Micro-Dosing of MDMA with Trauma Reminders Specifically Attenuates Conditioned Fear and Hyperarousal by Modulating Neural Circuits in a Predator Scent Stress Rat Model

Yarden Viktor Dejorno, **Hagit Cohen**

Faculty of Health Sciences, Ben-Gurion University of the Negev; Beer-Sheva Mental Health Center, Beer-Sheva, Israel

ABSTRACT

Background: Post-traumatic stress disorder (PTSD) is a chronic psychiatric condition characterized by intrusive memories, avoidance behaviors, and hyperarousal following traumatic experiences[1]. Lifetime prevalence is approximately 6–9% in the general population but notably higher in high-risk groups such as military personnel and first responders[2][3]. Core symptoms include re-experiencing trauma (e.g., intrusive thoughts, nightmares), persistent avoidance of trauma-related stimuli, and heightened physiological arousal[1][4]. Current therapeutic interventions—including cognitive-behavioral therapy and selective serotonin reuptake inhibitors (SSRIs)—often provide only partial symptom relief and rarely achieve full remission. High dropout rates and treatment resistance remain significant challenges[5–11].

Objective: 3,4-Methylenedioxymethamphetamine (MDMA) has shown promise in enhancing emotional openness, reducing fear during traumatic memory processing, and improving therapeutic engagement through its effects on serotonin, dopamine, norepinephrine, oxytocin, and cortisol[12–17]. However, the high doses used in MDMA-assisted psychotherapy raise concerns about adverse effects and neurotoxicity. This study aimed to assess whether a continuous micro-dosing regimen of MDMA could attenuate PTSD-like behaviors in a rodent model while minimizing side effects.

Methods: Using the predator scent stress (PSS) model, 78 adult male Sprague-Dawley rats were randomly assigned to six groups varying by PSS exposure, MDMA micro-dosing (200 μg/kg, subcutaneously), and whether drug administration was paired with trauma reminders. Treatments were given three times weekly for three weeks, and behavioral tests were conducted to evaluate conditioned fear (freezing behavior), hyperarousal (acoustic startle response, ASR), and anxiety (elevated plus maze, EPM). Immunohistochemistry was performed to measure expression of brain-derived neurotrophic factor (BDNF), tryptophan hydroxylase 2 (TPH2), oxytocin, and glucocorticoid receptor (GR) in relevant brain regions.

Results: Continuous MDMA micro-dosing significantly reduced conditioned freezing behavior one week after trauma (MDMA: 44 ± 1.2% freezing; Saline: 50 ± 1.2%; $p = 0.0015$). Across four weekly trauma reminders, MDMA-treated rats showed a blunted increase in freezing compared to controls, with residual fear attenuation even after treatment cessation. MDMA also attenuated hyperarousal, as indicated by lower startle response amplitudes (MDMA: 768 ± 52; Saline: 930 ± 25; $p = 0.035$). Notably, the therapeutic effects were most pronounced when MDMA dosing coincided with trauma cue exposure (paired reminders). In contrast, MDMA micro-dosing did not significantly reduce generalized anxiety in the EPM. MDMA-treated rats exhibited significantly elevated BDNF expression in the dorsal raphe nucleus (DRN), hippocampal CA1, and paraventricular nucleus (PVN) compared to controls, whereas oxytocin and GR expression in the PVN were unchanged.

Conclusion: Continuous micro MDMA administration after trauma exposure attenuated PTSD-like conditioned fear responses and hyperarousal in the PSS rat model. The benefits were specific to trauma-related behaviors and were contingent on pairing MDMA administration with trauma reminders. Enhanced BDNF levels in key brain regions suggest a neuroplastic mechanism underlying the observed behavioral improvements. These findings support further exploration of MDMA micro-dosing strategies, particularly in conjunction with therapeutic re-exposure to trauma cues, as a potential PTSD treatment approach.

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a chronic psychiatric condition that arises after traumatic events such as combat, severe accidents, or assault[1]. Lifetime prevalence ranges from approximately 6% to 9% in the general population, with considerably higher rates in populations with frequent trauma exposure (e.g., military personnel, first responders)[2][3]. Core PTSD symptoms include intrusive re-experiencing of the trauma (e.g., flashbacks, nightmares), persistent avoidance of trauma-related cues, and a state of hyperarousal or heightened reactivity[1][4]. Biologically, PTSD is associated with widespread alterations

across neural circuitry and neurochemical systems, as well as dysregulation of immune and endocrine function[4].

Current treatments for PTSD—primarily trauma-focused psychotherapies and pharmacotherapies such as SSRIs—often yield only partial relief, with many patients experiencing residual symptoms[5–11]. Treatment dropout rates are high, and a substantial subset of patients remain refractory to these interventions[8][10][11]. This therapeutic gap has prompted investigation into novel treatment modalities.

MDMA is a synthetic psychoactive compound known for enhancing empathy, mood, and emotional openness. In the context of psychotherapy, MDMA's pharmacological profile (releasing serotonin, dopamine, and norepinephrine, and stimulating oxytocin and cortisol release) can reduce anxiety and defensiveness during traumatic memory processing[12–16]. These properties have spurred interest in MDMA-assisted therapy for PTSD. Emerging clinical evidence demonstrates that a few supervised sessions of MDMA-assisted psychotherapy can produce significant and enduring reductions in PTSD severity among treatment-resistant patients[14][17–19]. Such therapy has also been associated with post-traumatic growth, improving patients' self-perception, interpersonal relationships, and overall life satisfaction compared to conventional treatments[20]. Animal studies mirror these findings: for instance, pairing MDMA administration with trauma reminders in rodents reduced PTSD-like behaviors and long-term stress pathology[21]. Notably, MDMA-assisted therapy tends to have higher response rates and lower dropout compared to standard care, contributing to its designation by the U.S. Food and Drug Administration as a breakthrough therapy for PTSD and its authorization for clinical use in treatment-resistant PTSD by Australia's Therapeutic Goods Administration[22–27].

Despite these encouraging outcomes, clinical protocols have generally employed full, psychoactive doses of MDMA. High-dose MDMA carries risks, including acute adverse effects and potential neurotoxicity[19][29]. These safety concerns have led to growing interest in "micro-dosing" MDMA—using sub-perceptual doses intended to confer therapeutic benefits without significant psychoactive effects or toxicity[29, 30]. However, empirical research on MDMA micro-dosing, especially in the context of PTSD, remains sparse.

Preclinical models offer a controlled avenue to investigate MDMA micro-dosing. The predator scent stress (PSS) paradigm in rodents is a well-validated model of PTSD: exposure to a predator odor (soiled cat litter) induces lasting behavioral and physiological disturbances analogous to PTSD symptoms[31–37]. In this study, we utilized the PSS model to evaluate whether

continuous post-trauma micro-dosing of MDMA (200 μg/kg) can alleviate PTSD-like behaviors, modulate responses to trauma reminders, and influence neurobiological markers of stress and plasticity[38][39].

We hypothesized that sustained MDMA micro-dosing after trauma would mitigate PTSD-like behavioral disturbances (reducing conditioned fear and hyperarousal), especially when administration is paired with trauma reminders. Furthermore, we expected MDMA-treated rats to exhibit altered neurobiological profiles, including enhanced expression of neurotrophic factors (e.g., BDNF) and markers of neural plasticity, consistent with the proposed mechanisms of MDMA-assisted therapy.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures were approved by the Animal Care and Use Committee of Ben-Gurion University of the Negev, Israel, and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Protocol IL-02-01-2023(c)).

Animals

Seventy-eight adult male Sprague-Dawley rats (200–250 g) were used in this study (two rats were excluded due to equipment malfunction, yielding a final $N = 76$). Rats were pair-housed in a temperature-controlled colony room on a 12-hour light/dark cycle, with food and water available ad libitum.

Experimental Design

Animals were randomly assigned to one of six experimental groups, differing in exposure to PSS, MDMA treatment regimen, and whether MDMA was paired with trauma reminders:

1. **Group 1 (Sham PSS + Saline, n = 7):** No predator stress; received saline injections (0.9% NaCl, 0.1 ml S.C.) three times per week for three weeks.
2. **Group 2 (Sham PSS + MDMA, n = 7):** No predator stress; received MDMA micro-dosing (200 μg/kg in 0.1 ml saline, S.C.) three times per week for three weeks.
3. **Group 3 (PSS + Saline, n = 18):** Exposed to predator scent stress; received saline injections (three times weekly for three weeks). A trauma reminder (fresh soiled cat

litter) was presented once weekly, 30 minutes after the injection, to reactivate the fear memory.

