MDMA administration attenuates hippocampal IL-β immunoreactivity and subsequent stress-enhanced fear learning: An animal model of PTSD

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A B S T R A C T

Post-traumatic stress disorder (PTSD) is a devastating disorder that involves maladaptive changes in immune status. Using the stress-enhanced fear learning (SEFL) paradigm, an animal model of PTSD, our laboratory has demonstrated increased pro-inflammatory cytokine immunoreactivity in the hippocampus following severe stress. Recent clinical trials have demonstrated 3,4-methylenedioxymethamphetamine (MDMA)-assisted psychotherapy as an effective novel treatment for PTSD. Interestingly, MDMA has been shown to have an immunosuppressive effect in both pre-clinical and clinical studies. Therefore, we predict MDMA administration may attenuate SEFL, in part, due to an immunosuppressive mechanism. The current studies test the hypothesis that MDMA is capable of attenuating SEFL and inducing alterations in expression of TNF-α, IL-1β, glial fibrillary acidic protein (GFAP), an astrocyte specific marker, and ionized calcium-binding adapter molecule 1 (IBA-1), a microglial specific marker, in the dorsal hippocampus (DH) following a severe stressor in male animals. To this end, experiment 1 determined the effect of MDMA administration 0, 24, and 48 h following a severe foot shock stressor on SEFL. We identified that MDMA administration significantly attenuated SEFL. Subsequently, experiment 2 determined the effect of MDMA administration following a severe stressor on the expression of TNF-α, IL-1β, GFAP, and IBA-1 in the DH. We found that MDMA administration significantly attenuated stress-induced IL-1β and stress-reduced IBA-1 but had no effect on TNF-α or GFAP. Overall, these results support the hypothesis that MDMA blocks SEFL through an immunosuppressive mechanism and supports the use of MDMA as a potential therapeutic agent for those experiencing this disorder. Together, these experiments are the first to examine the effect of MDMA in the SEFL model and these data contribute significantly towards the clinical PTSD findings.

1. Introduction

Post-traumatic stress disorder (PTSD) is a chronic and devastating psychological disorder resulting from a traumatic experience (Bremner et al., 1996). While some therapies exist, conventional psychotherapies and pharmacotherapies such as cognitive behavioral therapy and selective serotonin reuptake inhibitors (SSRIs) are ineffective and have high non-response and drop-out rates. The non-response rates for cognitive behavioral therapy may be as high as 50% (Schottenbauer et al., 1996), and only 20%–30% of patients achieve complete remission with SSRI use (Alexander 2012). Moreover, approximately 33% of those with PTSD are treatment resistant, which proves the development of successful treatments to be challenging (Boudewyns et al., 1991). Interestingly, recent clinical trials have demonstrated 3,4-methylenedioxymethamphetamine (MDMA)-assisted psychotherapy to be a highly efficacious, safe, and well-tolerated treatment for individuals with severe PTSD (Ot’alora et al., 2018; Mitchell et al. 2021). MDMA was initially developed for pharmaceutical purposes and was later discovered to have psychoactive properties, which prompted its popular use as a recreational party drug by the late 1980s (de Almeida and Silva 2000). Despite the illicit drug’s legal status, MDMA is also demonstrated to have therapeutic properties. Clinically, MDMA can assist psychotherapy by reducing a patient’s anxiety and increasing relaxation (Morgan 2020, Borissova et al., 2021), which can allow a therapist and patient to speak about difficult topics in-depth. An MDMA-assisted psychotherapy session consists of a patient revisiting traumatic memories and working through their emotions (Morgan 2020). The MDMA assists with this process by reducing anxiety associated with recalling traumatic experiences, increasing insight and memory, and allowing for empathic self-reflection without being overwhelmed by the traumatic memories (Sessa 2017; Morgan 2020). A randomized phase 2 controlled trial of MDMA-assisted psychotherapy demonstrated that 76% of trial participants...
participants with chronic PTSD that underwent the treatment no longer met PTSD criteria at a 12-month follow-up (Ot’alora et al., 2018; Mitchell et al., 2021). These promising results were expanded to a randomized, double-blind, placebo-controlled phase 3 clinical trial to test the efficacy and safety of MDMA-assisted therapy for the treatment of severe PTSD (Mitchell et al., 2021). Excitingly, MDMA was found to induce significant and robust attenuation of PTSD symptoms compared with placebo, suggesting that MDMA-assisted therapy is highly efficacious as well as safe and well-tolerated (Mitchell et al., 2021). Although these results are promising, the mechanism underlying MDMA-assisted psychotherapy remains unclear.

MDMA has multiple mechanisms of action in the human body such as increasing the release of monoamine neurotransmitters (serotonin, noradrenaline, and dopamine) through the binding and blocking of the transporter involved in reuptake (Kalant, 2001; Green et al. 2003; de la Torre et al., 2004), which physically produces an increase in wakefulness, energy, as well as some sense of euphoria and well-being (Simmler and Liechti, 2018). However, a mechanism of action infrequently discussed and associated with PTSD is MDMA’s immunomodulatory effects, as PTSD is associated with biological alterations of immune function (Koo and Duman, 2008; Smid et al., 2015; Neigh and Ali, 2016; Deslauriers et al., 2017). One such alteration of immune status following stress exposure is elevated levels of pro-inflammatory cytokines in the peripheral and central nervous system (Gill et al., 2009; Muhie et al., 2017; Kim et al., 2020; Munshi et al., 2020). In particular, the pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are associated with stress exposure in animal models (Casas et al., 2007; Cheng et al., 2015; Hussein et al., 2017; Barrett et al., 2021) and clinical presentations of PTSD (Passos et al., 2015; Hussein et al., 2017).

