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GluN2D expression is regulated by restraint stress and supports active stress coping bouts

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Abstract

Stress coping strategies represent critical responses to environmental challenges, and active coping has been linked to stress resilience in humans. Understanding the neuroadaptations that support these strategies may provide insights into adaptive and maladaptive stress responses. NMDA receptors (NMDARs) play key roles in neuroadaptation, and NMDARs have been specifically implicated in stress responsiveness. Constitutive knockout mice have been used to implicate the GluN2D NMDAR subunit in regulation of stress-sensitive and affective behavior, but the brain regions in which GluN2D expression changes drive these effects remain unknown. Here we report that following an acute restraint stressor, GluN2D subunit expression is specifically decreased in the bed nucleus of the stria terminalis (BNST), a key region involved in stress processing, in male but not female mice, with no differences found in the thalamus or ventral hippocampus in either sex. Rodents engage in active struggling events during restraint stress that may represent active coping strategies to stress. Thus, we assessed active coping bouts during acute and chronic restraint stress sessions in GluN2D knockout mice. During the first restraint session, GluN2D knockout mice exhibited a pronounced decrease in struggling bouts during restraint stress relative to wild-type littermates, consistent with a role of GluN2D in active coping responses to stress. Repeated, daily restraint sessions revealed a sex-specific role of GluN2D expression on certain aspects of active coping behaviors, with male GluN2D KO mice exhibiting a decrease in total coping bouts measured across five sessions. However, BNST-specific knockdown of GluN2D in male mice did not alter active coping bouts, suggesting either a multi-synaptic role of GluN2D

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and/or a developmental role of GluN2D in this behavior. Altogether, these data are consistent with a growing literature suggesting that exploration of GluN2D control of stress circuit actions may lead to a novel therapeutic target to consider for stress-related mood disorders.

Keywords

BNST; GluN2D; Restraint stress; Coping strategies

1. Introduction

Stress is a contributor to a variety of neuropsychiatric diseases. The method by which organisms respond and adapt to stressors may in part determine their susceptibility or resilience to pathological outcomes (Koolhaas et al., 1999; Wood and Bhatnagar, 2015). Stress coping strategies can be divided into active and passive behaviors. Active coping requires the organism to engage its own resources in an attempt to reduce the harmful impact of the stressor while passive coping instead relies on external sources (Folkman and Lazarus, 1980). This selection is significant, as active coping has been linked to stress resilience in humans (Southwick et al., 2005) and active vs. passive coping strategies have been associated with differences in hypothalamic–pituitary–adrenal (HPA) activity in animal models (De Boer et al., 1990; Koolhaas et al., 1999; Veenema et al., 2003). Understanding the neural mechanisms by which these strategies arise may provide insight into treatments that support stress resilience.

N-methyl-D-aspartate receptors (NMDARs) have emerged as potential therapeutic targets for the treatment of mood disorders and have been implicated in stress responsiveness (Hashimoto, 2009; Sanacora et al., 2008). NMDARs are ionotropic, heteromeric complexes composed of two GluN1 subunits with two GluN2 and/or GluN3 subunits (Traynelis et al., 2010). Specific subunit composition of NMDARs plays a major role in defining functionality, as specific subunits impart unique biophysical properties for the receptors (Paoletti et al., 2013; Traynelis et al., 2010). Stress is capable of inducing neuroadaptations in NMDAR subunit expression. For example, chronic restraint stress has been shown to drive changes in glutamatergic subunit expression in the hippocampus of juvenile rats (Sun et al., 2020) and acute and chronic restraint stress are capable of altering hippocampal NMDAR function in adult rats (Tse et al., 2021). While other GluN subunits, such as GluN2A and GluN2B, have been studied in this context, little is known about the GluN2D subunit.

The GluN2D subunit exhibits substantially restricted CNS expression during adulthood compared to expression during early development (Monyer et al., 1994b; Traynelis et al., 2010; Vyklicky et al., 2014). During development, *grin2d* is highly expressed, particularly in midbrain regions, but expression significantly decreases as animals age into adulthood and is hypothesized to be restricted to key populations of cells (Monyer et al., 1994b). Of particular interest to the field has been GluN2D-expressing populations in the adult hippocampus, cerebellum, and bed nucleus of the stria terminus (BNST), given GluN2D's ability to alter cellular function in these regions (Dubois et al., 2016; Dubois and Liu, 2021;

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Eapen et al., 2021; Perszyk et al., 2016; Salimando et al., 2020). Specifically, these subunits confer highly unique biophysical properties to NMDARs, including slow decay kinetics, magnesium insensitivity, heightened glutamate sensitivity, and low probability of opening (Qian et al., 2005; Vicini et al., 1998). Further, deletion of GluN2D disrupts long term potentiation as well as alters excitatory and inhibitory transmission in these regions (Dubois et al., 2016; Dubois and Liu, 2021; Eapen et al., 2021; Perszyk et al., 2016; Salimando et al., 2020). The limited expression of GluN2D in the adult brain and unique function of GluN2D-containing NMDARs highlight these receptors as potential therapeutic targets; however, our understanding of their role in stress responses is currently limited.

Changes in NMDA receptor composition have been shown to be relevant for animal behavior. This is true for GluN2D-containing NMDARs as well, as deletion of GluN2D is known to drive an increase in anxiety- and depressive-like behaviors in adult mice, as indicated by significant increases in time spent immobile in both forced swim and tail suspension tasks and decreases in time spent in the center of an open field arena, time spent in the open arms of an elevated zero maze, and sucrose intake during a sucrose preference test (Hagino et al., 2010; Salimando et al., 2020; Shelkar et al., 2019; Yamamoto et al., 2017). Together, these data suggest the contribution of GluN2D-containing NMDARs to stress-sensitive behaviors. Of note are the forced swim and tail suspension data (Salimando et al., 2020; Yamamoto et al., 2017), as these suggest that GluN2D may decrease active coping bouts exhibited during an acute stressor. Given the ability for acute restraint stress to regulate the expression of other NMDAR subunits and the contribution of GluN2D expression in driving negative affective behaviors, we hypothesized that GluN2D may be a key protein in modifying coping behaviors during a restraint stressor. Thus, we explored the impact of restraint stress on GluN2D expression and used constitutive and conditional deletion of GluN2D to assess the impact of GluN2D expression on active coping bouts exhibited during acute restraint stress.

2. Methods

2.1. Animals

Male and female mice of at least 8 weeks of age were used in these studies. For western blot experiments, male and female C57BL/6J mice (8 weeks of age) were purchased from Jackson Laboratory (#000664). Prior to the start of experiments, mice were allowed to habituate to the animal facility for at least 7 days.

For GluN2D manipulations, constitutive GluN2D knockout mice were originally purchased from RIKEN Experimental Animal Division Repository (RBRC, #01840) and bred heterozygously in house. Conditional GluN2D knockdown mice (*Grin2d*$^{\text{tm1c[EUCOMM]Wtsi}}$, EMMA ID:04857) were rederived and bred homozygously in house. Transgenic lines were maintained on a C57BL/6J background and genotyped at 3 weeks of age using the following primers (Salimando et al., 2020), respectively:

GluN2D Primer 1: 5’- GCA GGC CCC TGC CTC CTC GCT C -3’

GluN2D Primer 2: 5’- CTG ACC TCA TCC TCA GAT GAG -3’

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GluN2D Neo: 5’- TGG ATT GCA CGC AGG TTC TC -3’

FlxGluN2D Forward: 5’- GTG TGA CCA GGA AGC CAC TT -3’

FlxGluN2D Reverse: 5’- TCC TTG ATC CCG TCC CTC AA -3’

Unless otherwise noted, all mice were group housed on a standard 12 hr light-dark cycle at 22–25°C with food and water available *ad libitum*. All behavioral assays took place during the light phase. All experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC) and were carried out in accordance with the guidelines set in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2. Viral-mediated gene transfer

2.2.1. Surgical procedure—Stereotaxic surgeries were performed following established procedures (Salimando et al., 2020). Mice were anesthetized with isoflurane (initial dose: 3%, maintenance dose: 1.5%) and received bilateral intra-dlBNST infusions (0.3 μL) of rAAV5/CMV-Cre-recombinase (Cre)-GFP or rAAV5/TR-eGFP (University of North Carolina GTC Vector Core) at established coordinates (from bregma: A/P +0.14, M/L ±0.88, D/V −4.24, 15.03° tilt) (Salimando et al., 2020). Mice were allowed to recover for at least 21 days before the start of behavioral testing to allow for Cre-mediated gene deletion and the degradation of remaining GluN2D in infected cells. Following the completion of behavioral assays, viral targeting was confirmed using standard histological methods.

