Impaired Ethanol-Induced Sensitization and Decreased Cannabinoid Receptor-1 in a Model of Posttraumatic Stress Disorder

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Abstract

Background and Purpose
Impaired striatal neuroplasticity may underlie increased alcoholism documented in those with posttraumatic stress disorder (PTSD). Cannabinoid receptor-1 (CB1) is sensitive to the effects of ethanol (EtOH) and traumatic stress, and is a critical regulator of striatal plasticity. To investigate CB1 involvement in the PTSD-alcohol interaction, this study measured the effects of traumatic stress using a model of PTSD, mouse single-prolonged stress (mSPS), on EtOH-induced locomotor sensitization and striatal CB1 levels.

Methods
Mice were exposed to mSPS, which includes: 2-h restraint, 10-min group forced swim, 15-min exposure to rat bedding odor, and diethyl ether exposure until unconsciousness or control conditions. Seven days following mSPS exposure, the locomotor sensitizing effects of EtOH were assessed. CB1, post-synaptic density-95 (PSD95), and dopamine-2 receptor (D2) protein levels were then quantified in the dorsal striatum using standard immunoblotting techniques.

Results
Mice exposed to mSPS-EtOH demonstrated impaired EtOH-induced locomotor sensitization compared to Control-EtOH mice, which was accompanied by reduced striatal CB1 levels. EtOH increased striatal PSD95 in control and mSPS-exposed mice. Additionally, mSPS-Saline exposure increased striatal PSD95 and decreased D2 protein expression, with mSPS-EtOH exposure alleviating these changes.

Conclusions
These data indicate that the mSPS model of PTSD blunts the behavioral sensitizing effects of EtOH, a response that suggests impaired striatal neuroplasticity. Additionally, this study
demonstrates that mice exposed to mSPS and repeated EtOH exposure decreases CB1 in
the striatum, providing a mechanism of interest for understanding the effects of EtOH follow-
ing severe, multimodal stress exposure.

Introduction

A high comorbidity exists between posttraumatic stress disorder (PTSD) and alcoholism [1].
Specifically, among military personnel, over 60% of those that develop an alcohol use disorder
during or after deployment are also diagnosed with PTSD [2]. Like other addictions, the severity
of alcoholism is exacerated by stress [3–6], however the mechanisms of this underlying
interaction are not fully understood. Those with PTSD and alcoholics display abnormal striatal
activity [7–12], suggesting dysregulated striatal neuroplasticity may underlie the effects of
PTSD on alcohol-associated addictive behaviors.

The endocannabinoid system, including cannabinoid receptor-1 (CB1) and its endogenous
endocannabinoid ligands, anandamide and 2-arachidonoylglycerol (2-AG), is an important
modulator of dorsal striatal neuroplasticity and has known involvement in fear processing and
PTSD diagnoses. Endocannabinoids regulate long-term depression (LTD) of pre-synaptic neu-
rons through retrograde activation of CB1 receptors [13], which are found on a variety of cell
types throughout the brain including both gamma-aminobutyric acid (GABA) interneurons
and glutamatergic terminals in the dorsal striatum [14]. The endocannabinoid system is partic-
ularly important in regulating fear extinction [15, 16], modulating the hypothalamic-pituitary-
adrenal axis [17–20], and influencing amygdala habituation to fear as well as stress-reactivity
personality traits [21]. Cannabis use amongst those with PTSD is elevated, which is at least
partly motivated by symptom coping [22–24]. Neumeister and colleagues identified elevated
CB1 availability throughout the brain, including the caudate, and decreased peripheral ananda-
mide levels in PTSD patients suggesting altered endocannabinoid tone [25]. Additionally, a
small clinical trial documented that a CB1 agonist improved nightmare-related symptoms and
reported well-being of those with PTSD [26]. Those with PTSD exhibit dysregulation of the
endocannabinoid system [25, 27], similar to that observed in rodent stress models, including
the rat single-prolonged stress (SPS) model of PTSD (for review see [17]). The SPS rat, and the
modified mouse SPS (mSPS) models of PTSD both cause neuroendocrine changes that lead to
enhanced negative feedback of the hypothalamic-pituitary-adrenal axis, and impaired fear
extinction [28, 29]. These behavioral and endocrine changes that reflect symptoms of PTSD,
were prevented by injection of a CB1 receptor agonist peripherally or directly into the amyg-
dala [30] or hippocampus [31] within 24 h of SPS exposure. Similarly, genetic or drug-induced
enhancement of endocannabinoids reduced behavioral symptoms, endocrine responses, and
dendritic hypertrophy in a variety of PTSD-like models [17, 30, 31].