Not only are IL-1β and TNF-α correlated with stress exposure, they also have well-established roles in learning and memory, further implicating these cytokines in the development and maintenance of PTSD (Habbas et al., 2015; Ma et al., 2015; Noorbakhshnia and Karimi-Zandi 2017; Takemiyai et al., 2017; Bourgognon and Cavanagh, 2020). Although a basal level of these cytokines is necessary for learning and memory, both insufficient and excessive levels lead to deficits in these processes. For example, infusion of TNF-α into the dorsal hippocampus (DH) is shown to block the retrieval and reconsolidation of contextual fear memories (Takahashi et al., 2021), and enhanced TNF inflammation is associated with functional modulation of hippocampal excitatory synapses (Habbas et al., 2015). IL-1β is also associated with hippocampal-dependent memory tasks (Takemiyai et al., 2017), and IL-1β injections into the dorsal hippocampus have been shown to impair contextual learning (Barrientos, Sprunger et al. 2004) as well as fear conditioning (Pugh et al., 1999).

Along with the substantiated correlation between stress, a pro-inflammatory response, and impaired learning and memory, upregulated pro-inflammatory cytokines also play a mechanistic role in the behavioral consequences of stress, such as the anxiety and depressive symptoms that are prominent in PTSD. For example, peripheral administration of IL-1β has been shown to enhance anxiety-like behavior in the elevated plus maze (Swiergiel and Dunn, 2007), and inhibition of IL-1β blocks the effects of stress on depressive-like behaviors (Koo and Duman, 2008). Additionally, systemic TNF-α blockade has been shown to attenuate anxiety and depressive-like behaviors in mice (Alshammari et al., 2020), as well as decrease anxiety-like behaviors in a preclinical model of PTSD (Hill et al., 2021). Our laboratory has also investigated the association of pro-inflammatory cytokines with severe stress in the stress-enhanced fear learning (SEFL) paradigm, an animal model of fear-related features of PTSD (Rau et al., 2005). While it is difficult to incorporate all the symptoms of PTSD into a preclinical animal model, SEFL effectively demonstrates hyperarousal and greater susceptibility to future fear learning, a prominent component of clinical PTSD. In the SEFL paradigm, rats previously exposed to a severe stressor (inescapable foot shocks) show an exaggerated or enhanced fear response to a mild form of stress in a separate, distinct context (Rau et al., 2005). This is critical and unique to the SEFL paradigm, because this indicates a form of non-associative learning, while many animal models of PTSD capture a form of associative learning (Whitaker et al., 2014; Richter-Levin et al., 2019). The hyperarousal and enhanced reactivity response that is captured using the SEFL paradigm offers the opportunity to investigate an important, critical symptom of clinical PTSD. Consistent with the literature, we have found that the severe stressor in the SEFL paradigm induces a time-dependent region-specific increase in dorsal hippocampal (DH) IL-1β in astrocytes (Jones et al., 2015; Jones et al., 2018a).

Further, we have also shown a mechanistic role of IL-1β in the behavioral consequences of stress, as blockade of IL-1β attenuates enhanced fear learning, suggesting that IL-1β signaling is necessary for the development of enhanced fear learning (Jones et al., 2015; Jones et al., 2018a; Parekh et al., 2020). Remarkably, we have also demonstrated that TNF-α signaling plays a role in the development of an enhanced fear learning paradigm (Parekh et al., 2021).

When examining the cellular sources of these pro-inflammatory cytokines, glial cells such as microglia and astrocytes are potential candidates, as they regulate the immune response and can release cytokines when activated (Kim and Joh 2006; Cekanaviciute and Buckwalter 2016; Enomoto and Kato 2021, Li et al., 2022). Microglia and astrocytes are also thought to be implicated in the development of PTSD, and one such mechanism may be through cytokine signaling, as they are shown to be primary cellular sources of inflammatory cytokine production following stress (Wang et al., 2018; Himmerich et al., 2019; Johnson et al., 2019). Furthermore, our laboratory has shown that DH astrocytes are the predominant source of stress-induced IL-1β in the SEFL paradigm (Jones et al., 2018a). Microglia are also altered following stress and are implicated in fear learning, as a single session of stress provoked robust morphological microglial activation in several brain regions including the hippocampus (Sugama et al., 2007), and depleting or inhibiting microglia before foot-shock exposure alleviated PTSD-associated anxiety and contextual fear in an animal model (Li et al., 2021). Such evidence of glial cell involvement in anxiety, fear learning, and memory following stress suggests a mechanistic role of glial cells in the development of PTSD.

Despite the strong evidence for involvement of proinflammatory cytokines and glial cells in PTSD, most current treatments do not target these mechanisms. Interestingly, MDMA has been shown to have an immunomodulatory effect in both pre-clinical and clinical trials (Connor 2004). For example, MDMA inhibits the immune response by suppressing neutrophil phagocytosis and reducing lymphocyte numbers (Connor et al., 2000). Further, MDMA is shown to alter cytokine production by inducing an increase of anti-inflammatory and immunosuppressive cytokines, including transforming growth factor-beta and interleukin-10 (Pacifi et al., 2001), as well as reducing pro-inflammatory cytokines such as IL-1β and TNF-α (Connor et al., 2000; Connor et al., 2001). However, this previous literature has exclusively examined peripheral immune responses, necessitating investigation into the neuroimmune response.

Therefore, the current studies test the hypothesis that MDMA is capable of attenuating stress-enhanced fear learning and inducing alterations in TNF-α, IL-1β, glial fibrillary acidic protein (GFAP), and ionized calcium-binding adapter molecule-1 (IBA-1) expression in the DH. We chose to examine TNF-α and IL-1β due to their demonstrated roles in stress, fear learning, and memory. As glial cells are shown to be implicated in astrocyte activation and production, we also examined GFAP, a marker of astrocyte activation, as well as IBA-1, a marker of microglia activation. Our analysis focused on the DH, as this region has been shown to be critical to context-dependent fear learning and conditioning (Anagnostaras et al., 2001; Acheson et al., 2012; Huckleberry et al., 2018) and to the SEFL paradigm (Szczypkowski-Thomson et al., 2013; Jones et al., 2015; Jones et al., 2018a). We predict that MDMA-assisted psychotherapy treatment for PTSD may be effective through an immunosuppressive mechanism as our previous experiments have
demonstrated that pro-inflammatory signaling proteins are necessary for the development of stress-enhanced fear learning, which supports this hypothesis. Experiment 1 determined the effect of MDMA administration 0, 24, and 48 h following a severe stressor on stress-enhanced fear learning. We identified that MDMA administration significantly attenuated stress-enhanced fear learning. Subsequently, experiment 2 determined the effect of MDMA administration 0, 24, and 48 h following a severe stressor on the expression of TNF-α, IL-1β, GFAP, and IBA-1 in the DH. We found that MDMA administration significantly attenuated IL-1p and rescued the shock-induced reduction of IBA-1, however, there were no effects on TNF-α or GFAP. Together, these experiments are the first to examine the effect of MDMA in the SEFL model.