2.2.2. Viral targeting—At the completion of behavioral studies, mice were sacrificed and perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Following cryopreservation in 30% sucrose-PBS, brains were sliced into 30 μm sections and GFP labeling was used to confirm viral targeting. Mice with GFP expression outside of the dlBNST or with unilateral hits were excluded from analysis.

2.3. Restraint stress protocols

2.3.1 Restraint stress for the generation of western blot tissue—Following previously published methods (Luchsinger et al., 2021), mice were handled for 3 days prior to the start of restraint stress procedures. Acute stress groups were restrained in a conical tube with air holes for 30 minutes. Control groups were handled only. Immediately following the restraint stressor or control handling, all mice were singly housed in a clean cage and placed in a sound attenuating chamber for 60 minutes prior to sacrifice. Following this period, mice were sacrificed and the dorsal BNST, thalamus (midline nuclei), and ventral hippocampus (vHPC) were microdissected and stored at −80°C until processing.

2.3.2 Restraint stress for active coping bout measures—Mice were control handled for 3 days prior to the start of restraint stress procedures. During the restraint stressor, mice were placed in a RESTRAINT device (Luchsinger et al., 2021), with two devices arranged side-by-side. Their behavior was filmed from above, with the restrainers occupying as much of the viewing field as possible. The restraint stress occurred for 30 minutes each day on five consecutive days. Videos were analyzed using EthoVision XT

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software (Noldus) using modified methods (Grissom et al., 2008). Briefly, struggle bout number and duration were detected using the activity parameter for analysis. The arena encompassed the mouse from tip of nose to tip of tail. The detection threshold for a bout (movement) was determined as greater than 1% pixel change between video frames. For high activity bout measurements, the detection threshold was set as greater than 5% change was recorded, and low activity bout measurements were measured as between 1 and 5% change. Inactivity was recorded as less than 1% change.

2.3.4 Calculating change in body weight—The change in body weight over five consecutive restraint stress sessions were calculated as the percent change in weight from days one to five of restraint stress. The weight on both days one and five were taken just prior to placing the mice in the restraint devices.

2.4. Western blots

For western blot analysis of total levels of GluN2D and GluN2B subunit expression, tissue punches were homogenized in RIPA buffer containing phosphatase and protease inhibitors (Sigma), and the lysate was centrifuged at 20,000g (4°C) for 15 minutes. The supernatants were collected, and protein concentrations were determined by BCA Assay (ThermoFisher). Laemmli buffer was added, and samples (20 μg) were loaded into precast SDS 4–15% gradient gels, electrophoresed, and transferred to PVDF membranes. Membranes were blocked in 5% nonfat dairy milk (NDFM) in a 0.1% Tween 20 phosphate buffered saline (PBST) solution for 1 h at 25°C prior to an overnight incubation at 4°C with primary antibodies in a 5% bovine serum albumen (BSA) PBST solution. The following day, PBST was used to wash the membranes before incubating them in secondary antibody conjugated to horseradish peroxidase for 1 h at 25 °C in 5% NFDM in PBST. Membranes were washed in PBST, and enhanced chemiluminescence (ThermoFisher) was used to visualize protein bands. All data are normalized to the GAPDH loading control. Primary antibodies were used as follows: GluN2D (Millipore, MAB5578, 1:3,000), GluN2B (BD Biosciences, 610416, 1:3,000), GAPDH (Millipore, MAB374, 1:20,000). The secondary antibody was used as follows: anti-mouse HRP (Vector Labs, PI-2000, 1:40,000).

2.6. Statistics

Full statistical analyses and results are listed in Supplemental Table 1. All statistical analyses were performed using GraphPad Prism, and all values are represented as mean ± SEM. An unpaired t-test was used to compare means of two groups. A two-way analysis of variance (ANOVA) was used when comparing two independent variables followed by a Sidak post-hoc test when appropriate, and a two-way ANOVA with repeated measures (RM) was used when comparing two independent variables across multiple time points. A three-way ANOVA with repeated measures (RM) was used when comparing three independent variables across multiple time points. Significance was defined as *$p<0.05$ and **$p<0.01$.

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3. Results

3.1. Regulation of GluN2D and GluN2B expression in stress-sensitive brain regions.

To assess potential changes in NMDA receptor subunit expression in the context of stress, male and female mice underwent an acute 30-minute restraint stress, and total GluN2D and GluN2B subunit expression was measured via western blot. The BNST, thalamus, and ventral hippocampus were chosen due to their known roles in stress behaviors as well as expression of GluN2D in adulthood. Alterations in GluN2D or GluN2B expression were not observed in the thalamus or ventral hippocampus following acute restraint stress (Figure 1B–C). However, in the BNST we found a significant main effect of stress ($F_{(1,23)}$=5.23, p=0.03) on GluN2D subunit expression and an interaction of stress × sex ($F_{(1,23)}$=7.52, p=0.01). Specifically, GluN2D expression was found to be decreased in stressed males compared to control handled males, with no effect observed in female mice (p=0.008, Fig. 1A), suggesting that regulation of this subunit may be sex-dependent. Interestingly, no effect of stress ($F_{(1,24)}$=1.0, p=0.33) or interaction ($F_{(1, 24)}$=1.0, p=0.33) was observed for GluN2B regulation in the BNST of male or female mice (Fig. 1A). These results hint at potential sex differences in the regulation of GluN2D by stress and suggest that the BNST may be a key region in which this neuroadaptation occurs.

3.2. Constitutive GluN2D knockout mice exhibit altered active coping bouts during acute restraint stress.

As GluN2D was found to be regulated by acute restraint stress, we next sought to define the effects of altered gene expression on restraint stress-related behaviors. Stress coping strategies have previously been characterized during restraint stress, specifically with measures of struggling movements interpreted as active coping bouts (Brown et al., 2022; Grissom et al., 2008; Luchsinger et al., 2021; Patel et al., 2005). These movements were interpreted as behaviorally meaningful, as bout activity adapted over the time course of the stress (Patel et al., 2005), was sensitive to previous stress experiences (Grissom et al., 2008), and was correlated with neuroadaptations over repeated stress exposures (Brown et al., 2022; Luchsinger et al., 2021). As constitutive GluN2D knockout has been shown to induce negative affective behaviors, including decreased mobility in both forced swim and tail suspension tests (Salimando et al., 2020; Shelkar et al., 2019; Yamamoto et al., 2017), we used this mouse model to determine the impact of GluN2D deletion on active coping bouts exhibited during restraint stress. Specifically, we predicted that as GluN2D KO mice display enhanced anxiety- and depressive-like behaviors, these mice may also show deficits in active coping behaviors during a stressor. Male and female GluN2D knockout mice and their wild-type (WT) littermates underwent a single 30-minute restraint stress. Struggle movements were recorded during the stress and analyzed using EthoVision software to determine the total number and duration of active coping bouts. Here, a main effect of genotype ($F_{(1, 43)}$=9.15, p=0.004) was observed on total bouts exhibited during an acute restraint stress, with a decrease in active coping bouts observed in GluN2D KO mice compared to WT littermates (Fig. 2A). These effects were consistent when observed as the number of bouts across the 30 minute restraint session, with main effects of genotype ($F_{(1, 43)}$=9.08, p=0.004) and time ($F_{(3.596, 154.6)}$=3.19, p=0.02) (Fig. 2A). Next, we analyzed the total time spent in active coping bouts during this acute stressor and found a main

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effect of genotype ($F_{(1, 43)}$=7.88, p=0.008). Consistent with the number of bouts, GluN2D KO mice spent less time in active coping bouts as compared to their WT littermates (Fig. 2B). When the duration of coping bouts was binned across time, a main effect of genotype was observed ($F_{(1, 43)}$=7.08, p=0.01) (Fig. 2B). Together, these data indicate that mice lacking GluN2D show deficits in active coping behaviors, suggesting a potential role for GluN2D-subunit expressing NMDARs in stress resilience.

3.3. Breakdown of active coping bouts into high and low levels of activity reveals a potential shift in coping strategies.

Active coping bouts have been previously broken down into high and low level movements, as more strongly active bouts have been shown to be associated with greater calcium transients in stress sensitive brain regions (Luchsinger et al., 2021). Additionally, low vs. high mobility during active bouts are sensitive to change when mice underwent repeated restraint stress (Grissom et al., 2008). These data suggest that the types of movements exhibited during coping bouts may be behaviorally meaningful. To explore this idea further, we applied a cutoff for low vs. high mobility during the measured active coping bouts to divide identified bouts in high and low activity portions. Interestingly, no effects were observed in high activity duration (Fig. 2C); however, a main effect of genotype ($F_{(1, 43)}$= 9.54, p=0.004) was observed on low activity duration (Fig. 2D). Though changes in time spent in high activity movements were not observed, these data may indicate a shift in coping strategies exhibited by the GluN2D KO mice.