CB1 receptors are also involved in regulating EtOH response, are impacted by EtOH, and
those within the dorsal striatum appear to especially affect EtOH preference in pre-clinical
studies. In an electrophysiology study, CB1 receptor agonists attenuated the effects of EtOH on
GABAergic activation of the central amygdala [32]. In addition, activation of CB1 receptors is
required for the EtOH-induced depression of glutamatergic neurons in both the dorsal stria-
tum and the hippocampus [13, 33–35]. Clinical studies indicate a decrease in CB1 and dysregu-
lation of endocannabinoids in the ventral striatum of alcoholics [36]. Similarly, preclinical
studies indicate decreases in CB1 receptor levels, G-protein binding to CB1 receptors, and
increases in 2-AG within the dorsal striatum after EtOH vapor exposure in mice [37, 38], as
well as a decrease in CB1 signaling after chronic intermittent voluntary EtOH drinking in rats.
Interestingly, while CB1 receptor antagonists have been shown to increase EtOH preference if given during a 30-day EtOH exposure, they decrease EtOH preference if given after 30 days of EtOH exposure [40].

In conjunction with CB1 receptor dysregulation, chronic intermittent EtOH vapor exposure leads to dendritic hypertrophy within the mouse striatum, as well as impaired striatal learning in mice [37]. In mouse studies, psychomotor sensitization to EtOH [41, 42] may be representative of neuroplastic change [43], is related to stress-associated mechanisms [43], and is considered to be striatal-dependent [44]. In this procedure, mice are exposed to repeated administration of a locomotor activity (LMA)-inducing dose of EtOH, and over time, display an increase in behavioral sensitivity to the drug [41, 42]. CB1 receptor involvement in EtOH locomotor sensitization is not fully understood, however Coelho and colleagues have shown that high-responders to EtOH-sensitization show increased CB1 receptors [45] and numerous reports suggest endocannabinoids, their receptors, and catabolic enzyme, fatty acid amide hydrolase (FAAH), are critical in the response to EtOH and EtOH preference [40, 45–49]. This CB1-EtOH interaction is likely due to CB1 receptor regulation of LTD on both GABAergic and glutamatergic targets within the striatum (for review [50]).

Additionally, there is an established modulatory relationship between striatal dopamine-2 (D2) and CB1 receptors. The in vivo knockdown of either receptor in the striatum, leads to decreased protein expression, mRNA expression, and receptor-mediated G-protein activation of the opposing receptor [51]. This modulatory relationship also appears to impact acute striatal signaling [52, 53]. In D2 receptor expressing medium spiny neurons (MSNs), activation of D2 receptors increases the release of endogenous anandimide, while CB1 receptor antagonists increases spontaneous excitatory postsynaptic currents (sEPSC) and partially prevents D2-agonist induced decreases in EPSCs in these MSNs [52, 53]. Additionally, CB1 receptors are required for pre-synaptic striatal D2 autoreceptor inhibition of glutamatergic neurons [54]. D2 receptors are also involved in the response to EtOH, however their involvement appears to be complex and is not fully understood. Deletion of D2 receptors decreases EtOH preference [55] and the antagonism of striatal D2 receptors in vivo decreases habit-based EtOH-seeking in mice [56]. In contrast, overexpression of D2-receptors in the ventral striatum reduces EtOH preference in rats [57], D2 receptor agonists decrease EtOH preference of mice [58], and striatal D2-receptor binding positively correlates with behavioral sensitization to EtOH in mice [44]. In clinical studies, alcoholics show decreases in D2 protein in the striatum and nucleus accumbens of rats exposed to SPS [61]. Genes related to D2 receptors have also been associated with increased risk of PTSD, but the results are varied (for review see [62]). Interestingly, this D2-related genetic risk factor appears to be specifically relevant to cases that also include heavy alcohol consumption, indicating a possible mechanism of the PTSD-alcoholism association [63].