4. **Group 4 (PSS + MDMA Paired, n = 20):** Exposed to predator scent stress; received MDMA (200 µg/kg, S.C.) three times weekly for three weeks, with a trauma reminder presented 30 minutes post-injection once per week (MDMA administration paired with memory reactivation).
5. **Group 5 (PSS + MDMA Unpaired, n = 18):** Exposed to predator scent stress; received MDMA (200 µg/kg, S.C.) three times weekly for three weeks, but trauma reminders were presented on a separate day each week (24 hours after the nearest MDMA injection, i.e., unpaired with acute drug effects).
6. **Group 6 (PSS + MDMA Weekly, n = 6):** Exposed to predator scent stress; received MDMA (200 µg/kg, S.C.) only once per week for three weeks, with each MDMA injection immediately followed by a trauma reminder (paired administration, but lower dosing frequency).

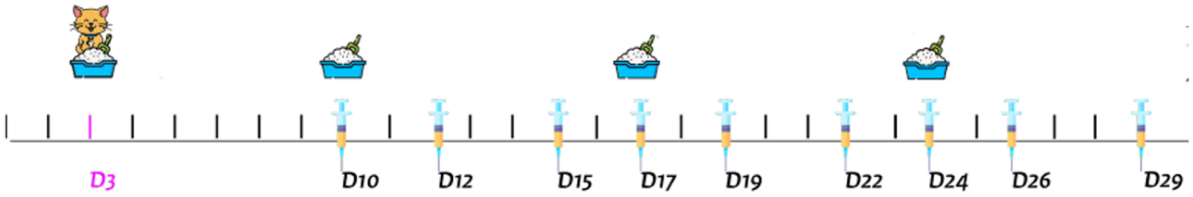


Figure 1: Schematic representation of the experimental timeline, illustrating the timing of predator scent stress exposure, MDMA or saline injections, and presentations of trauma reminders (cat litter).

Predator Scent Stress (PSS) Procedure

One week after acclimation, rats in the PSS groups (Groups 3–6) underwent the predator scent stress procedure to induce trauma. Each rat was placed individually in a cage containing soiled cat litter (previously used by an adult cat) for 20 minutes. This exposure to predator odor served as a psychological stressor. Sham-stressed control rats (Groups 1–2) were placed in a cage with clean, unused cat litter for an equivalent duration.

Drug Administration

(±)MDMA (Sigma-Aldrich) was dissolved in sterile 0.9% saline. Rats received subcutaneous (S.C.) injections of MDMA at a dose of 200 µg/kg (injection volume 0.1 ml) according to their group assignment. Control animals received equivalent volume S.C. injections of saline. In groups with thrice-weekly dosing, injections were given on a Monday-Wednesday-Friday

schedule. In the once-weekly group (Group 6), injections were given on the same day each week. Trauma reminder exposures (fresh cat litter) were presented either 30 minutes post-injection (for "paired" groups) or 24 hours post-injection (for the unpaired group), as detailed above.

Behavioral Assessments

All behavioral tests were conducted during the light phase (0900–1700 h) by an experimenter blinded to group assignments. Behavior was video-recorded and analyzed using EthoVision XT software (Noldus Information Technology).

Open Field and Contextual Freezing Test

Locomotor activity and freezing behavior were assessed in an open-field arena (45 × 45 cm) that also served as the context for trauma reminders. Rats were placed in the arena for a 10-minute session, 30 minutes after MDMA or saline injection, to measure baseline locomotion and conditioned freezing behavior in the presence of a trauma cue (fresh cat litter placed in a container within the arena). Freezing (cessation of all movement except respiration) was quantified as a percentage of total observation time.

Rectal Temperature Monitoring

To monitor potential MDMA effects on physiology, rectal temperatures were measured in all rats once weekly using a digital thermometer probe, immediately before drug or saline administration and again 1 hour post-injection. After two weeks, when no significant treatment-related temperature differences were observed, temperature monitoring was discontinued to minimize stress.

Elevated Plus Maze (EPM)

The EPM was used to evaluate anxiety-like behavior. The apparatus consisted of two open arms and two closed arms (50 cm above the floor). One week after the final injection, each rat was placed in the center of the EPM and allowed to explore for 5 minutes. An anxiety index was calculated for each animal as:

$$Anxiety\ Index = 1 - \left[\frac{\left(\frac{Time\ spent\ in\ the\ open\ arms}{Total\ time\ on\ the\ maze}\right) + \left(\frac{Number\ of\ entries\ to\ the\ open\ arms}{Total\ exploration\ on\ the\ maze}\right)}{2}\right]$$

where higher values indicate greater anxiety-like behavior (i.e., less open-arm exploration).

Acoustic Startle Response (ASR)

Hyperarousal and exaggerated startle responses were measured using an automated startle reflex chamber. One week after the final trauma reminder, rats were placed in the chamber and acclimated for 5 minutes. They were then presented with 30 trials of a 110 dB white-noise burst (40 ms) at random intertrial intervals. The peak startle amplitude (force) was recorded for each trial, and a mean startle amplitude was calculated for each animal.

Contextual Freezing (Trauma Reminder Test)

To specifically assess conditioned fear responses to trauma cues, a contextual freezing test was performed in which rats were re-exposed to the trauma context (fresh cat litter) without any injection. This final freezing test took place two weeks after the last MDMA/saline treatment. Rats were placed in a cage with fresh cat litter for 5 minutes, and freezing behavior was measured as described above. This test evaluated the lasting effects of MDMA micro-dosing on fear memory retention after treatment cessation.

Immunohistochemistry

Tissue Preparation

One hour after the final injection (on the last day of the experiment), a subset of rats (n = 4 per group, randomly selected) were deeply anesthetized with sodium pentobarbital and transcardially perfused with cold saline followed by 4% paraformaldehyde in phosphate buffer. Brains were removed, post-fixed overnight, and cryoprotected in 30% sucrose. Coronal brain sections (10 μm thick) were cut on a cryostat and mounted onto gelatin-coated slides for immunofluorescence staining.

Immunofluorescence Staining

Sections were washed in phosphate-buffered saline (PBS) and incubated in blocking solution (5% normal goat serum and 0.3% Triton X-100 in PBS) for 1 hour. Slides were then incubated overnight at 4°C with the following primary antibodies:

- **Glucocorticoid Receptor (GR):** rabbit monoclonal anti-GR (Abcam, ab183127)
- **Tryptophan Hydroxylase 2 (TPH2):** rabbit monoclonal anti-TPH2 (Abcam, ab184505)

- **Oxytocin Receptor:** rabbit polyclonal anti-oxytocin receptor (Abcam, ab300443)
- **Brain-Derived Neurotrophic Factor (BDNF):** rabbit polyclonal anti-BDNF (Alomone Labs, ANT-010)

After rinsing, sections were incubated for 2 hours at room temperature with Alexa Fluor-conjugated goat anti-rabbit IgG secondary antibodies. Slides were then counterstained with DAPI (4′,6-diamidino-2-phenylindole) to visualize cell nuclei and coverslipped with an antifade mounting medium.

For each brain region of interest (dorsal raphe nucleus, hippocampal CA1 and CA3, and hypothalamic paraventricular nucleus), images were captured at 20× magnification using a fluorescence microscope under identical exposure settings. ImageJ software (NIH) was used to quantify the number of positively stained cells (or average fluorescence intensity) for each marker in standardized regions of interest. All quantification was performed by an observer blinded to the treatment groups.

Golgi-Cox Staining and Neuronal Morphology

A separate subset of rats (n = 3 per group) was used for Golgi-Cox staining to assess structural neuronal changes. Brains were processed using a Golgi-Cox kit (FD NeuroTechnologies, USA) following the manufacturer's protocol. Briefly, freshly dissected brains were immersed in Golgi-Cox solution for 2 weeks in the dark, then transferred to a sucrose-based solution for 3 days. Brains were subsequently sectioned at 100 μm thickness on a vibratome. Sections were mounted on gelatin-coated slides, developed, fixed, and dried according to standard procedures.

Granule neurons in the suprapyramidal blade of the dentate gyrus (DG) were selected for dendritic morphology analysis. Neurons with well-impregnated, complete dendritic trees were traced under 400× magnification using a camera lucida. For each traced neuron, total dendritic length and the number of dendritic branch points were measured as indicators of dendritic complexity. At least 5 neurons per animal were analyzed. All analyses were done by an experimenter blind to the treatment condition.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Behavioral data (freezing percentage, anxiety index, startle amplitude) were analyzed using two-way analysis of variance

(ANOVA) with between-subject factors of PSS exposure (yes/no) and Treatment (MDMA vs. Saline), as well as their interaction. Repeated-measures ANOVA was used to evaluate freezing behavior across multiple trauma reminder sessions. Immunohistochemical and Golgi-Cox data were analyzed with one-way ANOVAs (treatment as factor) for each relevant comparison. When ANOVAs yielded significant effects, Bonferroni-corrected post-hoc tests were applied for pairwise comparisons. Statistical significance was set at $p < 0.05$ for all analyses.