2. Materials and methods

2.1. Animals

A total of 90 adult male Sprague Dawley rats (230–250 g, Charles River Laboratories, Raleigh, NC) were individually housed under a reversed 12-hr light-dark cycle. Rats were given ad libitum access to food and water and were regularly handled throughout experimentation. All procedures were conducted with approval from the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

2.2. Drug administration

MDMA (3,4-methylenedioxy-methamphetamine, National Institute on Drug Abuse (NIDA) Drug Supply Program, Bethesda, MD, USA) was dissolved in sterile 0.9% saline to produce a 10.0-mg/mL solution and stored at 4°C until time of injection. MDMA or saline was administered subcutaneously (Sub-Q) 0-hr, 24-hr, and 48-hr following the removal of the DH. We found that MDMA administration significantly attenuated stress-enhanced fear learning. Subsequently, experiment 2 determined the effect of MDMA administration 0, 24, and 48 h following the removal from Context A. Six days following Context A, animals were exposed to Context B and the home cage. Context B is associated with distinct textile, olfactory, and auditory characteristics from both Context A and the home cage. Freezing behavior was recorded in Context B using a video recording system (GoPro HERO9). Animals were exposed to Context B for 10 min to allow for habituation to the new context. On Day 8, animals were placed back into Context B, and all animals received a single, 1 mA scrambled foot shock, 3 min, 12 s after being placed into the context. On Days 9, 10, 15, and 22 (Test Days 1, 2, 7, and 14), animals were placed into Context B for 8 min, 23 s and were video recorded to analyze freezing behavior, a measure of learned fear. Automated video tracking and analysis software was used to quantify percent freezing behavior in all sessions (Ethovision XT, Activity Feature with Threshold = 10, Noldus Information Technology Inc., Netherlands) as previously analyzed (Jones et al., 2018b; Parekh et al., 2020; Parekh et al., 2021). Ethovision XT is a video tracking software that tracks and analyzes behavior, movement, and activity of any animal. The activity analysis feature in the software analyzes overall activity per arena based on pixel changes, which is useful for freezing detection (Pham et al., 2009). Freezing behavior is defined by the absence of detected movement of the animal. Therefore, the program determines the number of seconds during which the duration of levels of activity within the arena is inactive based on the threshold. This inactive number is divided by the total number of seconds of video time and multiplied by 100 to achieve percent freezing [(Ethovision Inactive Seconds/Total Seconds of Video Time) × 100].

2.3. Stress enhanced fear learning paradigm

All animals were assigned to a Context A treatment (foot shock or no foot shock) and a drug treatment (MDMA or saline) and were exposed to the SEFL paradigm. This paradigm has been previously described at length (Szczytkowski-Thomson et al., 2013; Jones et al., 2015; Jones et al., 2018b), and is described in Fig. 1a. Briefly, on Day 1, animals are exposed to Context A (BRS/LVE, Laurel, MD; H 26.7 cm x D 24.8 cm x W 30.7 cm) which is housed in a separate room with distinct textile, olfactory, and auditory characteristics from the home cage. Animals assigned to the foot shock condition received 15x2 mA scrambled foot shocks over 90 min on a 6 min variable interval schedule while control animals were exposed to the context for the same amount of time without foot shocks being delivered. MDMA or saline was administered 0-hr, 24-hr, and 48-hr following the removal from Context A. Six days following Context A, animals were exposed to Context B (Med Associates, St. Albans, VT), which is housed in a separate room from Context A and the home cage. Context B is associated with distinct textile, olfactory, and auditory characteristics from both Context A and the home cage. Freezing behavior was recorded in Context B using a video recording system (GoPro HERO9). Animals were exposed to Context B for 10 min to allow for habituation to the new context. On Day 8, animals were placed back into Context B, and all animals received a single, 1 mA scrambled foot shock, 3 min, 12 s after being placed into the context. On Days 9, 10, 15, and 22 (Test Days 1, 2, 7, and 14), animals were placed into Context B for 8 min, 23 s and were video recorded to analyze freezing behavior, a measure of learned fear. Automated video tracking and analysis software was used to quantify percent freezing behavior in all sessions (Ethovision XT, Activity Feature with Threshold = 10, Noldus Information Technology Inc., Netherlands) as previously analyzed (Jones et al., 2018b; Parekh et al., 2020; Parekh et al., 2021). Ethovision XT is a video tracking software that tracks and analyzes behavior, movement, and activity of any animal. The activity analysis feature in the software analyzes overall activity per arena based on pixel changes, which is useful for freezing detection (Pham et al., 2009). Freezing behavior is defined by the absence of detected movement of the animal. Therefore, the program determines the number of seconds during which the duration of levels of activity within the arena is inactive based on the threshold. This inactive number is divided by the total number of seconds of video time and multiplied by 100 to achieve percent freezing [(Ethovision Inactive Seconds/Total Seconds of Video Time) × 100].