Post-synaptic density 95 (PSD95) has also been implicated in the effects of EtOH and stress exposure, and has recently been associated with the endocannabinoid system. As a post-synaptic scaffolding protein, PSD95 is important in regulating neuroplastic changes through its ability to accumulate N-methyl D-aspartate (NMDA) receptors on post-synaptic neurons [64, 65]. It is also important in balancing homeostatic disruptions in neuronal excitation and inhibition, specifically observed in drug responses [64, 65]. An in vitro study of PSD95 type 3 in MSNs, the most common type of PSD95 within the striatum [66], indicates that PSD95 type 3 is important in regulating endocannabinoid striatal plasticity through its modulation of glutamatergic receptor-5 (mGlu5) [67]. When striatal MSN mGlu5 is activated, there is a post-synaptic release of endocannabinoids, activating presynaptic CB1 receptors and inducing LTD of presynaptic glutamatergic cortical-striatal neurons [50]. Studies show that PSD95-related genes
are upregulated in the lower midbrain of mice in response to \textit{in vivo} EtOH vapor exposure \cite{68}, is important in fear conditioning \cite{69} and is increased in several stress models \cite{70, 71}. However, the effects of EtOH and/or severe multimodal stress on regulating striatal PSD95 and endocannabinoid interactions remain unidentified.

In order to define more clearly the striatal mechanisms involved in the PTSD-alcohol interaction, the present study measured the effects of severe, multimodal stress exposure using the mSPS model of PTSD on EtOH-induced locomotor sensitization, as well as on striatal CB1, D2, and PSD95 protein levels. It was hypothesized in the present study that mSPS would increase EtOH-induced locomotor sensitization in association with a decrease in CB1 and D2 and an increase in PSD95 protein expression in the dorsal striatum.

\textbf{Materials and Methods}

\textbf{Mice}

Male C57BL/6 mice (\textit{mus musculus}) were housed in groups of 2–5 mice with standard mouse chow and water available \textit{ad libitum} when not being tested. All procedures were approved by the Wayne State University Institutional Animal Care and Use Committee. Mice were tested in 2 cohorts, where the first (BEC-Test) were treated in the test room and trunk blood was utilized for blood ethanol content (BEC) \(n=36\), divided equally into 4 groups, naïve mice treated with saline (Control-Sal), naïve mice treated with EtOH (Control-EtOH), mSPS-exposed mice treated with saline (mSPS-Sal), and mSPS-exposed mice treated with EtOH (mSPS-EtOH). The second cohort was treated in their home cages and their brains were utilized for immunoassays (Immuno-Home) \(n=42\), with groups including Control-Sal \(n=10\), Control-EtOH \(n=11\), mSPS-Sal \(n=10\), and mSPS-EtOH \(n=11\).

\textbf{Mouse Single Prolonged Stress (mSPS)}

mSPS procedures were adapted from Eagle and colleagues \cite{28} and outlined with all other behavioral procedures in Fig 1. Briefly, mSPS mice were habituated to the testing room for 1 h, and then were individually restrained by loose immobilization in ventilated 50 mL tubes for 2 h. Next, the mSPS mice were exposed to a forced group swim for 10 min, where 4–5 randomly selected mice swam in cylinders (18 cm diameter) filled with water (25°C) to 14 cm. After the forced group swim, the mice were quickly towel dried and placed into home cages. Next, during a 15 min rest period, the mice were exposed to odors from soiled rat bedding as a predator scent. Lastly, the mSPS mice were exposed to diethyl ether on soaked cotton balls (no direct contact) in an enclosed chamber until loss of consciousness (approximately 5 min), and placed on warming pads until active and alert. Control mice were brought to a separate testing room.
for 2 h where they were weighed and handled. All mice were then returned to the vivarium and left undisturbed for a total of 7 days after mSPS or control exposure, aside from 1 cage-change on day 5 post-exposure.

