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*Grin*1 deletion in CRF neurons sex-dependently enhances fear, sociability, and social stress responsivity

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Abstract The corticotropin releasing factor (CRF) system plays a critical role in responses to stressful stimuli, and is expressed in many areas of the brain involved in processing fear, anxiety, and social behaviors. To better understand the mechanisms by which the CRF system modulates responses to stressful events and social stimuli, we employed a mouse model that selectively disrupts NMDA receptor function via NMDA receptor subunit NR1 (*Grin*1) knockout specifically in Cre-expressing CRF neurons. These animals (Cre+/$^{fGrin1+}$) were compared with littermates lacking Cre expression (Cre−/$^{fGrin1+}$). Following cue discrimination fear conditioning, male Cre+/$^{fGrin1+}$ mice showed increased fear expression to the tone paired with a foot shock (CS+) while still discriminating the CS+ from a tone never paired with a foot shock (CS−). In contrast to males, female mice learned and discriminated fear cues equivalently across the genotypes. Similarly, no genotype differences in sociability or social novelty were observed in female mice, but Cre+/$^{fGrin1+}$ males displayed greater naive sociability and preference for social novelty than Cre−/$^{fGrin1+}$ littermates. Furthermore, the level of social withdrawal exhibited by male Cre+/$^{fGrin1+}$ mice susceptible to social defeat stress relative to same genotype controls was significantly more pronounced than that displayed by susceptible Cre−/$^{fGrin1+}$ mice compared to control Cre−/$^{fGrin1+}$ mice. Together, these results demonstrate increased fear, social, and stress responsiveness specifically in male Cre+/$^{fGrin1+}$ mice. Our findings indicate that NMDA-mediated glutamatergic regulation of CRF neurons is important for appropriately regulating fear and social responses, likely functioning to promote survival under aversive circumstances.

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1. Introduction

One of the key regulators of stress responsiveness across species is the corticotropin releasing factor (CRF) system (Backström and Winberg, 2013; Hostetler and Ryabinin, 2013; Jasnow et al., 2004b; Rodrigues et al., 2009; van der Kooij and Sandi, 2012). Yet, how the CRF system is modulated to elicit such stress responses remains unclear. Previously we have demonstrated the necessity of the central amygdala (CeA), a CRF neuron-rich brain region (Martin et al., 2010; Potter et al., 1994; Swanson et al., 1983), in the acquisition and expression of social defeat stress in Syrian hamsters (Jasnow and Huhman, 2001). Further, we and others have reported that CRF antagonism in the bed nucleus of the stria terminalis (BNST) can block the behavioral expression of social defeat (Cooper and Huhman, 2005; Jasnow et al., 2004b). This brain region receives heavy innervation from the CeA by neurons co-expressing CRF and *Grin*1 (glutamate receptor, ionotropic N-methyl-D-aspartate 1; NR1) receptor subunits (Beckerman et al., 2013). Blockade of this CRF-ergic projection also attenuates the behavioral effects of social defeat (Jasnow et al., 2004b). The CeA is also important for the acquisition, consolidation and expression of Pavlovian fear conditioning (Wilensky et al., 2006; Zimmerman et al., 2007) through NMDA receptor-dependent mechanisms (Goosens and Maren, 2003). Both CRF and NMDA receptors have been implicated in fear learning as well as acquisition and expression of responses to social defeat (Campeau et al., 1992; Fanselow and Kim, 1994; Goosens and Maren, 2003; Jasnow et al., 2004a; Zimmerman and Maren, 2010), suggesting considerable overlap between these systems and regulation of learning, memory, and stress responses.