2.4. Tissue collection and histology

In experiment 2, animals were sacrificed by transectional perfusion 1 h following the last MDMA or saline injection. Animals were terminally anesthetized with 9:1 (vol/vol) ketamine hydrochloride (100 mg/mL) mixed with xylazine (100 mg/mL), and transcardially perfused with ice-
cold phosphate buffer (PB; pH = 7.4) followed by 4% paraformaldehyde in 0.1 M PB. Brains were extracted and post-fixed in 4% paraformaldehyde for 6 h, and 30% sucrose with 0.1% sodium azide was used for cryoprotection at 4°C. Once the brains were saturated with sucrose, brains were cut into 40-µm coronal sections on a cryostat (Leica CM 3050 S, Leica Microsystems, Buffalo Grove, IL, USA).

2.5. Immunohistochemistry

Experiment 2 used fluorescent immunohistochemistry (IHC) to examine alterations in DH IL-1β, TNF-α, GFAP, and IBA-1 in the DG. The IHC protocol used here has been described previously (Jones et al., 2018a; Parekh et al., 2020; Parekh et al., 2021). The following primary antibodies were used: rabbit anti-IL-1β (1:500, Abcam, Cambridge, MA, Cat # Ab9722, USA), rabbit anti-TNF alpha (1:1000, Abcam, Cambridge, MA, Cat # ab66579), mouse anti-GFAP (1:1000, ThermoFisher Scientific, Waltham, MA, Cat # MS-1376P, USA), and rabbit anti-IBA-1 (1:500, Wako, Richmond, VA, Cat # 019–19741, USA). To visualize IL-1β, TNF-α, and IBA-1, Alexa Fluor conjugated secondary antibody, goat anti-rabbit 488 (1:1000, ThermoFisher Scientific, Waltham, MA, Cat # A11008) was used. To visualize GFAP, Alexa Fluor conjugated secondary antibody, goat anti-mouse 594 (1:1000, ThermoFisher Scientific, Waltham, MA, Cat # A11005) was used. Briefly, tissue sections were washed three times for 10 min in 0.1M phosphate buffer (PB, pH = 7.4) followed by a 1 h incubation in 5% normal goat serum (NGS) and 0.5% TritonX100 in 0.1M PB at room temperature. Tissue was incubated in primary antibody, 5% NGS, and 0.5% TritonX100 in 0.1M PB overnight at 4°C, washed three times for 10 min in 0.1M PB, and incubated in secondary antibody, 5% NGS, and 0.5% TritonX100 in 0.1M PB for 1 h at room temperature. Primary antibodies were verified by no primary control stains. Sections were mounted onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) using Vectorshield with DAPI hardset mounting medium (Vector Laboratories, Burlingame, CA).

2.6. Microscopy and image analysis

Fluorescent microscopy (Leica DM6000 B widefield light microscope, Leica Microsystems, Buffalo Grove, IL, USA) was used to capture color images (20x). Positive fluorescence in images was quantified using automatic Image J (NIH) triangle thresholding feature. The implementation of the ImageJ automatic triangle algorithm has been previously described (Zack et al., 1997). Briefly, the algorithm assumes a maximum peak near one end of the histogram and searches for intensity toward the end of the histogram bins. Three to five sections were analyzed bilaterally per animal for the dorsal dentate gyrus and values were averaged and expressed as percent positive stain. In addition, the number of positive cells overlaid with DAPI, an indicator of cellular nuclei, in all the images taken was counted manually for all four immunohistochemistry stains. All analyses including thresholding and counting were made blind to treatment condition. Tissue from poor perfusions that yielded high nonspecific background which interfered with thresholding was dropped from the analysis. Moreover, images where automatic thresholding failed were also excluded from analysis. The decision to exclude these samples was made blind to the treatment group. Publication images were compiled with the Adobe Photoshop CS software (Creative Cloud Photoshop v22.1, San Jose, CA, USA). Color levels and background were reduced for optimal representation with level tools. Images from all experimental groups were treated equally.

2.7. Statistical analysis

For Experiment 1, a 2 (Shock, No-Shock) x 2 (MDMA, Saline) ANOVA was used to analyze baseline freezing data. A 2 (Shock, No-Shock) x 2 (MDMA, Saline) x 4 (Test Day) repeated measures ANOVA was used to analyze freezing behavior. Significant interactions for freezing behaviors were examined using Tukey’s post-hoc comparisons. For Experiment 2, a 2 (Shock, No-Shock) x 2 (MDMA, Saline) ANOVA was used to analyze alterations in DH IL-1β, TNF-α, GFAP, and IBA-1 immunoreactivity as well as cell counts. Significant interactions for both immunoreactivity and cell counts were examined using Tukey’s post-hoc comparisons.

3. Results

3.1. Experiment 1: effect of MDMA administration on stress-enhanced fear learning

Fig. 1a depicts the design of Experiment 1. Three subcutaneous injections of MDMA prevented enhanced fear learning following a severe stressor (N = 59, n = 15–16). There was no effect of shock treatment or MDMA treatment on baseline contextual freezing (F(1,58) = 0.837, p = .479), indicating there were no differences in freezing at baseline. Additionally, the low freezing levels indicate that there was no generalization fear to the novel context B. A 2 x 2 x 4 repeated measures ANOVA revealed a significant main effect of MDMA treatment (F(1,57) = 22.291, p < .001) on contextual freezing. There was also a significant effect of test day (F(3,171) = 72.705, p < .001), indicating that conditioned freezing behavior diminished over time, thus suggesting extinction of contextual conditioning. Importantly, there was a significant shock treatment by MDMA treatment interaction (F(1,57) = 9.201, p = .004). On test days 1 and 2, Tukey’s post hoc comparisons revealed foot-shock, saline-treated animals exhibited significantly higher freezing behavior compared to animals that received no-foot shock and saline on test days 1 (p < .001) and 2 (p < .05), replicating enhanced fear learning. Foot-shock, MDMA-treated animals exhibited significantly less freezing than foot-shock, saline treated controls on test days 1 (p < .001) and 2 (p < .001). Furthermore, foot-shock, MDMA-treated animals exhibited significantly less freezing behavior compared to both no-foot shock groups (p < .05), indicating that MDMA may decrease freezing below control levels (Fig. 1b). These results indicate that MDMA administration following severe shock prevented future enhanced fear learning.