Ethanol Sensitization
In previous sensitization studies in our laboratory, mice received injections in the test room on habituation, acute-EtOH and challenge days, while injections in between the acute and challenge days were conducted in their home cages. In order to confirm that sensitization was not confounded by a change in context, we tested the mice in 2 cohorts. First, in the BEC-Test cohort the mice were always injected in the testing room and received daily exposure to the LMA chambers on days 2–10. After the completion of the BEC-Test cohort’s assessments, the Immuno-Home cohort began testing. The Immuno-Home cohort was injected in the test room on days when LMA was assessed (habituation, acute-EtOH, and challenge), but was injected in the home cages on days 2–10 as done in previous work. On day 8 post mSPS or control condition exposure, all mice were habituated to the OPTO-M3 LMA chambers (Coulborn Instruments, Whitehall, PA). The LMA chambers have 16 light beam sensors on the x-axis, with 2.46 cm between each sensor, which scan at a rate of 160 Hz in the arena measuring 21.5 x 44 cm. The LMA chambers were kept in isolation cubes with a house fan for ventilation (62 db) and overhead lighting (approximately 200 lumens). Mice were injected intraperitoneally (i.p. 0.01 mL/g body weight) with sterile saline (0.9% NaCl) and immediately placed into the LMA chambers where total activity (total beam breaks) was measured. The first 50 minutes served as habituation to the apparatus and the final 10 minutes served as their baseline activity. Mice were then returned to their home cages. The following day the mice began Day 1 of EtOH sensitization. On Day 1, the mice were injected with 2.0 g/kg EtOH (20% v/v, i.p.) dissolved in saline or saline alone as a control. LMA was immediately assessed for 10 min. On Days 2–10 of EtOH sensitization, the same procedure was repeated, except mice receiving EtOH were administered a 2.5 g/kg dose. Finally, on Day 11 of EtOH sensitization, EtOH groups were injected with a challenge dose of 2.0 g/kg of EtOH and all animals were tested for LMA for 10 min.

Sample Extraction & Preparation
Immediately following the final LMA test session, the mice were euthanized by cervical dislocation, and brains were removed and stored at -80°C until microdissection to obtain striatal tissue samples. Trunk blood samples from the BEC-Test cohort were also collected and centrifuged at 5,000 rpm for 10 min to obtain plasma, which was stored at -80°C until use. Brains from the Immuno-Home cohort were sliced into 2 mm sections using a rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA), and unilateral 1.5 mm tissue punches (Miltex, Inc., York, PA) from the left anterior-dorsal striatum at 1.5 to -0.5 mm anterior to bregma were taken and stored at -80°C until immunoassay. A unilateral punch was then homogenized for approximately 20 seconds in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2mM EDTA, 1% triton X-100, 10% glycerol with Phosphatase Inhibitor Cocktail 2 P5726, Phosphatase Inhibitor Cocktail 3 P0044 and Protease Inhibitor P8340 from Sigma Aldrich, St. Louis, MO), then centrifuged at 4°C for 10 min at 10,000 x g, and the protein was quantified using a Pierce 660 nm protein assay using a bovine serum albumin standard (Thermo Scientific, Rockford, IL).

Blood Ethanol Content
Samples from the BEC-Test cohort were diluted 1:1 with saline. Solutions containing 0.5% EtOH standard, saline control, or sample in Alcohol Reagent Set according to manufacturer
instructions (Pointe Scientific Inc., Canton, MI) were incubated at 37°C for 5 min, and then measured at 340 nm using a BioMate 3S UV-Visible Photospectrometer (Thermo Scientific, Rockford, IL). One mSPS-EtOH sample was not included due to insufficient plasma collection.

