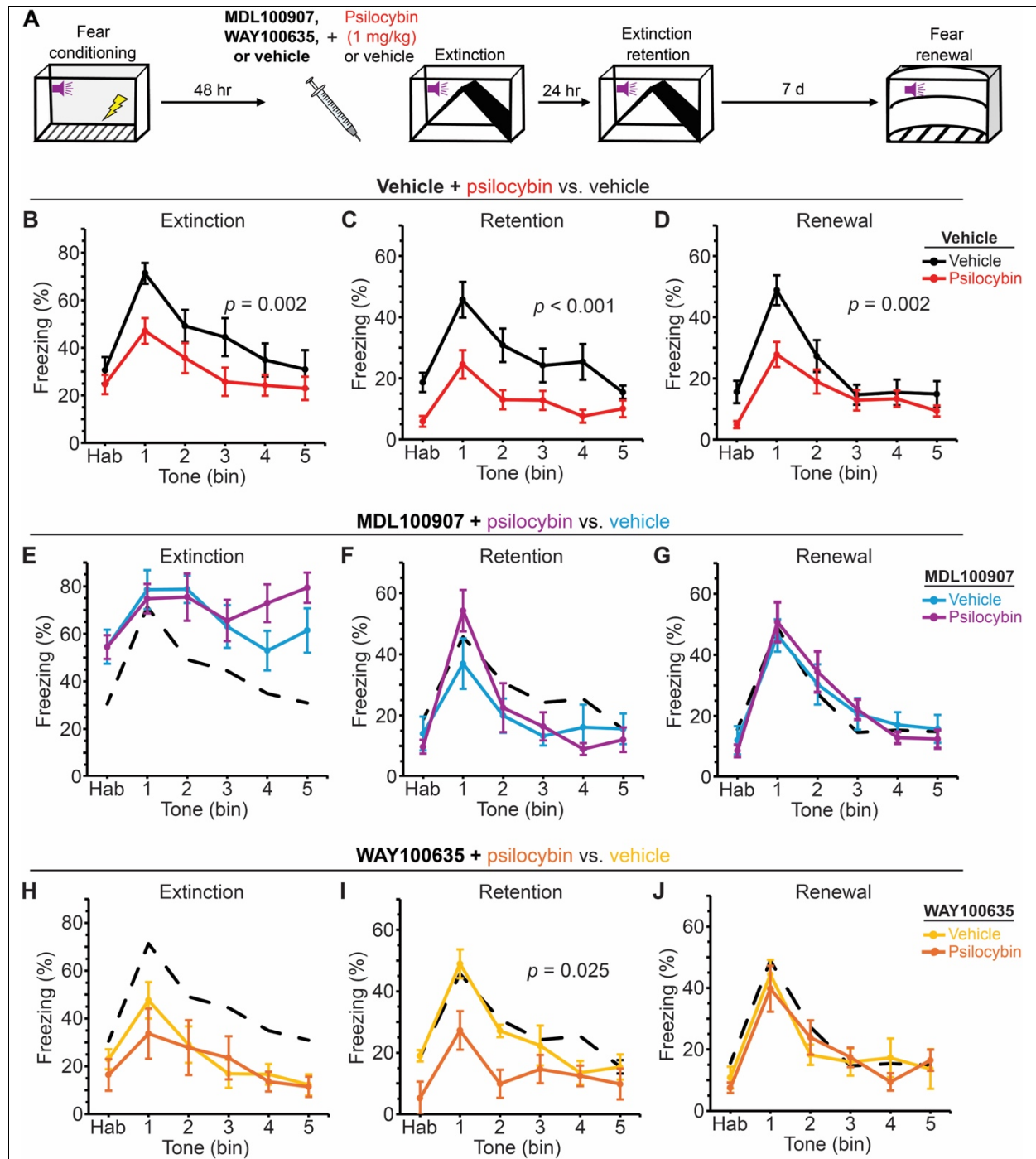
Figure 4. Effects of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor antagonists on psilocybin-enhanced fear extinction.