3.4. Repeated restraint stress reveals a sex-specific influence of GluN2D expression on total active coping bouts.

As there was no main effect of sex observed following a single restraint stress session, assessment of coping bouts was extended to a total of five consecutive days of restraint stress to test the hypothesis that chronic restraint stress may reveal sex differences in coping behavior associated with a repeated stressor. With respect to the total number of coping bouts exhibited during repeated restraint stress sessions, there were main effects of sex ($F_{(1, 43)}$=8.59, p=0.005) and genotype ($F_{(1, 43)}$=23.91, p<0.0001) as well as an interaction of sex × genotype ($F_{(1, 43)}$=8.36, p=0.006) (Fig. 3A) on bout number, and there were main effects of time ($F_{(3.246, 139.6)}$=8.18, p<0.0001) and genotype ($F_{(1, 43)}$=10.85, p=0.002) as well as an interaction of sex × genotype ($F_{(1, 43)}$=17.47, p=0.0001) and a nonsignificant trend of a time × sex × genotype interaction ($F_{(4, 172)}$=2.14, p=0.078) on the total duration of time spent in active coping bouts across repeated restraint stress sessions (Fig. 3B). Due to the identified sex difference in total coping bout number (Fig. 3A), we separated the sexes to further compare genotypes within each sex. In males, there was a main effect of genotype ($F_{(1, 21)}$= 25.39, p<0.0001), yielding fewer coping bouts as a result of GluN2D deletion (Fig. 3C, left). Females showed no main effect of genotype ($F_{(1, 22)}$= 2.42, p=0.13) (Fig. 3C, right), indicating that gene deletion did not alter active coping bout number in this sex. Finally, as mice have been shown to lose body weight over the course of chronic restraint stressors (Bollinger et al., 2016), we calculated a percent change in body weight over the five sessions. Males and females were analyzed separately due to known sex differences in body weight and weight loss induced by repeated restraint stress (Bollinger et al., 2016). There was a nonsignificant trend toward male GluN2D KO mice losing less

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body weight compared to WT littermates (p=0.07), while no difference was seen between female groups (Fig. 3D). These data hint at another aspect of maladaptive behavior present in the male GluN2D KO mice; however, more experiments are needed to determine the source. Together, these data indicate that GluN2D expression alters specific measures of active coping bout behaviors during repeated restraint stressors in a sex-specific manner.

3.5. Decreases in active coping bouts are not driven by changes in BNST GluN2D expression.

Though data collected from the constitutive GluN2D KO mice have been critical in defining a role for GluN2D expression in active coping strategies, it remains unknown where this change in gene expression may be critical for driving the observed deficit in behavior. Therefore, we used a floxed GluN2D mouse line to begin to assess the role of gene knockdown on behavior in a region-specific manner. As male mice showed a significant decrease in total GluN2D expression in the BNST following an acute restraint stress (Fig. 1A) and gene knockdown is known to disrupt synaptic transmission in this region (Salimando et al., 2020), we sought to define the role of BNST GluN2D expression on active coping behaviors during acute restraint stress. This idea is further supported by data showing that BNST knockdown of GluN2D is sufficient to drive increased negative affect, including the ability for BNST GluN2D knockdown to replicate constitutive KO effects of increased mobility in a single forced swim test (Salimando et al., 2020). This experiment used only male mice, as female mice did not show changes in BNST GluN2D expression following an acute restraint stressor (Fig. 1A) and no effect of genotype was found on coping bout number in female GluN2D KO mice (Fig. 3). An AAV containing Cre-recombinase or a GFP control was bilaterally injected into the BNST of male GluN2D floxed mice (Fig. 4A). Following three weeks to allow for viral expression and degradation of GluN2D protein, mice underwent a 30 minute session of restraint stress to assess changes in active coping bouts. Movements were recorded from above and analyzed using EthoVision software, as described above. Following behavioral analysis, no statistical differences were observed in the BNST GluN2D KD mice compared to GFP controls in either the total number of active coping bouts or the total duration of time spent in active coping bouts (Fig. 4B–C); however, there was a main effect of time ($F_{(5, 60)}= 8.416$, $p<0.0001$) on the total number of coping bouts measured, with mice engaging in more bouts during the 15, 20, 25, and 30 minute timepoints compared to those measured in the first 5 minutes. Similarly, no differences were observed in time spent in high or low activity (Fig. 4D–E). These data indicate that while GluN2D expression may be decreased in the BNST of male mice following acute restraint stress (Fig. 1A), this expression is not sufficient to induce a decrease in active coping bouts during an acute stressor.

4. Discussion

Stress coping strategies represent critical responses to environmental challenges, therefore, understanding the neuroadaptations that support these strategies can provide insights into adaptive and maladaptive stress responses. In the present study we found acute restraint stress produces region and sex-specific alterations in GluN2D expression, with a decrease in GluN2D protein observed in male BNST. Further, our data suggest that GluN2D expression

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is a contributor to active coping bout responses to stress. Constitutive GluN2D knockout mice displayed fewer coping bouts during an acute restraint stressor as compared to WT littermates. Then, when expanded out to a chronic restraint context, male mice specifically displayed a decrease in total measured coping bouts across repeated stressors, while there was not a main effect of sex on total time spent in coping bouts. These data led us to hypothesize that BNST-specific regulation of GluN2D in male mice may contribute to the observed behavioral effects. However, expression in the BNST itself may not drive these changes in coping behavior, as male mice with a BNST-specific knockdown of GluN2D did not show altered active stress coping compared to GFP controls.

4.1. Regulation of NMDAR subunits by restraint stress exposure.

NMDAR subunits are known to be regulated by restraint stress. We showed that total protein expression of GluN2D is significantly decreased in the BNST of male mice following an acute restraint stressor. The literature has previously explored GluN2A and GluN2B expression following restraint stress, but to our knowledge this is the first measurement of GluN2D subunit expression following any stressor. Previous studies assaying changes in GluN2A and GluN2B subunits also raise interesting points related to receptor trafficking. For example, surface expression vs. total protein expression is differently regulated in the PFC by restraint stress, as increases in GluN2A- and GluN2B-containing NMDAR surface expression are observed following acute restraint stress without changes in total protein levels (Yuen et al., 2009; Yuen et al., 2012). Additionally, synaptic vs. extrasynaptic expression of NMDARs is differently regulated by acute and chronic restraint in the hippocampus of adult rats (Tse et al., 2021), suggesting yet another level of regulation in NMDAR surface expression. These changes may further be measured by subunit phosphorylation, as GluN2B phosphorylation at key residues is modified by acute restraint stress (Ai et al., 2017) and has been linked to depressive-like behaviors (Shi et al., 2021). Together, these findings suggest that while no changes in total GluN2B expression in the BNST, thalamus, or ventral hippocampus of mice were observed following acute restraint stress, other types of regulation may have occurred.

GluN2D expression is likely also regulated by a number of factors. In the hippocampus, assembly of NMDARs with a GluN2D subunit is hypothesized to affect membrane trafficking, thus potentially modifying where these receptors are expressed and altering synaptic signals (Perszyk et al., 2016). GluN2D-containing NMDARs are believed to be largely extrasynaptic in localization (Brickley et al., 2003; Harney et al., 2008; Yao et al., 2022); however, these receptors can be recruited to the synapse in a transient manner (Harney et al., 2008). Additionally, given the capacity for phosphorylation to alter function of other GluN subunits, it could be predicted that GluN2D phosphorylation would also contribute to changes in receptor function or localization. While three serine residues have been identified as phosphorylation sites via mass spectrometry (Traynelis et al., 2010) and GluN2D is known to undergo tyrosine phosphorylation *in vivo* (Dunah et al., 1998), the functionality of these modifications remains unknown. Unfortunately, no commercially available tools are currently available to assay phosphorylated GluN2D, however, this remains a key area of future investigation.

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4.2. Characterization of stress coping behaviors during restraint stressors.

