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The effects of predator odor (TMT) exposure and mGlu₃ NAM pretreatment on behavioral and NMDA receptor adaptations in the brain

Ryan E. Tyler^{1,2}, Maya N. Bluitt^{1,2}, Julie L. Engers³, Craig W. Lindsley³, Joyce Besheer^{1,2,4}

¹Neuroscience Curriculum, School of Medicine, University of North Carolina – Chapel Hill, Chapel Hill, NC

²Bowles Center for Alcohol Studies, School of Medicine, University of North Carolina, Chapel Hill, NC

³Warren Center for Neuroscience Drug Discovery, Vanderbilt University, Nashville, TN

⁴Department of Psychiatry, School of Medicine, University of North Carolina, Chapel Hill, NC

Abstract

A stressor can trigger lasting adaptations that contribute to neuropsychiatric disorders. Predator odor (TMT) exposure is an innate stressor that may activate the metabotropic glutamate receptor 3 (mGlu₃) to produce stress adaptations. To evaluate functional involvement, the mGlu₃ negative allosteric modulator (NAM, VU6010572; 3 mg/kg, i.p.) was administered before TMT exposure in male, Long Evans rats. Two weeks after, rats underwent context re-exposure, elevated zero maze (ZM), and acoustic startle (ASR) behavioral tests, followed by RT-PCR gene expression in the insular cortex and BNST to evaluate lasting behavioral and molecular adaptations from stressor. Rats displayed stress-reactive behaviors in response to TMT exposure that were not affected by VU6010572. Freezing and hyperactivity were observed during the context re-exposure, and mGlu₃-NAM pretreatment during stressor prevented the context freezing response. TMT exposure did not affect ZM or ASR measures, but VU6010572 increased time spent in the open arms and ASR habituation regardless of stressor treatment. In the insular cortex, TMT exposure increased expression of mGlu (*Grm3*, *Grm5*) and NMDA (*GriN2A*, *GriN2B*, *GriN2C*, *GriN3A*, *GriN3B*) receptor transcripts, and mGlu₃-NAM pretreatment blocked *GriN3B* upregulation. In the BNST, TMT exposure increased expression of *GriN2B* and *GriN3B* in vehicle-treated rats, but decreased

Correspondence: Joyce Besheer, Ph.D., Professor, Bowles Center for Alcohol Studies, Department of Psychiatry, University of North Carolina at Chapel Hill Chapel Hill, NC 27599-7171 joyce_besheer@med.unc.edu.

Ryan E. Tyler: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing – Original draft, Writing – Review and editing, Supervision, Project Administration. **Maya N. Bluitt:** Methodology, Validation, Formal Analysis, Investigation, Data Curation. **Julie L. Engers:** Methodology, Validation. **Craig W. Lindsley:** Methodology, Validation, Writing – Review and editing, Resources. **Joyce Besheer:** Conceptualization, Methodology, Resources, Writing – Review and editing, Supervision, Project Administration, Funding Acquisition.

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expression in the mGlu₃-NAM group. Similar to the insular cortex, mGlu₃-NAM reversed the stressor-induced upregulation of *GriN3B* in the BNST. mGlu₃-NAM also upregulated *GriN2A*, *GriN2B*, *GriN3B* and *Grm2* in the control group, but not the TMT group. Together, these data implicate mGlu₃ receptor signaling in some lasting adaptations of predator odor stressor and anxiolytic-like effects.

Keywords

stress; glutamate; NR3B; VU6010572; Insular Cortex; BNST

1. Introduction

Stress-related neuropsychiatric disorders are associated with dysregulated glutamatergic function [1–6]. Stress directly alters glutamatergic neurotransmission through glucocorticoid-receptor (GR) binding on glutamate neurons [7]. For example, stress models (e.g., forced-swim, tail-suspension, foot shock, glucocorticoid treatment) have repeatedly been shown to acutely increase presynaptic glutamate release in the brain [7–14]. Further, acute stressor exposure (e.g., forced-swim, tail suspension, elevated-plus maze) potentiates *N*-methyl-D-aspartate receptor (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor currents and surface expression through a GR-dependent mechanism on medial prefrontal cortex (mPFC) pyramidal neurons [15]. Overall, stress has been shown to have a multitude of acute and lasting effects specifically on cortico-limbic glutamate function that potentially contribute to maladaptive responses that increase vulnerability for the development of neuropsychiatric disorders [7, 16, 17].

Metabotropic glutamate (mGlu) receptors are potential therapeutic targets for many neuropsychiatric disorders [1–3, 6, 18], and modulate excitatory cell function through intracellular cascade events that can result in lasting adaptations to the cell in response to stressors [6, 19]. Group II mGlu receptors (mGlu₂ and mGlu₃) are coupled to Gi-proteins that reduce cellular activity as a result of activation [6]. Traditionally, Group II mGlu receptors are considered predominantly located on presynaptic terminals and their activation attenuates presynaptic neurotransmitter release [6]. However, mGlu₃ receptors have been found on both pre- and post-synaptic neurons, as well as astrocyte projections in the brain [20, 21]. Regardless of localization, mGlu_{2/3} receptor blockade has consistently been shown to have antidepressant- and antianxiety-like behavioral effects in rodents induced by stress with overlapping brain mechanisms to that of ketamine and other glutamate-targeting therapeutics [16, 17, 22–27]. Furthermore, the glutamate system has been shown to play an important role in anti-depressant drug effects [12, 28–30]. Therefore, mGlu_{2/3} receptor blockade has been proposed as an alternative to ketamine for its therapeutic potential without the psychogenetic and addiction-liability risks associated with ketamine [17]. The mechanisms by which mGlu₂ and mGlu₃ signaling exert their therapeutic effects are important for understanding novel mechanisms of glutamate-targeting antidepressant-like effects. However, prior to the recent development of highly specific negative allosteric modulators (NAMs) for mGlu₃ receptors [31, 32], the specific role of mGlu₃ vs. mGlu₂ receptors in these effects had been elusive. The same drug class of mGlu₃ NAMs that

were used in these experiments (VU6010572, first generation: VU0650786) have recently been employed to show that mGlu₃ mediates a form of synaptic plasticity in cortico-limbic circuitry that is sensitive to stress [19, 20, 27, 31] and exhibits rapid anti-depressant-like effects [27]. Restraint stress before electrophysiological measurements occluded mGlu₃-mediated long-term depression (LTD) in a manner consistent with mGlu₃ loss of function. Prior studies show that blocking mGlu₃ activation *in vivo* prior to restraint stress restored mGlu₃-LTD [19]. As previously mentioned, mGlu_{2/3} receptor blockade reverses stress-induced depressive-like phenotypes in rodents [17]. Therefore, the hypothesis tested here is that synaptic plasticity induced by mGlu₃-signaling during a stress event plays a role in the lasting behavioral and glutamatergic adaptations of stressor exposure.

Cortico-limbic circuitry is implicated in stress-related neuropsychiatric disorders. In animal models, the insular cortex and the bed nucleus of the stria terminalis (BNST) both play an important role in stress responses [33, 34]. The insular cortex is commonly associated with the integration of sensory and interoceptive processing [35, 36], which is highly sensitive to stress and anxiety in preclinical models [37, 38] and implicated in the etiology of neuropsychiatric disorders [39]. For example, inactivation of the anterior insular cortex produced anxiogenic-like effects, whereas activation of the insular cortex produced anxiolytic-like effects as assessed by the elevated plus maze test [37]. Clinically, anticipation of unpredictable stress increased BOLD signal in the insular cortex as assessed by fMRI [34]. The BNST is also associated with stress-related neuropsychiatric disorders [40] and animal models of stress adaptations [41]. For example, restraint stress in mice increased c-Fos immunoreactivity in CRF-expressing neurons of the dorsal BNST [42]. Additionally, inactivation of the BNST during predator odor exposure stress blocked stressor-induced freezing behavior in rodents [43], indicating involvement in the fear-like response to TMT. Finally, the insular cortex and BNST show reciprocal projections that are likely involved in the lasting plasticity from a stress response [44]. Therefore, the focus of the present work is on glutamatergic gene expression adaptations following stressor exposure in the insular cortex and BNST. Genes that encode for glutamate receptors were evaluated: NMDA: *GriN1*, *GriN2A*, *GriN2B*, *GriN2C*, *GriN2D*, *GriN3A*, *GriN3B*, and mGlu: *Grm2*, *Grm3*, and *Grm5*.

Animal models have used exposure to the scent of a predator as a stressor that produces lasting behavioral consequences with relevance to stress-related neuropsychiatric disorders [45–58]. For example, exposure to the synthetically derived fox odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) produces avoidance and freezing behaviors, indicative of a fear-like response and increased serum corticosterone, reflecting a stress response [50, 53, 59, 60]. Other studies have showed lasting anxiety-like and hyperarousal behavior, as well as avoidance of a TMT-paired context or cue [46, 54]. Predator odor animal models also capture the acute and long-term effects of glutamatergic adaptation to stress. For example, a ferret predator odor was shown to increase glutamate efflux in the CeA of rats [61]. Furthermore, predator odor adaptations to NMDA receptors in CeA circuitry mediate some of the lasting behavioral effects of the stressor [62]. Our lab recently showed that TMT exposure also produces lasting adaptations (4 weeks after TMT) in glutamate-related gene expression (*Grm2*, *Grm5*, *Grm7*, *Shank3*, *Homer1*, *Slc1A3*) in corticolimbic brain regions [63]. Of relevance to the present work, we have reported decreased *Grm3* gene expression

2 days after TMT exposure [63]. Given recent physiology findings showing that mGlu₃ activation results in subsequent loss of function [29], we hypothesized that the decrease in *Grm3* following TMT exposure may reflect a transcriptional mechanism for loss of function following stressor-induced *activation* of mGlu₃. Furthermore, mGlu₃ receptor activation during stressor may be involved in lasting adaptations to glutamatergic cortico-limbic circuitry [16, 17, 23–26]. Therefore, the present study blocks mGlu₃ signaling during the TMT exposure to evaluate the functional involvement of mGlu₃ activation during a stressor.