Immunoblotting

Immunoblotting was conducted as described previously [72]. Protein (20 μg) was separated on a NuPAGE 4–12% Bis-Tris Gel using MOPS running buffer, and then transferred to a nitrocellulose membrane using NuPAGE transfer buffer on a semi-dry transfer apparatus at 15 V for 40 min (all from Life Technologies, Carlsbad, CA). One membrane was prepared for detection of CB1, PSD95 and actin, with a second membrane prepared for detection of D2 and actin proteins. After 3x10 min rinses in Tris-buffered saline containing 0.1% Tween 20 (TBST), nonspecific binding was blocked for 1 h using 5% non-fat dry milk in TBST. Antibodies against CB1 (1:1000; 10006590; Cayman Chemical, Saxtons River, VA), D2 (1:2000; Alomone labs, Jerusalem, Israel), PSD95 (1:5000; MA1-045; Thermo Scientific, Waltham, MA), and actin (1:5000; A5060, Sigma-Aldrich, St. Louis, MO) were applied individually overnight in a solution of TBST and 5% dry milk. CB1, D2, and actin were detected using 1:1000, 1:2000, or 1:2000 anti-rabbit IgG HRP-linked antibody, respectively (7074, Cell Signaling Technology, Beverly, MA). PSD95 was detected using 1:5000 anti-mouse IgG HRP-linked antibody (NA931V; GE Healthcare UK Limited, Little Chalfont Buckinghamshire HP7 9NA, UK). All secondary antibodies were developed with Super Signal West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA). Membranes were stripped of proteins between exposures to antibodies using Restore TM PLUS Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA) for 2 min followed by 3 x 1 sec rinses followed by 3 x 10 min rinses in TBST according to manufacturer instructions. Densiometric analysis using Image J software (NIH) was used to quantify CB1, D2, and PSD95 immunoblot signals which were normalized to actin.

Statistical Analyses

All analyses were conducted using IBM Statistical Package for the Social Sciences (SPSS) version 22 (IBM Corporation, Armonk, NY). Three-way mixed-design analyses of variance (ANOVA) were used to determine if within-subjects factor LMA sensitization test day (day 1, challenge) and between subjects factors EtOH (Saline, EtOH) and mSPS (Control, mSPS) had effects on the dependent variables, raw LMA and percent baseline LMA. An independent-t test was used to determine if mSPS had an effect on BECs. Two-way ANOVAs were used to assess the effects of EtOH and mSPS on striatal CB1, PSD95, and D2. Pearson’s partial correlation coefficient analyses were utilized to determine relationships between percent baseline LMA on challenge and striatal CB1. Alpha was set at 0.05 for all analyses.

Results

EtOH-induced LMA Sensitization and Blood Ethanol Content

Because the cohort’s day interaction was not significant in the BEC-Test and Immuno-Home control groups, the cohorts were analyzed as a single study, F(1,70) = 0.350, p = 0.556. Two mSPS-exposed mice died, 1 during forced-swim and 1 after ether exposure. Additionally, Control-Sal (n = 1), Control-EtOH (n = 2), and mSPS-EtOH (n = 2) were removed due to a technical malfunction during data collection in LMA. Final groups included: Control-Sal (n = 18), Control-EtOH (n = 18), mSPS-Sal (n = 18), and mSPS-EtOH (n = 18), for a total (n = 72).

A two-way ANOVA revealed an overall effect of mSPS during habituation, which indicated that mSPS-exposed mice were significantly more active compared to control mice, F(1,68) =
6.286, \( p = 0.015 \) (Fig 2). Because the mSPS and control groups were significantly different prior to EtOH exposure, and to provide a more representative visualization, the last 10 minutes of the LMA habituation were used to calculate percent baseline LMA when assessing the EtOH sensitization paradigm in addition to assessments of raw data.

When assessing raw LMA, a three-way ANOVA also revealed a significant mSPS × EtOH × Day interaction, \( F(1, 68) = 5.943, p = 0.017 \), with increases in Control-EtOH mice on challenge compared to Control-EtOH mice on Day 1, \( p < 0.001 \), Control-Sal mice on challenge, \( p < 0.001 \), and mSPS-EtOH on challenge, \( p = 0.034 \), as well as a significant increase in mSPS-EtOH mice compared to Control-EtOH mice on Day 1, \( p = 0.034 \) (Fig 3). A three-way ANOVA also revealed a significant mSPS × EtOH × Day interaction in percent baseline LMA assessed in the

Fig 2. Activity was significantly increased during habituation in mSPS-exposed mice compared to control mice prior to EtOH exposure (\(^* \) \( p < 0.05 \)).