Apart from biochemical mechanisms discussed above, few studies have characterized stress coping strategies that occur during restraint stress. Of particular interest are active coping behaviors, where an organism attempts to reduce the harmful impact of the stressor using its own resources, as these actions have been linked to stress resilience in humans. A small number of previous studies have similarly identified changes in active coping bout behaviors during restraint stress in rat models. In two studies conducted by Patel et al. and Grissom et al., a decrease in active coping bouts was identified over the time-course of an acute restraint stressor (Grissom et al., 2008; Patel et al., 2005). These studies highlight the first 5 minutes as a significant window for behavioral responses. Additionally, Grissom et al. defined time spent highly active as a key behavioral marker, as time spent highly mobile could be facilitated by prior stress exposure (Grissom et al., 2008). Interestingly, our studies here did not replicate a decrease in duration spent in active coping bouts over the course of the restraint stressor (Fig. 2B and Fig. 4C). Additionally, our studies indicated that time spent in low, but not high, activity bouts drove genotype effects on overall activity (Fig. 2C). These differences suggest a potential species difference in how active coping bouts are performed in rodent models, as previous work was performed in rats. Alternatively, differences in how bouts were defined and measured may have yielded the discrepancies. Specifically, previous studies have utilized hand scoring (Patel et al., 2005), behavioral analysis software (EthoVision) (Grissom et al., 2008), and machine learning approaches (DeepLabCut) (Brown et al., 2022; Luchsinger et al., 2021) to detect and measure animal behavior, each of which have distinct advantages and disadvantages. It is possible that nuances of active coping bouts may have been better detected in one method compared others.

4.3. Contribution of GluN2D-containing NMDARs to stress- and negative affect-related behaviors.

In the present study, we found that constitutive knockout of GluN2D reduced both the number of and duration of active coping bouts exhibited during a single restraint stress session (Fig. 2), and this decrease in bout duration was driven by a decrease in time spent specifically in low activity movement (Fig. 2). However, there was no main effect of sex in this acute context. When mice underwent five consecutive restraint stress sessions, a sex-dependent effect of GluN2D KO on behavior was observed (Fig. 3A). Specifically, male GluN2D KO mice showed fewer active coping bouts compared to their WT littermates, with no effect of genotype observed in females (Fig. 3C). However, not all measures of active coping were similarly affected, as a main effect of sex was not observed in the total bout duration across repeated restraint stress sessions (Fig. 3B). There was also a trend toward male GluN2D KO mice losing less weight than their WT littermates, with no effect detected in female mice (Fig. 3D). Together, these data suggest that GluN2D may contribute to aspects of active coping behaviors, particularly those driven by repeated stress exposure, in a sex-specific manner.

Our data demonstrating decreased active coping measures in GluN2D knockout mice are consistent with other measures of negative affect assessed in these mice. Previous work in this mouse line has demonstrated that GluN2D deletion induces a significant increase in

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anxiety- and depressive-like behaviors. Specifically, these mice display an increase in time spent immobile in both forced swim and tail suspension tasks and decreases in time spent in the center of an open field arena, time spent in the open arms of an elevated zero maze, and sucrose intake during a sucrose preference test (Hagino et al., 2010; Salimando et al., 2020; Shelkar et al., 2019; Yamamoto et al., 2017). Of note, the forced swim data (Salimando, 2020; Shelkar et al., 2019) and tail suspension data (Yamamoto et al., 2017) are consistent with GluN2D KO mice displacing decreased active coping behaviors.

It is important to note that GluN2D KO mice exhibit a decrease in locomotor activity during some behavioral test, including an open field task (Hagino et al., 2010; Salimando et al., 2020; Yamamoto et al., 2017); however, we do not believe that this drove the decrease in active coping bouts observed during restraint stress. First, while Hagino et al. showed a decrease in locomotor activity during an open field task, these mice did not show any motor deficits in a rotarod test (Hagino et al., 2010), highlighting intact motor coordination and learning in constitutive GluN2D KO mice. Though Salimando et al. similarly reported a decrease in locomotor activity during an open field task, they also reported no differences in activity during light/dark box and elevated zero maze tasks (Salimando et al., 2020), indicating that hypolocomotion is not observed in all assays. Finally, Yamamoto et al. reported a decrease of locomotor activity during a marble burying test; however, no differences were observed in the number of marbles buried between genotypes (Yamamoto et al., 2017), suggesting that altered locomotion in this test did not prevent the mice from preforming the assay. In the model of BNST-specific GluN2D knockdown, male mice showed no differences in open field locomotor behavior; however, knockdown mice still showed decreased mobility in a forced swim test (Salimando et al., 2020), consistent the idea that overall locomotor deficits did not drive decreased active coping behaviors.

4.4. The BNST is engaged during restraint stress.

The BNST is a region highly studied for its role in negative effect (Centanni et al., 2019; Harris and Winder, 2018; Kim et al., 2013; Lebow and Chen, 2016). BNST cFos expression is increased by acute restraint stress (Fetterly et al., 2019; Kim and Chung, 2021), suggesting that cells in the BNST are active during this stressor. Further, the ventral BNST has been previously implicated in passive stress coping (Johnson et al., 2019) and the dorsal BNST in active coping (Brown et al., 2022; Luchsinger et al., 2021). Building from these findings, recent work from our lab characterized an increase in both BNST calcium and glutamatergic signaling at the onset of active coping bouts exhibited during restraint stress (Luchsinger et al., 2021). Though we found altered GluN2D expression in the BNST of male mice following acute restraint stress (Fig. 1A) and a BNST-specific approach was previously successful in generating an increase in negative affective behaviors (Salimando et al., 2020), BNST knockdown of GluN2D was not sufficient to replicate behavioral effects observed in the whole body knockout line (Fig. 2 and 4). These data suggest that GluN2D expression in brain regions not sampled by this study or in concert with expression changes across multiple regions may be necessary for the regulation of active coping bouts. Specifically, while the hippocampus and thalamus also express significant levels of GluN2D expression and electrophysiological effects of GluN2D expression in the hippocampus has been recently explored (Berg et al., 2013; Booker et al., 2021; Eapen et al., 2021; Perszyk

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et al., 2016), we did not observe a regulation of subunit expression by acute restraint stress, leading us to believe that these regions may not be key sites contributing to the decrease in bouts observed in the constitutive GluN2D KO model; however, given their position within stress-sensitive neurocircuitry, this possibility cannot be ruled out. In addition to these regions, GluN2D expression in the cerebellum influences GABAergic plasticity and release (Dubois et al., 2016; Dubois and Liu, 2021) and has been shown to regulate fear extinction learning (Dubois and Liu, 2021). Given these data, future studies will seek to identify which region(s) contributes to the bout effects observed in the constitutive KO model.

Further, there are significant advantages and disadvantages in using constitutive and conditional knockout/down models. Developmental vs. adult deletion of GluN2D in the two mouse models could contribute to the differences in behavior observed. The expression of *grin2d* is greatest during early development but decreases over time, ultimately yielding low levels in the adult brain (Monyer et al., 1994a). Therefore, deletion of GluN2D during early development in the constitutive knockout mouse line (Fig. 2) may have contributed to phenotypic differences not observed when knockdown was both region-constrained and only occurred during adulthood in the conditional model (Fig. 4). Additionally, as viral-based approaches in floxed mouse lines do not affect all cells in a region, failure to entirely knockout GluN2D expression in the BNST may have contributed to the lack of effect observed in this model. This highlights an advantage of a constitutive approach, though only the conditional model gains region specificity.

4.5. Sex differences in GluN2D expression and coping behaviors during stressors.

To date, few studies have directly analyzed sex differences in response to acute restraint stress. For example, an acute restraint stressor induces divergent effects on medial prefrontal cortex microglial activation in male and female rats as well as results in differentially expressed immune factors (Bollinger et al., 2016). In the present study, we identified a significant decrease in BNST GluN2D expression in male, but not female, mice in response to an acute restraint stressor (Fig 1A). This sex difference in molecular response to restraint stress may in part be driven by differences in neuroendocrine regulation. For example, female rats show higher plasma corticosterone levels both at baseline and in response to a single restraint stressor compared to males (Galea et al., 1997). Additionally, estrogen is able to directly affect the expression of *grin2d*, the GluN2D gene (Watanabe et al., 1999). Neuroendocrine differences may drive differential gene expression or regulation of GluN2D in female mice compared to males.

Further, preclinical studies have revealed sex-dependent stress coping strategies. One prominent study revealed distinct coping responses used by male vs. female rats in a fear conditioning paradigm (Gruene et al., 2015). Gruene et al. identified an active “darting” behavior primarily used by female rats in response to a conditioned fear stimulus (Gruene et al., 2015). Importantly, rats that displayed darting behaviors showed less freezing behavior during extinction conditions (Gruene et al., 2015), consistent with the hypothesis that active stress coping is associated with stress resilience. Recently, additional studies have followed sex-dependent patterns of coping strategies (see (Colom-Lapetina et al., 2019; Kokras and Dalla, 2017; Ornelas et al., 2021)); however, these experiments also highlight the complexity

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of stress-related behaviors when more than traditional measures of anxiety- and depressive-like behaviors are examined. Interestingly, the current study did not find significant sex-specific behavioral effects during the single restraint stressor (Fig. 2), with no main effect of sex observed on the number or duration of active struggle bouts measured during an acute restraint stressor (Fig. 2). However, data collected during a repeated restraint stressor suggest that GluN2D deletion contributes to aspects of maladaptive coping behaviors in male mice that were not observed in females lacking GluN2D (Fig. 3). It is possible that females engaged in coping behaviors not measured in the current study, and it may be that these missed behaviors are sensitive to GluN2D modification. Altogether, continued investigation is necessary to better understand these potentially translationally relevant insights.