RESULTS

MDMA Micro-Dosing Reduces Conditioned Freezing After Predator Stress

One week after the predator scent stress, we assessed conditioned freezing behavior in the context associated with the trauma (cat litter). Rats exposed to PSS and treated with MDMA (Group 4) exhibited significantly less freezing compared to PSS-exposed rats that received saline (Group 3). Specifically, MDMA-treated rats froze for 44 ± 1.2% of the observation time versus 50 ± 1.2% in saline-treated rats ($F_{(1,36)} = 12.5$, $p = 0.0015$). This reduction in freezing suggests that MDMA micro-dosing attenuated the conditioned fear response (Figure 2).

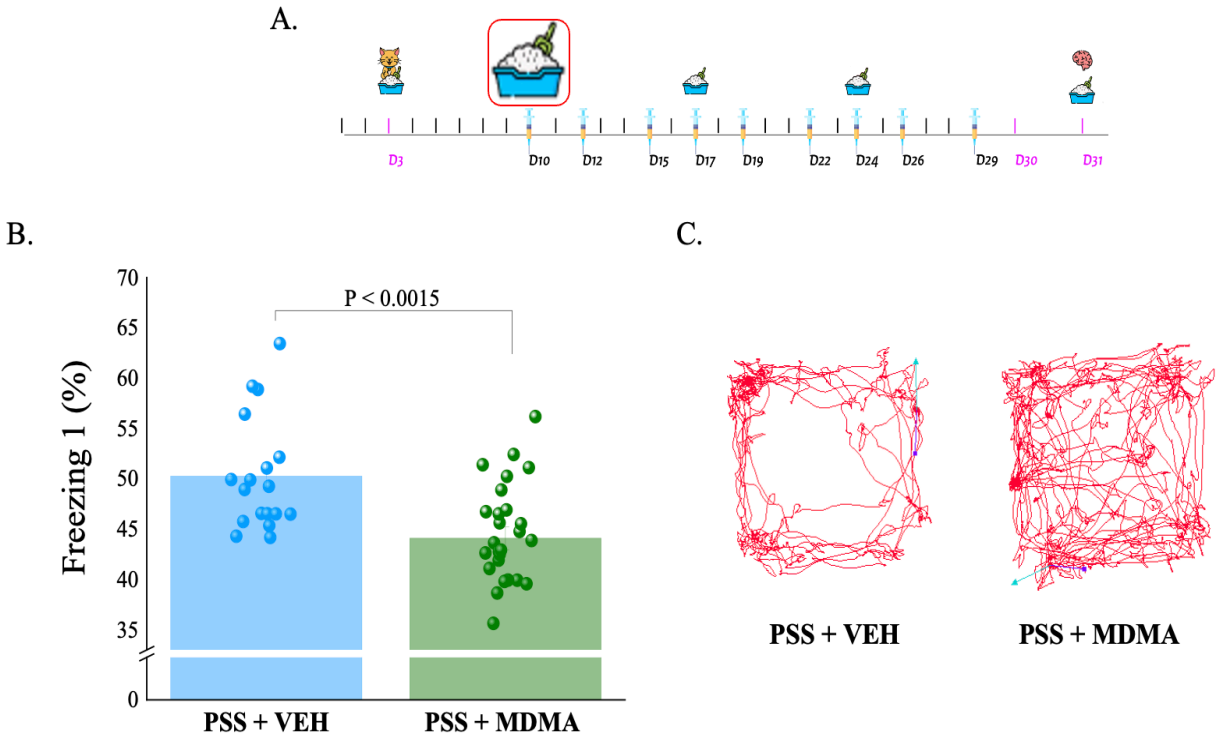


Figure 2: (A) Freezing behavior (% of time) during the first trauma reminder session for PSS-exposed rats treated with saline (Group 3) or MDMA (Group 4). (B) Percentage of time spent freezing at the first trauma reminder, showing a significant reduction in the MDMA group. (C) Representative locomotor activity traces during the 10-minute context

test, illustrating greater exploratory movement (and less freezing) in the MDMA-treated rat.

Repeated MDMA Treatments Limit the Increase in Freezing with Serial Trauma Reminders

To evaluate the persistence of MDMA's effect, PSS-exposed rats in Groups 3 and 4 were subjected to a trauma reminder (context re-exposure) once per week for four consecutive weeks, with Groups 3 and 4 continuing their assigned saline or MDMA treatments during the first three reminder trials. Both groups displayed increased freezing over repeated reminders (reminder session effect: $F_{(3,108)} = 42.11$, $p < 0.0001$), reflecting a reinforcement of fear with repeated memory reactivation in saline-treated animals. However, MDMA treatment significantly blunted this escalation of freezing (treatment main effect: $F_{(1,36)} = 6.32$, $p = 0.016$; treatment × reminder interaction: $F_{(3,108)} = 3.30$, $p = 0.023$). Post-hoc comparisons showed that Group 4 (PSS + MDMA) froze less than Group 3 (PSS + Saline) at the first reminder (Week 1, $p = 0.0011$), the third reminder (Week 3, $p = 0.032$), and even at the fourth reminder (Week 4, one week after discontinuing MDMA, $p = 0.023$). These results indicate that continuous MDMA micro-dosing impeded the progressive increase in conditioned fear normally seen with repeated trauma reminders, and that its fear-attenuating effects persisted at least one week after the end of treatment (Figure 3).

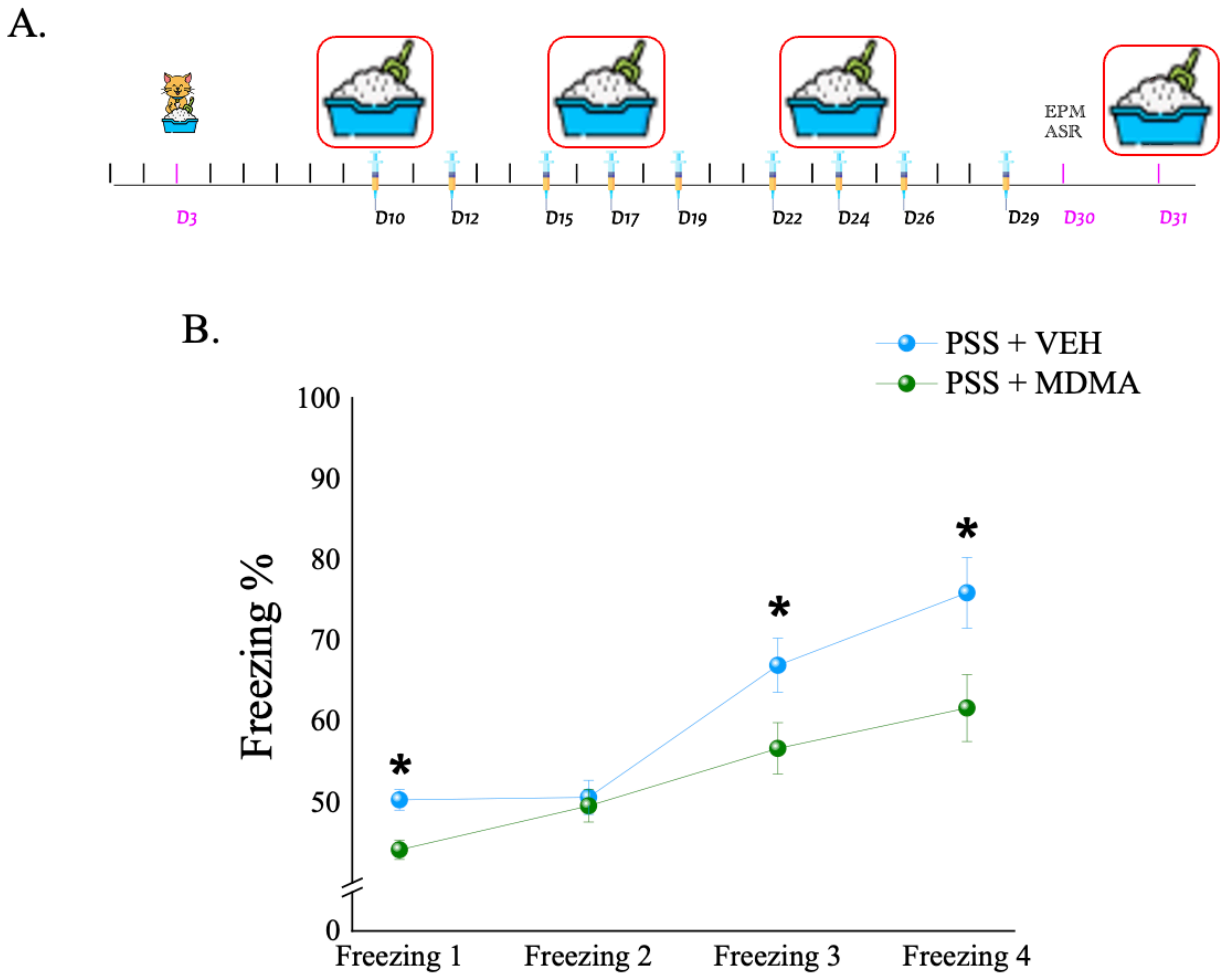


Figure 3: Freezing behavior across four weekly trauma reminder sessions for PSS-exposed rats receiving saline (Group 3) versus MDMA (Group 4). (A) Time course of freezing (% of session) over the reminders, demonstrating a slower increase in freezing in the MDMA group. (B) Comparison of freezing levels at each reminder ($p < 0.05$ for Group 4 vs. Group 3 at Weeks 1, 3, and 4). Notably, even after MDMA treatment ceased (Week 4), previously MDMA-treated rats continued to exhibit lower freezing than controls.