3.2. Experiment 2: analysis of IL-1β, TNF-α, GFAP, and IBA-1 immunoreactivity and cell counts following MDMA administration and severe stress

3.2.1. IL-1β immunoreactivity and cell counts

Fig. 2 depicts the design of Experiment 2. A 2 X 2 ANOVA revealed a significant main effect of shock (F(1,28) = 12.446, p = .002), and a significant shock treatment by MDMA treatment interaction (F(1,28) = 9.216, p = .006) with IL-1β immunoreactivity (N = 29, n = 7–8). Tukey’s post hoc comparisons revealed shock-saline animals had significantly increased IL-1β immunoreactivity compared to both no-shock groups (p < .05), indicating that IL-1β immunoreactivity increased following shock treatment, which replicates our previous findings (Jones et al., 2015; Jones et al., 2018a). Furthermore, MDMA-treated shock animals had significantly decreased IL-1β immunoreactivity compared to the shock-saline animals (p < .05) and had comparable IL-1β immunoreactivity levels (no significant differences) compared to both no-shock groups (p > .05), indicating that IL-1β immunoreactivity decreased following MDMA treatment (Fig. 3a). Four animals were excluded due to multiple images containing high background staining and failure of automatic thresholding. Exclusions were made blind to treatment group and prior to any statistical analysis.

A 2 x 2 ANOVA performed on IL-1β-positive cell counts revealed similar results. A significant main effect of shock (F(1,29) = 20.387, p < .001) and a significant main effect of MDMA treatment (F(1,29) = 39.298, p < .001) was found. Additionally, a significant shock treatment by MDMA treatment interaction (F(1,29) = 9.325, p = .005) was revealed. Tukey’s post hoc comparisons revealed shock-saline animals had significantly increased IL-1β-positive cells in the DH compared to both no-shock groups (p < .001), indicating that IL-1β cell counts increased...
following shock treatment, as well as replicating our previous findings (Jones et al., 2015; Jones et al., 2018a). Furthermore, MDMA-treated shock animals had significantly decreased IL-1β-positive cells compared to the shock-saline animals (p < .001) and had comparable IL-1β-positive cell counts (no significant differences) compared to both no-shock groups (p > .05), indicating that IL-1β immunoreactivity decreased following MDMA treatment (Fig. 3b). Three animals were excluded due to multiple images containing high background staining and failure of automatic thresholding. Exclusions were made blind to treatment group and prior to any statistical analysis.

3.2.2. TNF-α immunoreactivity and cell counts

A 2 x 2 ANOVA revealed no significant main effects of shock treatment ($F_{(1,31)} = .018$, $p = .894$) or MDMA treatment ($F_{(1,31)} = 1.562$, $p = .222$), indicating that MDMA or shock does not play a role in TNF-α immunoreactivity ($N = 30$, $n = 7–8$) (Fig. 4a).

A 2 x 2 ANOVA performed on TNF-α positive cell counts revealed similar results. No significant main effects of shock treatment ($F_{(1,31)} = 3.604$, $p = .068$) or MDMA treatment ($F_{(1,31)} = .372$, $p = .547$) were found, further indicating that MDMA or shock does not play a role in TNF-α-positive cells (Fig. 4b).
3.2.3. GFAP immunoreactivity and cell counts

A 2 x 2 ANOVA revealed no significant main effects of shock treatment ($F_{(1,29)} = .005, p = .943$) or MDMA treatment ($F_{(1,29)} = .573, p = .456$), indicating that MDMA or shock does not play a role in GFAP immunoreactivity ($N = 31, n = 7–8$). Tukey’s post-hoc comparisons revealed no differences between saline treated no-shock animals and saline treated shock animals ($p > .05$), replicating our previous findings (Jones et al., 2015; Jones et al., 2018a) (Fig. 5a). Three animals were excluded, for multiple images containing high background staining and failure of automatic thresholding. Exclusions were made blind to treatment group and prior to any statistical analysis.

A 2 x 2 ANOVA performed on GFAP positive cell counts revealed similar results. No significant main effects of shock treatment ($F_{(1,31)} = 3.052, p = .092$) or MDMA treatment ($F_{(1,31)} = .297, p = .590$) were

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Fig. 4. MDMA has no effect on TNF-α immunoreactivity and cell counts in the dentate gyrus of the dorsal hippocampus following severe stress. Quantification of positive fluorescence stain of TNF-α (Alexa-488) (A). Quantification of TNF-α positive cells (B) ($N = 30, n = 7–8$). Representative images (×20) for saline and MDMA animals taken within the DG of the DH. (C). * , statistically significant difference relative to respective control. Error bars indicate SEM.

Fig. 5. MDMA has no effect on GFAP immunoreactivity and cell counts in the dentate gyrus of the dorsal hippocampus following severe stress. Quantification of positive fluorescence stain of GFAP (Alexa 594) (A). Quantification of GFAP positive cells (B) ($N = 31, n = 7–8$). Representative images (×20) for saline and MDMA animals taken within the DG of the DH. (C). * , statistically significant difference relative to respective control. Error bars indicate SEM.
found, further indicating that MDMA or shock does not play a role on GFAP-positive cells (Fig. 5b).

3.2.4. IBA-1 immunoreactivity and cell counts
A 2 x 2 ANOVA revealed a significant main effect of shock ($F_{(1,30)} = 22.635, p < .001$) on IBA-1 immunoreactivity ($N = 30, n = 7–8$). Tukey’s post hoc comparisons showed that there was no effect of MDMA in the non-shocked animals. The comparisons revealed that animals that received shock and were saline treated had significantly lower IBA-1 immunoreactivity compared to both no-shock groups ($p < .001$), replicating our previous findings (Jones et al., 2015; Jones et al., 2018a). Furthermore, there were no differences between MDMA-treated shock animals compared to the both no-shock groups ($p > .05$), suggesting that MDMA attenuated the shock induced reduction in IBA-1 reactivity. However, a direct comparison of both shock groups did not show a significant difference between IBA-1 immunoreactivity levels with MDMA or saline treatment, although there was a trend ($p = .079$) suggesting an effect of MDMA on IBA-1 immunoreactivity (Fig. 6a). One animal was excluded due to multiple images containing high background staining and failure of automatic thresholding. Exclusions were made blind to treatment group and prior to any statistical analysis.

