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Effects of a psychedelic 5-HT_{2A} receptor agonist on anxiety-related behavior and fear processing in mice

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Abstract

Psychedelic-assisted psychotherapy gained considerable interest as a novel treatment strategy for fear-related mental disorders. Promising clinical results have been obtained with 3,4-methylenedioxyamphetamine (MDMA)-assisted psychotherapy in patients with post-traumatic stress disorder (PTSD) but the underlying mechanism remains poorly understood. The serotonin 2A (5-HT_{2A}) receptor is a key target underlying the effects of psychedelics on emotional arousal but its role in fear processing remains controversial. Using the psychedelic 5-HT_{2A}/5-HT_{2C} agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) and 5-HT_{2A} receptor knockout (KO) mice we investigated the effect of 5-HT_{2A} receptor activation on emotional processing. We show that DOI administration did not impair performance in a spontaneous alternation task but reduced anxiety-like behavior in the elevated plus maze task. Moreover, we found that DOI did not block memory recall but diminished fear expression in a passive avoidance task. Similarly, we found that DOI reduced fear expression in an auditory fear conditioning paradigm, while it did not affect fear extinction learning in wild type (WT) mice. The effect of DOI on fear expression was abolished in 5-HT_{2A} receptor KO mice. Administration of DOI induced a significant increase of c-Fos expression in specific amygdalar nuclei. Moreover, local infusion of the 5-HT_{2A} receptor antagonist M100907 into the amygdala reversed the effect of systemic administration of DOI on fear expression while local administration of DOI into the amygdala was sufficient to suppress fear expression. Our data demonstrate that activation of 5-HT_{2A} receptors in the amygdala suppresses fear expression, but provide no evidence for an effect on fear extinction.

Introduction

Post-traumatic stress disorder (PTSD) is a severe mental disorder, that can develop following exposure to a traumatic event [1], affecting between 1.3% and 12.2% of individuals during their lifetime [2]. Patients typically suffer from intrusive thoughts and re-experience the trauma, while suffering long-term changes in arousal, mood and cognition [1]. The first-line treatment for PTSD consists of trauma-focused psychotherapies, such as exposure-based therapy, and pharmacotherapy with selective serotonin reuptake inhibitors [3-5]. While psychotherapy typically yields greater effects than pharmacotherapy [4], dropout rates are high [6,7] and up to 60-72% of patients fail to achieve full remission [3,6]. This has been conceptualized as a failure of fear extinction, the process in which subjects learn that a past fearful experience no longer poses an acute threat [8]. This has led to an ongoing search for pharmacological agents that improve the tolerability and efficacy of psychotherapy, especially of drugs that improve extinction [9,10].

Psychedelic-assisted psychotherapy gained considerable interest for the treatment of mental disorders [11-15] and 3,4-methylenedioxymethamphetamine (MDMA) has given promising clinical results in patients with PTSD [16,17]. The main rationale behind MDMA-assisted psychotherapy is that it may reduce aversion to fear-provoking thoughts while promoting the extinction of fearful memories [15,18,19]. Fear extinction is the learning process by which exposure to fear-provoking cues reduces fear responses. As such, MDMA may influence memories associated with the traumatic events in a clinical setting [20]. Preclinical studies indicate that MDMA suppresses fear expression during extinction training, but its effects on extinction learning remain unclear [21-24]. MDMA shares several properties with psychostimulants, and classic psychedelics such as psilocybin, lysergic acid dimethylamide (LSD) and dimethyltryptamine (DMT) [25]. MDMA stimulates norepinephrine, serotonin and dopamine release by reversing the activity of their transporters [26-31]. Interestingly, 5-HT_{2A} antagonism was shown to attenuate the effects of MDMA on emotional arousal in humans [18,32,33] and reversed the effect of MDMA on fear processing in rats [23].

Serotonin 2A (5-HT_{2A}) receptors are a key target for classic serotonergic psychedelics [34,35]. While classic psychedelics were explored in psychodynamic therapies of trauma, no formal clinical trial has examined their therapeutic potential in the treatment of PTSD [15]. In preclinical studies, the psychedelics psilocybin and TCB-2 enhanced the acquisition of trace fear extinction in mice [36,37]. However, the effect of these compounds on the retention of fear extinction remain unclear. Interestingly, a recent study showed that a single dose of DMT decreased fear expression during extinction training and improved the retention of extinction [38]. Moreover, while chronic intermittent administration of DMT did not affect fear expression, it enhanced extinction retention in rats [39]. However, given that besides the 5-HT_{2A} receptor, most psychedelics, including DMT, also target other receptors such as the 5-HT_{1A} receptor, the molecular targets that drive the effects of classic psychedelics on fear processing is not clearly established.

In the present study, we explored the effects of the psychedelic 5-HT_{2A}/5-HT_{2C} receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) in wild type and 5-HT_{2A} receptor knockout (KO) mice to determine the impact of 5-HT_{2A} receptor activation on anxiety and fear. We first studied the effects of DOI administration on exploratory behavior in spontaneous alternation task and exploratory behavior in an elevated plus maze task. Next, we determined the effects of DOI

treatment on memory recall and fear expression in passive avoidance task. Finally, we investigated whether DOI affects fear expression and facilitates fear extinction in an auditory fear conditioning paradigm. We used c-Fos analysis and local administration of the 5-HT_{2A} antagonist M100907 to identify key anatomical targets for the effect of DOI on fear expression.

Materials and Methods

Animals

Male C57BL/6J wild type mice or 5-HT_{2A} receptor KO mice on C57BL/6J background, aged 8-12 weeks at the time of testing, were group housed (2-4 per cage) and maintained on a 12/12h day-night cycle under standard laboratory conditions with controlled temperature (20-24°C) and humidity (30-60%). Food and water were available ad libitum. Mice were habituated to the housing facility for at least one week and to handling procedures on at least three consecutive days before starting behavioral experiments. All procedures were in strict compliance with the animal use and care guidelines of Montpellier University (authorization A34-518/A34-172-13) and the guidelines of the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (ECD 17-213-3).