Trauma Reminder Pairing Is Critical for MDMA's Efficacy

After completing the reminder trials for Groups 3 and 4, a final contextual freezing test was conducted for all experimental groups (Groups 1–6) to compare their responses in the trauma context (Figure 4). Two-way ANOVA revealed a significant main effect of PSS exposure ($F_(1,48) = 24.55$, $p < 0.001$), confirming that predator stress increased freezing overall. There was no significant main effect of treatment when averaging across all groups ($F_(1,48) = 0.05$, $p = 0.829$), but importantly, there was a significant PSS × Treatment interaction ($F_(1,48) = 6.24$, $p =$

0.016). Post-hoc tests showed that PSS-exposed rats given saline froze significantly more than sham-stressed controls (Group 3 vs. Group 1, $p < 0.0001$). Within the PSS condition, however, MDMA treatment paired with reminders (Group 4) led to significantly less freezing than saline (Group 3, $p = 0.015$). Furthermore, among MDMA-treated groups, those that received trauma reminders in conjunction with MDMA (Group 4) froze less than those that received reminders unpaired from MDMA (Group 5, $p = 0.003$). Increasing the frequency of MDMA dosing beyond the paired reminder sessions (e.g., thrice-weekly in Group 5 vs. once-weekly in Group 6) did not confer additional reduction in freezing. These findings underscore that MDMA's fear-attenuating effect in this model was contingent on both the occurrence of prior trauma (PSS) and the temporal pairing of MDMA administration with trauma memory reactivation.

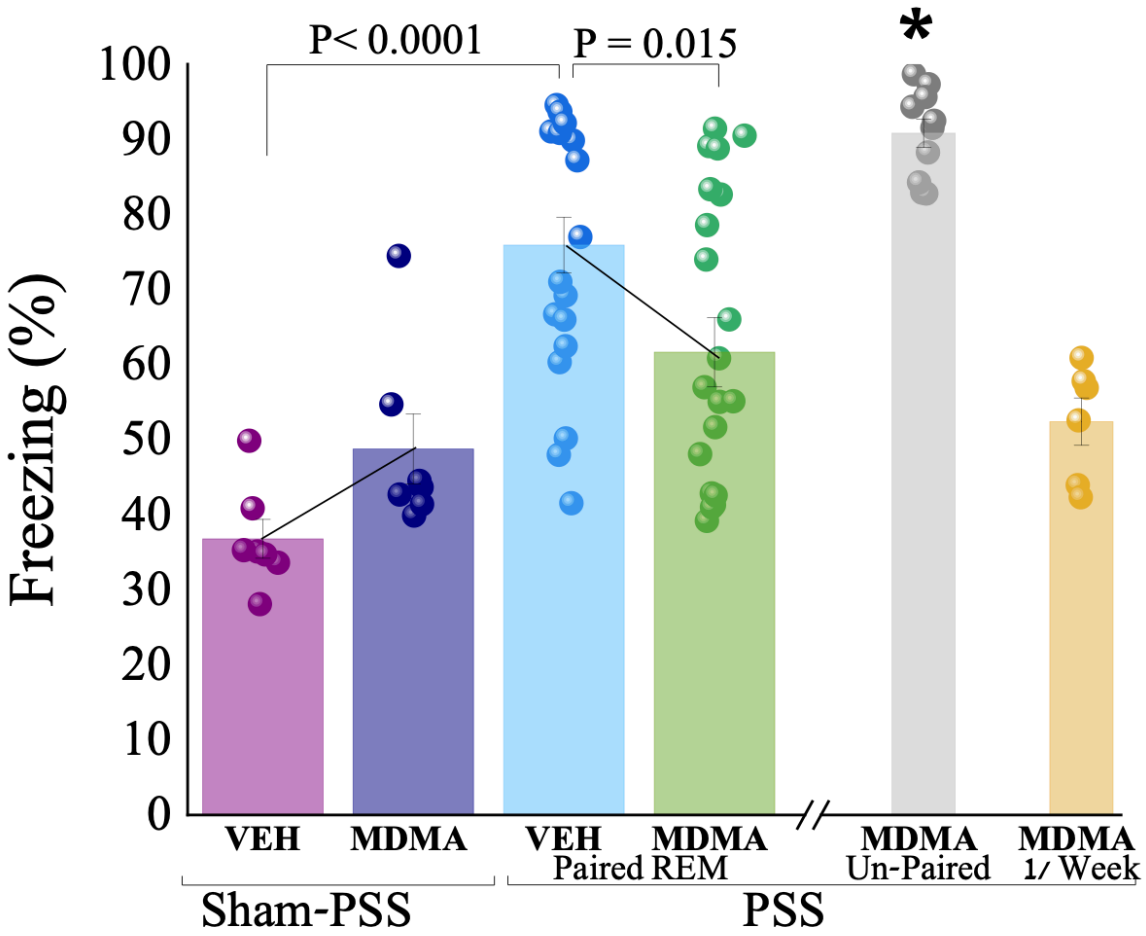


Figure 4: Final contextual freezing test across all groups. Bars represent mean (± SEM) percentage of time spent freezing during re-exposure to the trauma context (cat litter) after treatments. PSS exposure increased freezing in saline-treated rats (Group 3 vs. Group 1). MDMA paired with trauma reminders (Group 4) significantly reduced freezing compared to PSS + Saline (Group 3, $p < 0.05$). MDMA given without paired reminders (Group 5) was less effective, resulting in higher freezing than the paired condition

(Group 5 vs. Group 4, $p < 0.01$). No difference was observed between the two MDMA dosing schedules (thrice weekly vs. once weekly) when reminders were paired.

MDMA Micro-Dosing Fails to Reduce PSS-Induced Anxiety in the EPM

We next assessed whether MDMA micro-dosing affected generalized anxiety-like behavior using the elevated plus maze. PSS exposure had a robust anxiogenic effect on this measure (Figure 5). Two-way ANOVA of the anxiety index showed a significant main effect of PSS ($F_{(1,48)} = 24.55$, $p < 0.001$), with PSS-exposed rats displaying higher anxiety indices (i.e., less open-arm exploration) than non-stressed controls. In contrast, there was no significant main effect of MDMA treatment ($F_{(1,48)} = 1.92$, $p = 0.172$) and no PSS × MDMA interaction ($F_{(1,48)} = 0.61$, $p = 0.440$). Thus, MDMA micro-dosing did not appreciably ameliorate the heightened anxiety caused by predator stress. All PSS-exposed groups (Groups 3–6) exhibited similarly elevated anxiety indices compared to sham groups, regardless of MDMA treatment. These results suggest that the beneficial effects of MDMA in this paradigm are specific to conditioned fear responses and hyperarousal, rather than broadly anxiolytic.