A 2 x 2 ANOVA performed on IBA-1 positive cell counts revealed a significant main effect of shock ($F_{(1,30)} = 13.142, p = .001$), a significant main effect of MDMA treatment ($F_{(1,30)} = 6.752, p = .015$), as well as a significant shock by MDMA treatment interaction ($F_{(1,30)} = 6.106, p = .020$). Tukey’s post hoc comparisons revealed shock-saline animals had significantly decreased IBA-1-positive cell counts compared to both no-shock groups ($p < .01$), indicating that IBA-1-positive cells decreased following shock treatment. Furthermore, MDMA-treated shock animals had significantly increased IBA-1-positive cells compared to the shock-saline animals ($p = .001$) and had comparable IBA-1-positive cells (no significant differences) compared to both no-shock groups ($p > .05$), indicating that IBA-1 increased following MDMA treatment. These data show that shock reduces the number of IBA-1-positive cells, and MDMA attenuates this reduction (Fig. 6b). One animal was excluded due to multiple images containing high background staining and failure of automatic thresholding. Exclusions were made blind to treatment group and prior to any statistical analysis. Overall, the combination of immunoreactivity and cell count data suggests that shock treatment reduced microglia immunoreactivity as well as the number of microglia cells, and MDMA attenuated this reduction.

These data indicate that blocking stress-induced increase of IL-1β and decrease of microglia may act as neuroimmune mechanisms by which MDMA prevents the development of enhanced fear learning (see Fig. 7).

4. Discussion
The current study demonstrates for the first time that MDMA administration following severe stress significantly blocks the development of stress-enhanced fear learning in the SEFL paradigm. Excitingly, MDMA administration following a severe stressor attenuated stress-induced increases in both IL-1β immunoreactivity and IL-1β-positive cell counts within the DG of the DH. Furthermore, MDMA administration also prevented the severe stress-induced decrease in IBA-1 positive cell counts within the DG of the DH. These findings provide new evidence of MDMA’s ability to prevent the development of stress-enhanced fear learning and support the hypothesis that MDMA’s immunomodulatory properties are a potential mechanism for its efficacy in treating PTSD.

Our novel findings reveal that MDMA prevents the development of stress-enhanced fear learning, as MDMA administration following a severe stressor significantly decreased freezing behavior, even below levels of control groups. Although control groups do not experience severe stress, all the animals are exposed to the milder stressor in Context B. This mild stressor does promote some level of freezing behavior, and therefore, we speculate that MDMA may be promoting a neuroprotective process that is sustained even following drug metabolism and excretion. The overall attenuation of stress-enhanced fear learning we observed suggests that MDMA may affect fear learning and memory processes. This is supported by the literature, as MDMA...
administration following fear conditioning enhanced the process of fear memory extinction in an animal model of cued fear conditioning (Young et al., 2015). Further, Hake and colleagues demonstrate that MDMA administration immediately following a brief fear memory retrieval session reduced later expression of fear in both contextual and cued fear conditioning models (Hake et al., 2019). While these studies implement cue- and context-conditioned Pavlovian models of fear learning and SEFL stimulates non-associative fear learning, the maladaptive fear learning observed in PTSD manifests in both associative and non-associative forms. Therefore, MDMA’s suppression of the fear response across several animal models of PTSD demonstrates that MDMA can counteract a variety of fear learning types and symptomatology involved in PTSD. For example, reexperiencing and avoidance symptoms are thought to stem from associative learning, while anxious and reactive symptoms are correlated with non-associative learning (Lissek and van Meurs, 2015). In turn, our observations further establish the efficacy of MDMA in attenuating fear behaviors by demonstrating its ability to reduce stress-enhanced fear learning, a form of non-associative learning that is a key component of clinical PTSD.

The present study demonstrates that MDMA can attenuate enhanced fear learning in the SEFL paradigm. Our lab has previously shown neuroinflammation to be functionally relevant in the development of enhanced fear learning (Jones et al., 2015), and therefore, we examined the immunoreactivity of several pro-inflammatory cytokines, including IL-1β, following severe stress and MDMA administration. Excitingly, we demonstrated that MDMA administration significantly reduced both stress-induced IL-1β immunoreactivity and IL-1β-positive cell counts in the DG of the DH. This is consistent with previous literature that demonstrates the ability of MDMA to suppress peripheral IL-1β in response to a lipopolysaccharide challenge (Connor et al., 2000). The present study addresses a shortcoming in the literature by extending these peripheral findings (Connor et al., 2000; Connor et al., 2001) to the brain, specifically, in the dorsal hippocampus. MDMA’s suppression of IL-1β signaling in the hippocampus represents a potential mechanism for MDMA’s attenuation of fear learning, as IL-1β signaling in the hippocampus is critical for learning and memory processes (Goshen et al.,

Fig. 7. Schematic of experimental results and candidate mechanisms. Results from experiment 1 indicate severe shock leads to the development of enhanced fear learning and MDMA blocks the development of enhanced fear learning (A, B). Results from experiment 2 indicate that severe shock increases IL-1β immunoreactivity and decreases microglial counts in the dorsal hippocampus (C, E). Administration of MDMA blocks the stress-induced increase of IL-1β and decrease of microglia in the dorsal hippocampus (D, F). Overall, we believe that blocking stress-induced increase of IL-1β and decrease of microglia may act as neuroimmune mechanisms by which MDMA prevents the development of enhanced fear learning (G, H). Created with BioRender.com.
Further, excessive IL-1β is shown to disrupt normal memory formation (Moore et al., 2009). As IL-1β expression is elevated following severe stress, increased IL-1β may be implicated in the development of maladaptive fear learning. Indeed, our previous findings show that IL-1β signaling is necessary for the development of enhanced fear learning, as blocking the action of IL-1β following the severe stressor with IL-1 receptor antagonist (IL-1Ra) prevented the development of SEFL (Jones et al., 2015; Jones et al., 2018a). In turn, we hypothesize that MDMA also attenuates fear learning through inhibiting IL-1β expression, thus blocking the maladaptive action of IL-1β on future fear learning.