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Fig 3. Mice exposed to mSPS followed by chronic EtOH (mSPS-EtOH) demonstrate impaired EtOH-LMA sensitization. \(^* \) \( p < 0.05 \) Control-EtOH mice on challenge compared to Control-Sal on Day 1, as well as Control-Sal and mSPS-EtOH on Challenge. \(^\dagger \) \( p < 0.05 \) mSPS-EtOH mice compared to Control-EtOH mice on Day 1.

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sensitization paradigm, $F(1, 68) = 12.664, p = 0.001$ (Fig 3). Percent baseline LMA activity was significantly increased in Control-EtOH mice on challenge compared to Control-EtOH mice on Day 1, $p<0.001$, Control-Sal mice on challenge, $p = 0.011$, and mSPS-EtOH on challenge, $p = 0.002$. An independent t-test indicated that BEC did not differ significantly between mSPS-EtOH and Control-EtOH mice in the BEC-Test cohort, $t(16), p = 0.749$ (Fig 4).

### Immunoblot Assays

A total of 20 samples from the Immuno-Home cohort were utilized for CB1 assays (2 samples (1 Control-Sal and 1 mSPS-EtOH) were removed from the original 22 samples due to insufficient protein collection). Final groups included, Control-Sal ($n = 4$), Control-EtOH ($n = 7$), mSPS-Sal ($n = 4$), and mSPS-EtOH ($n = 5$). Analysis of CB1 protein levels with a two-way ANOVA revealed a significant mSPS/C3EtOH interaction, $F(3,16) = 6.169, p = 0.024$ (Fig 5A).

![Graph showing CB1 receptor protein levels](image)

**Fig 5.** CB1 receptor protein was significantly decreased in mice exposed to mSPS followed by chronic EtOH in the anterior striatum compared to all other treatments. (A) $*p<0.05$; au, arbitrary units. Representative samples inserted in upper right corner (kDa, kilodaltons). (B) LMA movements at challenge compared to LMA during last 10 min of habituation (% Baseline) positively correlated with striatal CB1 receptor protein ($p<0.05$), suggesting that LMA decreased as CB1 receptors decreased.

![Graph showing LMA movements and CB1 correlation](image)

**Fig 4.** mSPS did not significantly alter BEC compared to control ($p>0.05$).

![Bar graph comparing BEC in Control-EtOH and mSPS-EtOH](image)

**Fig 5.** CB1 receptor protein was significantly decreased in mice exposed to mSPS followed by chronic EtOH in the anterior striatum compared to all other treatments. (A) $*p<0.05$; au, arbitrary units. Representative samples inserted in upper right corner (kDa, kilodaltons). (B) LMA movements at challenge compared to LMA during last 10 min of habituation (% Baseline) positively correlated with striatal CB1 receptor protein ($p<0.05$), suggesting that LMA decreased as CB1 receptors decreased.

![Graph showing LMA movements and CB1 correlation](image)
Pair-wise comparisons indicated that mSPS-EtOH mice showed significantly decreased CB1 compared to Control-EtOH mice, \( p < 0.001 \), and to mSPS-Sal mice, \( p = 0.002 \). CB1 positively correlated with percent baseline LMA, such that percent baseline LMA decreases as CB1 receptor levels decrease, \( r(18) = 0.581, p = 0.009 \) (Fig 5B).