Drugs and administration

Stock solutions of the 5-HT_{2A}/5-HT_{2C} agonist 2,5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI, Tocris Bioscience) and the 5-HT_{2A} receptor antagonist (R)-(+)-alpha-(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol (M100907, Tocris Bioscience) were prepared in 99.9% dimethylsulfoxide (DMSO; Sigma-Aldrich Chemicals) and stored at -20°C. Working solutions were freshly prepared by diluting the stock solutions in sterile saline (0.9% NaCl, Baxter). We based these doses on previous studies showing robust effects on 5-HT_{2A} receptors in mice [40,41]. For intraperitoneal (i.p.) administration, DOI was diluted to a dose of 2 mg/kg for an injection volume of 10 ml/kg. The working solution contained 5% V/V DMSO in sterile saline and control mice received the same volume of 5% V/V DMSO in sterile saline. For targeted intracranial (i.c.) administrations, working solutions were prepared by diluting a stock solution to a final dose of 2.5 µg per site for DOI and 0.3 µg per site for M100907. Working solutions were adjusted to pH 5.5-6.5 and contained 20% DMSO. Control mice were injected with the same volume of 20% DMSO in sterile saline. Infusion needles (33G, PlasticsOne) with 0.5 mm protrusion (prefrontal cortex, PFC) or 1 mm protrusion (amygdala) were inserted into each guide cannulae for i.c. administration. DOI and the corresponding vehicle were infused at a flow rate of 0.5 µl/min for 1 min. M100907 and the corresponding vehicle were infused at a flow rate of 1 µl/min for 1 min. The injector was left in the cannulae for 1 min after infusion to allow for diffusion of the compounds and to avoid back-flow.

Stereotactic surgery

Cannula implantations were carried out as previously described [42]. Briefly, stainless steel guide cannulae (26 gauge, Plastics One) were implanted bilaterally above the medial prefrontal cortex (mPFC, coordinates: 2.00 mm anterior, 0.40 mm lateral, 2.00 mm ventral from bregma) or amygdala (coordinates: 1.80 mm posterior, 3.40 mm lateral, 3.90 mm ventral from bregma). Correct cannula placement was evaluated post-mortem, using a dissecting microscope and anatomical reference atlas [43]. Animals with misplaced or blocked cannulae were excluded from analysis.

Y-maze spontaneous alternation

The apparatus consisted of three enclosed arms (LxWxH: 30x6x15 cm). No intramaze cues were provided while extramaze cues were placed in the room. Mice were placed in a random arm of the maze and explored it for 5 min with all three arms open. Trials were recorded using an overhead camera and video files were scored manually by an observer blinded to treatment. The percentage alternation (i.e., the number of alternations divided by the total possible alternations and multiplied by 100) was used as a measure of spatial working memory and executive function [44,45]. One alternation was counted when mice visited the three different arms consecutively. Immediate reentries were discounted.

Elevated plus maze

The apparatus consisted of a plus-shaped maze with two opposite open (LxW: 23.5x8 cm) and enclosed arms (LxWxH: 23.5x8x20 cm high). The arms extended from a central platform (LxW: 8x8 cm) and the maze was elevated to a height of 50 cm above the floor. Each mouse was placed at the center of the maze and could freely explore it for a total duration of 10 min. Trials were recorded using an overhead camera and the behavior of mice was scored by an observed blinded to treatment. The time spent in open arms and the number of entries in both arms were recorded manually and expressed as a percentage of total time spent and total entries. Propensity of mice to explore the open arms was used as a measure of anxiety-like behavior.

Passive avoidance

The procedure comprised training, short-term memory (STM) performed 60 min after the training, and long-term memory (LTM) performed 24 h after training. Mice were placed in a dark room for 30-180 min before the start of the experiments. The training sessions began by placing the mice in a brightly lit (700 lux) corridor (LxWxH: 50x10x40 cm Plexiglas walls), separated from a dark chamber (LxWxH: 10x10x40 cm) by a guillotine door. When mice entered the dark chamber, the door was shut followed by foot-shock (2 s, 0.6 mA) delivery. Training ended after foot-shock delivery or after 5 min. STM and LTM sessions were identical to the training session, but no foot-shock was given in the LTM test. Latency to chamber entry was recorded for all mice. Additionally, the corridor was divided into 4 areas (L1-L4) based on their distance from the dark chamber (L1: closest, L4: furthest). Latency to explore, time spent in each area and freezing were quantified. An overhead camera recorded all trials and behavior was analyzed using an automated video monitoring system (Ethovision XT, Noldus).

Auditory fear processing

Auditory fear processing followed previously described procedures with minor modifications [46]. Different context configurations were used for fear conditioning (context A: metal grid on black floor, gray walls, cleaned with 1% acetic acid between trials) and for fear extinction, extinction recall or fear recall tests (context B: white rubber floor, checkerboard-pattern walls, cleaned with 3% hospital antiseptic concentrate between trials). Each session began with 2 min habituation to the context and ended with 1 min cool down. During conditioning, mice received 3 (moderate conditioning) or 5 (strong conditioning) pairings (60 s inter-stimulus interval) of the conditioned stimulus (CS, 4 kHz, 80 dB, 30 s tone) co-terminating with a 0.6 mA (moderate conditioning) or 1.0 mA (strong conditioning) unconditioned stimulus (US, 2 s, scrambled footshock). Fear extinction followed one day after conditioning and consisted of 40 CS

presentations (5 s inter-stimulus interval). Extinction recall was tested one day after extinction by 4 CS presentation (60 s inter-stimulus interval). Fear recall was tested one day after fear conditioning by 2 CS presentations (60 s inter-stimulus interval). Freezing behavior was defined as a lack of detectable movements other than breathing for 1 s or more. Freezing was assessed using an automated video monitoring system (Ethovision XT, Noldus: Fig 1E, 1F, 1G, 2D, 3B, 4C, 4E) or load-cell coupler detection system (Freezing, Panlab: Fig 2B, 2C, 4G).