4.6. Conclusions

In summary, the present study supports GluN2D as a key NMDAR subunit that supports active coping behaviors during restraint stress, with chronic restraint stress revealing sex-depenent effect of GluN2D deletion on aspects of stress coping behaviors. Interestingly, though GluN2D protein expression was decreased by acute restraint stress in the BNST of male mice, BNST-specific knockdown of GluN2D in male mice did not recapitulate effects on active coping bouts observed in constitutive GluN2D KO mice, suggesting that expression changes in this region alone do not drive changes in active stress coping in male mice. Future studies seek to define region- and circuit-specific contributions of GluN2D function in stress-related behaviors and to better assess potential sex differences in coping strategies exhibited during restraint stressors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References:

Ai H, Shi XF, Hu XP, Fang WQ, Zhang B, Lu W, 2017. Acute stress regulates phosphorylation of N-methyl-d-aspartate receptor GluN2B at S1284 in hippocampus. Neuroscience 351, 24–35. [PubMed: 28359951]

Berg LK, Larsson M, Morland C, Gundersen V, 2013. Pre- and postsynaptic localization of NMDA receptor subunits at hippocampal mossy fibre synapses. Neuroscience 230, 139–150. [PubMed: 23159309]

Bollinger JL, Bergeon Burns CM, Wellman CL, 2016. Differential effects of stress on microglial cell activation in male and female medial prefrontal cortex. Brain Behav Immun 52, 88–97. [PubMed: 26441134]

Booker SA, Sumera A, Kind PC, Wyllie DJA, 2021. Contribution of NMDA Receptors to Synaptic Function in Rat Hippocampal Interneurons. eNeuro 8.

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Brickley SG, Misra C, Mok MH, Mishina M, Cull-Candy SG, 2003. NR2B and NR2D subunits coassemble in cerebellar Golgi cells to form a distinct NMDA receptor subtype restricted to extrasynaptic sites. J Neurosci 23, 4958–4966. [PubMed: 12832518]

Brown JA, Petersen N, Centanni SW, Jin AY, Yoon HJ, Cajigas SA, Bedenbaugh MN, Luchsinger JR, Patel S, Calipari ES, Simerly RB, Winder DG, 2022. An ensemble recruited by alpha2a-adrenergic receptors is engaged in a stressor-specific manner in mice. Neuropsychopharmacology.

Centanni SW, Bedse G, Patel S, Winder DG, 2019. Driving the Downward Spiral: Alcohol-Induced Dysregulation of Extended Amygdala Circuits and Negative Affect. Alcohol Clin Exp Res 43, 2000–2013. [PubMed: 31403699]

Colom-Lapetina J, Li AJ, Pelegrina-Perez TC, Shansky RM, 2019. Behavioral Diversity Across Classic Rodent Models Is Sex-Dependent. Front Behav Neurosci 13, 45. [PubMed: 30894806]

De Boer SF, Slangen JL, Van der Gugten J, 1990. Plasma catecholamine and corticosterone levels during active and passive shock-prod avoidance behavior in rats: effects of chlordiazepoxide. Physiol Behav 47, 1089–1098. [PubMed: 2395914]

Dubois CJ, Lachamp PM, Sun L, Mishina M, Liu SJ, 2016. Presynaptic GluN2D receptors detect glutamate spillover and regulate cerebellar GABA release. J Neurophysiol 115, 271–285. [PubMed: 26510761]

Dubois CJ, Liu SJ, 2021. GluN2D NMDA Receptors Gate Fear Extinction Learning and Interneuron Plasticity. Front Synaptic Neurosci 13, 681068. [PubMed: 34108872]

Dunah AW, Yasuda RP, Wolfe BB, 1998. Developmental regulation of tyrosine phosphorylation of the NR2D NMDA glutamate receptor subunit in rat central nervous system. J Neurochem 71, 1926–1934. [PubMed: 9798917]

Eapen AV, Fernandez-Fernandez D, Georgiou J, Bortolotto ZA, Lightman S, Jane DE, Volianskis A, Collingridge GL, 2021. Multiple roles of GluN2D-containing NMDA receptors in short-term potentiation and long-term potentiation in mouse hippocampal slices. Neuropharmacology 201, 108833. [PubMed: 34637787]

Fetterly TL, Basu A, Nabit BP, Awad E, Williford KM, Centanni SW, Matthews RT, Silberman Y, Winder DG, 2019. α 2A-Adrenergic Receptor Activation Decreases Parabrachial Nucleus Excitatory Drive onto BNST CRF Neurons and Reduces Their Activity In Vivo. J Neurosci 39, 472–484. [PubMed: 30478032]

Folkman S, Lazarus RS, 1980. An analysis of coping in a middle-aged community sample. J Health Soc Behav 21, 219–239. [PubMed: 7410799]

Galea LA, McEwen BS, Tanapat P, Deak T, Spencer RL, Dhabhar FS, 1997. Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. Neuroscience 81, 689–697. [PubMed: 9316021]

Grissom N, Kerr W, Bhatnagar S, 2008. Struggling behavior during restraint is regulated by stress experience. Behav Brain Res 191, 219–226. [PubMed: 18466984]

Gruene TM, Flick K, Stefano A, Shea SD, Shansky RM, 2015. Sexually divergent expression of active and passive conditioned fear responses in rats. Elife 4.

Hagino Y, Kasai S, Han W, Yamamoto H, Nabeshima T, Mishina M, Ikeda K, 2010. Essential role of NMDA receptor channel ε4 subunit (GluN2D) in the effects of phencyclidine, but not methamphetamine. PLoS One 5, e13722. [PubMed: 21060893]

Harney SC, Jane DE, Anwyl R, 2008. Extrasynaptic NR2D-containing NMDARs are recruited to the synapse during LTP of NMDAR-EPSCs. J Neurosci 28, 11685–11694. [PubMed: 18987204]

Harris NA, Winder DG, 2018. Synaptic Plasticity in the Bed Nucleus of the Stria Terminalis: Underlying Mechanisms and Potential Ramifications for Reinstatement of Drug- and Alcohol-Seeking Behaviors. ACS Chem Neurosci 9, 2173–2187. [PubMed: 29851347]

Hashimoto K, 2009. Emerging role of glutamate in the pathophysiology of major depressive disorder. Brain Res Rev 61, 105–123. [PubMed: 19481572]

Johnson SB, Emmons EB, Lingg RT, Anderson RM, Romig-Martin SA, LaLumiere RT, Narayanan NS, Viau V, Radley JJ, 2019. Prefrontal-Bed Nucleus Circuit Modulation of a Passive Coping Response Set. J Neurosci 39, 1405–1419. [PubMed: 30573644]

Kim SY, Adhikari A, Lee SY, Marshel JH, Kim CK, Mallory CS, Lo M, Pak S, Mattis J, Lim BK, Malenka RC, Warden MR, Neve R, Tye KM, Deisseroth K, 2013. Diverging neural pathways

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assemble a behavioural state from separable features in anxiety. Nature 496, 219–223. [PubMed: 23515158]

Kim W, Chung C, 2021. Brain-wide cellular mapping of acute stress-induced activation in male and female mice. FASEB J 35, e22041. [PubMed: 34780680]

Kokras N, Dalla C, 2017. Preclinical sex differences in depression and antidepressant response: Implications for clinical research. J Neurosci Res 95, 731–736. [PubMed: 27870451]

Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, Hopster H, De Jong IC, Ruis MAW, Blokhuis HJ, 1999. Coping styles in animals: current status in behavior and stress-physiology. Neurosci Biobehav Rev 23, 925–935. [PubMed: 10580307]

Lebow MA, Chen A, 2016. Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. Mol Psychiatry 21, 450–463. [PubMed: 26878891]

Luchsinger JR, Fetterly TL, Williford KM, Salimando GJ, Doyle MA, Maldonado J, Simerly RB, Winder DG, Centanni SW, 2021. Delineation of an insula-BNST circuit engaged by struggling behavior that regulates avoidance in mice. Nat Commun 12, 3561. [PubMed: 34117229]