Functional assembly of NMDA receptors requires *Grin*1 subunits (Monyer et al., 1992), therefore knockout of *Grin*1 produces a loss of NMDA receptor function. Recently, *Grin*1 deletion specifically in CRF neurons of male mice was found to increase fear memory without affecting anxiety, locomotion, pain sensitivity, or novel object exploration (Gafford et al., 2014). This effect was attributed to deletion of *Grin*1 specifically within CRF neurons of the CeA, suggesting NMDA-dependent regulation of CRF neurons in this region may play an important role in regulating appropriate fear responses. Global *Grin*1 hypomorphic mice show reduced social engagement accompanied by deficiencies in aggression (Duncan et al., 2009, 2004). Decreases in fos activation in the CeA and BNST were also observed in these socially stressed *Grin*1 hypomorphs (Duncan et al., 2009). Conversely, social isolation of wildtype mice during adolescence elevates membrane levels of adulthood *Grin*1 expression in the basolateral amygdala (Gan et al., 2014). Such findings indicate that *Grin*1 expression facilitates neural activation after social interactions and is upregulated in the absence of social stimuli.

Based on these data, we hypothesized that removal of NMDA functionality from CRF-expressing neurons would attenuate stress-mediated behavioral expression of social defeat. We utilized the Cre/*loxP* system to explore how NMDA-dependent activation of CRF neurons influences fear, naïve social behavior, and responses to social stressors. Mice with homozygous floxed *Grin*1 alleles (Tsien et al., 1996) were crossed with mice expressing Cre driven by the CRF promoter (Martin et al., 2010). This permitted precise cell-specific ablation of NMDA receptor function exclusively in CRF-expressing neurons (Gafford et al., 2014), as the *Grin*1 subunit is required for NMDA receptor functionality (Moriyoshi et al., 1991; Nakanishi, 1992). First, we extended previous work that detected enhanced fear expression in knockout (Cre+/$^{fGrin1+}$) male mice compared to their control littermates (Cre−/$^{fGrin1+}$; Gafford et al., 2014). Using different shock levels, we evaluated the ability of both male and female knockouts and controls to discriminate auditory cues paired or unpaired with foot shocks. This permits characterization of whether functional ablation of NMDA receptors in CRF neurons influences ability to differentiate between auditory stimuli that were or were not associated with physical stressors of varying intensity (Ito et al., 2009; Tang et al., 2003; Verma et al., 2012). We also assessed the naïve social characteristics of male and female knockout mice, as well as the response of male knockout mice to social defeat stress. Here, our goal was to identify how NMDA receptors specifically in CRF-expressing neurons influence social behaviors and responsiveness to defeat stress.

2. Materials and methods

2.1. Animals

All mice were bred in-house. Male and female experimental mice (6–8 weeks of age) on a C57BL/6 background were generated by crossing mice expressing Cre driven by the CRF promoter (CRF-Cre) (Martin et al., 2010) with mice possessing the *Grin*1 gene flanked by *loxP* sites (floxed NR1, *fGrin*1). Offspring from this first cross were hemizygous negative (Cre−) or positive for Cre (Cre+), and heterozygous for *fGrin*1. These F1 offspring were then crossed to produce an F2 generation with homozygous *fGrin*1 mice that were Cre+ or Cre−. Pairing of F2 homozygous f*Grin*1 mice that are Cre+ or Cre− then produced experimental mice that were ≥F3.

Experimental mice tested for locomotor and anxiety-related measures underwent testing in the open field, followed 48 h later by testing in the elevated plus maze. Mice tested for social behavior underwent all three phases of naïve social testing (habituation, sociability, social novelty) but did not undergo any other tests. Separate groups of mice were used for each shock level tested during fear conditioning, i.e., mice trained at 0.6 mA were different from those mice trained at 0.8 mA. Similarly, experimental male mice that underwent social defeats followed by subsequent post-defeat social interaction testing were not used in any fear conditioning, naïve social behavior testing, or locomotor/anxiety-related measures.

Male aggressor or control CD-1 mice (Charles River, Wilmington, MA) were singly housed, while male and female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) for naïve social interaction targets were group housed. Male CD-1 mice used for post-defeat social interaction were group housed. All mice were housed on a 12:12 light:dark cycle with *ad libitum* access to food and water. All experiments were approved by the Kent State Institutional Animal Care and Use Committee and conducted in accordance with the