Immunofluorescence and confocal microscopy

Tissue processing and image acquisition were carried out as previously described [47]. In brief, mice were anaesthetized with sodium pentobarbital i.p. (200 mg/ml) and transcardially perfused with phosphate-buffered saline (PBS, Sigma-Aldrich) and ice-cold 4% paraformaldehyde (PFA, VWR Chemicals), 90 min after the behavioral procedure. 40-um thick slices were prepared on a vibratome (Leica VT1000S, Leica Biosystems), rinsed in tris-buffered saline (TBS, Sigma-Aldrich) and blocked for 1h with 0.3% Triton-X (Sigma-Aldrich) and 3% bovine serum albumin (Sigma-Aldrich) in TBS at room temperature. Slices were incubated in blocking buffer overnight at 4°C with rabbit anti-c-Fos (1:500; #2250, Cell Signaling) and mouse anti-parvalbumin (1:1000; #PV235, Swant) antibodies or for 72 h in primary rabbit anti-5-HT2A antibodies (1:100; #24288 ImmunoStar Inc.) in blocking buffer at 4 °C. Sections were rinsed in TBS with 0.1% Triton-X and incubated with secondary CyTM3-labeled goat anti-rabbit (1:500; #111–165-003, Jackson ImmunoResearch Laboratories) and Alexa Fluor 488-labeled donkey anti-mouse (1:400; #711–175-152, Jackson ImmunoResearch Laboratories) antibodies in TBS for 45 min in room temperature. Finally, slices were rinsed in TBS and tris buffer before mounting on microscopy slides. Confocal microscope images (Zeiss, Axio Observer with LSM 710-6NLO configuration, Zeiss International) were processed and analyzed with ImageJ software (v. 1.53b). Prefrontal cortex and amygdala nuclei were manually marked after applying an automatic Li threshold algorithm using the channel corresponding to parvalbumin (PV) [48]. Expression of c-Fos was analyzed using the *Find Maxima* ImageJ plugin, after preprocessing the images with a median filter [48,49]. Cells expressing PV were counted manually. Final analysis was performed on average measurements of 2-4 images per region per mouse.

Statistical analysis

Data were expressed as mean values \pm SEM. Statistical analysis was performed using Graphpad Prism (v. 9.1.1). Threshold for statistical significance was set at $\alpha = 0.05$. Data were analyzed by unpaired two-tailed t-tests (with Welsch's correction if the compared datasets had significantly different variations) or by repeated measures (RM) two-way ANOVA (with Greenhouse-Geisser correction for interpretation of within-group effects [50] and Bonferroni adjustment for post-hoc analysis) as appropriate.

Results

DOI does not impair exploratory behavior and aversive memory recall but suppresses anxiety and conditioned avoidance behavior

We selected a dose of DOI (2 mg/kg i.p.) that was previously shown to induce a robust 5-HT2A-dependent head-twitch response in C57BL/6J mice [51]. DOI had no significant effect on spontaneous alternation (Fig. 1A; $t(21) = 0.77$, $p = 0.45$), demonstrating intact executive performance, but increased exploratory activity in the Y-maze (Fig. 1A, $t(21) = 2.27$, $p < 0.05$).

Increased locomotor activity was associated with decreased anxiety-like behavior in the elevated plus maze, demonstrated by increased open arm entries (Fig. 1B; $t(15) = 3.33, p < 0.01$) and time spent in the open arms (Fig. 1B; $t(15) = 3.25, p < 0.01$). In the passive avoidance task, mice were placed in a brightly lit corridor and trained to avoid an adjacent dark chamber (Fig. 1C). During the short-term memory test (STM), one hour after training, none of the mice entered the dark chamber (Fig. 1D). One day later, mice were injected with DOI or vehicle and were subjected to a long-term memory test (LTM). During LTM, none of the mice entered the dark chamber (Fig. 1D), demonstrating intact aversive memory recall. Interestingly, DOI significantly lowered freezing (Fig. 1E; $t(20.19) = 5.20, p < 0.0001$), decreased the latency to explore areas of the corridor closest to the dark chamber (Fig. 1F; treatment: $F(1, 31) = 3.91, p = 0.06$, area: $F(1.314, 40.73) = 14.08, p < 0.001$, interaction: $F(2, 62) = 6.22, p < 0.01$) and decreased time in the area furthest away from the dark chamber (Fig. 1G; treatment: $F(1, 31) = 0.20, p = 0.66$, area: $F(1.171, 36.29) = 416.4, p < 0.0001$, interaction: $F(3, 93) = 7.94, p < 0.0001$). This demonstrates that DOI suppresses conditioned avoidance behavior.

DOI suppresses conditioned freezing through activation of 5-HT_{2A} receptors

We next investigated the effects of DOI (2 mg/kg i.p.) in auditory fear processing paradigm in which we subjected mice to fear conditioning, fear extinction training and an extinction recall test on three consecutive days (Fig. 2A). Effective randomization was demonstrated by lack of significant group differences during conditioning on the first day (Supplementary Table 1). Mice received treatment before extinction training on the second day. Freezing during the extinction recall test on the third day was used as a measure for efficacy of the extinction learning process. In mice that underwent moderate conditioning (3 CS-US pairings at 0.6 mA), the administration of DOI before extinction training significantly reduced conditioned freezing in wild type mice (Fig. 2B; treatment: $F(1, 17) = 23.37, p < 0.001$) but not in 5-HT_{2A} receptor KO mice (Fig. 1C; treatment: $F(1, 16) = 1.037, p = 0.32$). During extinction recall we observed no significant treatment effect in wild type (Fig. 2B; $t(17) = 0.28, p = 0.79$) or 5-HT_{2A} receptor KO mice (Fig. 2C; $t(16) = 0.32, p = 0.76$). This demonstrates that DOI had no significant effect on the extinction learning process. We carried out an experiment with strong conditioning (5 CS-US pairings at 1 mA). While DOI abolished freezing during extinction training (Fig. 2D; treatment: $F(1, 26) = 49.33, p < 0.0001$), we again found no significant treatment effect during extinction recall (Fig. 2D; $t(26) = 0.22, p = 0.83$). Under these experimental conditions, freezing during extinction recall was significantly higher than freezing at the end of extinction training in control animals (Fig. 2D time: $F(1, 26) = 88.45, p < 0.0001$). This shows a lack of retention of fear extinction, which was not improved by administration of DOI.