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH, 1994a. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12, 529–540. [PubMed: 7512349]

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH, 1994b. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12, 529–540. [PubMed: 7512349]

Ornelas LC, Tyler RE, Irukulapati P, Paladugu S, Besheer J, 2021. Increased alcohol self-administration following exposure to the predator odor TMT in active coping female rats. Behav Brain Res 402, 113068. [PubMed: 33333108]

Paoletti P, Bellone C, Zhou Q, 2013. NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. Nat Rev Neurosci 14, 383–400. [PubMed: 23686171]

Patel S, Roelke CT, Rademacher DJ, Hillard CJ, 2005. Inhibition of restraint stress-induced neural and behavioural activation by endogenous cannabinoid signalling. Eur J Neurosci 21, 1057–1069. [PubMed: 15787710]

Perszyk RE, DiRaddo JO, Strong KL, Low CM, Ogden KK, Khatri A, Vargish GA, Pelkey KA, Tricoire L, Liotta DC, Smith Y, McBain CJ, Traynelis SF, 2016. GluN2D-Containing N-methyl-d-Aspartate Receptors Mediate Synaptic Transmission in Hippocampal Interneurons and Regulate Interneuron Activity. Mol Pharmacol 90, 689–702. [PubMed: 27625038]

Qian A, Buller AL, Johnson JW, 2005. NR2 subunit-dependence of NMDA receptor channel block by external Mg2+. J Physiol 562, 319–331. [PubMed: 15513936]

Salimando GJ, 2020. Investigating the role of GluN2D-containing NMDARs in BNST excitatory signaling: Implications for regulating cell-specific synaptic function and the modulation of affective behaviors. Vanderbilt University.

Salimando GJ, Hyun M, Boyt KM, Winder DG, 2020. BNST GluN2D-Containing NMDA Receptors Influence Anxiety- and Depressive-like Behaviors and ModulateCell-Specific Excitatory/Inhibitory Synaptic Balance. J Neurosci 40, 3949–3968. [PubMed: 32277042]

Sanacora G, Zarate CA, Krystal JH, Manji HK, 2008. Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. Nat Rev Drug Discov 7, 426–437. [PubMed: 18425072]

Shelkar GP, Pavuluri R, Gandhi PJ, Ravikrishnan A, Gawande DY, Liu J, Stairs DJ, Ugale RR, Dravid SM, 2019. Differential effect of NMDA receptor GluN2C and GluN2D subunit ablation on behavior and channel blocker-induced schizophrenia phenotypes. Sci Rep 9, 7572. [PubMed: 31110197]

Shi X, Zhang Q, Li J, Liu X, Zhang Y, Huang M, Fang W, Xu J, Yuan T, Xiao L, Tang YQ, Wang XD, Luo J, Yang W, 2021. Disrupting phosphorylation of Tyr-1070 at GluN2B selectively produces resilience to depression-like behaviors. Cell Rep 36, 109612. [PubMed: 34433031]

Southwick SM, Vythilingam M, Charney DS, 2005. The psychobiology of depression and resilience to stress: implications for prevention and treatment. Annu Rev Clin Psychol 1, 255–291. [PubMed: 17716089]

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Sun DS, Zhong G, Cao HX, Hu Y, Hong XY, Li T, Li X, Liu Q, Wang Q, Ke D, Liu GP, Ma RH, Luo DJ, 2020. Repeated Restraint Stress Led to Cognitive Dysfunction by NMDA Receptor-Mediated Hippocampal CA3 Dendritic Spine Impairments in Juvenile Sprague-Dawley Rats. Front Mol Neurosci 13, 552787. [PubMed: 33192290]

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R, 2010. Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev 62, 405–496. [PubMed: 20716669]

Tse YC, Nath M, Larosa A, Wong TP, 2021. Opposing Changes in Synaptic and Extrasynaptic N-Methyl-D-Aspartate Receptor Function in Response to Acute and Chronic Restraint Stress. Front Mol Neurosci 14, 716675. [PubMed: 34690693]

Veenema AH, Meijer OC, de Kloet ER, Koolhaas JM, Bohus BG, 2003. Differences in basal and stress-induced HPA regulation of wild house mice selected for high and low aggression. Hormones and Behavior 43, 197–204. [PubMed: 12614650]

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, Grayson DR, 1998. Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. J Neurophysiol 79, 555–566. [PubMed: 9463421]

Vyklicky V, Korinek M, Smejkalova T, Balik A, Krausova B, Kaniakova M, Lichnerova K, Cerny J, Krusek J, Dittert I, Horak M, Vyklicky L, 2014. Structure, function, and pharmacology of NMDA receptor channels. Physiol Res 63, S191–203. [PubMed: 24564659]

Watanabe T, Inoue S, Hiroi H, Orimo A, Muramatsu M, 1999. NMDA receptor type 2D gene as target for estrogen receptor in the brain. Brain Res Mol Brain Res 63, 375–379. [PubMed: 9878838]

Wood SK, Bhatnagar S, 2015. Resilience to the effects of social stress: evidence from clinical and preclinical studies on the role of coping strategies. Neurobiol Stress 1, 164–173. [PubMed: 25580450]

Yamamoto H, Kamegaya E, Hagino Y, Takamatsu Y, Sawada W, Matsuzawa M, Ide S, Yamamoto T, Mishina M, Ikeda K, 2017. Loss of GluN2D subunit results in social recognition deficit, social stress, 5-HT2C receptor dysfunction, and anhedonia in mice. Neuropharmacology 112, 188–197. [PubMed: 27480795]

Yao L, Rong Y, Ma X, Li H, Deng D, Chen Y, Yang S, Peng T, Ye T, Liang F, Xu N, Zhou Q, 2022. Extrasynaptic NMDA Receptors Bidirectionally Modulate Intrinsic Excitability of Inhibitory Neurons. J Neurosci 42, 3066–3079. [PubMed: 35197319]

Yuen EY, Liu W, Karatsoreos IN, Feng J, McEwen BS, Yan Z, 2009. Acute stress enhances glutamatergic transmission in prefrontal cortex and facilitates working memory. Proc Natl Acad Sci U S A 106, 14075–14079. [PubMed: 19666502]

Yuen EY, Wei J, Liu W, Zhong P, Li X, Yan Z, 2012. Repeated stress causes cognitive impairment by suppressing glutamate receptor expression and function in prefrontal cortex. Neuron 73, 962–977. [PubMed: 22405206]

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Highlights

- Total GluN2D protein levels are decreased in the BNST of male, but not female, mice following an acute restraint stressor.
- Constitutive GluN2D knockout mice show a deficit in active coping bout behavior during a single restraint stressor.
- Male constitutive GluN2D knockout mice display fewer active coping bouts across repeated restraint stress sessions.
- Knockdown of GluN2D in the BNST of male mice does not recapitulate deficits in active coping bout behaviors observed during a single restraint session.

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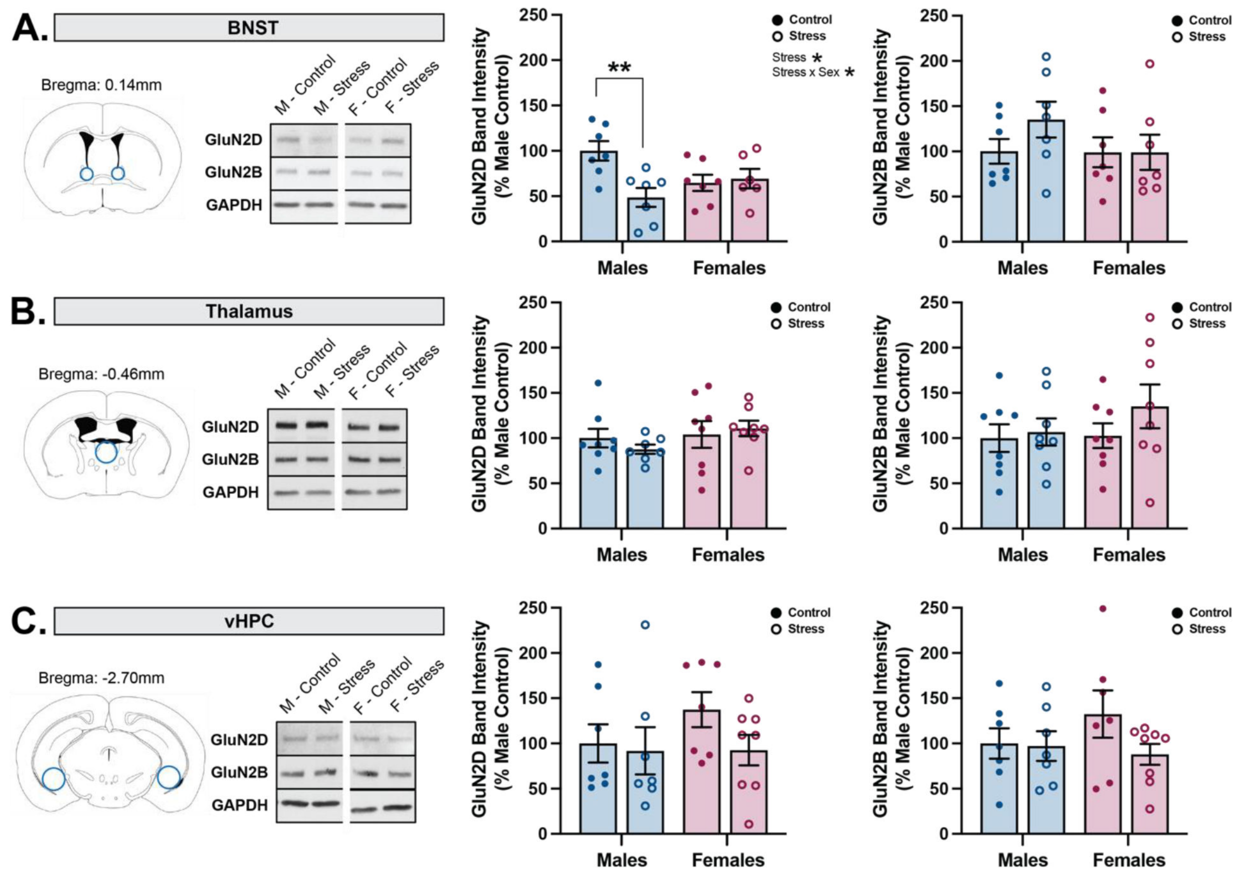