On a separate blot, a total of 19 samples were utilized for analysis of D2 receptors (1 mSPS-EtOH sample had been removed from the original 20 samples due to insufficient protein collection). Final groups included, Control-Sal (\( n = 5 \)), Control-EtOH (\( n = 5 \)), mSPS-Sal (\( n = 5 \)), and mSPS-EtOH (\( n = 4 \)). Analysis of D2 receptors with a two-way ANOVA revealed a significant mSPS-EtOH interaction, \( F(3, 15) = 9.381, p = 0.008 \) (Fig 6A). Pair-wise comparisons indicated that D2 receptors were significantly decreased in mSPS-Sal mice compared to Control-Sal, \( p = 0.032 \), and mSPS-EtOH, \( p = 0.028 \), mice. Control-EtOH mice did not significantly differ from Control-Sal, \( p = 0.080 \), or mSPS-EtOH, \( p = 0.066 \), mice.

A total of 22 samples were utilized for PSD95 assays on the same blot as the CB1 assays. Final groups included, Control-Sal (\( n = 5 \)), Control-EtOH (\( n = 7 \)), mSPS-Sal (\( n = 4 \)), and mSPS-EtOH (\( n = 6 \)). Analysis of PSD95 using a two-way ANOVA indicated a significant

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Fig 6. Interactions between mSPS and EtOH on D2-receptor and PSD95 protein levels. (A) Striatal D2 receptors were significantly decreased in mSPS-Sal mice compared to both Control-Saline and mSPS-EtOH mice (*\( p < 0.05 \)). Representative samples inserted in upper right corner (kDa, kilodaltons). (B) PSD95 was significantly increased in Control-EtOH, mSPS-Sal, and mSPS-EtOH mice compared to Control-Sal mice (*\( p < 0.05 \)). PSD95 was also significantly decreased in mSPS-EtOH mice compared to mSPS-Sal mice (*\( p < 0.05 \)).

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mSPS’ EtOH interaction, $F(3,17) = 15.390, p = 0.001$ (Fig 6B). Pair-wise comparisons indicated that PSD95 was significantly increased in Control-EtOH mice compared to Control-Sal mice, $p = 0.045$. Additionally, PSD95 was significantly increased in mSPS-Sal mice compared to Control-Sal, $p < 0.001$, and mSPS-EtOH, $p = 0.004$, mice.

**Discussion**

Overall, the present study demonstrates evidence of impaired striatal neuroplasticity in mSPS mice, as reflected by a disruption in EtOH-induced locomotor sensitization. The impaired behavioral response observed in mice exposed to both mSPS and EtOH was associated with a decrease in striatal CB1 receptors. Repeated EtOH exposure increased striatal PSD95 in accordance with previous reports [68, 73, 74]. Additionally, increases in striatal PSD95 and decreases in striatal D2 receptor proteins induced by mSPS were reversed in mice exposed to mSPS and EtOH.

Although the inability of mSPS mice to sensitize to EtOH suggests impaired striatal neuroplasticity, most studies of psychostimulant sensitization imply heightened sensitization is predictive of addictive behaviors [75–77]. However, several studies show that EtOH sensitization can also be reduced in rodents with increased EtOH preference or voluntary intake [78–81]. Specifically, C57BL/6 mice, the mice used in this study, are typically more resistant to EtOH sensitization compared to DBA/2J mice [80], despite having increased EtOH preference (for review [82]). The reduced sensitization of mSPS mice may reflect an already maximized stress response. EtOH sensitization is modified by corticosterone and corticotrophin-releasing factor-1 receptors- both of which are important mechanisms of the stress response [83, 84]. SPS-exposed rats display elevated glucocorticoid receptor mRNA expression in several regions of the brain and elevated corticotrophin releasing factor in the hypothalamus, and thus far our results indicate that glucocorticoid receptor mRNA is upregulated in the hippocampus of mSPS-exposed mice [28, 85–87]. While mild stressors or injections of corticosterone enhance ethanol sensitivity [84], perhaps the severity of the mSPS combined with the stress of EtOH exposure has induced a ceiling effect, where EtOH-induced behavioral sensitization can no longer be detected. Correspondingly, what appears to be partial attenuation of increases in PSD95 in mSPS-EtOH exposed mice, may indicate that PSD95 can no longer compensate for the reduced glutamatergic tone, similar to that observed in chronic stress models of depression [88, 89].