DOI increases c-Fos expression in the amygdala upon auditory fear memory recall

To identify potential brain regions involved in DOI-induced fear expression, we administered DOI (2 mg/kg i.p.) or vehicle before fear recall (Fig. 3A) and sacrificed mice 90 min later for analysis of c-Fos expression (Fig. 3E), a marker for neuronal activity [52]. We focused our analysis on brain regions expressing 5-HT_{2A} receptors (Supplementary Figure 1) and involved in auditory fear processing [53], namely the prelimbic (PL) and infralimbic (IL) subregions of the mPFC, and the lateral amygdala (LA), basolateral amygdala (BLA), lateral division of the central amygdala (CeL) and medial division of the central amygdala (CeM). As expected, DOI suppressed conditioned freezing (Fig. 3B; $t(12) = 5.46, p = 0.0001$). DOI also induced an overall

increase in c-Fos expression (Fig. 3C; treatment: $F(1, 12) = 16.28, p < 0.01$) and post-hoc analysis revealed significant increase in the LA (Fig. 3B; $t(9.747) = 4.89; p < 0.01$) and CeL (Fig. 3B; $t(7.735) = 3.70; p < 0.05$). Activation of 5-HT_{2A} receptors was previously reported to increase firing of PV interneurons in the amygdala, suggesting a potential mechanism through which 5-HT_{2A} receptor activation may suppress fear expression [54,55]. However, we found no significant treatment effect (Fig. 3D; $F(1, 12) = 1.33, p = 0.27$), but significant regional differences (Fig. 3D; $F(2.314, 27.77) = 25.79, p < 0.0001$) and a significant treatment by region interaction (Fig. 3D; $F(3, 36) = 4.88, p < 0.01$) when analyzing c-Fos expression in PV⁺ interneurons (Fig. 3D). This suggests that DOI may increase c-Fos expression in PV⁺ interneurons in some brain regions, but this was not confirmed in post-hoc analysis given the low number of co-labeled cells (Fig. 3D and E).

5-HT_{2A} receptors in the amygdala drive the effect of DOI on conditioned freezing

To further investigate the neuroanatomical substrate for the observed effect of DOI on conditioned freezing, we carried out an experiment where we administered DOI (2 mg/kg i.p.) systemically and the selective 5-HT_{2A} receptor antagonist M100907 (0.3 µg per injection site) [54] or vehicle locally either in the mPFC or the amygdala 30 min before fear recall (Fig. 4A). Low freezing following DOI administration was not significantly affected when M100907 was infused into the mPFC (Fig 4B-C; $t(8) = 0.85, p = 0.42$) but was reversed when M100907 was delivered into the amygdala (Fig. 4D-E; $t(4.030) = 3.40, p < 0.05$). Finally, we demonstrated that local infusion (Fig. 4F) of DOI (2.5 µg per injection site) into the amygdala 30 min before fear recall significantly suppressed freezing (Fig. 4G; $t(18) = 4.47, p < 0.001$).

Discussion

We found that the psychedelic 5-HT_{2A}/5-HT_{2C} receptor agonist DOI decreased anxiety-like behavior and conditioned passive avoidance behavior. In addition, DOI suppressed conditioned freezing during extinction training without significant effects on the retention of extinction. The effect of DOI on conditioned freezing was mediated by activation of 5-HT_{2A} receptors in the amygdala.

We found no evidence for impaired execution of the spontaneous alternation task following the administration of DOI at the selected dose. While other studies have found effects of DOI on executive functions in a similar task in rats [56,57] and on spontaneous alternation in mice [58], these effects appear to be dependent on serotonergic tone and activation of 5-HT_{2C} receptors by the high DOI doses [58]. Consistent with this notion, the selective 5-HT_{2A} receptor agonist 25CN-NBOH did not affect spontaneous alternation in mice but restored deficits induced by the 5-HT_{1A} receptor agonist 8-OH-DPAT [58]. Similarly, spontaneous alternation impairments induced by the non-selective 5-HT receptor agonist meta-chlorophenylpiperazine (mCPP) were reversed by the 5-HT_{2C} receptor antagonist SB242084 but not by the 5-HT_{2A} receptor antagonist M100907 in rats [59].

While DOI decreases open field exploration in rats [60-63] it appears to have dose-dependent effects in mice, with lower doses (up to 1 mg/kg) increasing exploration through activation of 5-HT_{2A} receptors and higher doses (from 10 mg/kg) decreasing exploration through 5-HT_{2C} receptors [64]. We found a significant increase in Y-maze exploration, confirming previous observations [58], but this may reflect decreased anxiety rather than a psychostimulant effect.

Indeed, DOI also increased open arm exploration in the elevated plus maze indicating lower anxiety-like behavior. DOI was previously reported to affect anxiety-like behavior in the elevated plus maze task in mice and rats [65,66]. These effects were dose-dependent, with 5-HT_{2A} receptor-dependent anxiolytic effects for lower doses of DOI and potential 5-HT_{2C}-dependent anxiogenic effects for higher doses of DOI [65]. 5-HT_{2A} receptor involvement in the anxiolytic effects of DOI was previously observed in other behavioral tests [67].

The notion that DOI reduces anxiety-like behavior was further confirmed in the passive avoidance task. Surprisingly, we found that DOI had no effect on aversive memory retention, given that none of the mice entered the compartment where they previously received a shock. Nevertheless, DOI significantly reduced avoidance behavior and conditioned freezing. In the auditory fear conditioning paradigm, we found that DOI suppressed conditioned freezing but had no significant effect on extinction learning. A previous study suggested that stimulation of 5-HT_{2A} receptors may facilitate the acquisition fear extinction but retention of extinction was not investigated [37]. Our findings indicate an effect on fear expression rather than on fear extinction.

DOI significantly increased c-Fos expression in the LA and CeL. While this effect may occur in cells and brain regions that do not express the 5-HT_{2A} receptor [68,69], we found expression of 5-HT_{2A} receptors in LA (Supplemental Figure 1). Moreover, local antagonism of 5-HT_{2A} receptors in the amygdala reversed the effect of systemic DOI administration on fear expression. Corroborating this observation, direct infusion of DOI into the amygdala similarly suppressed fear expression. Together, this demonstrates that 5-HT_{2A} receptors in the amygdala may be a critical target through which psychedelics affect fear expression. Interestingly, a previous study found that impairment of 5-HT_{2A} signaling in PV interneurons in the amygdala may drive stress-induced changes in GABAergic transmission and behavior. However, we found no significant effects of DOI on c-Fos expression in PV interneurons. This may be due to the low number of PV interneurons in the amygdala and relatively small group sizes. Importantly, 5-HT_{2A} receptors expressed in other brain regions, such as the ventral hippocampus or periaqueductal gray matter may contribute to the anxiolytic effects of DOI [70,71].