Figure 1. Acute restraint stress downregulates GluN2D expression in the BNST of male, but not female, mice.

A. Expression of the GluN2D subunit is significantly decreased in the BNST of male mice following acute restraint stress (n=6–7, two-way ANOVA followed by a Sidak post hoc test, main effect of stress and an interaction of stress × sex, control male vs. stress male p=0.008) with no effect of GluN2B expression (n=7, two-way ANOVA).

B. Acute restraint stress did not alter GluN2D (n=7–8, two-way ANOVA) or GluN2B (n=8, two-way ANOVA) expression in the thalamus.

C. GluN2D (n=7–8, two-way ANOVA) and GluN2B (n=7–8, two-way ANOVA) expression were also not altered following restraint stress in the ventral hippocampus (vHPC).

*p<0.05, **p<0.001. All data are represented as mean ± SEM.

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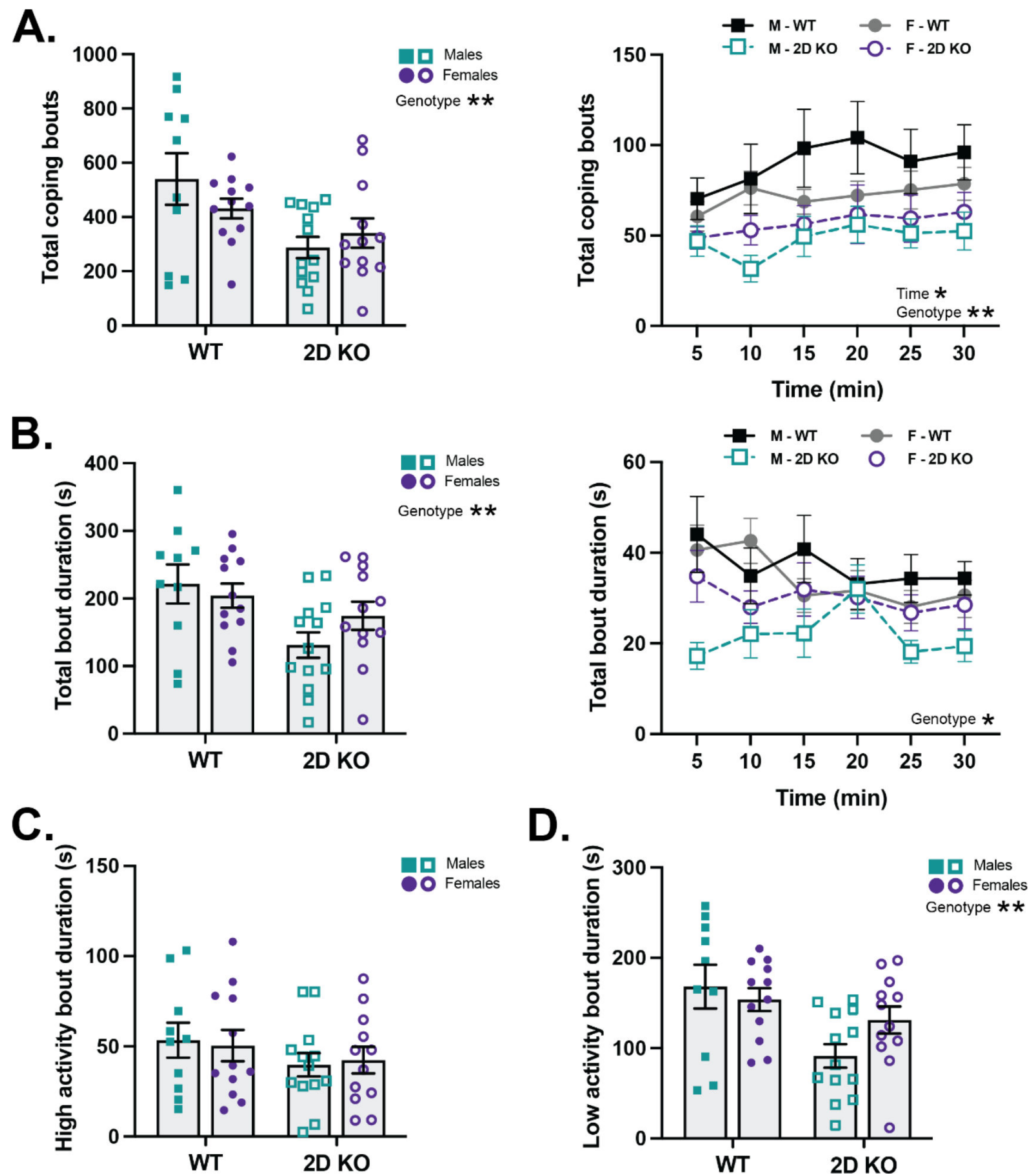

Figure 2. Constitutive GluN2D KO decreases active coping bouts exhibited during acute restraint stress.

A. (Left) GluN2D KO mice showed a decrease in the number of active coping bouts exhibiting during an acute restraint stressor (n=10–13, two-way ANOVA, main effect of genotype); (Right) Main effects of time and genotype were observed over the course of the 30 minute stressor on the total number of active coping bouts per 5 minute bin (n=10–13, three-way ANOVA with repeated measures).

B. (Left) Total duration spent in active coping bouts was altered in KO mice compared to WT littermates (n=10–13, two-way ANOVA, main effect of genotype); (Right) 5 minute bins during the restraint stressor displayed a main effect of genotype on total time spent in active coping bouts (n=10–13, three-way ANOVA with repeated measures).

*p<0.05, **p<0.001. All data are represented as mean ± SEM.

C. No changes in time spent highly active were observed across sex or genotype during restraint stress (n=10–13, two-way ANOVA).

D. Mice displayed a main effect of genotype on time spent in low activity states during the acute restraint stressor (n=10–13, two-way ANOVA, main effect of genotype).

*p<0.05, **p<0.001. All data are represented as mean ± SEM.