Excessive IL-1β expression has been shown to impair neuronal plasticity and memory, indicating its role in the enhanced fear learning paradigm (Goshen et al., 2007; Goshen and Yirmiya 2009). In turn, MDMA may prevent enhanced fear learning by rescuing IL-1β’s suppression of neuronal plasticity. For example, MDMA has been shown to increase the expression of activity-regulated cytoskeleton-associated protein (Arc), which is a well-established marker of neuroplasticity (Abad et al., 2019). Our results support this possible mechanism as the focus of our analysis is the dentate gyrus of the dorsal hippocampus, which is a prominent site of neurogenesis and neuroplasticity even in the adult brain. It is possible that MDMA may be altering synaptic plasticity within the hippocampus of adult humans during MDMA-assisted psychotherapy. In line with this, a pre-clinical study indicated that MDMA re-opens a critical period of learning in adult mice that is similar to the period of neuroplasticity observed in adolescent mice (Nardou, Lewis et al. 2019). This has strong implications for the use of MDMA in treating psychiatric conditions as a strong bond between a psychotherapist and patient is well-known to be important for successful treatment (Ardisio and Rabellino 2011) and therefore MDMA may be altering synaptic plasticity to strengthen the psychotherapist-patient bond. Additionally, MDMA’s alteration of synaptic plasticity may also allow for re-encoding of previously formed maladaptive fear learning associations. These studies warrant MDMA’s importance in neuroplasticity and support further studies to elucidate its role in these processes. Future studies could examine neuroplastic changes following MDMA administration in the SEFL paradigm by assessing neuroplastic markers, such as brain-derived neurotrophic factor (BDNF), immediate early genes (IEG), and the postsynaptic density protein 95 (PSD95).

One mechanism we have previously identified to be integral to increases in IL-1β following severe stress is astrocytic production of IL-1β. In turn, MDMA’s effects on astrocytes could be a potential mechanism through which it inhibits enhanced IL-1β. Astrocytes produce a number of pro-inflammatory cytokines (Lau and Yu 2001), and this astrocyte-derived inflammation can contribute to neurological diseases (Choi et al., 2014). In the pathogenesis of PTSD, changes in astrocyte morphology and neuroinflammatory functioning are involved in the development of maladaptive fear memories (Izquierdo et al., 2016; Li et al. 2020; Li et al., 2022). In agreement with this literature, our previous studies have demonstrated that astrocytes are mechanistically implicated in maladaptive fear responses, as the majority of IL-1β immunoreactivity, a cytokine critical to the development of SEFL, is colocalized with GFAP following severe stress (Jones et al., 2015; Jones et al., 2018a). Collectively, these findings indicate that increased IL-1β following severe stress is primarily produced by astrocytes, and is functionally relevant to SEFL. However, we unexpectedly did not observe significant differences in GFAP immunoreactivity between shock and no-shock animals (Jones et al., 2018a). Nonetheless, increased IL-1β expression is seen in astrocytes independently of a GFAP increase, as GFAP is only expressed within a small region of an astrocyte’s total area, and is also not expressed in all astrocytes (Khakh and Sofroniew 2015). In line with these previous findings, we also saw an increase in IL-1β following severe stress, but not an increase in GFAP in the present study.

Following MDMA administration, we also did not observe changes in GFAP immunofluorescence or number of GFAP-positive cells. However, this does not necessarily eliminate an astrocytic mechanism for MDMA’s immunosuppressive effects. For example, the literature indicates mixed findings when examining MDMA’s effect on GFAP. Although other studies have also found no change in GFAP following MDMA administration (Pubill et al., 2003), repeated administration of MDMA has also been shown to cause a decrease in the number of hippocampal astrocytes (Jahanshahi et al., 2013). Conversely, increases in GFAP-positive cell number and GFAP immunoreactivity have also been observed in brain areas such as the striatum and hippocampus following MDMA treatment (Aguirre et al., 1999). These conflicting results may suggest that measures of GFAP immunoreactivity may not adequately or reliably capture MDMA’s effects on astrocytes. MDMA may affect other astrocyte-specific markers of astrocyte reactivity that are influenced by stress, such as S100 beta (Wallensten et al., 2022). Our laboratory’s previous findings have demonstrated that stress-induced IL-1β was primarily colocalized with astrocytes in the hippocampus (; Parekh et al., 2020), and therefore it is possible that MDMA does not alter astrocyte reactivity or astrocyte cytokine production directly, but instead suppresses IL-1β by altering astrocytes’ indirect effects on immune molecules. For example, MDMA administration could stimulate serotonin 1A receptors on astrocytes, which is shown to promote upregulation of antioxidant molecules (Miyazaki and Amanuma 2016). Increased oxidative stress, which is associated with PTSD (Wilson et al., 2013), suppresses the synthesis and secretion of IL-1Ra following depletion of the antioxidative response (Carta et al., 2017). A MDMA-induced anti-oxidant increase could rescue IL-1Ra production, in turn, suppressing IL-1β production following severe stress. Furthermore, MDMA also increases synaptic availability of neurotransmitters by increasing serotonin, norepinephrine, and dopamine release (Green et al., 2003), and in turn may suppress enhanced astrocyte-derived IL-1β (Tomaz et al. 2020).