No formal clinical trials have investigated classic psychedelics in PTSD. However, psilocybin and LSD showed promising effects in cancer-related depression and anxiety [72-76]. Interestingly, psilocybin reduced threat-induced modulation of amygdala connectivity in healthy volunteers [77]. Similarly, LSD suppressed amygdala reactivity to fearful stimuli [78] and impaired fear recognition in healthy volunteers [79]. This indicates that classic psychedelics affect fear processing and may be useful for treating fear-related disorders such as PTSD. In mice, the psychedelic 5-HT_{2A}/5-HT_{2C} receptor agonists TCB-2 and psilocybin reduced freezing during trace fear extinction in mice [36,37]. Recently, DMT, a 5-HT_{2A}/5-HT_{1A} agonist, was shown to decrease fear expression during extinction training and to improve the retention of fear extinction [38]. This suggests that 5-HT_{2A} receptors are a key target for the effects of psychedelics on fear expression, but other receptors such as the 5-HT_{1A} receptor may contribute to their effects on fear processing. Notably activation of 5-HT_{1A} receptors was previously shown to reduce fear expression and to facilitate fear extinction in rodents [80-83].

It was recently proposed that the clinical effect of MDMA-assisted psychotherapy for PTSD treatment may result from reduction of fear during fearful memories [15,17,18] or from enhanced extinction of fearful memories [20]. In line with this notion, MDMA was reported to suppress freezing during extinction training and to improve retention of fear extinction in mice [21,22].

However, a recent study showed that while MDMA suppressed fear expression during extinction training, it interfered with the retention of extinction in rats [24]. Therefore, it remains unclear whether facilitation of extinction is the underlying mechanism of MDMA-assisted psychotherapy [20]. Moreover, it remains uncertain whether the psychedelic properties of MDMA are required for its therapeutic effects. While MDMA is not a classic psychedelic, many of its psychotropic effects involve 5-HT release and activation of 5-HT_{2A} receptors [33,84-88]. In this context, activation of 5-HT_{2A} receptors may indeed be a mechanism through which MDMA modulates fear processing [23], consistent with our data demonstrating that 5-HT_{2A} receptor activation by a psychedelic suppresses fear expression.

We conclude that the psychedelic DOI suppresses fear expression in mice through activation of amygdalar 5-HT_{2A} receptors while it does not affect extinction learning. Our study is limited to a single administration of DOI but it provides insight into the mechanism through which acute psychedelic effects may yield therapeutic outcomes for psychedelic-assisted psychotherapy. Indeed, if psychedelics suppress fear expression without impairing recall and extinction of fearful memories, this may enable psychotherapy in patients for whom the experience would otherwise be too aversive as described by a PTSD patient who underwent MDMA-assisted psychotherapy: *"It allowed me to see my trauma without fear or hesitation and finally process things and move forward"* [20].

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Author contributions

D.D.B., E.V. and C.B. conceived the original idea. J.B. and P.M. contributed to conception of the study. D.D.B., E.V. and B.P. designed the experiments. D.D.B., B.P. and A.V.S. carried out the experiments. D.D.B. and B.P. analyzed the data. A.P provided technical training and contributed to supervising the experiments. C.B. contributed essential materials. D.D.B. and B.P. wrote the manuscript. All authors contributed to interpretation of the results and provided critical feedback on the manuscript.