In this paper we used the newly developed mGlu₃ NAM VU6010572 to block mGlu₃ receptor signaling. Prior reports demonstrate that VU6010572 shows high specificity for mGlu₃ receptors and high CNS permeability (brain/plasma $K_p = 1.2$), overcoming a common problem in the investigation of the specific involvement of mGlu₃ receptor signaling from that of mGlu₂ receptor signaling [32]. High selectivity for mGlu₃ receptors has been shown (mGlu₃ $IC_{50} = 245$ nM; mGlu₂ $IC_{50} > 30$ μ M; mGlu₅ $IC_{50} > 30$ μ M), which also did not have off-target effects in “a Eurofins radioligand binding panel of 68 GPCRs, ion channels, transporters, and nuclear hormone receptors.” [32] Additionally, studies show that this drug class blocks LTD induced by mGlu_{2/3} agonists [19, 20] and produces acute antidepressant like effects (tail suspension test) in mice at a dose of 3 mg/kg [32]. Therefore, this was the justification for choosing the 3 mg/kg dose in these studies. Together, these data demonstrate high confidence that VU6010572 can be used as a tool to reduce mGlu₃ signaling in the brain.

The goal of the present work was to block mGlu₃ receptor activation (using VU6010572) *during* the TMT stressor and evaluate its functional implications on the *lasting* adaptations as a consequence of stressor. To capture the *lasting* behavioral and molecular consequences of the TMT stressor, rats remained undisturbed in the home cage for 2 weeks (operationally defined here as an “incubation period”) before behavioral testing (context re-exposure, zero maze, and acoustic startle response) and molecular assessments (gene expression for glutamate receptor transcripts) were conducted. Therefore, these experiments provide insight into the role of mGlu₃-signaling *during* a stressor on *lasting* stressor-induced adaptations, and in doing so also evaluate the potential long-term therapeutic effects of the mGlu₃-NAM VU6010572. These data could provide us with greater mechanistic insight into how stressor may influence vulnerability to the development of a neuropsychiatric disorder and the adaptations that result from mGlu₃ signaling during stressor exposure.

2. Methods

2.1 Animals

Male Long-Evans rats ($n = 48$; Envigo, Indianapolis, IN) were used for all experiments. Rats arrived at 7 weeks and were handled for at least 1 minute daily for 1 week prior to experiments. To obviate potential stress-transfer effects, all rats arrived and remained single-housed throughout the duration of experiments, consistent with our previous publications [63]. Rats were housed in ventilated cages with access to food and water ad libitum and maintained in a temperature and humidity-controlled vivarium with a 12-hour light/dark cycle. All experiments were conducted during the light cycle. Rats were under continuous care and monitoring by veterinary staff from the Division of Comparative Medicine at UNC-

Chapel Hill. All procedures were conducted in accordance with the NIH Guide to Care and Use of Laboratory Animals and institutional guidelines.

2.2 Compounds

VU6010572 was provided by Dr. Craig Lindsley and synthesized according to [32]. VU6010572 was dissolved in 45% β -cyclodextrin. A 1.5 mg/mL solution was made and injected at a volume of 2 mL/kg intraperitoneally (i.p.) to achieve a 3 mg/kg dose. An equal volume of 45% β -cyclodextrin was used as vehicle. The predator odor used was 2,5-dihydro- 2,4,5-trimethylthiazoline (TMT; 97% purity; SRQ Bio, Sarasota, FL).

2.3 Assessment of mGlu₃-NAM pretreatment on TMT exposure stress reactivity

Rats (n=12/grp) were injected with VU6010572 (0 or 3 mg/kg, i.p.) and returned to the home cage for 45 mins prior to TMT-exposure testing. TMT exposure test chambers and experimental set-up were identical to those used in [59, 63]. Briefly, rats were transported from the vivarium in the home cage to a separate, well-ventilated room that contained the test chambers in which rats were exposed to TMT (45.72 × 17.78 × 21.59 cm; UNC Instrument Shop, Chapel Hill, NC). Only one rat was placed in each chamber. The length of the back wall of the test chambers was opaque white with two opaque black side walls and a clear, plexiglass front wall to enable video recordings and a clear sliding lid. The bottom of the chamber was covered with a layer of white bedding [63]. A small, metal basket was hung on the right- side wall (17.8 cm above the floor) to hold a piece of filter paper. 10 μ L of TMT (2,5-dihydro- 2,4,5-trimethylthiazoline) or water for controls was pipetted onto the filter paper in the metal basket immediately prior to putting the rat in the chamber. The control group was always run before the TMT group to prevent odor contamination. The odor exposure session lasted 15 mins and was video recorded for evaluation of behavior using ANY-maze Video Tracking System (Version 6.12, Stoelting Co. Wood Dale, IL). After TMT exposure, rats were returned to the home cage and remained undisturbed in the home cage for 2 weeks as an incubation period prior to starting behavioral assessments. Two rats that were initially assigned to the TMT/mGlu₃-NAM group were moved to the TMT/vehicle group prior to injection due to unintentional loss of mGlu₃-NAM solution. One other animal in the TMT/mGlu₃-NAM group was removed from the study due to a failed i.p. injection. Final sample sizes: (n=12 - ctrl/vehicle; n=12 - ctrl/mGlu₃-NAM; n=14 - TMT/vehicle; n=9 - TMT/mGlu₃-NAM).

2.4 Context Re-exposure

Two weeks (14 days) following TMT exposure, rats were returned to the same test chambers in which they had been previously exposed to TMT. No TMT was used for this context re-exposure test. Identical to the TMT exposure, this test lasted 15 min in duration and was video recorded for behavioral assessments identical to the TMT exposure behavioral measures.

2.5 Elevated Zero Maze

24 hours after the context re-exposure test (15 days post-TMT exposure), rats were evaluated on an elevated zero maze. Rats were transported in their home cages to the behavioral

testing room and allowed to acclimate for 30 min prior to testing. The zero maze was made up of a circular platform with a diameter of 99 cm raised above the floor to a height of approximately 70 cm. The maze was divided equally into four quadrants/arms. Two enclosed arms contained two walls with one front wall [33.02 cm (H)] and back wall [64.52 cm (H)]. The exposed arms were located on opposite sides of the circular platform and were bordered with approximately 5 cm rim to prevent the rat from falling off the circular platform. Rats were placed in one open arm at the start of the 5 min test. These experiments were video recorded for analysis.

2.6 Acoustic Startle Response

24-hours after the zero-maze test (16 days pots-TMT exposure), rats were evaluated on an acoustic startle response test using an acoustic startle response system (S-R Lab; San Diego Instruments, San Diego, CA). Rats were transported in their home cages to the behavioral testing room and allowed to acclimate for 30 min prior to testing. Rats were placed in a cylinder Plexiglas animal enclosure located within a sound-attenuating test chamber that included an exhaust fan, a sound source, and an internal light that was turned off during the test. At the start of each test, rats underwent a 5-min habituation period during which 60 dB of background white noise was present. The background noise was present during the entire test session. The test session consisted of 30 trials of a 100 ms burst of a 110 dB startle. Each trial was separated by a 30 to 45-s randomized intertrial interval. Startle response (amplitude) was measured with a high-accuracy accelerometer mounted under the animal enclosure and analyzed with SR-Lab software.

2.7 Brain tissue collection and sectioning

24 hours after the acoustic startle test (17 days post-TMT exposure), a sub-set of rats (n=8 control/vehicle, n=5 control/mGlu₃-NAM, n=12 TMT/vehicle, n=9 TMT/mGlu₃-NAM) were sacrificed for qPCR analysis. More rats were allocated to the TMT group because previous studies showed that TMT exposure can produce distinct sub-groups within the TMT-exposed group that differ in brain gene expression [54]. Rats were anesthetized in 5% isoflurane immediately prior to brain extraction. Brains were rapidly extracted, and flash frozen with isopentane (Sigma- Aldrich, MI), then stored at -80 °C until brain region sectioning. Brains were sectioned on a cryostat (-20 °C) up to a predetermined bregma for each region of interest (ROI) according to [64]. Then, a micropunch tool was used to remove tissue specific to each brain region as illustrated in Table 1. Brain sections were stored at -80°C until qPCR analysis.

qPCR Analysis

2.8 RNA Extraction—RNA was extracted from brain tissue using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. RLT lysis buffer containing β- mercaptoethanol (Sigma Aldrich) was used for tissue homogenization. RNA concentration and purity for each sample were determined using a Spectrophotometer (Nanodrop 2000, ThermoScientific).

2.9 Reverse Transcription—RNA was reverse transcribed into cDNA using the SuperScript™ III First-Strand Synthesis System (ThermoFisher Scientific) according to the

manufacturer's instructions. Following reverse transcription, all samples were diluted 1:5 with water and stored at -20°C before RT-PCR experiments.

2.10 RT-PCR—The QuantStudio3 PCR machine (ThermoFisher) was used for all experiments. Using a 96-well plate, each sample was run in triplicate using 10 μL total volume per well with the following components: PowerUp Syber green dye (ThermoFisher, containing ROX dye for passive reference), forward and reverse primers (Eton Biosciences Inc., NC) and cDNA template. The PCR was run with an initial activation for 10 minutes at 95°C , followed by 40 cycles of the following: denaturation (95°C for 15 seconds), annealing/extension (60°C for 60 seconds). Melt curves were obtained for all experiments to verify synthesis of a single amplicon. All primers targeted genes that encode for glutamate receptors and include: *GriN1*, *GriN2A*, *GriN2B*, *GriN2C*, *GriN2D*, *GriN3A*, *GriN3B*, *Grm2*, *Grm3*, and *Grm5*. β -actin was used as the housekeeper gene. All primer sequences are displayed in Table 1.