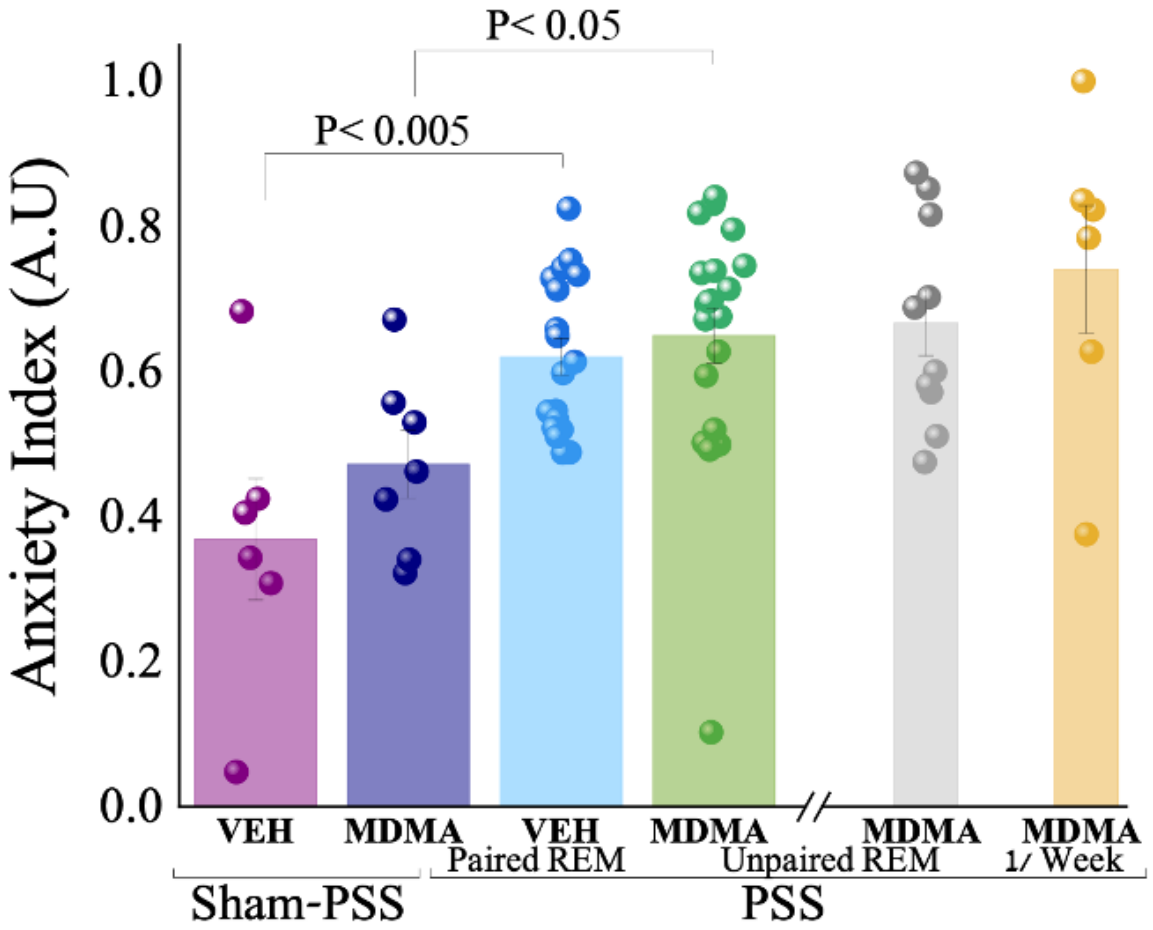


Figure 5: Anxiety-like behavior in the elevated plus maze across experimental groups. Data are presented as anxiety index (mean ± SEM). PSS exposure (Groups 3–6) led to higher anxiety indices compared to non-stressed controls (Groups 1–2, $p < 0.005$). MDMA treatment had no significant effect on the anxiety index in either sham or PSS-exposed rats.

MDMA Attenuates Hyperarousal and Startle Responses

We assessed acoustic startle response (ASR) to determine the impact of MDMA on hyperarousal (Figure 6). PSS exposure greatly increased startle reactivity: PSS + Saline rats had markedly higher startle amplitudes than sham + Saline controls ($p < 0.0001$). Two-way ANOVA confirmed a significant main effect of PSS ($F_(1,48) = 41.1$, $p < 0.0001$) and a significant PSS × MDMA interaction ($F_(1,48) = 5.40$, $p = 0.025$) on startle amplitude. Although the overall MDMA main effect was not significant ($p = 0.119$), planned comparisons revealed that MDMA treatment did mitigate the PSS-induced hyperarousal. Specifically, PSS-exposed rats given MDMA showed lower mean startle amplitude (767.6 ± 51.6) than PSS rats given saline (930.1 ± 25.5; $p = 0.035$). Moreover, the attenuation of startle was influenced by the dosing regimen: rats receiving MDMA three times per week (Group 4) had significantly lower startle responses than those receiving MDMA only once weekly (Group 6, $p = 0.045$). Additionally, PSS rats that received MDMA paired with reminders (Group 4) had lower startle amplitudes than those receiving the same MDMA dosing frequency without paired reminders (Group 5, $p = 0.045$). These findings indicate that MDMA micro-dosing can counteract trauma-induced hyperarousal, and that more frequent, reminder-linked dosing produces the strongest reduction in startle reactivity.

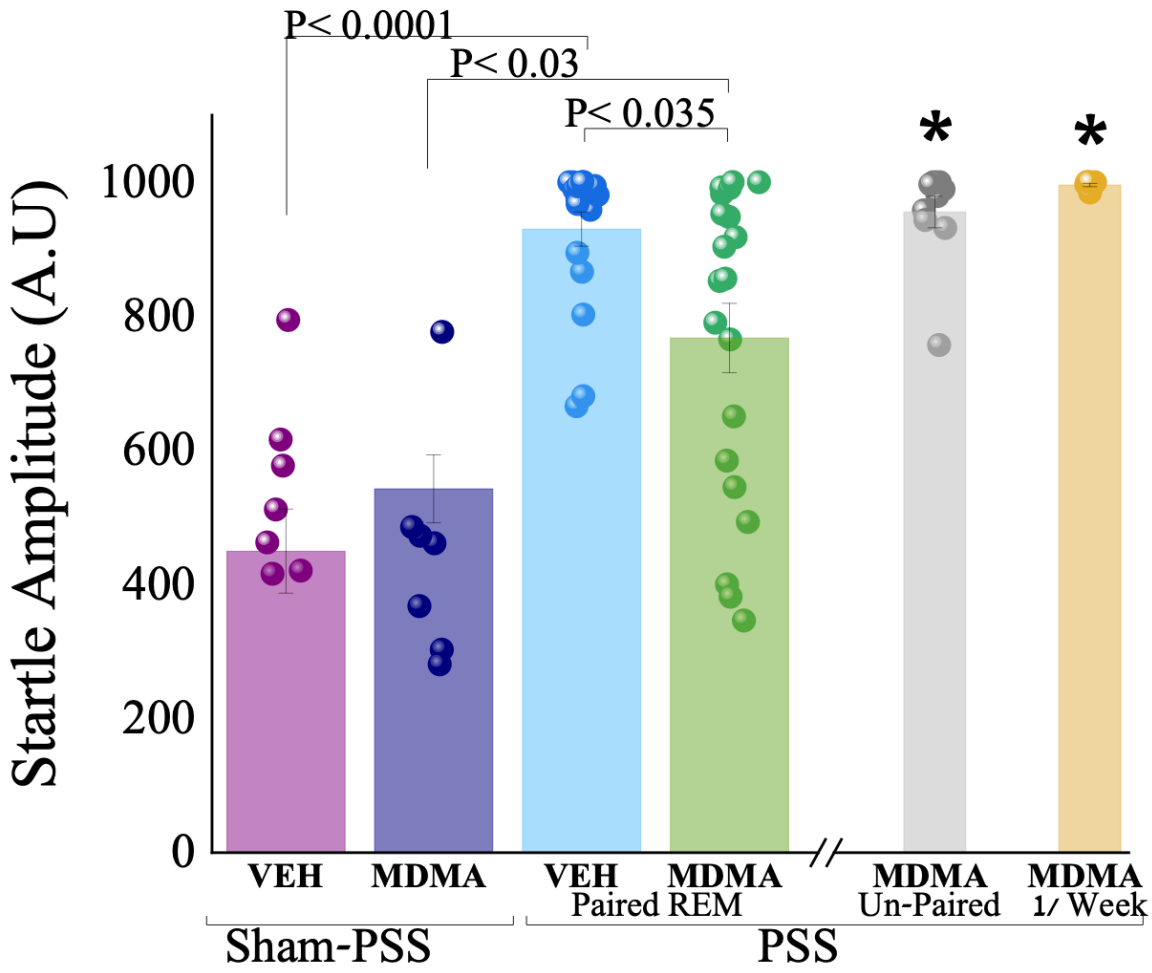


Figure 6: Acoustic startle response (mean startle amplitude ± SEM) for each group. PSS exposure significantly increased startle in saline-treated rats (Group 3 vs. Group 1, *$p <$ 0.0001). MDMA treatment in PSS rats (Group 4) reduced startle amplitude compared to saline (Group 3, $p < 0.05$). MDMA's effect was greater with thrice-weekly dosing (Group 4 vs. Group 6, $p < 0.05$) and when paired with trauma reminders (Group 4 vs. Group 5, $p < 0.05$).

Serotonergic Neuron Density in the Dorsal Raphe

We next examined neurobiological changes that might underlie the behavioral effects. In the dorsal raphe nucleus (DRN), we quantified neurons immunoreactive for tryptophan hydroxylase-2 (TPH2), a marker of serotonergic neurons, in PSS-exposed rats with and without MDMA treatment (Groups 3 and 4). MDMA micro-dosing was associated with a higher density of TPH2-positive cells in the mid-rostrocaudal DRN compared to saline controls (mean counts per 0.5 mm²: 18,265 ± 4,487 vs. 12,108 ± 4,317, respectively), corresponding to an approximately

51% increase. Due to variability, this difference did not reach statistical significance ($F_{(}1,6_{)} = 3.909$, $p = 0.0953$). The trend nonetheless suggests that sustained MDMA micro-dosing may enhance serotonergic tone in the DRN (Figure 7).