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Figures and figure legends

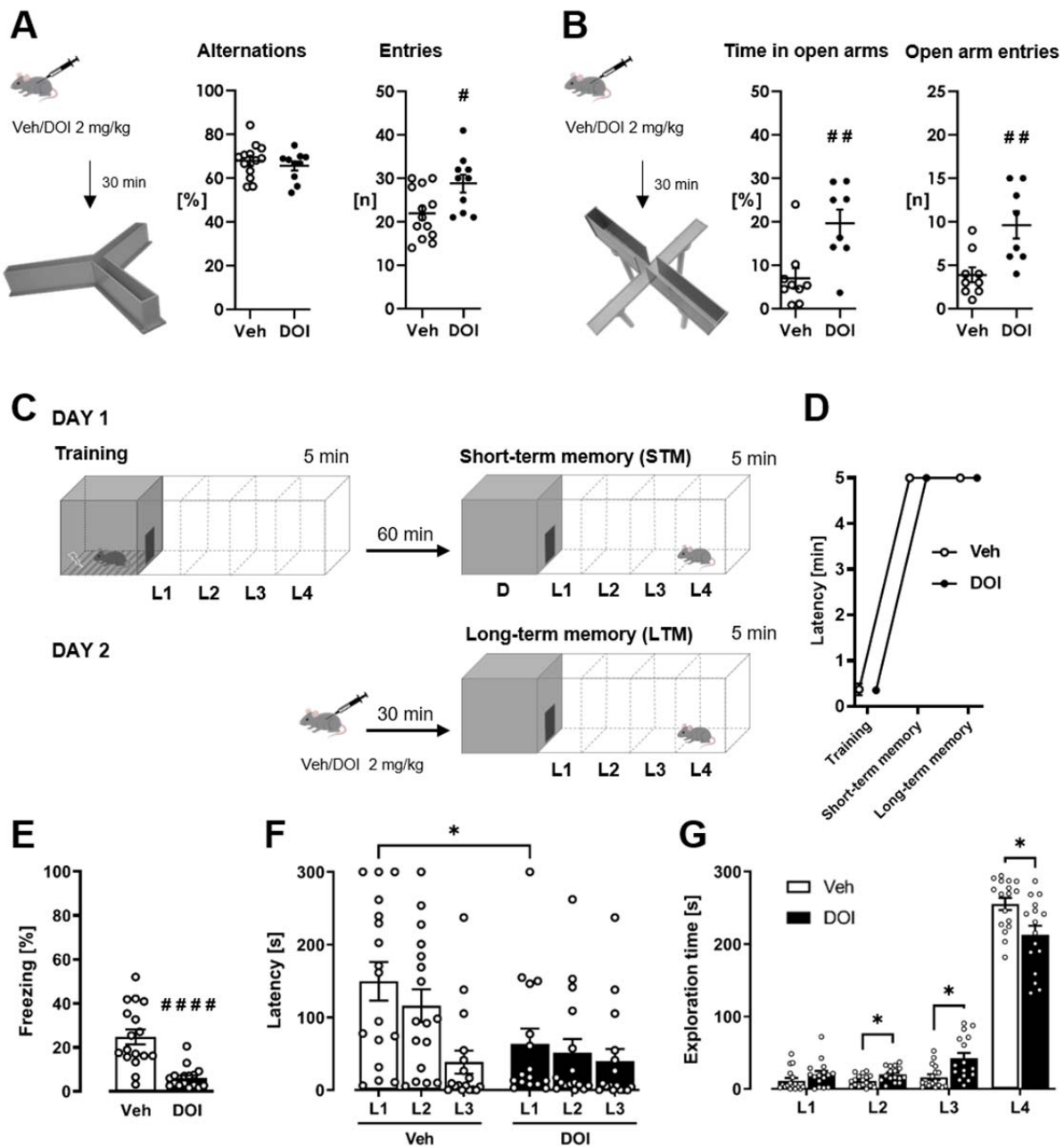


Fig. 1. Effects of DOI in the passive avoidance, spontaneous alternation and elevated plus maze tasks. **A** Effect of DOI on the number of alternations (unpaired two-sided t-test: $t(21) = 0.77$, $p = 0.45$) and number of arm entries (unpaired two-sided t-test, $t(21) = 2.66$, $p < 0.05$) in the Y-maze spontaneous alternation test. **B** Effect of DOI on the number of open arm entries (unpaired two-sided t-test, $t(15) = 3.33$, $p < 0.01$) and the percentage of time spent in open arms (unpaired two-sided t-test, $t(15) = 3.25$, $p < 0.01$). **C** Schematic representation of the experimental design for passive avoidance testing in a light corridor and dark chamber setup. Areas of the light compartment were designated L1-L4 from closest to furthest away from the

dark compartment. **D** Following training, none of the mice ($n_{veh} = 17; n_{DOI} = 16$) entered the dark chamber. **E** Effect of DOI on freezing during the long-term memory test (unpaired t-test with Welsch correction $t(20.19) = 5.20, p = 0.0001$). **F** Effect of DOI on latency to enter areas of the brightly-lit corridor: two-way RM ANOVA with treatment effects ($F(1, 31) = 3.90, p = 0.06$), area effects ($F(1.314, 40.73) = 14.08, p < 0.001$) and interaction ($F(2, 62) = 6.22, p < 0.01$). **G** Effect of DOI on time spent in areas of the brightly-lit corridor: two-way RM ANOVA with treatment effects ($F(1, 31) = 0.20, p = 0.66$), area effects ($F(1.171, 36.29) = 416.4, p = 0.0001$) and interaction ($F(3, 93) = 7.94, p = 0.0001$). Data are presented as mean values \pm SEM (* $p < 0.05$; ## $p < 0.01$; ##### $p < 0.0001$ vs. Veh group).

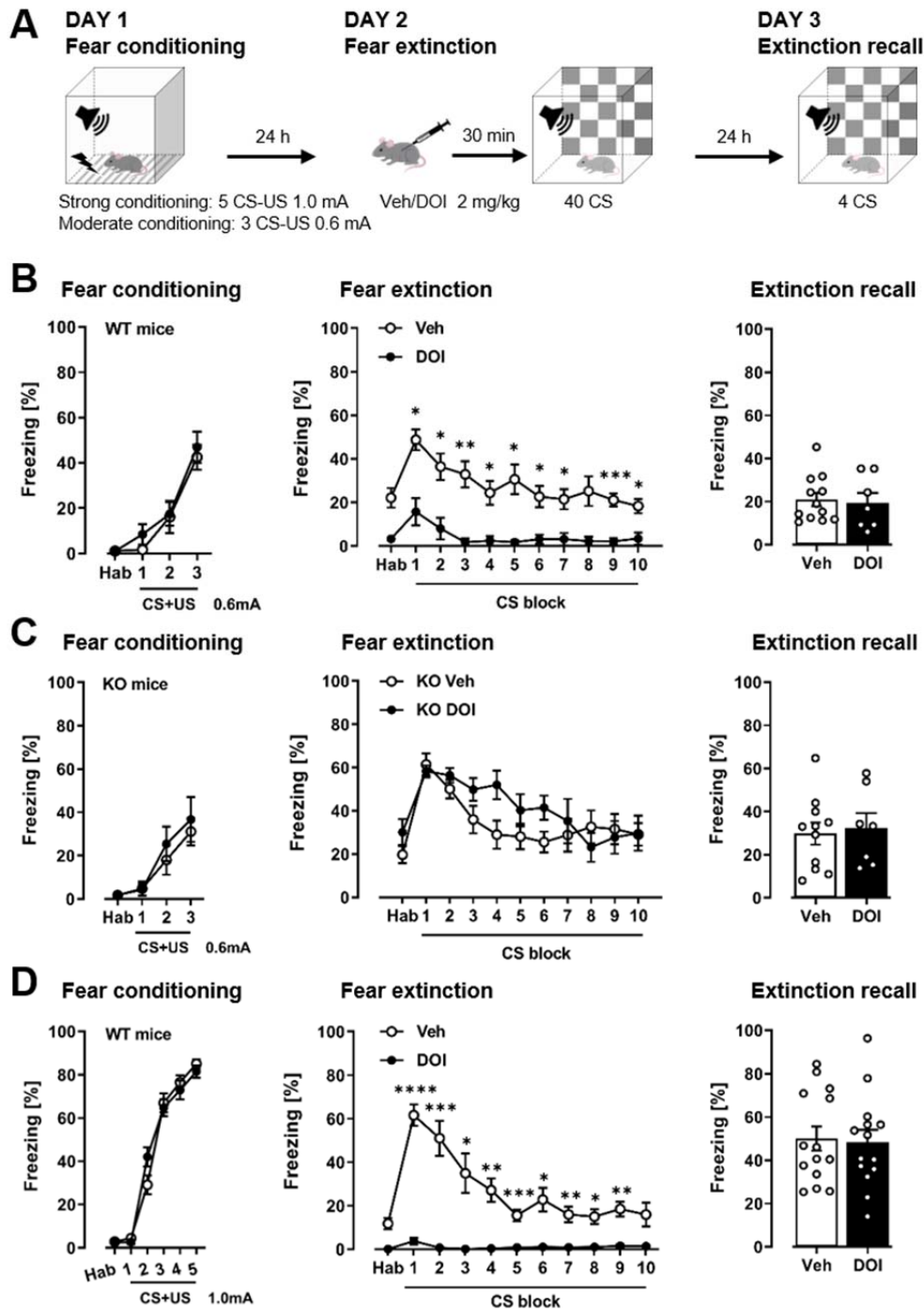


Fig. 2 Effects of DOI on auditory fear extinction. **A** Schematic representation of the experimental design for testing the effect of DOI on auditory fear extinction. **B** Effect of DOI on fear extinction following moderate conditioning in wild type mice ($n_{Veh} = 12$; $n_{DOI} = 7$). Fear conditioning: two-way RM ANOVA with CS effect ($F(1.757, 29.86) = 38.67, p < 0.0001$), treatment effect ($F(1, 17) = 0.46, p = 0.51$) and interaction ($F(2, 34) = 0.18, p = 0.84$). Fear extinction: two-way RM ANOVA with CS effect ($F(2.861, 48.64) = 5.06, p < 0.01$), treatment effect ($F(1, 17) = 23.37, p < 0.001$) and interaction ($F(9, 153) = 1.13, p = 0.35$). Extinction