3. Data Analysis

3.1 TMT Exposure and Context Re-exposure.

Analysis of TMT exposure and context re-exposure behaviors are identical to [63] except for freezing analysis included in the present work. Using ANY-maze, the length of the rectangular TMT exposure chamber was divided into two compartments for analysis (TMT side and non-TMT side). The basket containing TMT was located on the far end of the TMT side. Dependent measures assessed via ANY-maze included time spent of the TMT side of the chamber, time spent digging, time spent grooming, distance traveled and midline crossings (the number of times the animal crossed between the TMT and non-TMT side). Freezing was operationally defined as the absence of movement except respiration while the animal was not resting/sleeping, identical to [54]. Time spent digging in the bedding (see [59, 63]), time spent freezing, and time spent grooming were quantified manually by an experimenter blind to experimental conditions. For the context re-exposure, two rats were removed from analysis due to a camera recording error. Context re-exposure sample sizes: (n=11 - control/vehicle; n=12 - control/mGlu₃-NAM; n=13 - TMT/vehicle; n=9 - TMT/mGlu₃-NAM).

These dependent measures were analyzed by two-way ANOVAs and Sidak's multiple comparison test was used for all post hoc analyses, other than for freezing analysis in the context re-exposure. Based on the a priori hypothesis and the literature that TMT exposure would increase freezing upon re-exposure to the context, planned comparisons were conducted to investigate the effects of TMT exposure in both the vehicle and mGlu₃-NAM treated groups (student's t-test). Pearson correlational analyses were conducted between stress-reactive behaviors to the TMT exposure and to the context re-exposure reactivity in the TMT/Vehicle group. Positive correlations identified in the TMT/Vehicle group were evaluated in all other experimental groups.

3.3 Zero Maze.

The time spent in the open arms, number of open and closed arm entries, total number of entries (open + closed), and total distance traveled were quantified using ANY-maze software and analyzed by two- way ANOVA with Sidak's multiple comparison test for post hoc analyses. Three rats were excluded from this analysis – two due to falling off of the zero maze, and another was determined to be a statistically significant outlier. Sample sizes: (n=10 - control/vehicle; n=12 - control/mGlu₃-NAM; n=13 - TMT/vehicle; n=9 - TMT/mGlu₃-NAM).

3.4 Acoustic Startle Response.

The peak startle response (amplitude) was averaged for all 30-trials. In order to assess how startle response changes over time, and to evaluate habituation, we averaged the first and last 15-trials. Next, we calculated the percent change between the average of the first 15 trials and last 15 trials as follows:

$$\text{Percent Change} = \left(\frac{\text{Last 15} - \text{First 15}}{\text{First 15}} \right) \times 100$$

This measure was defined as “Habituation.” Two rats were removed from this analysis due to an experimental error. Sample sizes: (n=11 - control/vehicle; n=11 - control/mGlu₃-NAM; n=13 - TMT/vehicle; n=9 - TMT/mGlu₃-NAM). Data were analyzed by two- way ANOVA with Sidak's multiple comparison test for post hoc analyses.

3.5 Gene expression assessments.

The Ct method was used to determine fold change relative to controls using *β-actin* as the housekeeper gene [65]. Fold changes were normalized so that average control fold change equaled 1. Samples sizes for gene expression analysis started as: n=8 control/vehicle; n=5 control/mGlu₃-NAM; n=12 TMT/vehicle; n=9 TMT/mGlu₃-NAM (see methods). Two rats were removed from analysis due to brain extraction errors. Additionally, two of the control/vehicle samples ran out of cDNA before testing all genes, making the final samples sizes for the insular cortex: n=6–8 control/vehicle; n=5 control/mGlu₃-NAM; n=12 TMT/vehicle; n=7 TMT/ mGlu₃-NAM. For the BNST, 4 additional samples were lost as a result of RNA isolation errors, resulting in n=8 - control/vehicle; n=4 control/mGlu₃-NAM; n=9 TMT/vehicle; n=7 TMT/mGlu₃-NAM. Data were analyzed using 2-way ANOVAs, with Sidak's multiple comparison test for post hoc analyses. All gene expression data are reported as mean fold change ± SEM. For all analyses, significance was set at P = 0.05.

3.6 Power Analysis and Effect Sizes

Sample sizes (N=12/group; total 48) were determined using G-power analysis incorporating both predicted variability and effect size necessary to detect statistically significant differences based on our published work and other data from the lab. With a large effect size of f=0.45 (determined from prior effects of TMT exposure), α set at 0.05, a power of 0.80, and 4 groups, a total sample size of 41 rats was determined using G-plot. Therefore, these experiments started with 12 rats per group (total 48) to account for potential animal loss. All statistically significant results include effect sizes as eta squared (η²). η² is used in

multi-factor analyses as a metric of the proportion of the total variability that is attributable to the factor of interest [66]. Small, medium, and large sample size delineation is based off of Cohen's d definitions of effect size magnitude that correspond with η^2 . Effects are defined as a small effect ($\eta^2=0.009$), medium effect ($\eta^2=0.0588$), and large effects ($\eta^2=0.1379$) [66].

4. Results

4.1 Timeline of experiment

Figure 1 shows the experimental timeline.

4.2 TMT exposure produces stress-reactive behaviors that are not affected by mGlu₃-NAM

Figure 2 A–F shows the behavioral responses during TMT exposure. All behavioral measures (e.g., digging, freezing, time spent on the TMT side, grooming, distance traveled and midline crossings) showed a main effect of TMT exposure and no significant effect of mGlu₃-NAM. TMT exposure increased time spent digging (Fig. 2A, $F(1, 43) = 38.32$, $p < 0.0001$, $\eta^2=0.46$) and time spent freezing (Fig. 2B, $F(1, 43) = 210.10$, $p < 0.0001$, $\eta^2=0.77$). For freezing, we observed a significant interaction effect between TMT exposure and mGlu₃-NAM pretreatment ($F(1, 43) = 4.92$, $p = 0.03$, $\eta^2=0.02$), with both the vehicle ($p < 0.0001$) and mGlu₃-NAM ($p < 0.0001$) groups that were exposed to TMT showing increased freezing compared to respective control groups. TMT exposure decreased time spent on the TMT side of the test chamber (Fig. 2C, $F(1, 43) = 61.55$, $p < 0.0001$, $\eta^2=0.72$) and time spent grooming (Fig. 2D, $F(1, 43) = 6.02$, $p = 0.01$, $\eta^2=0.12$). Distance traveled (Fig. 2E, $F(1, 43) = 7.15$, $p = 0.01$, $\eta^2=0.13$) and midline crossings (Fig. 2F, $F(1, 43) = 10.44$, $p = 0.002$, $\eta^2=0.19$) were decreased as a main effect of TMT exposure. These results demonstrate robust behavioral stress-reactivity to TMT exposure that is not affected by pretreatment with the mGlu₃-NAM.

4.3 TMT exposure produces freezing and hyperactivity in response to the TMT-paired context: role for mGlu₃ signaling

Figure 3 A–F shows the behavioral response during the context re-exposure. For digging (Fig. 3A), a main effect of TMT exposure was found to increase time spent digging ($F(1, 41) = 4.58$, $p=0.038$, $\eta^2=0.10$). For freezing (Fig. 3B), a main effect of TMT exposure was found ($F(1, 41) = 5.70$, $p=0.022$, $\eta^2=0.11$). No effect of mGlu₃-NAM or interaction observed for freezing. Planned comparisons showed an increase in time spent freezing between the water and TMT group in the vehicle condition ($p<0.05$), but not the mGlu₃-NAM condition. No effect of TMT exposure or mGlu₃-NAM was observed for time spent on the TMT side (Fig. 3C). For time spent grooming (Fig. 3D), a main effect of TMT showed reduced grooming behavior ($F(1, 41) = 5.73$, $p=0.02$, $\eta^2=0.12$), but no significant main effect of mGlu₃-NAM or interaction was observed.

For distance traveled (Fig. 3E), a main effect of TMT exposure ($F(1, 41) = 13.36$, $p=0.0007$, $\eta^2=0.22$) and mGlu₃-NAM ($F(1, 41) = 9.57$, $p=0.004$, $\eta^2=0.15$) were observed, both showing increases in distance traveled. No interaction effect was found. For the number

of midline crossings (Fig. 3F), a main effect of TMT exposure ($F(1, 41) = 11.15$, $p=0.0018$, $\eta^2=0.17$), mGlu₃-NAM ($F(1, 41) = 6.43$, $p=0.02$, $\eta^2=0.10$) and a TMT x mGlu₃-NAM interaction effect was observed ($F(1, 41) = 5.77$, $p=0.02$, $\eta^2=0.09$). The TMT/vehicle group displayed greater midline crossings compared to the control/vehicle group ($p=0.0003$). Interestingly, this difference was not observed in the animals that received mGlu₃-NAM pretreatment ($p > 0.05$). This lack of effect was likely driven by an increase in midline crossings in the control/mGlu₃-NAM group compared to the control/vehicle group ($p=0.002$). Together, these data indicate that TMT exposure produces freezing and hyperactivity in the previously paired TMT context, and that mGlu₃ signaling during the TMT exposure contributes to the freezing response in the context re-exposure.

Understanding individual differences in response to stressor may prove valuable in understanding vulnerability to stress-related disorders [67] and behavioral outcomes in rodents [45, 59]. Therefore, correlational analyses were conducted between stress-reactive behaviors during the TMT exposure (Fig. 2) and behavioral responses to the context re-exposure 2 weeks later (Fig. 3). Interestingly, in the TMT/vehicle group, time spent grooming during the context re-exposure correlated with 3 distinct stress-reactive behaviors during the TMT exposure. First, time spent digging (Fig. 3G) negatively correlated with time spent grooming ($r^2=0.39$, $p=0.02$). This correlation was not observed in the TMT/mGlu₃-NAM group (Fig. 3H). The number of midline crossings during the TMT exposure negatively correlated with time spent grooming during the context re-exposure ($r^2=0.51$, $p=0.008$) in the TMT/vehicle group (Fig. 3I), but not in the TMT/mGlu₃-NAM group (Fig. 3J). These correlations were not observed in either control group (control/vehicle: Digging X Grooming: $r=-0.07$; Midline Crossings X Grooming: $r=-0.11$; or the Control/mGlu₃-NAM: Digging X Grooming: $r=-0.019$; Midline Crossings X Grooming: $r=-0.16$) group. These correlations suggest an association between the degree of stress-reactivity to the TMT exposure with the degree of engagement in grooming behavior during the context re-exposure. Interestingly, these associations were not observed in the TMT-exposed rats that were pretreated with mGlu₃-NAM suggesting that the mGlu₃-NAM may disrupt this association.