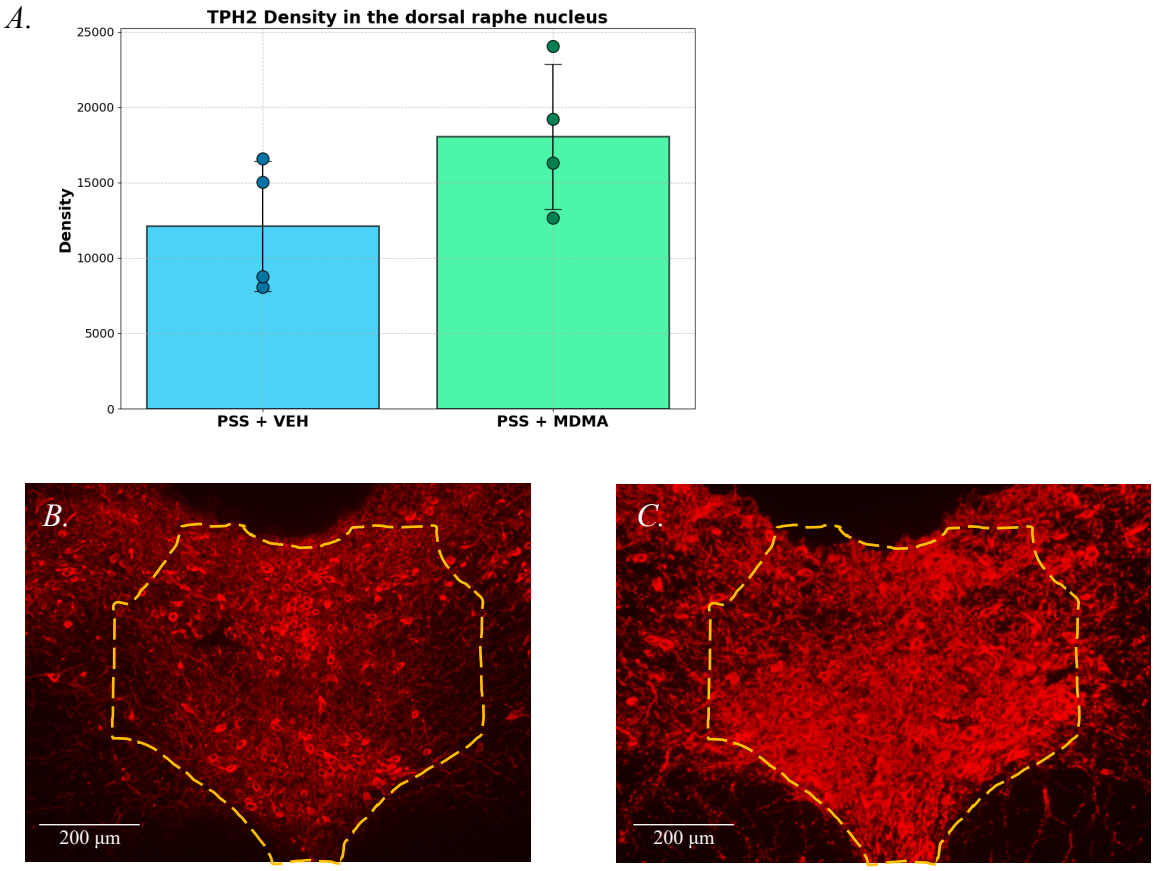


Figure 7: (A) Density of TPH2-immunoreactive serotonergic neurons in the dorsal raphe nucleus (DRN) of PSS-exposed rats with saline vs. MDMA treatment. (B) Representative DRN section from a PSS + Saline rat stained for TPH2 (red fluorescence). (C) Representative DRN section from a PSS + MDMA rat showing more extensive TPH2 staining (red).

MDMA Increases BDNF Expression in Key Brain Regions

Given the role of brain-derived neurotrophic factor (BDNF) in neural plasticity and stress resilience, we assessed BDNF immunoreactivity (BDNF-ir) in several regions implicated in PTSD: the DRN, hippocampus, and hypothalamic paraventricular nucleus (PVN). MDMA micro-dosing led to significantly higher BDNF expression in multiple areas compared to saline treatment.

In the **dorsal raphe nucleus (DRN)**, MDMA-treated rats had a greater number of BDNF-positive cells (1050 ± 61) than saline-treated rats (714 ± 151; $F_{(}1,6_{)} = 6.959$, $p = 0.0062$; Figure 8A).

In the **hippocampus**, MDMA selectively increased BDNF in the CA1 subregion. MDMA-treated rats showed 103 ± 10 BDNF-positive cells in CA1 versus 87 ± 3 in controls ($F_{(}1,6_{)} = 8.454$, $p = 0.027$; Figure 9A). By contrast, in the CA3 subregion, BDNF-positive cell counts did not differ significantly between MDMA and saline groups (88 ± 12 vs. 77 ± 6; $F_{(}1,6_{)} = 2.58$, $p = 0.158$).

In the **paraventricular nucleus (PVN)** of the hypothalamus, MDMA treatment also enhanced BDNF expression, with MDMA rats exhibiting 516 ± 93 BDNF-positive cells compared to 351 ± 79 in saline controls ($F_{(}1,6_{)} = 7.273$, $p = 0.036$; Figure 10A).

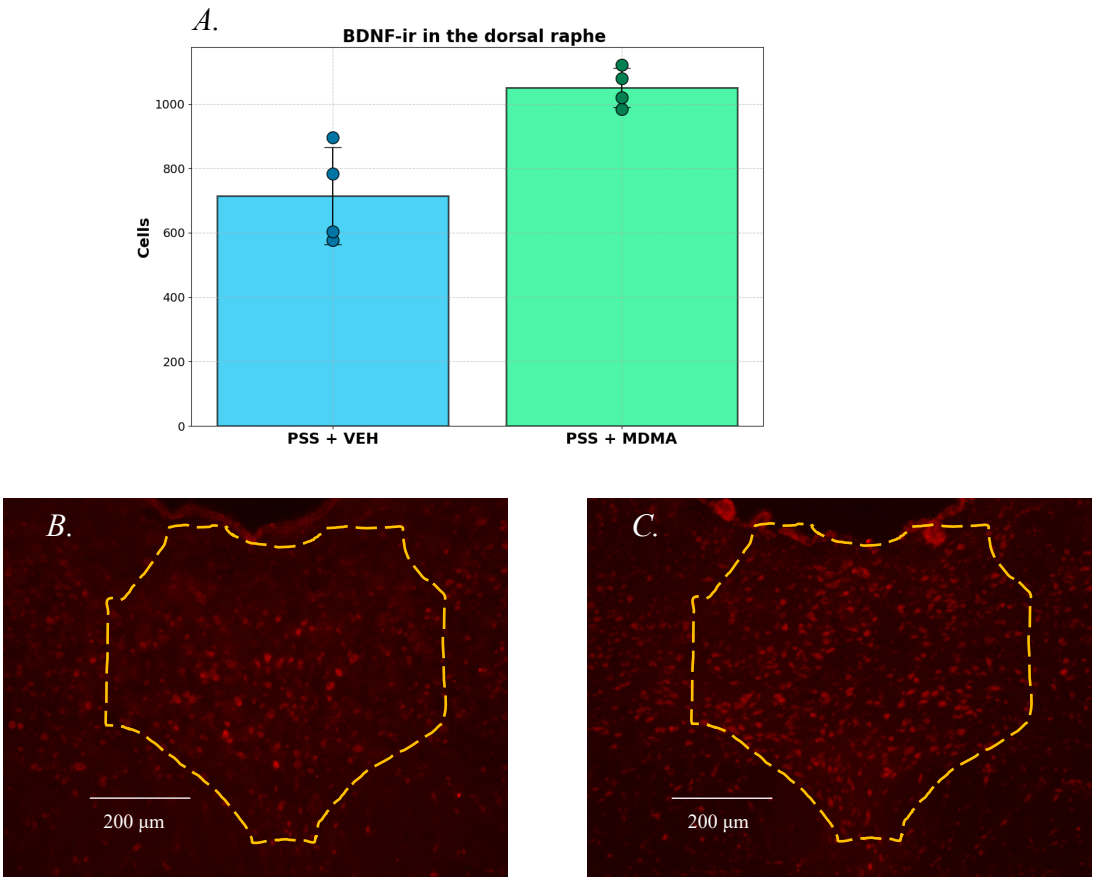


Figure 8: (A) Quantification of BDNF-immunoreactive cells in the dorsal raphe nucleus (DRN), showing a significant increase with MDMA micro-dosing compared to saline ($p < 0.01$). (B) Representative DRN section from a PSS + Saline rat (BDNF staining in red). (C) Representative DRN section from a PSS + MDMA rat (BDNF in red), illustrating the higher density of BDNF-positive cells.