recall: unpaired two-tailed t-test ($t(17) = 0.28, p = 0.79$). **C** Effect of DOI on fear extinction following moderate conditioning in 5-HT_{2A} receptor KO mice ($n_{veh} = 10; n_{DOI} = 7$). Fear conditioning: two-way RM ANOVA with CS effect ($F(1.853, 27.79) = 15.69, p = 0.0001$), treatment effect ($F(1, 15) = 0.11, p = 0.74$) and interaction ($F(2, 30) = 0.18, p = 0.83$). Fear extinction: two-way RM ANOVA with CS effect ($F(3.214, 51.42) = 8.34, p = 0.0001$), treatment effect ($F(1, 16) = 1.04, p = 0.33$) and interaction ($F(9, 144) = 1.84, p = 0.07$). Extinction recall: unpaired two-tailed t-test ($t(16) = 0.32, p = 0.76$). **D** Effect of DOI on fear extinction following strong conditioning in wild type mice ($n_{veh} = n_{DOI} = 14$). Fear conditioning: two-way RM ANOVA with CS effects ($F(3.185, 82.82) = 237.8, p < 0.0001$), treatment effects ($F(1, 26) = 0.01, p = 0.92$) and interaction ($F(4, 104) = 2.77, p < 0.05$). Fear extinction: two-way RM ANOVA with CS effect ($F(2.338, 60.78) = 16.30, p < 0.0001$), treatment effect ($F(1, 26) = 49.33, p < 0.0001$) and interaction ($F(9, 234) = 14.46, p < 0.0001$). Extinction recall: unpaired two-tailed t-test ($t(26) = 0.22, p = 0.83$). Data are presented as values \pm SEM (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs. Veh group).