4.4 mGlu₃-NAM produces lasting anxiolytic-like behavioral effects

Figure 4 shows analysis of the zero-maze test and acoustic startle response test approximately 2 weeks after TMT exposure and mGlu₃-NAM pretreatment. For percent time spent in the open arms of the zero maze (Fig. 4A), a main effect of mGlu₃-NAM was found ($F(1, 40) = 6.19$, $p=0.01$, $\eta^2=0.13$), indicating greater percent time spent in the open arms, but no effect of TMT exposure or interaction was observed. Analysis of the total distance traveled in the zero maze showed no effect of TMT exposure or mGlu₃-NAM (control/vehicle – 3.97 ± 0.18 ; control/mGlu₃-NAM – 4.19 ± 0.26 ; TMT/vehicle – 3.85 ± 0.26 ; TMT/mGlu₃-NAM – 4.80 ± 0.53). Analysis of the number of entries into the open arm showed no main effect of TMT exposure or mGlu₃-NAM (control/vehicle – 10.2 ± 1.67 ; control/mGlu₃-NAM – 12.54 ± 1.63 ; TMT/vehicle – 7.61 ± 1.75 ; TMT/mGlu₃-NAM – 9.8 ± 1.48). Interestingly, TMT exposure decreased the number of closed arm entries ($F(1, 40) = 4.24$, $p = 0.04$, $\eta^2=0.09$; control/vehicle: 10.3 ± 1.9 ; control/mGlu₃-NAM: 12.6 ± 1.7 ; TMT/vehicle: 6.84 ± 1.7 ; TMT/mGlu₃-NAM: 8.9 ± 1.4). mGlu₃-NAM did

not affect the number of closed arm entries. No effect of mGlu3-NAM or TMT exposure was found for total arm entries (control/vehicle: 20.5 ± 3.57 ; control/mGlu3-NAM: 25.18 ± 3.38 ; TMT/vehicle: 14.46 ± 3.47 ; TMT/mGlu3-NAM: 18.7 ± 2.93). In the acoustic startle test, there were no significant effects of TMT exposure or mGlu₃-NAM on average peak startle response (mV, control/vehicle - 770.81 ± 128.30 ; control/mGlu₃-NAM - 957.31 ± 310.94 ; TMT/Vehicle - 481.33 ± 83.06 ; TMT/mGlu₃-NAM - 1125.47 ± 404.21). However, mGlu₃-NAM treated rats displayed greater habituation (Fig. 4B) across the 30-trials ($F(1, 40) = 4.95, p=0.03, \eta^2=0.12$), with no significant effect of TMT or interaction. These results indicate that mGlu₃-NAM produces lasting anxiolytic-like effects and facilitates habituation to the startle response two weeks after the mGlu₃-NAM injection. Changes were not observed as an effect of TMT exposure, but as main effects of mGlu₃-NAM itself, demonstrating that these drug effects are not stressor-related.

4.5 TMT exposure produces lasting increases in glutamate receptor gene expression in the insular cortex

Figure 5 shows the effects of TMT exposure and mGlu₃-NAM on glutamate receptor transcripts in the insular cortex. Neither TMT exposure nor mGlu₃-NAM affected the expression of the house-keeper gene (*β-actin*). For NMDA receptor transcripts, no effect of TMT exposure, mGlu₃-NAM or interaction was found for *GriN1* expression (Fig. 5A). For *GriN2A* (Fig. 5B), *GriN2B* (Fig. 5C), and *GriN2C* (Fig. 5D), a main effect of TMT exposure showed increased expression (*GriN2A* - $F(1, 25) = 4.41, p = 0.04, \eta^2 = 0.15$; *GriN2B* - $F(1, 25) = 8.02, p=0.009, \eta^2 = 0.22$; *GriN2C* - $F(1, 26) = 14.50, p=0.0008, \eta^2 = 0.32$). There was no main effect of the mGlu₃-NAM or interaction. For *GriN2D* (Fig. 5E), there was no effect of TMT exposure, mGlu₃-NAM or interaction. A main effect of TMT exposure showed increased expression of *GriN3A* ($F(1, 25) = 5.09, p=0.03, \eta^2 = 0.16$; Fig. 5F), but did not show an effect of the mGlu₃-NAM or interaction. *GriN3B* expression (Fig. 5G) was increased by TMT exposure ($F(1, 27) = 6.98, p=0.013, \eta^2 = 0.17$), but not affected by mGlu₃-NAM, and a significant TMT exposure x mGlu₃-NAM interaction was observed ($F(1, 27) = 4.02, p=0.05, \eta^2 = 0.10$). Post-hoc analyses showed that *GriN3B* expression was increased in the TMT/vehicle group compared to the control/vehicle group ($p=0.002$), an effect that was prevented by Glu₃-NAM pretreatment (i.e., no difference between TMT/mGlu₃-NAM vs. control/mGlu₃-NAM groups).

For mGlu receptor transcripts, *Grm2* (Fig. 5H) was not affected by TMT exposure or mGlu₃-NAM. *Grm3* (Fig. 5I) and *Grm5* (Fig. 5J) were upregulated as shown by a main effect of TMT exposure (*Grm3* - $F(1, 25) = 4.39, p=0.04, \eta^2 = 0.13$; *Grm5* - $F(1, 25) = 6.01, p=0.02, \eta^2 = 0.18$) with no effect of the mGlu₃-NAM or interaction. These data indicate that TMT exposure increases gene expression of several NMDA and mGlu receptor sub-units in the insular cortex, and that mGlu₃ signaling during TMT exposure may mediate the stressor-induced increase of *GriN3B*.

4.6 TMT exposure and mGlu3-NAM produce lasting changes in glutamate receptor transcript levels in the BNST

Figure 6 shows the gene expression changes in the BNST following TMT exposure and mGlu₃-NAM pretreatment. Neither TMT exposure nor mGlu₃-NAM affected the expression

of the house-keeper gene (β -actin). For NMDA receptor, *GriN1* (Fig. 6A) showed no effect of TMT or mGlu₃-NAM. For *GriN2A* (Fig. 6B), there was a main effect of TMT exposure ($F(1, 24) = 10.79, p=0.003, \eta^2 = 0.23$), no main effect of mGlu₃-NAM, but a significant interaction effect ($F(1, 24) = 17.35, p=0.0003, \eta^2 = 0.36$). mGlu₃-NAM increased *GriN2A* expression in controls ($p=0.001$), but not in the TMT group. *GriN2A* expression was decreased by TMT exposure in the mGlu₃-NAM group ($p=0.0002$), but not changed in vehicle-treated group. For *GriN2B* (Fig. 6C), there was a significant main effect of mGlu₃-NAM ($F(1, 24) = 12.71, p=0.0016, \eta^2 = 0.26$), no main effect of TMT exposure, and a significant interaction effect ($F(1, 24) = 16.45, p=0.0005, \eta^2 = 0.33$). mGlu₃-NAM increased expression in controls ($p < 0.0001$) but not in the TMT group. TMT exposure decreased expression in mGlu₃-NAM treated rats ($p=0.016$), but increased expression in vehicle treated rats ($p=0.015$). *GriN2C* (Fig. 6D) was downregulated as a main effect of TMT exposure ($F(1, 24) = 7.399, p=0.011, \eta^2 = 0.22$), but no effect of mGlu₃-NAM or significant interaction was found. No effect of TMT exposure, mGlu₃-NAM, or interaction was found for *GriN2D* expression (Fig. 6E) or *GriN3A* expression (Fig. 6F). For *GriN3B* (Fig. 6G), no main effect of TMT or mGlu₃-NAM was observed, but a significant interaction effect was found ($F(1, 24) = 23.75, p < 0.0001, \eta^2 = 0.49$). TMT exposure increased expression of *GriN3B* in the vehicle group ($p=0.005$), but decreased expression in the mGlu₃-NAM group ($p=0.0033$). mGlu₃-NAM increased *GriN3B* expression in controls ($p=0.0007$), but decreased expression in the TMT group ($p=0.028$).

For mGlu receptors, *Grm2* (Fig. 6H), no main effect of TMT or mGlu₃-NAM was observed, but a significant TMT x mGlu₃-NAM interaction was found ($F(1, 24) = 11.25, p=0.0026, \eta^2 = 0.31$). Post hoc analyses showed that mGlu₃-NAM increased *Grm2* expression in control rats ($p=0.0075$) but did not affect the TMT group. TMT exposure decreased *Grm2* expression in the mGlu₃-NAM group ($p=0.012$) but did not affect the vehicle group. *Grm3* and *Grm5* (Fig. 6I and Fig. 6J, respectively) expression were not affected by TMT exposure or mGlu₃-NAM.

5. Discussion

These studies show that TMT exposure produces behavioral (context re-exposure) and molecular adaptations (insular cortex and BNST glutamate transcript upregulation) 2 weeks after the exposure, demonstrating lasting behavioral and molecular phenotypes from this stressor model. Furthermore, mGlu₃ signaling during exposure to the TMT predator odor stressor contributes to some extent to both behavioral (freezing response during the context reactivity) and molecular (*GriN3B* upregulation in the insular cortex and BNST) adaptations approximately 2 weeks after the stressor exposure in male rats.