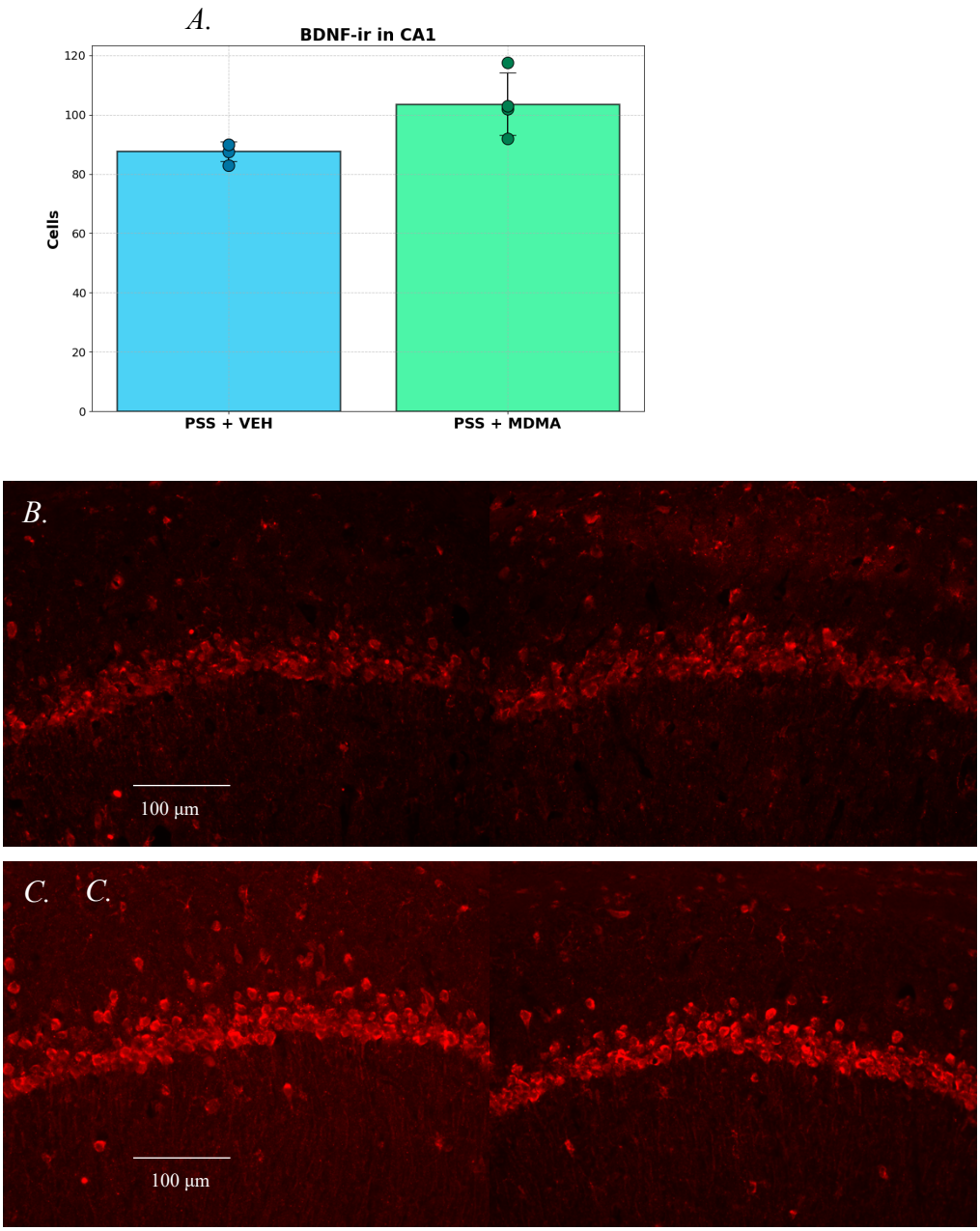


Figure 9: (A) BDNF-immunoreactive cell counts in the hippocampal CA1 region. MDMA-treated rats had significantly more BDNF-expressing cells in CA1 than saline controls ($p < 0.05$). (B) Representative CA1 section from a PSS + Saline rat (BDNF in red). (C) Representative CA1 section from a PSS + MDMA rat (BDNF in red).

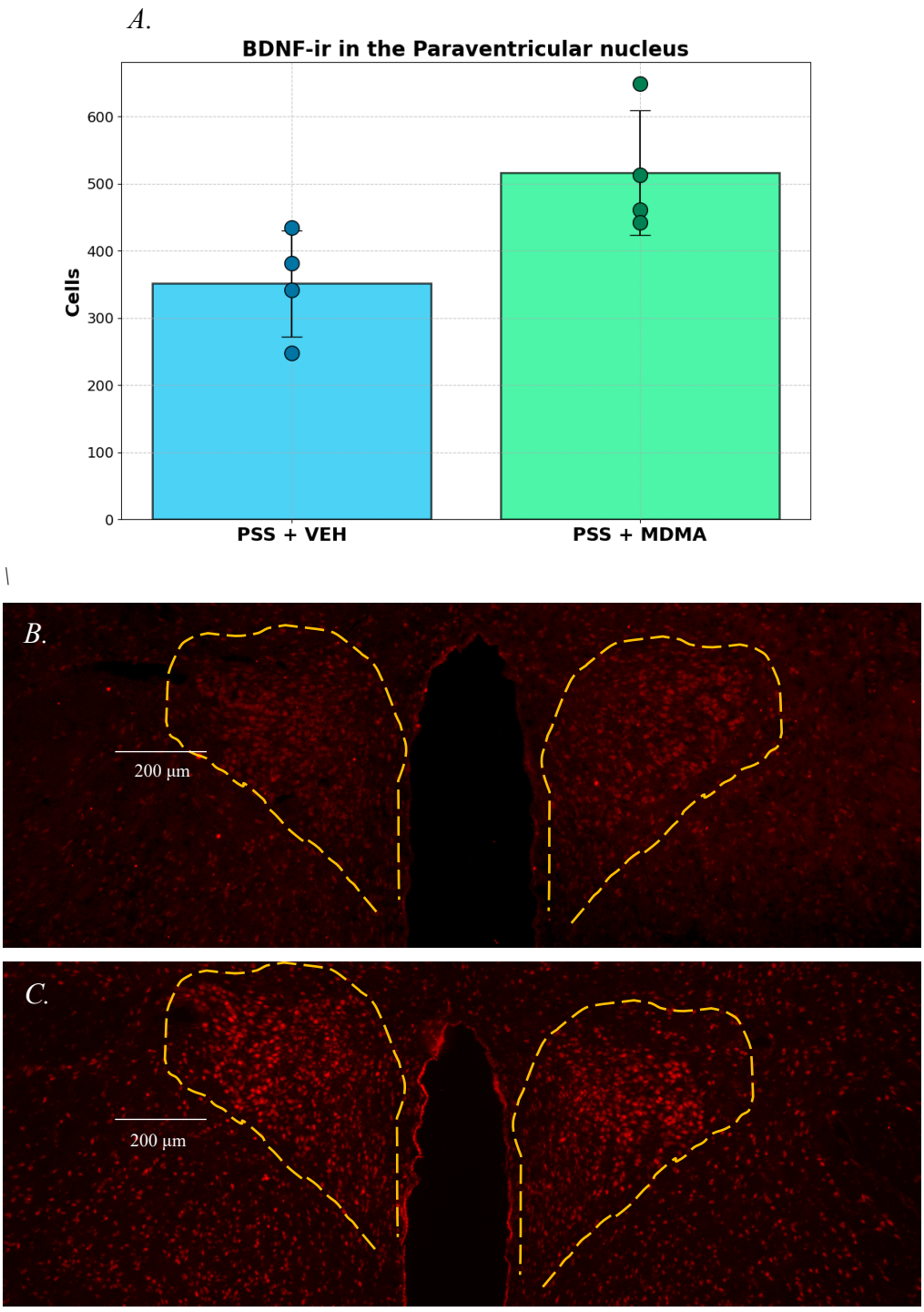


Figure 10: (A) BDNF-immunoreactive cell counts in the paraventricular nucleus (PVN) of the hypothalamus, showing higher BDNF expression in MDMA-treated rats ($p < 0.05$ vs. saline). (B) Representative PVN section from a PSS + Saline rat (BDNF in red). (C) PVN section from a PSS + MDMA rat (BDNF in red).

Oxytocin and Glucocorticoid Receptor Expression in the PVN

We measured oxytocin-producing cells and glucocorticoid receptor (GR) levels in the PVN to determine if MDMA micro-dosing influenced these systems, given their known roles in stress and social bonding. There were no significant differences between MDMA and saline-treated PSS rats in the number of oxytocin-immunoreactive neurons ($F_(1,6) = 0.131$, $p = 0.730$) or in GR immunoreactivity ($F_(1,6) = 0.261$, $p = 0.627$). Thus, chronic micro MDMA did not detectably alter oxytocin or GR expression in the PVN under these conditions. The absence of changes in these markers suggests that the behavioral effects of MDMA were not mediated by modulation of hypothalamic oxytocinergic activity or by altering glucocorticoid feedback pathways.

Dendritic Morphology in the Dentate Gyrus

Golgi-Cox analysis of dentate gyrus granule neurons revealed no significant structural differences between MDMA and saline-treated rats. Total dendritic length per neuron in the DG did not differ between treatments ($F_(1,3) = 1.885$, $p = 0.263$), and there was no significant difference in dendritic branching (as indicated by number of branch points; $F_(1,3) = 0.511$, $p = 0.526$). Thus, within the three-week treatment period, MDMA micro-dosing did not produce overt changes in the dendritic architecture of hippocampal neurons.