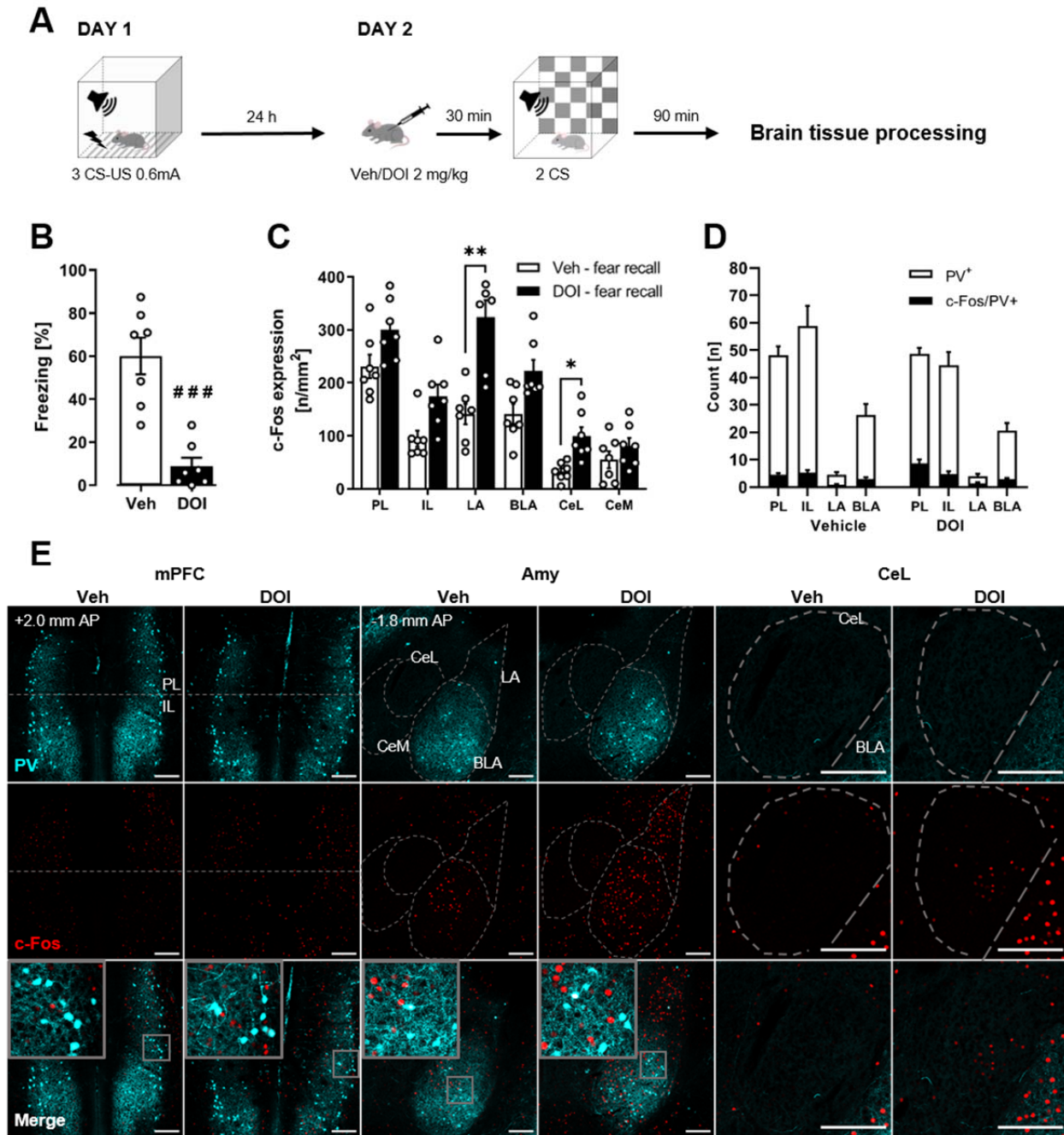


Fig. 3. Effects of DOI on neuronal activity during fear memory recall. **A.** Schematic representation of the experimental design for investigating the effect of DOI on c-Fos expression in mice subjected to fear conditioning. Regions examined: prelimbic (PL) and infralimbic (IL) cortices of the medial prefrontal cortex; lateral (LA), basolateral (BLA), lateral (CeL) and medial (CeM) division of the central amygdala. **B** Effect of DOI on freezing during fear memory recall ($n_{\text{Veh}} = n_{\text{DOI}} = 7$): unpaired two-tailed t-test ($t(12) = 5.46, p = 0.0001$). **C** Effect of DOI on c-Fos expression during fear memory recall: two-way RM ANOVA treatment effect ($F(1, 12) = 16.28, p < 0.01$), region effect ($F(3.539, 42.47) = 66.17, p < 0.0001$) and interaction ($F(5, 60) = 6.55, p < 0.0001$). **D** Effect of DOI on c-Fos expression in PV⁺ interneurons during fear memory recall. Two-way RM ANOVA for PV⁺ interneurons with treatment effect ($F(1, 12) = 2.50, p =$

0.14), region effect ($F(1.793, 21.51) = 77.23, p = 0.0001$) and interaction ($F(3, 36) = 1.49, p = 0.23$). Two-way RM ANOVA for c-Fos expression in PV⁺ neurons with treatment effect ($F(1, 12) = 1.33, p = 0.27$), region effect ($F(2.314, 27.77) = 25.79, p = 0.0001$) and interaction ($F(3, 36) = 4.88, p < 0.01$). **E** Representative confocal microscopy images labeled for c-Fos (red) and PV (cyan). Scale bar = 200 μm . Data are presented as mean values \pm SEM (* $p < 0.05$; ** $p < 0.01$; ### $p < 0.001$. vs *Veh* group).

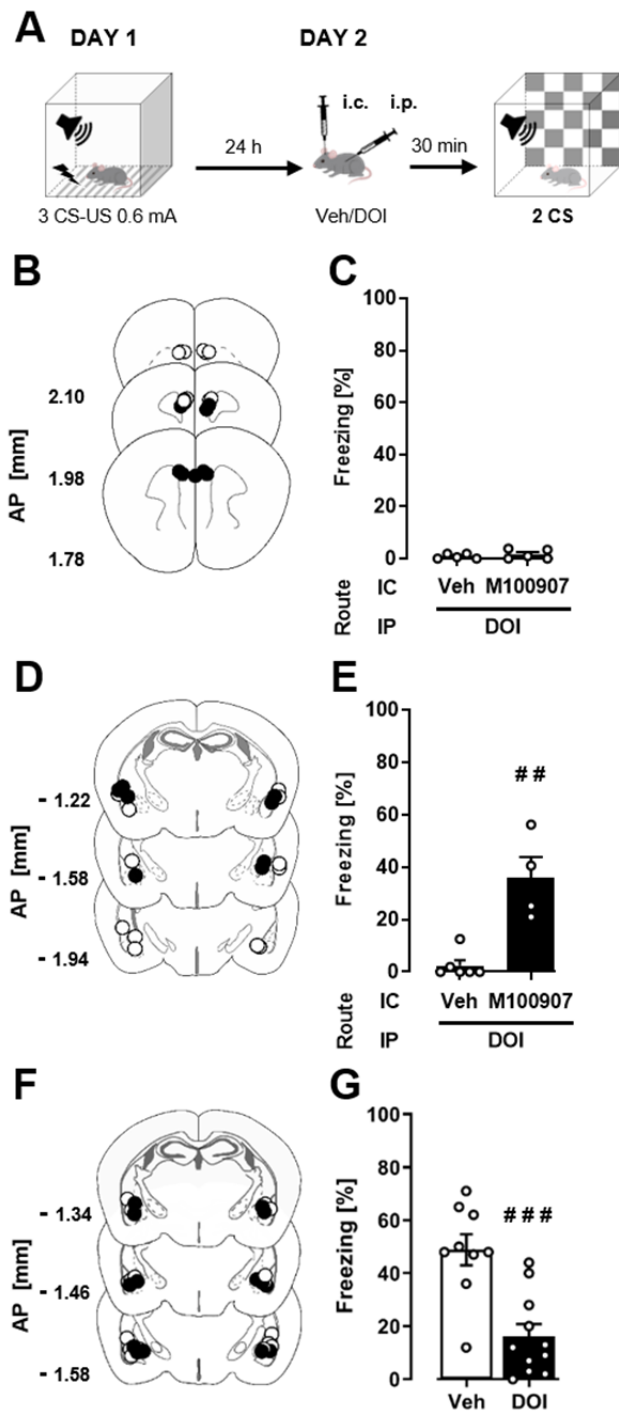


Fig. 4. Brain region-specific role of 5-HT_{2A} receptors in the effects of DOI on freezing during fear memory recall

A Schematic representation of the experimental design used to investigate how 5-HT_{2A} receptors expressed in discrete brain regions control freezing behavior during fear memory recall. **B** Cannula placement sites for infusion of M100907 (black) or the corresponding vehicle (white) in

the mPFC. **C** Modulation of freezing during fear memory recall by systemic DOI (2 mg/kg i.p.) and local infusion of M100907 (0.3 µg per injection site) or the corresponding vehicle ($n_{veh} = n_{DOI} = 5$): unpaired two-sided t-test ($t(8) = 0.85, p = 0.42$). **D** Cannula placement sites for infusion of M100907 (black) or the corresponding vehicle (white) in the amygdala. **E** Modulation of freezing during fear memory recall by systemic DOI (2 mg/kg i.p.) and local infusion of M100907 (0.3 µg per injection site) or the corresponding vehicle into amygdala ($n_{veh/DOI} = 6; n_{M100907/DOI} = 4$): unpaired two-sided t-test with Welch correction ($t(4.04) = 3.40, p < 0.05$). **F** Cannula placement sites for infusion of DOI (black) or the corresponding vehicle (white) in the amygdala. **G** Modulation of freezing during fear memory recall by local infusion of DOI (2.5 µg per injection site) or the corresponding vehicle into the amygdala ($n_{veh} = 8; n_{doi} = 10$): unpaired two-tailed t-test ($t(18) = 4.465; p < 0.001$). Data are presented as mean values \pm SEM (### $p < 0.01$, #### $p < 0.001$ vs Veh group).