Although we have previously shown enhanced IL-1β production to primarily originate in astrocytes, the role of microglia is less clear and not well understood in the SEFL paradigm. It is possible that MDMA suppresses increased IL-1β production through its effects on microglia. In the current study, MDMA prevented a stress-induced decrease in IBA-1 positive cell counts, suggesting that MDMA may influence the number of microglia in the DG of the DH following severe stress exposure. This stress-induced effect on microglia is consistent with our previous findings, as our lab has shown that foot shock stress significantly attenuates hippocampal IBA-1 immunoreactivity (Jones et al., 2018a). The attenuation of IBA-1 positive cell counts following stress is also consistent with literature suggesting microglia activation, specifically in the hippocampus, three days following MDMA treatment (Pubill et al., 2003). Although activated microglia can produce pro-inflammatory cytokines, a large body of evidence has demonstrated that microglia also produce cytokines with anti-inflammatory activity, such as TGF-β, IL-10, and IL-1Ra (Kim and Joh 2006). Therefore, it is possible that MDMA could suppress IL-1β by preventing a decrease in microglial anti-inflammatory activities or decrease astrocyte-derived IL-1β through its effects on microglia.

Although MDMA’s immunomodulatory effects could be mediated by glial cells such as microglia and astrocytes, there are also other potential mechanisms for MDMA’s immunomodulatory effects. Cytokines can interact with many neurochemicals, such as neurotransmitters. MDMA enhances serotonin release and prevents serotonin reuptake (Green et al. 2003). Since many immune cells express serotonin receptors, serotonergic interactions may mediate MDMA’s suppression of IL-1β. There is also evidence that suggests enhanced dopamine could also elicit MDMA’s immunosuppressive effects, as d-amphetamine is demonstrated to have immunosuppressive effects in rodents (Freire-Garabai et al., 1992). Further, the release of serotonin and dopamine can also have indirect effects on the immune system that could account for MDMA’s effect on IL-1β. While these studies do implicate a relationship between MDMA’s effects on neurotransmitters and cytokines, the molecular mechanisms involved are not fully understood. Further studies should investigate whether IL-1β and TNF-α as well as dopamine and
serotonin play a synergistic role following MDMA treatment. The current study focused on characterizing the effects of MDMA on IL-1β and TNFα, as these cytokines are critical to the development of enhanced fear learning (Connor et al., 2000; Parekh et al., 2020) and MDMA has been shown to suppress these cytokines (Connor et al., 2001). However, IL-1β and TNFα are not the only cytokines that MDMA can affect. For instance, it has been shown that MDMA suppresses IFN-γ and increases the anti-inflammatory cytokine IL-10 in the periphery (Boyle and Connor 2007). Future studies should assess the expression of multiple pro-inflammatory and anti-inflammatory cytokines following MDMA administration after severe stress, as it is possible that other cytokines may be involved in MDMA’s suppression of SEFL. Additionally, the findings of the current study are limited to the DG of the DH. While we focused our investigations on the DG due to our previous work that has shown cytokines in this region are critical to enhanced fear learning (Jones et al., 2015; Parekh et al., 2020), it is possible that MDMA may induce changes in the neuroimmune system in other brain regions. For instance, MDMA administration normalized morphological changes of neurons in both the DG and the basolateral amygdala following predator scent stress exposure (Arluk et al., 2022). Additionally, a variety of changes in gene expression in the amygdala have been shown following SEFL (Ponomarev et al, 2010) and amygdalar activity has been shown to modulate learning and memory processes in the hippocampus (Tsoory et al., 2008). Therefore, future studies should address the effect of MDMA on the amygdala following fear learning behavior.

This study is limited in its use of male, adult rats, as well as in the stressful exposure’s timeline. Our exclusion of female subjects overlooks the distinct sex-differences observed in clinical populations with PTSD. For example, regardless of trauma type or prevalence, women experience more chronic PTSD and different symptoms and comorbidities than men, and the literature suggests that neurobiological sex-differences underlie this occurrence (Pooley et al., 2018). In turn, these biological sex-differences could differentially influence MDMA’s treatment efficacy and outcomes in female subjects. To better elucidate the sex-differences following MDMA administration, further studies should include female subjects. Additionally, our use of adult rats has limited generalization of our findings to other populations, as there are specific considerations to evaluate when MDMA is implemented as a treatment in more vulnerable groups such as adolescents. In human populations, MDMA users under 18 years of age are likely to be especially vulnerable to the drug’s potential neurotoxic effects (Buchert et al., 2001). In turn, further examination of MDMA in adolescent rats to determine its effects and treatment efficacy for clinical adolescent PTSD is needed. Additionally, the timing of the stressful exposure could potentially influence the effects of MDMA administration. For example, the timing of a stressful exposure after the MDMA injection or at an earlier time could influence MDMA’s efficacy in suppressing SEFL. Future studies should assess MDMA’s effectiveness in preventing fear learning in the SEFL paradigm if the stressful event occurs at different timepoints.

Our exciting finding that MDMA significantly attenuates stress-enhanced fear learning provides essential preclinical data to support the recent successful clinical trials of MDMA-assisted psychotherapy for patients with PTSD (O’’alora et al., 2018). These data suggest that MDMA may attenuate PTSD symptoms by decreasing enhanced fear learning and hyperreactivity to stressors experienced by patients, a hallmark symptom of PTSD that the SEFL paradigm is designed to capture. Furthermore, our data demonstrating MDMA’s reversal of a severe stress-induced increase in IL-1β supports the hypothesis that MDMA may decrease stress-enhanced fear learning, at least in part, through immunosuppressive actions. These results further emphasize the importance of using pharmacotherapies that target proinflammatory cytokines and the neuroimmune system in treating PTSD, and support the use of MDMA as a potential therapeutic agent for those experiencing this disorder.

Studies in animals statement

All animal experiments comply with the ARRIVE guidelines and were carried out in accordance with and approval from the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. This manuscript used male animals.

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Declaration of competing interest

The authors declare no conflict of interests.

Data availability

Data will be made available on request.

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