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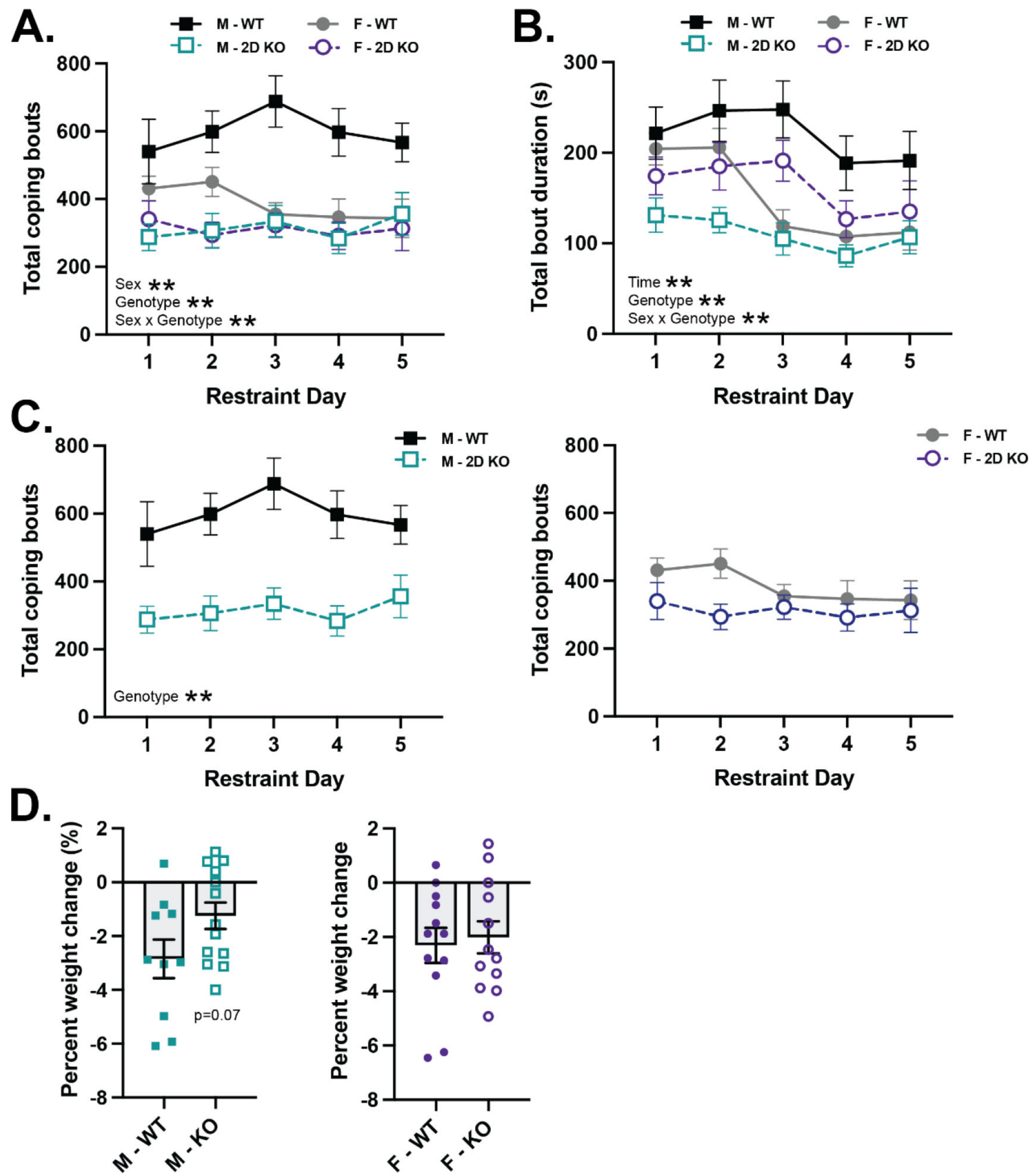

Figure 3. Chronic restraint stress reveals a sex-dependent contribution of GluN2D expression to aspects of active coping behaviors.

A. Main effects of time and genotype and an interaction of sex × genotype were observed on total active coping bouts measured across repeated restraint stress sessions (n=10–13, three-way ANOVA with repeated measures).

B. Main effects of time and genotype and an interaction of sex × genotype were found on total bout duration were seen over the course of chronic restraint stressors (n=10–13, three-way ANOVA with repeated measures).

C. (Left) Male mice display a significant decrease in active coping bouts measured over the course of five restraint stressors compared to their WT littermates (n=12, two-way ANOVA with repeated measures, main effect of genotype). (Right) Female mice did not exhibit any

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differences in active coping bout number across chronic restraint stress sessions (n=10–13, two-way ANOVA with repeated measures).

D. Male GuN2D KO mice trended toward losing less weight compared to their WT littermates (n=12, unpaired t-test, p=0.07) and females showed no differences on weight loss between genotype groups (n=10–13, unpaired t-test).

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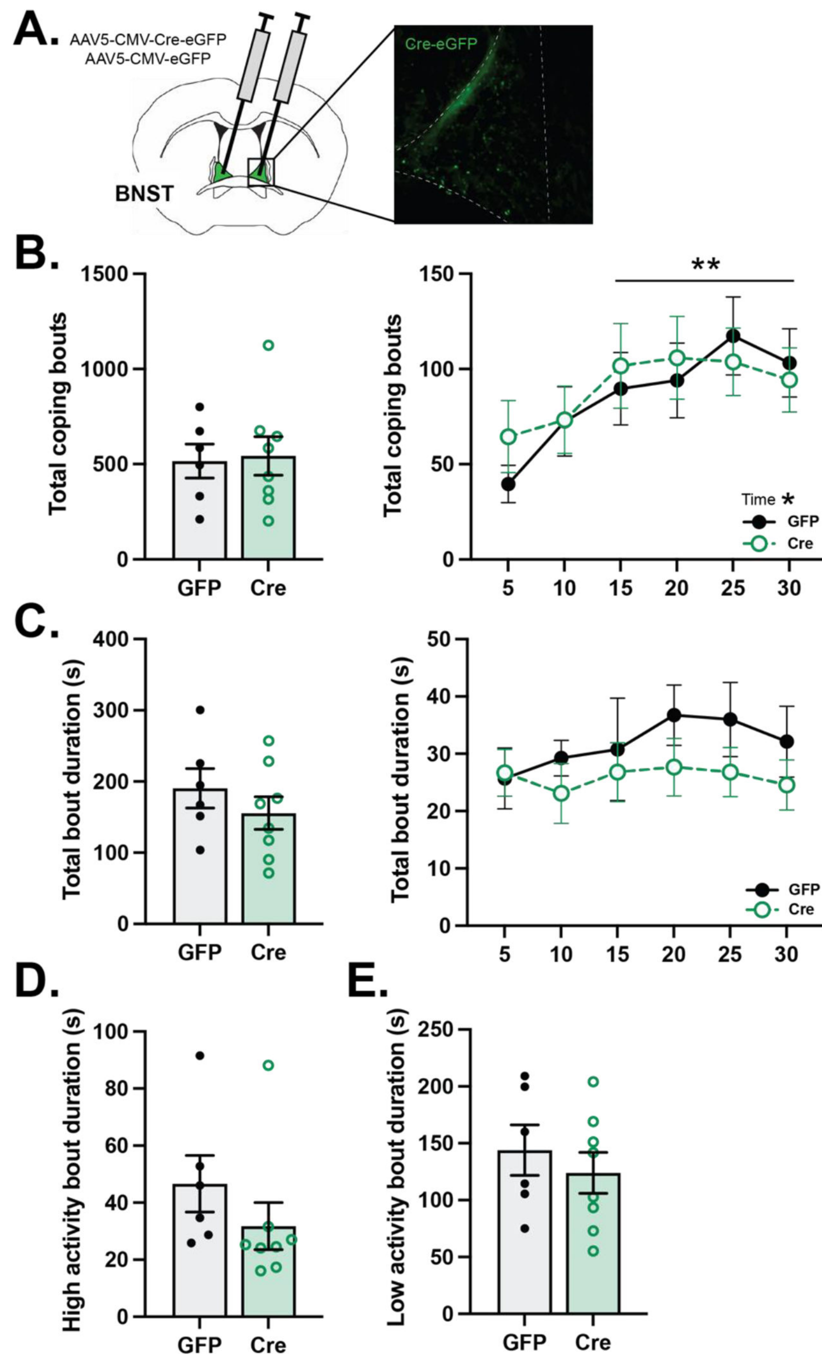

Figure 4. BNST knockdown of GluN2D does not alter active coping bouts during restraint stress.

A. Schematic of viral surgeries and representative image of Cre-eGFP expression.

B. (Left) BNST knockdown of GluN2D did not alter the total time spent in active coping bouts (n=6–8, unpaired t-test); (Right) A main effect of time but not virus was observed on binned coping bout number during the acute restraint stressor, with significantly more bouts occurring at timepoints 15–30 compared to in the first 5 minutes (n=6–8, two-way ANOVA with repeated measures followed by a Sidak post hoc test, main effect of time).

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C. (Left) Total time spent in active coping bout was similarly unchanged by BNST GluN2D knockdown (n=6–8, unpaired t-test); (Right) no differences were observed over time in total bout duration (n=6–8, two-way ANOVA with repeated measures).

D. Time spent in a highly active state was not different between viral groups (n=6–8, unpaired t-test).

E. No differences in time spent in a low activity state were induced by BNST GluN2D knockdown (n=6–8, unpaired t-test).

$^*p<0.05$, $^{**}p<0.001$. All data are represented as mean ± SEM.