Consistent with previous findings, the TMT exposure produced engagement in stress-reactive behaviors (freezing, digging, avoidance, and decreased grooming, distance traveled and midline crossings), indicating that TMT exposure is an acute stressor [54, 55, 59, 63, 68]. Pretreatment with the mGlu₃-NAM (VU6010572) did not affect any of these behaviors, suggesting that mGlu₃ signaling during the stressor does not contribute to the engagement of acute stress-reactive behaviors. Prior studies in mice show that a similar mGlu₃-NAM (VU0650786) administered prior to restraint stress prevented decreased sucrose intake

indicative of motivational behavior caused by the restraint stress [19] and that VU6010572 produced acute anti-depressant-like behavioral effects [32]. Together with the present findings, these data serve to specify the acute effects of mGlu₃-NAM, which may be related more to anti-depressant-like effects rather than effects on stress-reactivity.

TMT exposure increased time spent freezing during the context re-exposure test conducted 2 weeks after the stressor, which is consistent with prior reports using TMT exposure in rats [54, 55, 68]. Note that rats engaged in a very small amount of freezing (about 1% time spent freezing during the context re-exposure). As such, TMT exposure did not produce a high degree of freezing behavior during the context re-exposure. Interestingly, during the context re-exposure, rats in the TMT groups also showed increases in measures of locomotion and general activity (increased digging, distance traveled, midline crossings, and decreased grooming), which together with freezing may reflect a hypervigilant behavioral phenotype in response to the context re-exposure in rats previously exposed to TMT. Pretreatment with the mGlu₃-NAM VU6010572 before the TMT exposure that occurred 2 weeks earlier prevented the freezing response to the context re-exposure. This indicates that mGlu₃ activation as a consequence of the TMT stressor exposure contributes to the formation of a fear-like association with the stressor context. The context re-exposure did not show an avoidance phenotype (i.e., less time spent on the TMT side of the chamber), which indicates that this model does not capture the avoidance symptom cluster observed in PTSD, as some other predator odor models do [45]. However, the absence of an avoidance phenotype is consistent with our prior publications [59, 63, 69], and other published work using TMT exposure [57, 70, 71]. Correlational analyses in the TMT/vehicle group showed that time spent grooming during the context re-exposure negatively correlated with both time spent digging and the number of midline crossings during the TMT exposure. These data patterns suggest a potential association between stress-reactivity during the TMT exposure with the lasting behavioral adaptations in response the context re-exposure (specifically diminished grooming). Interestingly, these correlations in the TMT/vehicle group were not observed in the TMT/mGlu₃-NAM group, suggesting that mGlu₃-NAM pretreatment prior to stressor disrupted the associations between TMT exposure and some of the context re-exposure stress reactivity measures.

The mGlu₃-NAM pretreatment also produced other lasting behavioral changes. While TMT exposure did not affect zero maze and ASR measures, the mGlu₃-NAM produced anxiolytic effects on the zero maze and promoted greater habituation of arousal response in the ASR test. Because mGlu₃-NAM was administered 2 weeks prior to behavioral tests, these findings suggest a lasting effect of the mGlu₃-NAM on anxiety and arousal processes, which may contribute to the therapeutic potential of negative allosteric modulation of mGlu₃ as to date VU6010572 has been shown to have acute and rapid anti-depressant-like effects [32]. Further, mGlu_{2/3} antagonists have been proposed as potential alternative anti-depressants with similar acute and lasting efficacy and mechanisms to ketamine in the absence of abuse liability [17]. Therefore, these novel findings on anxiety-like behavior and arousal habituation show that a single administration of the mGlu₃-NAM VU6010572 is sufficient to produce lasting therapeutic effects, and therefore may have potential as a new glutamate-acting therapeutic. Note that these drug effects were not dependent on the stressor condition (i.e., main effect of mGlu₃-NAM pretreatment), but were observed in both the

control and TMT groups. Finally, while it is possible that the context re-exposure testing affected subsequent zero maze and ASR measures, these tests were separated by 24 hours to minimize this concern.

TMT exposure produced lasting increases of several glutamate receptor transcripts, including mGlu (*Grm3*, *Grm5*) and NMDA (*GriN2A*, *GriN2B*, *GriN2C*, *GriN3A*, *GriN3B*) in the insular cortex. Because many of these genes encode for synaptic glutamate receptors [72], these data may reflect changes to excitatory synaptic number, density, and/or receptor composition of glutamate receptors that potentially result in functional differences. Because the insular cortex is involved in negative affect, anxiety, fear memories and threat conditioning [34, 37, 38, 44, 73], and NMDA receptor adaptations are involved in synaptic plasticity in the insular cortex [74], these adaptations could in part underlie the hypervigilance and freezing phenotypes observed in the context re-exposure test.

Similar to the insular cortex, the BNST showed increased NMDA receptor transcripts (*GluN2B* and *GluN3B*) from TMT exposure. Interestingly, TMT exposure decreased expression of *GriN2C*. Collectively, these data suggest that TMT exposure may have altered the sub-unit composition of NMDA receptors in the BNST. Surprisingly, TMT exposure also *decreased* expression of some NMDA receptor transcripts only in the mGlu₃-NAM treated group, including *GriN2A*, *GriN2B*, *GriN3B* and *Grm2*. This effect may have been driven by the fact the mGlu₃-NAM increased expression of these transcripts, showing a similar effect as TMT exposure. Together, these data suggest that mGlu₃-NAM and TMT exposure affect glutamate receptor transcription. Prior data suggests that mGlu₃ receptors may be activated during stressor exposure [19, 63]. Therefore, the upregulation of these NMDA receptor transcripts induced by blocking mGlu₃ may be offset by the subsequent activation of mGlu₃ induced by the TMT exposure, thus preventing the upregulation of the NMDA receptor transcripts. Indeed, prior studies have also demonstrated involvement of mGlu_{2/3} signaling in stress-induced adaptations to the BNST. Specifically, mGlu_{2/3} agonism (LY379268) potentiated foot-shock induced c-Fos increases in the dorsolateral BNST [75].

Interestingly, mGlu₃-NAM pretreatment prior to TMT exposure blocked the upregulation of *GriN3B* (encodes GluN3B) induced by TMT exposure in both the insular cortex and the BNST. GluN3B is an NMDA receptor subunit expressed at low levels in the brain [76] but may play an important role in the lasting alterations from synaptic plasticity and synaptogenesis [77]. In contrast to canonical NMDA receptors (GluN1-GluN2 heterotetramers), GluN3-containing NMDA receptors (GluN1-GluN3A/B) have a smaller single channel conductance, lower Ca²⁺ permeability, less sensitivity to the Mg²⁺ block, and are located outside of the synapse [77].

Early studies indicated that the GluN3 subunits diminished glutamate-evoked NMDA currents [78], which was proposed to indicate a dominant-negative function of GluN3B on traditional GluN1-GluN2 currents. Therefore, these data could indicate that mGlu₃ signaling during the TMT predator odor stressor diminishes synaptic/phasic NMDA receptor function through sequestration of glutamate by the dominant-negative sub-unit GluN3B. Interestingly, GluN1-GluN3 heterotetramers are not sensitive to glutamate or NMDA, but all 4 subunits bind glycine [77]. Because endogenous concentrations of glycine constitutively

occupy these receptors, it has been proposed that GluN1/GluN3 heterotetramers remain open *in vivo* and serve to modulate tonic excitation [77, 79]. Therefore, the upregulation of *GriN3B* transcript expression in the insular cortex and BNST by stressor-induced mGlu₃ signaling might also reflect enhanced tonic excitation in these brain regions as a lasting adaptation to stressor. As such, important future directions will examine how these adaptations affect cellular physiology. Importantly, these findings are the first to show that TMT exposure produces lasting adaptations to excitatory gene expression in the insular cortex and BNST, and that mGlu₃ signaling during stressor mediates *GriN3B* transcriptional upregulation in both brain regions. Finally, given recent clinical findings implicating *GriN3B* expression levels as a predictive blood biomarker of PTSD outcomes following trauma [80], the mGlu₃-NAM VU6010572 may have potential therapeutic effects by attenuating stressor-induced *GriN3B* expression in the brain.

The insular cortex and BNST form reciprocal projections [81, 82] and both regions contribute to affective behavioral measures in rodents [37, 38, 83] and humans [34]. Recent studies have shown that insular cortex/BNST circuitry are involved in negative affective measures associated with alcohol withdrawal in rodents [44]. Therefore, these findings showing stressor- and mGlu₃-NAM-induced adaptations to glutamate receptors in the insular cortex and BNST could reflect adaptations to insular cortex/BNST excitatory circuitry as a result of stressor and mGlu₃-NAM, and further implicate this circuitry in stress-induced changes. Future work will investigate the functional involvement of insular cortex/BNST circuitry on lasting behavioral adaptations of the TMT exposure (i.e. context re-exposure hyperactivity, freezing and anxiolytic effects of mGlu₃-NAM).

Because glutamatergic signaling is involved in learning and fear-memory associations [84], these data may demonstrate that mGlu₃-NAM disrupted the formation of the stress/context association through its effects on NMDA receptor transcriptional regulation. mGlu₃ receptors have specifically been implicated in learning and cognition [85], further implicating mGlu₃ receptors in the learning or formation of stressor environments. mGlu_{2/3} activation potentiated NMDAR-currents in isolated hippocampal CA1 neurons [86]. Specific SNPs of the *Grim3* gene are associated with cognitive and working memory deficits in schizophrenia [87]. mGlu₃ may also have neuroprotective properties, showing that group II agonism protects against NMDAR-induced excitotoxic degeneration in cultured neurons [88]. Based on these studies demonstrating a role for mGlu₃ signaling in learning, cognition, and NMDA receptor function, the mGlu₃-NAM pretreatment may have partially disrupted the formation of the fear memory associated with the stressor context through its effects on NMDA receptors.