DISCUSSION

This study examined the effects of continuous micro MDMA administration on behavioral and neurobiological outcomes in a rat model of PTSD. We found that MDMA micro-dosing (200 µg/kg, thrice weekly) after a traumatic stressor significantly attenuated two hallmark PTSD-like symptoms in rats: conditioned fear (freezing to trauma-related cues) and hyperarousal (exaggerated startle response). These improvements were accompanied by increased expression of the neurotrophic factor BDNF in brain regions associated with stress and memory, suggesting a potential neuroplastic mechanism of action. Notably, the therapeutic effects of MDMA were contingent on its pairing with trauma reminders, highlighting the importance of context and timing in its efficacy.

Attenuation of Conditioned Fear Responses

MDMA-treated rats demonstrated markedly lower freezing in the trauma context compared to saline-treated rats, indicating a reduction in conditioned fear. An acute MDMA dose administered one week after trauma was sufficient to reduce freezing behavior, and this effect persisted with repeated weekly trauma re-exposures. In fact, MDMA not only reduced freezing during ongoing treatment but also showed a residual fear-suppressive effect even one week after

the treatment regimen ended (as evidenced in the final no-drug freezing test). These findings suggest that MDMA micro-dosing disrupted the reconsolidation of traumatic memory or facilitated extinction learning, thereby weakening the conditioned fear response. This is consistent with the idea that MDMA, by acutely reducing fear and anxiety during trauma recall[12–17], creates a favorable psychological state for memory updating, resulting in a lasting decrease in fear associated with the context.

Importance of Pairing MDMA with Trauma Reminders

A key insight from our experimental design is the importance of temporally pairing MDMA administration with trauma-related cues. MDMA's benefits were most pronounced in rats that received the drug in conjunction with memory reactivation (Group 4). When MDMA was given without a concurrent trauma reminder (Group 5), its efficacy was significantly diminished—those rats exhibited freezing levels closer to the saline-treated controls. This pattern underscores that the therapeutic potential of MDMA in this model relies on engaging memory processes: MDMA appears to enhance the processing or extinction of traumatic memories when those memories are activated during the drug's acute window of effect[22]. In our study, simply increasing the frequency of MDMA dosing (thrice weekly in Group 5) without synchronization to trauma cues did not improve outcomes, emphasizing that **when** the drug is given (relative to memory reactivation) is more critical than **how often**. Importantly, MDMA had no impact on freezing behavior in unstressed control rats, suggesting that its effects are specific to pathological fear memory traces and not due to non-specific changes in locomotion or baseline anxiety.

Effects on Hyperarousal and Startle

MDMA micro-dosing also mitigated the heightened startle response observed in PSS-exposed rats, indicating a reduction in hyperarousal. The degree of startle attenuation was influenced by the dosing schedule: more frequent MDMA administration (three times weekly) and pairing with trauma cues both enhanced the reduction in startle amplitude. This aligns with clinical observations that multiple MDMA-assisted sessions can yield cumulative improvements in hyperarousal symptoms[15][18–20]. Our results extend those findings by suggesting that even at sub-psychedelic doses, MDMA can dampen the hyper-reactivity of the defensive startle reflex, which is often elevated in PTSD. The fact that one weekly dose (Group 6) was less effective implies a frequency-dependent effect, where maintaining more continuous MDMA exposure (or repeatedly pairing MDMA with reminders) is necessary to observe robust effects on arousal circuits.

Lack of Generalized Anxiolytic Effect

Interestingly, despite its impact on conditioned fear and startle, MDMA micro-dosing did not alleviate general anxiety as measured on the EPM. All PSS-exposed rats showed an increased anxiety index, regardless of MDMA treatment. This suggests that the low MDMA dose used was insufficient to produce a broad anxiolytic effect in the absence of trauma-related cues, or that PTSD-related generalized anxiety (as modeled by the PSS effect in the EPM) is mediated by neural circuits not readily affected by this dosing regimen. MDMA's therapeutic effects at micro-doses may thus be more domain-specific, prominently affecting fear memory and hyperarousal but not baseline anxiety. This specificity could be advantageous in targeting the most debilitating PTSD symptoms while avoiding excessive dampening of normal anxiety responses.

Neurobiological Mechanisms: BDNF and Serotonergic Modulation

The behavioral benefits of MDMA micro-dosing were paralleled by increases in BDNF expression in the DRN, hippocampal CA1, and PVN. BDNF is crucial for synaptic plasticity and fear extinction; elevated BDNF in these regions suggests that MDMA created a neurobiological environment conducive to learning new, less fearful associations and recovering from stress[39]. Increases in DRN BDNF are particularly noteworthy, as the DRN houses the majority of serotonergic neurons. MDMA's ability to elevate BDNF in the DRN might reflect enhanced neurotrophic support for serotonin neurons, which could improve serotonergic neurotransmission to downstream areas (such as the amygdala and prefrontal cortex) involved in mood and anxiety regulation. Although the increase in TPH2+ neuron density in the DRN did not reach significance, the upward trend implies a possible MDMA effect on serotonin neuron viability or enzyme expression. Together, these neurochemical changes align with MDMA's known pharmacology of acutely boosting serotonin levels[13], and they hint at longer-term adaptations such as upregulation of growth factors that help rewire neural circuits after trauma.

The hippocampus is central to contextual memory, and BDNF within the hippocampus (especially CA1) has been implicated in fear extinction and antidepressant effects. The selective increase of BDNF in CA1 (but not CA3) observed in MDMA-treated rats may indicate region-specific synaptic potentiation related to processing the trauma context. Similarly, the PVN is a critical node in the stress response (governing hypothalamic-pituitary-adrenal axis activity), and increased BDNF there could influence neuroendocrine adaptations to stress. These findings collectively suggest that MDMA micro-dosing triggers adaptive plasticity in both the serotonergic system and stress-related neural circuits, which may underlie the observed reductions in fear and hyperarousal.

Oxytocin and GR Pathways

We did not find changes in oxytocin or glucocorticoid receptor levels in the PVN with MDMA micro-dosing. Oxytocin release is one of the hallmark acute effects of MDMA and has been theorized to contribute to its prosocial and anxiolytic properties[16][17]. However, at a micro-dose given chronically, it appears MDMA may not have sufficiently elevated oxytocin (or induced lasting changes in oxytocin-expressing neurons) to be detectable at the time points we examined. Similarly, GR expression, which can be affected by chronic stress or antidepressant treatment, was unchanged. It is possible that the dose was too low to engage these pathways, or that any changes in oxytocin/GR dynamics are transient and not captured by postmortem immunohistochemistry. The lack of effect on these measures suggests that the fear- and startle-reducing properties of MDMA micro-dosing in this model were not mediated by modulating the hypothalamic oxytocinergic system or altering HPA-axis feedback sensitivity via GR. Instead, as discussed above, the mechanism may rely more on neurotrophic and monoaminergic systems.

Limitations and Future Directions

This study focused on a single MDMA micro-dose (200 μg/kg) and a specific dosing schedule. It is possible that different doses or longer treatment durations could produce broader effects, including impacts on oxytocin or GR pathways, or greater anxiolytic outcomes. Future research should explore a range of micro-dosing regimens to determine the optimal balance between efficacy and safety. Additionally, while we observed positive effects during and shortly after the dosing period, we did not assess very long-term outcomes. It will be important to determine whether the benefits of MDMA micro-dosing persist beyond one week post-treatment and whether any delayed adverse effects or tolerance might develop with prolonged use. Neurotoxicity is a concern with higher MDMA doses[30], but the long-term safety of repeated micro-doses needs verification. Including molecular assays for potential neurotoxic markers or using in vivo neuroimaging in future studies could help address these safety questions.

Another limitation is that our behavioral testing largely focused on fear and startle responses in the presence of trauma-related cues, which maximized sensitivity to MDMA's effects on those domains. It remains to be seen whether micro-dosing MDMA would influence other PTSD-relevant behaviors (such as social interaction deficits or depressive-like symptoms) that were not assessed in this model. Broadening the behavioral battery and including female subjects (to account for sex differences in stress responses) are important future directions.

Conclusion

In conclusion, continuous post-trauma MDMA micro-dosing, especially when combined with trauma cue exposure, proved effective in reducing key PTSD-like symptoms in rats. The regimen specifically dampened conditioned fear responses and hyperarousal without reducing general anxiety, suggesting a focused therapeutic action. The concomitant upregulation of BDNF

in stress- and memory-related brain regions provides a neurobiological basis for these behavioral changes, pointing to enhanced neuroplasticity as a mechanism. These preclinical findings support the idea that strategically timed micro MDMA could be a promising adjunct to exposure-based therapies for PTSD, helping to safely facilitate the reprocessing of traumatic memories. Further research into dose optimization, underlying mechanisms, and long-term safety will be essential to determine the translational potential of MDMA micro-dosing in clinical settings.

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