(A) Experimental timeline. **(B)** Fraction of time spent freezing during habituation and different binned CS periods during extinction on day 3. Black, vehicle + vehicle. Red, vehicle + 1 mg/kg psilocybin. Line, mean. Error bars, ± s.e.m.. Shown is the p-value (if <0.05) for between-group post hoc analysis between vehicle + vehicle and vehicle + 1 mg/kg psilocybin performed after generalized linear mixed effects model with fixed factors of antagonist, psilocybin, time, as well as all 2- and 3-way interactions. **(C)** Similar to (B) for extinction retention on day 4. **(D)** Similar to (B) for fear renewal on day 11. **(E – G)** Similar to (B) – (D) for MDL100907 + vehicle (teal) and

MDL100907 + psilocybin (purple). Dashed line, the vehicle + vehicle data repeated from (B) – (D) for purpose of comparison. **(H – J)** Similar to (B) – (D) for WAY100635 + vehicle (yellow) and WAY100635 + psilocybin (orange). Dashed line, the vehicle + vehicle data repeated from (B) – (D) for purpose of comparison. N = 64 including 8 males and 8 females for vehicle + vehicle, 8 males and 8 females for vehicle + psilocybin, and 4 males and 4 females each for MDL100907 + vehicle, MDL100907 + psilocybin, WAY100635 + vehicle, and WAY100635 + psilocybin.

275

276   For extinction on day 3, the co-administration of a receptor antagonist altered the conditioned

277   freezing response (antagonist × drug interaction: $F_{2, 870}$ = 3.604, p = 0.028; generalized linear

278   mixed effects model). Replicating our earlier result in **Fig. 1E**, vehicle + psilocybin mice froze

279   significantly less than vehicle + vehicle controls (p = 0.002; **Fig. 4B**). Mice with WAY100635,

280   regardless of whether they received psilocybin, froze less than vehicle + vehicle controls (p =

281   0.001; **Fig. 4E**). Conversely, mice with MDL100907, regardless of whether they received

282   psilocybin, increased freezing compared to vehicle + vehicle controls (p = 0.001; **Fig. 4H**). Our

283   interpretation is that, at the doses used, the receptor antagonists themselves significantly

284   altered the measurement of freezing response. Indeed, it has been shown that WAY100635

285   increases rearing in light box[47] and MDL100907 reduces spontaneous motor activity in rats and

286   mice[46], which would affect the readout by our automated video analysis. In agreement with this

287   explanation, MDL100907-treated mice froze more than any other group during the 3-minute

288   habituation period prior to CS presentation (p < 0.01 in all cases; bin "Hab" in **Fig. 4E**).

289   Notwithstanding the confound of acute locomotor effects, mice that received receptor

290   antagonists appeared awake on day 3, and thus were expected to have still formed an

291   extinction memory.

292

293   For extinction retention on day 4, there was a significant antagonist × psilocybin × time

294   interaction ($F_{28, 870}$ = 1.791; p = 0.007). Replicating our previous finding in **Fig. 1F**, vehicle +

295   psilocybin mice froze less than their vehicle + vehicle treated counterparts (p < 0.001; **Fig. 4C**).

296   MDL100907 + psilocybin and MDL100907 + vehicle animals froze at similar levels (**Fig. 4F**),

297   indicating that 5-HT$_{2A}$ receptor contributes to psilocybin's effect on extinction retention. By

298   contrast, psilocybin remained effective relative to vehicle in mice that were co-administered with

299   WAY100635 (p = 0.025; **Fig. 4I**). For fear renewal on day 11, like **Fig. 1G**, vehicle + psilocybin

300   mice froze less than vehicle + vehicle mice (p = 0.002; **Fig. 4D**). No effect of psilocybin was

301   observed in MDL100907-treated mice, which froze at approximately control levels (**Fig. 4G**).

302   Similarly, no effect of psilocybin was observed in WAY100635-treated mice for fear renewal

303   (**Fig. 4J**). Collectively, these results provide evidence that psilocybin's long-term effects on

304 enhanced extinction relies on 5-HT$_{2A}$ receptors, with the suppression of fear renewal in

305 unfamiliar context additionally dependent on 5-HT$_{1A}$ receptors.

306

307 **DISCUSSION**

308 There are two main findings for this study. First, psilocybin enhances fear extinction, including

309 longer term effects in extinction retention and fear renewal in a novel context. Second, the effect

310 of psilocybin on fear extinction is dependent on dose, timing of administration, and contributions

311 from 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors.