This study indicates an interaction between mSPS and EtOH exposure, but the complexity of signaling within the striatum offers numerous interpretations of the data presented. First, CB1 receptors are localized to both glutamatergic and GABAergic synapses [90] suggesting that either or both cell populations may contribute to our observations. For instance, reductions of CB1 receptor expression on glutamatergic synapses would be expected to increase glutamatergic signaling, and therefore increase the activation of GABAergic MSNs. Alternatively, decreases in GABAergic CB1 receptor expression would lead to an increase in GABA release to MSNs, decreasing their activation. Second, CB1 receptors are thought to compensate for changes in endocannabinoid release [25], with a wide variety of mechanisms contributing to their release (for review see [50]). Finally, traumatic stress and chronic EtOH differentially impact the endocannabinoid system. In rodent studies, chronic EtOH exposure increases endocannabinoid release [37] while decreasing endocannabinoid signaling [39], and clinical studies show a decrease in CB1-binding in many brain regions, including the striatum of alcoholics [91]. Alternatively, stress rodent models suggest an initial increase in endocannabinoids with acute stress followed by decreases in endocannabinoids after chronic stress [17]. Further dissection of the effects of EtOH and traumatic stress on independent systems is needed to completely elucidate the mechanisms of this interaction.
Changes in PSD95 after both traumatic stress and EtOH exposure suggest the involvement of glutamatergic dysregulation. In response to decreases in glutamatergic signaling, PSD95 increases the clustering of NMDA receptors and the insertion and retention of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [64]. Elevated PSD95 with repeated EtOH corresponds to reported findings that PSD95 compensates for the ability of EtOH to antagonize NMDA receptors [73]. The increased PSD95 in Control-mSPS mice shown here is supportive of decreased glutamatergic tone in SPS rats reported in a previous study from our group [92]. Endocannabinoids are also known to indirectly alter NMDA receptors through their regulation of pre-synaptic glutamatergic neurons [93, 94], but additional studies are needed to determine if the decrease in CB1 occurs in response to decreased glutamatergic signaling or if it is related to another mechanism involved in endocannabinoid regulation, such as, changes in activation of adenyl cyclases, cholinergic input, or dopaminergic tone [50].

Decreases in striatal D2 receptors after mSPS-exposure in mSPS-Sal mice were alleviated similarly to PSD95 in mSPS-EtOH mice. The decrease in D2 receptor protein after mSPS exposure corresponds to reported findings in rats exposed to SPS [61] and chronic stress [95]. Similarly, clinical studies on those with PTSD suggest genetic involvement of genes related to D2 receptors [96] and demonstrate elevated striatal dopamine-transporter protein [97], implying corresponding decreases in D2 receptors. D2 receptors were not significantly reduced in the assessed Control-EtOH mice. While D2 receptors are often decreased in those with alcoholism, some studies do not detect decreases in striatal D2 receptors ([60, 98] for review [99]). The alleviation of SPS-induced D2 receptor loss in EtOH exposed mice was not expected, and continued study is needed to elucidate more fully the mechanisms involved. Others report increased striatal D2 receptors in CB1-knockout mice [100], suggesting that D2 receptors are compensating for the dramatic loss of CB1 receptors. Interestingly, a subpopulation of alcoholics that is at particularly high risk of relapse displays an attenuation of decreased D2 compared to alcoholics who are able to recover with rehabilitation [59]. Determining if this high-risk population of alcoholics also has a greater history of traumatic stress would be an important future direction of study.

Overall, the present study suggests that severe, multimodal stress exposure impairs the striatal neuroplasticity that develops with prolonged EtOH treatment and that altered CB1 receptor expression is associated with this change. Furthermore, these findings point to CB1 receptors as a potential therapeutic target for the treatment of alcoholism in those affected with PTSD.

Supporting Information

S1 Table. Locomotor activity data.
(DOCX)

S2 Table. Immunoblot data.
(DOCX)

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Author Contributions
Conceived and designed the experiments: JJM ACC SAP. Performed the experiments: JJM LLS BLS. Analyzed the data: JJM LLS. Contributed reagents/materials/analysis tools: ACC SAP. Wrote the paper: JJM ACC SAP LLS BLS.

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