An important consideration for the present findings is that like the TMT groups, the control groups experienced exposure to a novel environment (although in the absence of TMT). This experience was potentially a stressor and therefore, this feature of our experimental design should be considered when making conclusions about the effects of mGlu₃-NAM and the observed adaptations in behavior and gene expression. Furthermore, the effects of TMT exposure and mGlu₃ signaling were not evaluated in female rats, which is a limitation to these studies as sex differences may exist in response to TMT stressor and mGlu₃-NAM [59]. Furthermore, the BNST is sexually dimorphic [89], suggesting the possibility of sex

differences in the gene expression changes in the BNST specifically. Important follow-up studies will evaluate these effects in female rats. Finally, blockade of mGlu₃ receptors by negative allosteric modulation was chosen for these experiments based on the hypothesis that stressor activates mGlu₃ receptors, resulting in plasticity that has lasting effects [19, 20, 63]. However, it will also be important to evaluate the direct role of pharmacological mGlu₃ activation in the acute and lasting effects of TMT exposure for a more comprehensive evaluation of mGlu₃ signaling in the lasting effects of stress. The importance of evaluating mGlu₃ activation in stressor-related adaptations is reflected by work showing that mGlu_{2/3} activation by LY354740 produces anxiolytic effects on the elevated plus-maze (EPM) test and blocks the EPM-induced c-Fos expression in limbic brain regions [90]. This previous work suggests the possibility that mGlu₃ activation might attenuate the acute stress response to the TMT exposure.

These findings show that mGlu₃-NAM did not affect acute stress-reactivity in response to TMT exposure but did contribute to lasting phenotypes induced by the TMT exposure. TMT exposure produced a hypervigilant and freezing phenotype in response to the context re-exposure 2 weeks later, and mGlu₃-NAM pretreatment prevented the freezing response to the context re-exposure. Gene expression analysis show that TMT exposure produced adaptations to glutamate receptors in the insular cortex and BNST, and that mGlu₃-NAM during stressor mediated the transcriptional upregulation of *GriN3B* in the insular cortex and BNST. Finally, mGlu₃-NAM produced lasting anxiolytic-like behavioral phenotypes in both control and TMT groups. These findings show that mGlu₃ receptors are involved in some of lasting adaptations to stressor and may reflect a promising therapeutic target for neuropsychiatric disorders.

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Highlights

- mGlu₃-NAM pretreatment does not affect TMT exposure stress-reactive behaviors.
- TMT exposure produces freezing and hyperactivity in response to the TMT-paired context.
- mGlu₃-NAM pretreatment prevents freezing response to the context.
- mGlu₃-NAM pretreatment produces lasting anxiolytic-like effects.
- TMT exposure and mGlu₃-NAM affect glutamatergic gene expression in the insular cortex and BNST.

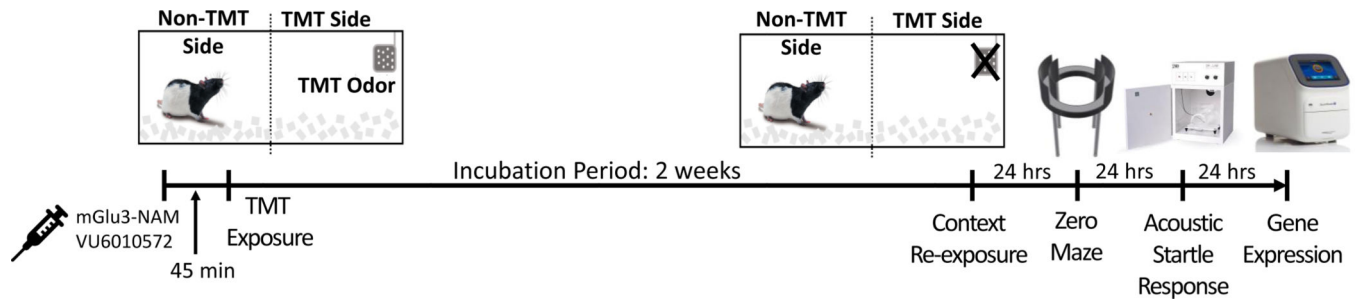


Figure 1.
Experimental Timeline

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TMT Exposure

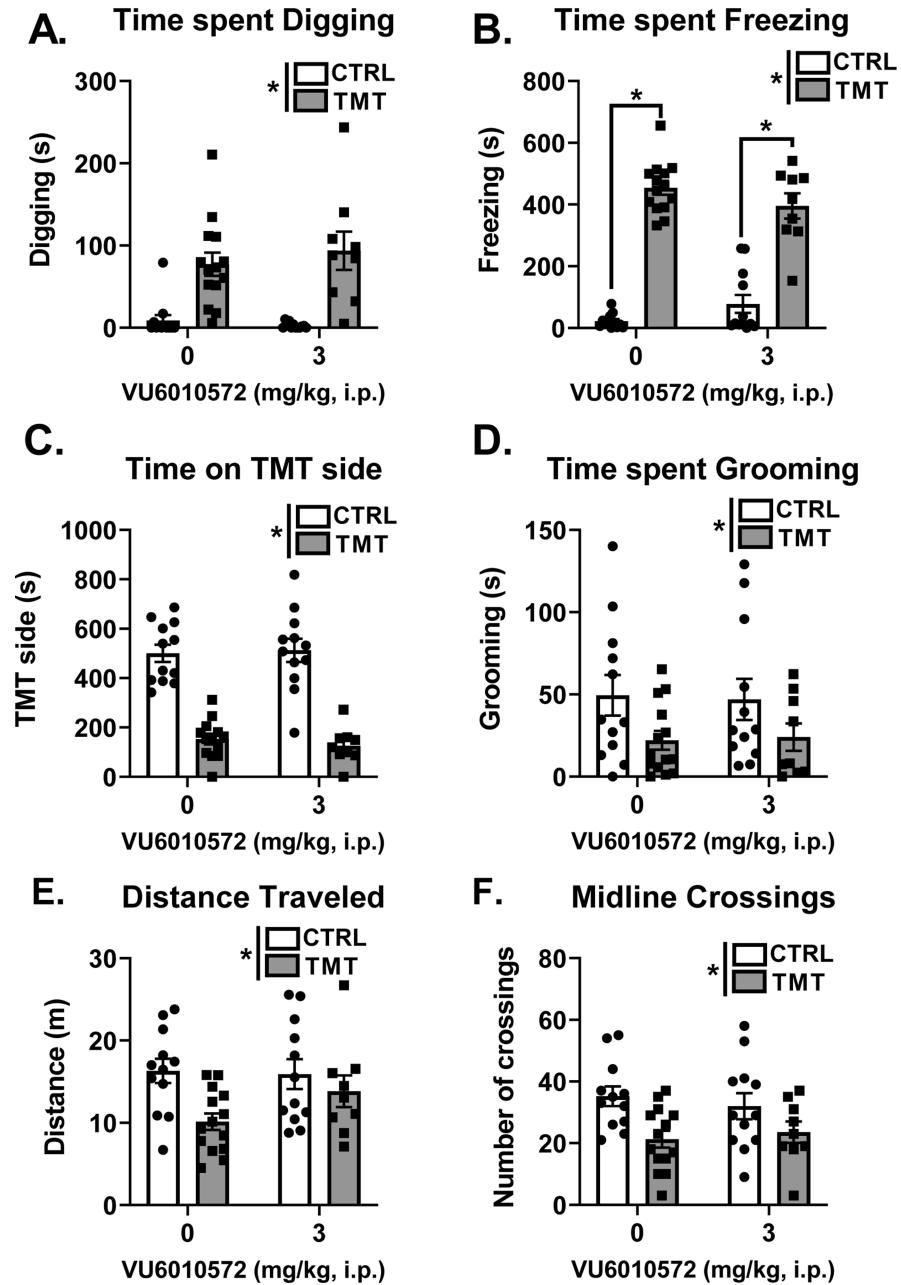


Figure 2. TMT exposure produces stress-reactive behaviors that are not affected by mGlu₃-NAM TMT exposure increased time spent digging (A) and time spent freezing (B). TMT exposure decreased time spent on the TMT side (C), time spent grooming (D), distance traveled (E) and the number of midline crossings (F). mGlu₃-NAM did not affect any of these measures * p 0.05 significantly different from Control.