312

313 We found a dynamic dose-response relationship for psilocybin-enhanced extinction, as 0.5, 1

314 and 2 mg/kg psilocybin all reduced conditioned freezing for extinction acutely, but only the 1

315 mg/kg dose showed persisting effects in extinction retention and fear renewal. The dose

316 dependence may arise because fear extinction is a complex behavior moderated by concerted

317 changes in attention, arousal, and memory, which often exhibit U-shaped dose-effect

318 relationships[48,49]. Interestingly, a previous study assessing psilocybin's impact on acoustic

319 startle in rats came to a similar conclusion, identifying 1 mg/kg as the dose causing the greatest

320 extent of behavioral change, while doses beyond 4 mg/kg failed to elicit lasting effects[50]. We

321 explored potential sex difference in psilocybin's dose-dependent behavioral effects. We

322 detected statistical differences but did not proceed to post hoc analyses because of few animals

323 per group when the data were fully segmented by sex and dose. Qualitatively, female mice had

324 a narrower dose-response relationship with 1 mg/kg as the optimal dose, whereas male mice

325 exhibited sensitivity to a wider range of psilocybin doses. These results highlight the importance

326 of testing different doses and sexes when evaluating psilocybin's effects using preclinical

327 behavioral assays.

328

329 Psilocybin was only effective when administered prior to extinction, as injection before fear

330 conditioning or following extinction testing yielded no behavioral change. The timing of

331 administration, and by extension the context following drug administration, is therefore crucial

332 for psilocybin. The timing requirement is consistent with previous studies investigating the

333 effects of MDMA and low dose of scopolamine on fear extinction[15,51], but differs sharply with

334 ketamine which can accelerate fear extinction and suppress fear renewal even when

335 administered well in advance[52]. Moreover, the behavioral domain may matter because prior

336 work showed that psilocybin rescued stress-induced deficits in sucrose preference[53] and

337 learned helplessness[34] when administered 24 hours prior to testing. This raises an intriguing

338   question of whether certain psychedelics and tasks may require concurrent behavioral

339   experience to produce their translationally important effects[54,55]. Thus, it is worth thinking

340   beyond the simple picture of drug-evoked synaptogenesis and ask how these novel synapses

341   are integrated into neural circuits in a behaviorally relevant manner for the appropriate context.

342

343   One caveat of the study is that although WAY100635 is a potent $5\text{-}HT_{1A}$ receptor antagonist[47], it

344   has the off-target effect of activating D_4 receptors[56]. The results in the WAY100635 experiment

345   may therefore be due in part to agonism of D_4 receptors. That said, there are previous studies

346   which corroborate the potential role of $5\text{-}HT_{1A}$ receptors in mediating psilocybin's action. Mice

347   lacking the $5\text{-}HT_{1A}$ receptor show heightened fear responses when encountering previously

348   conditioned cues in novel contexts[57,58]. Attenuating $5\text{-}HT_{1A}$ receptor activity in novel contexts,

349   but not familiar ones, blocks induction of long-term potentiation of medial perforant path inputs

350   to the dentate gyrus[59]. Yohimbine has been extensively tested as a potential agent for

351   enhancing fear extinction, and a potential target for its mechanism of action is $5\text{-}HT_{1A}$

352   receptors[60]. Moreover, our results agrees generally with prior studies showing the importance of

353    $5\text{-}HT_{2A}$ receptors in mediating psychedelics' effects on various fear-based assays[16,17,19,21,33,61],

354   but here more specifically we highlight the importance for extinction at the long time scale.

355   Another caveat is that we rely on conditioned freezing as the sole readout of learned fear. This

356   is advantageous because the measurement was automated to eliminate experimenter bias.

357   However, the readout ignores other potential response strategies, which may be differentially

358   employed by male and female rodents[36]. In the future, it will be useful to compare the effects of

359   psilocybin on fear extinction retention using other readout methods and in other species, such

360   as humans where MDMA has been tested in preliminary studies[62,63].

361

362   In summary, this study shows that psilocybin enhances fear extinction and suppresses fear

363   renewal long after the drug has been administered. Dose, context, and serotonin receptors are

364   contributing factors to psilocybin's effects. The suppression of fear renewal is particularly

365   relevant for translation because this is an unmet need in the clinic. Thus, the findings provide a

366   detailed characterization of psilocybin's effects on behavioral processes relevant to prolonged

367   exposure therapy. Moreover, the work adds further preclinical support for investigating the utility

368   of psilocybin as a pharmacological adjunct for extinction-based therapy for PTSD.

369

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378   **Contributions**

379   S.C.W. and A.C.K planned the study. S.C.W. performed experiments. C.M.L. and A.M.K.

380   assisted with the experiments. S.C.W. analyzed the data. S.C.W. and A.C.K. drafted the

381   manuscript. All authors reviewed the manuscript before submission.

382

383   **Competing interests**

384   A.C.K. has served as a scientific advisor for Empyrean Neuroscience, Freedom Biosciences,

385   and Psylo. A.C.K. has received research support from Intra-Cellular Therapies. The other

386   authors report no financial relationships with commercial interests.

387

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