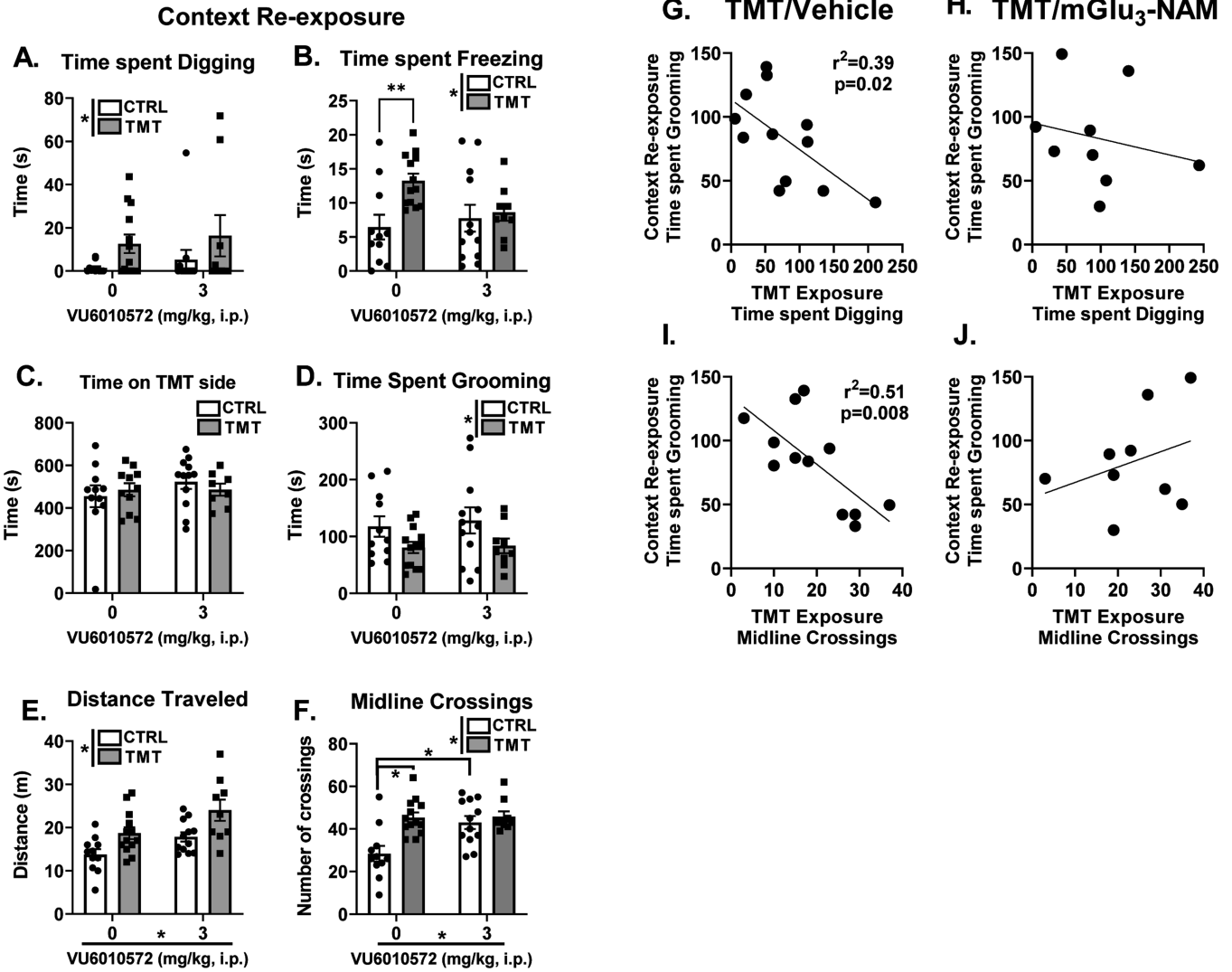


Figure 3. TMT exposure produces freezing and hyperactivity in response to the TMT-paired context: role for mGlu₃ signaling during stressor on the freezing response
 During the context re-exposure, a main effect of TMT exposure increased both time spent digging (A) and time spent freezing (B). A freezing response was observed in the control group but not in the mGlu₃-NAM group (B). Time spent on the TMT side of the chamber was not affected by TMT exposure or mGlu₃-NAM (C). Time spent grooming (D) was decreased as a result of TMT exposure. Distance traveled (E) was increased by the TMT exposure and mGlu₃-NAM pretreatment. Finally, midline crossings (F) were increased by both the TMT exposure and mGlu₃-NAM pretreatment. Time spent digging during the TMT exposure negatively correlated with time spent grooming during the context re-exposure in the TMT/vehicle group (G), but not the TMT/mGlu₃-NAM group (H). The number of midline crossings during the TMT exposure negatively correlated with time spent grooming during the context re-exposure in the TMT/vehicle group (I), but not the TMT/mGlu₃-NAM group (J). * p 0.05 significantly different from Control

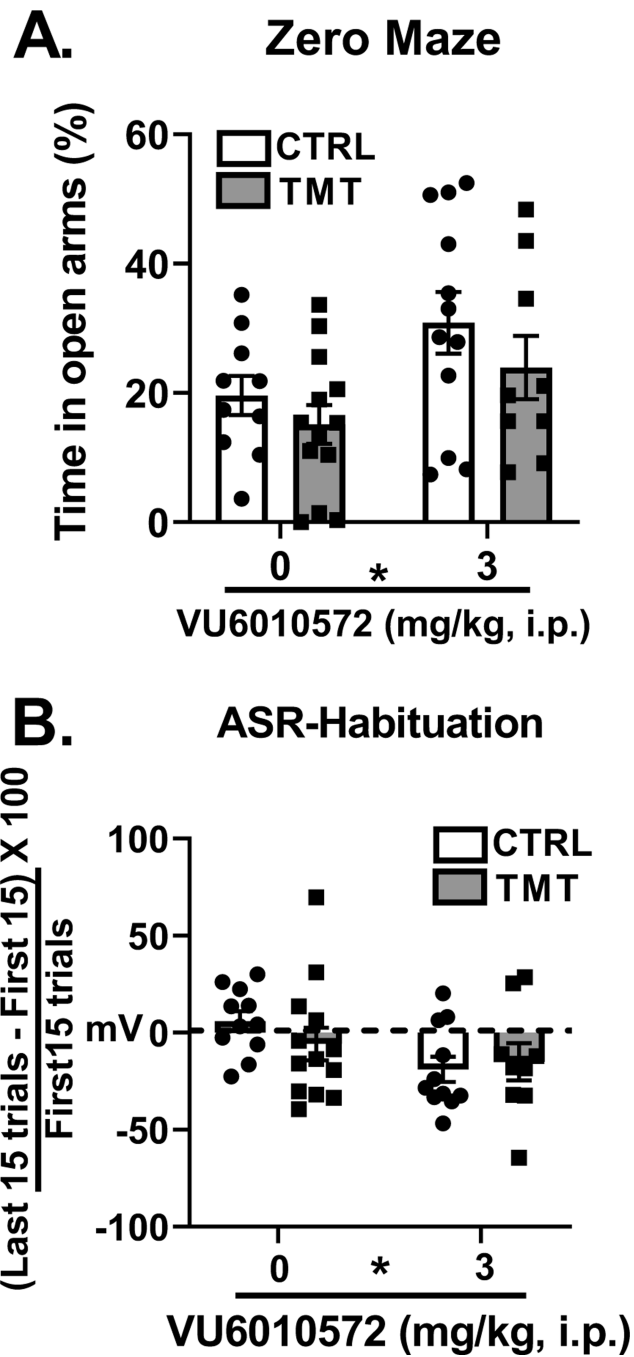


Figure 4. mGlu₃-NAM produces lasting anxiolytic-like behavioral effects
 mGlu₃-NAM treatment 2 weeks before testing resulted in increased time spent in the open arms of the zero-maze test (A) and increased habituation of acoustic startle responses (B). TMT exposure did not affect zero maze or ASR measures. * p < 0.05 significantly different from Control.

Insular Cortex Gene Expression

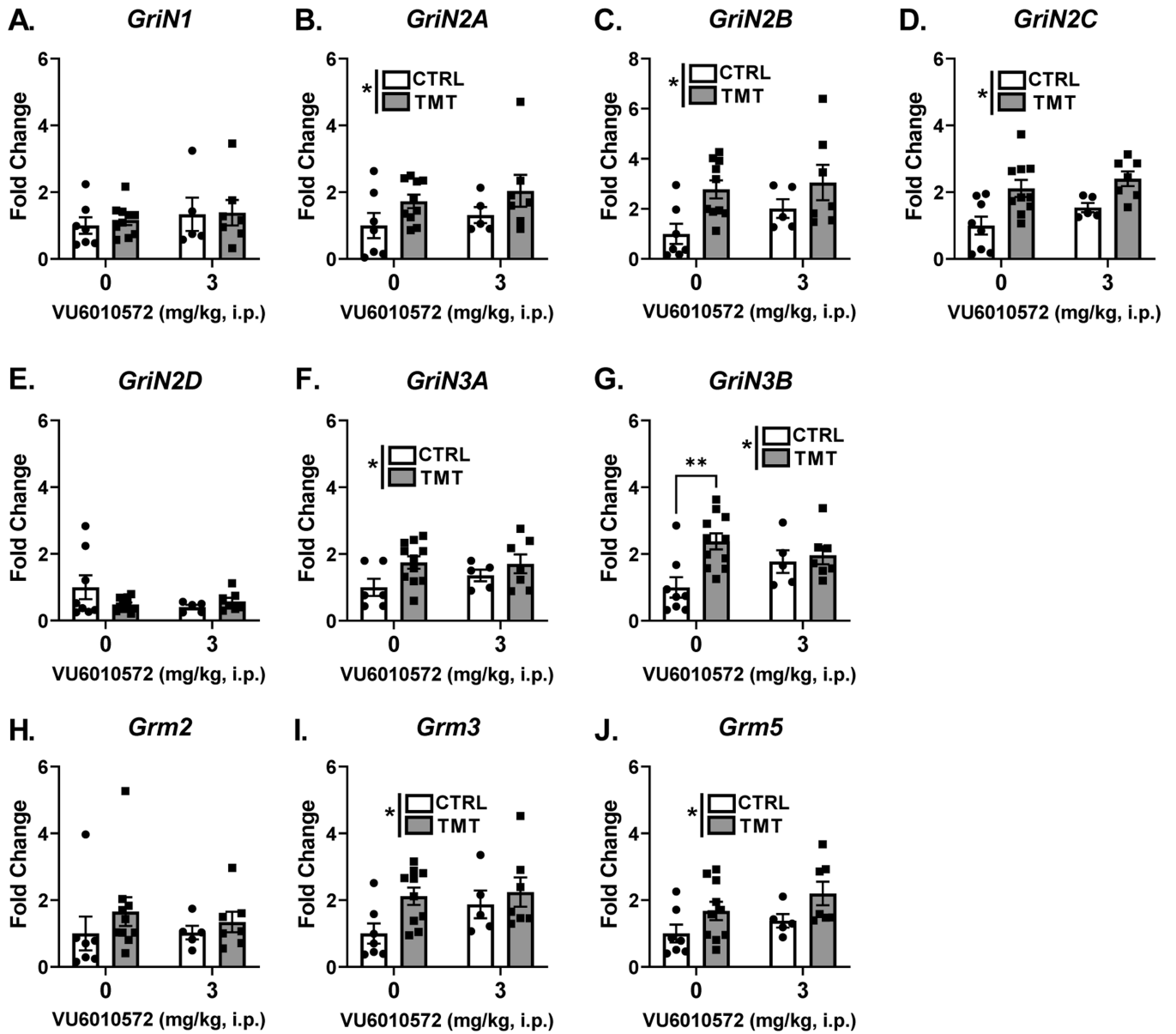


Figure 5. TMT exposure produces lasting increases in glutamate receptor gene expression in the insular cortex.

TMT exposure and mGlu₃-NAM did not affect *GriN1* expression (A). TMT exposure increased expression of *GriN2A* (B), *GriN2B* (C), and *GriN2C* (D). TMT exposure and mGlu₃-NAM did not affect expression of *GriN2D* (E). TMT exposure increased expression of *GriN3A* (F) and *GriN3B* (G). An interaction effect between TMT exposure and mGlu₃-NAM was found for *GriN3B*, and TMT exposure increased expression in the vehicle-treated group, but not the mGlu₃-NAM group (G). *Grm2* expression was not affected by TMT exposure and mGlu₃-NAM (H). TMT exposure increased expression of *Grm3* (I) and *Grm5* (J). * p 0.05 significantly different from Control.

BNST Gene Expression

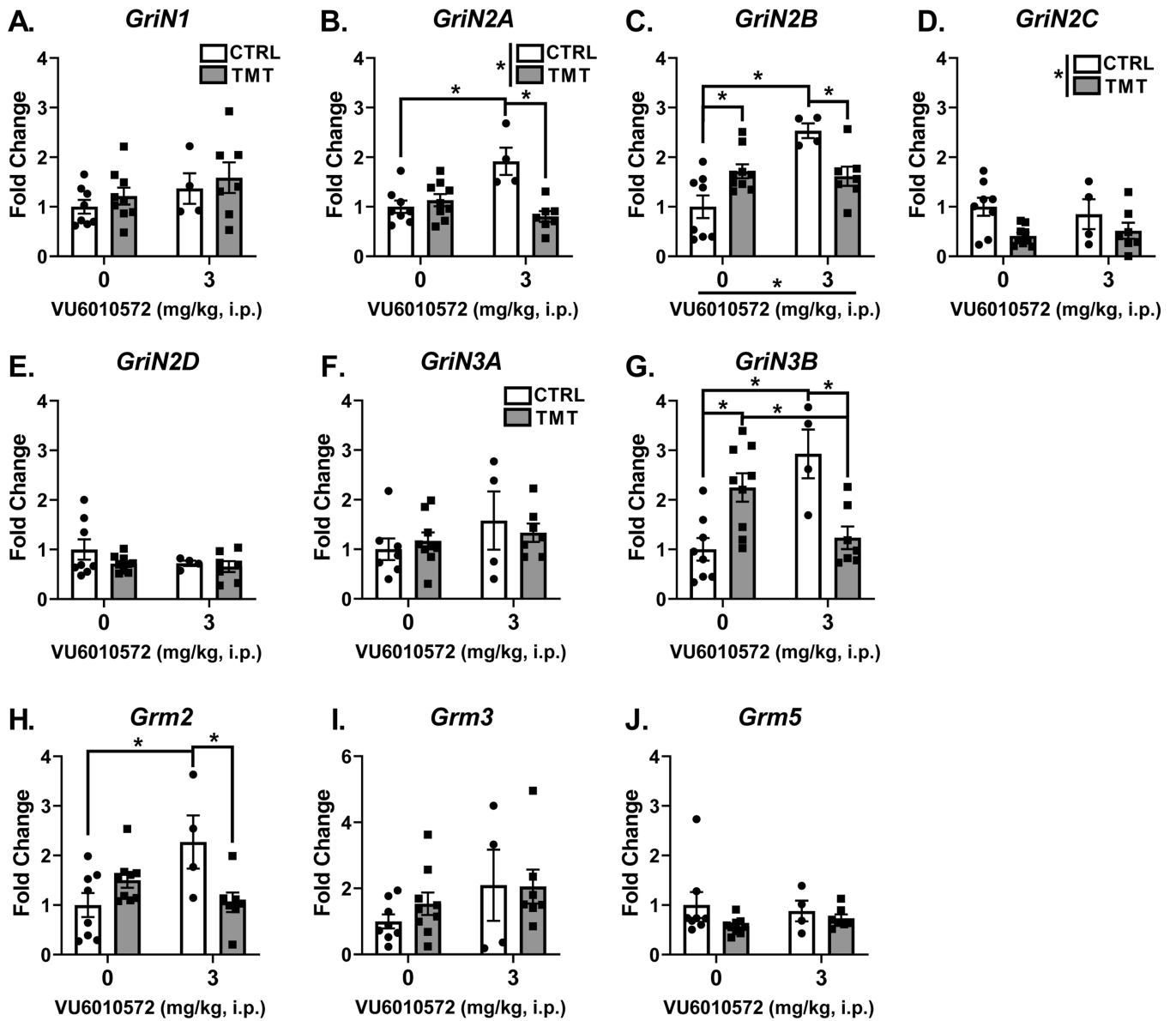


Figure 6. TMT exposure and mGlu₃-NAM produce lasting changes in glutamate receptor transcript levels in the BNST

The TMT exposure and mGlu₃-NAM did not affect *GriN1* (A) gene expression in the BNST. mGlu₃-NAM increased expression of *GriN2A* (B) and *GriN2B* (C) in the control group only. TMT exposure reduced the expression of *GriN2A* and *GriN2B* in the mGlu₃-NAM group. For *GriN2B*, TMT exposure increased expression in the vehicle group, but decreased expression in the mGlu₃-NAM group. TMT exposure decreased expression of *GriN2C* (Fig. 6D). TMT exposure and mGlu₃-NAM did not affect *GriN2D* (E) or *GriN3A* (F) expression. TMT exposure increased *GriN3B* (G) expression in the vehicle group, but decreased expression in the mGlu₃-NAM group. mGlu₃-NAM reversed the stressor-induced upregulation of *GriN3B* (G). mGlu₃-NAM increased *GriN3B* expression in the vehicle

group (G). mGlu₃-NAM increased *Grm2* (H) expression in the vehicle group, but TMT exposure prevented this effect. TMT exposure and mGlu₃-NAM did not affect expression levels of *Grm3* (I) and *Grm5* (J).

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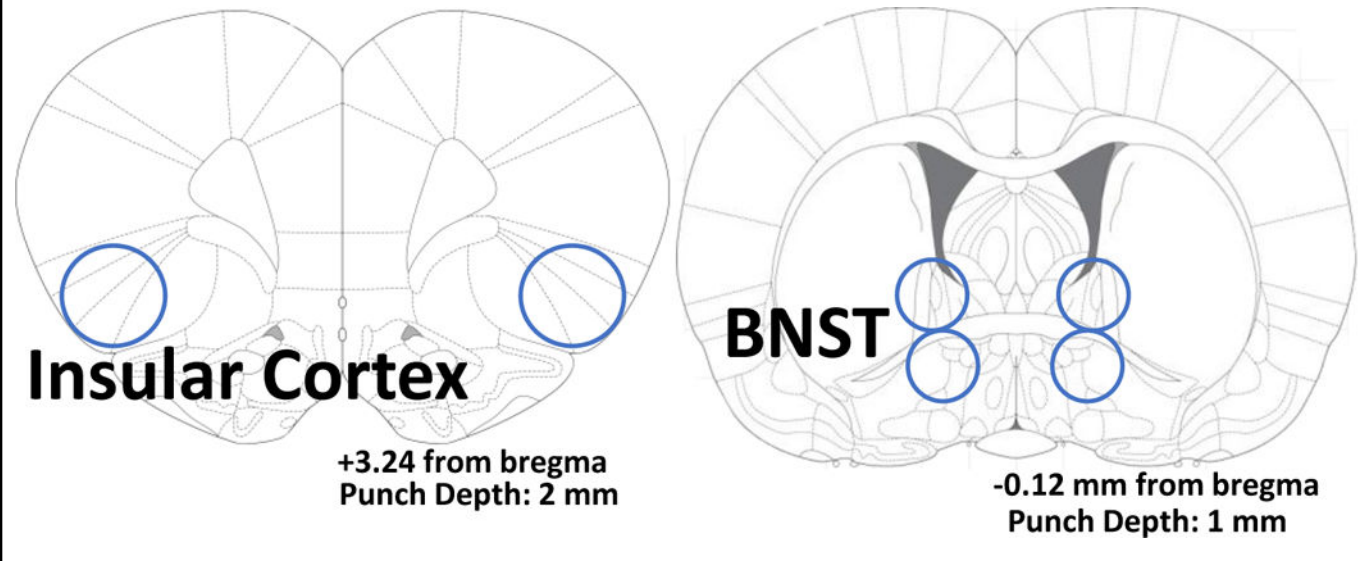
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Table 1 –

qPCR Tissue extraction and Primer Sequences



Primer	Forward (5'–3')	Reverse (5'–3')
<i>GriN1</i>	CTATGACAACAAGCGGGAC	GCCCGTCATGTTTCAGCATTG
<i>GriN2A</i>	GCGGGAACCCGCTAAACC	GCAATACCAGCAAGGTCCAGT
<i>GriN2B</i>	GGGTCACGCAAACCCCTTC	CCTTGTTTTTGACGCCCTG
<i>GriN2C</i>	CAACGTCTTGGTCCCTCA	CTGGGCTTCTCCTCAGC
<i>GriN2D</i>	CCTCATGGGTGGGAAGGAC	TCTGGCTCATAGTCCCA
<i>GriN3A</i>	TCTCTCCATCCTGACCACCAT	CCATCTTCCATCTGCTCTTCT
<i>GriN3B</i>	ACCTGTACGAATGGCGAAG	GTGCGTCAAAGAGAATGGC
<i>Gm2</i>	CCTACAATGTGCTCCTCATCGC	CTGACCTAAAGGCTGGGAATC
<i>Gm3</i>	ATTCTCAGTCTCTGCAAGC	AGACCCTGTCACCAATGCTC
<i>Gm5</i>	CTGGCCTTCGTGCCTATCTA	TTCCGTTGGAGCTTAGGGTT
<i>β-Actin</i>	CTACAATGAGCTGCGTGTGGC	CAGGTCCAGACGCAGGATGGC