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Research Report

Effects of prefrontal cortex and hippocampal NMDA NR1-subunit deletion on complex cognitive and social behaviors

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ABSTRACT

Glutamate *N*-methyl-D-aspartate receptors (NMDARs) in the medial prefrontal cortex (mPFC) and hippocampus may play an integral role in complex cognitive and social deficits associated with a number of psychiatric illnesses including autism, mood disorders, and schizophrenia. We used localized infusions of adeno-associated virus Cre-recombinase in adult, targeted knock-in mice with loxP sites flanking exons 11–22 of the NR1 gene to investigate the effects of chronic NMDAR dysfunction in the mPFC and CA3 hippocampus on cognitive and social behavior. A 5-choice serial reaction time task (5-CSRTT) was used to monitor aspects of cognitive function that included attention and response inhibition. Social behavior was assessed using Crowley's sociability and preference for social novelty protocol. Chronic NMDAR dysfunction localized to the anterior cingulate/prelimbic mPFC or dorsal CA3 hippocampus differentially affected the response inhibition and social interaction. mPFC NR1-deletion increased perseverative responding in the 5-CSRTT and enhanced preference for social novelty, whereas CA3 NR1-deletion increased premature responding in the 5-CSRTT and decreased social approach behavior. These findings suggest that mPFC and CA3 NMDARs play selective roles in regulating compulsive and impulsive behavior, respectively. Furthermore, these findings are consistent with emerging evidence that these behaviors are mediated by distinct, albeit overlapping, neural circuits. Our data also suggest that NMDARs in these regions uniquely contribute to the expression of normal social behavior. In this case, mPFC and CA3 NMDARs appear to inhibit and facilitate aspects of social interaction, respectively. The latter dissociation raises the possibility that distinct circuits contribute to the expression of social intrusiveness and impoverished social interaction.

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1. Introduction

Disrupted glutamatergic neurotransmission may represent a common substrate for cognitive and social behavioral deficits associated with a number of psychiatric illnesses including autism, mood disorders, and schizophrenia (Choudhury et al., 2012; Coyle et al., 2012; Lapidus et al., 2013). Because cognitive and social behavioral deficits can be debilitating and remain challenging to treat, it is important to further explore the pathophysiological substrates underlying these deficits. Early interest in glutamate's role in these deficits was generated by the observation that healthy human subjects given systemically administered glutamate N-methyl-D-aspartate receptor (NMDAR) antagonists exhibit social withdrawal and deficits in cognitive function, including deficits in sustained attention and response inhibition (Krystal et al., 1999, 1994; Lahti et al., 1995). Studies using rodent models have been valuable in further substantiating the role of NMDAR dysfunction in these deficits. In rodents, sustained attention and response inhibition have been assessed simultaneously using a 5-choice serial reaction time task (5-CSRTT) in which subjects attend to an array of nosepoke apertures; correct responses to an illuminated aperture are reinforced and incorrect responses, failures to respond, or responses during an intertrial interval (ITI) are punished with a timeout. Acute and repeated systemic NMDAR antagonists impair attention and response inhibition in the 5-CSRTT (Amitai et al., 2007; Greco et al., 2005; Higgins et al., 2003; Jin et al., 1997; Le Pen et al., 2003; Oliver et al., 2009; Pozzi et al., 2010). Drug-induced attention deficits are expressed as decreased response accuracy and increased omissions whereas response inhibition deficits are expressed as increased premature and perseverative responding (responding during an ITI and multiple responses to a single stimulus, respectively). Acute and repeated systemic NMDAR antagonists have also previously been shown to disrupt social interaction and social recognition memory in rodents (Boulay et al., 2004; Corbett et al., 1995; Sams-Dodd, 1996, 1998; Zimnisky et al., 2012). Most recently, behavioral effects of early developmental NMDAR dysfunction have been examined in mice with conditional, global Grin1 gene deletion or Grin1 deletion targeted to specific subpopulations of neurons. Behavioral deficits observed in these NR1-knockdown mice include spatial working-memory deficits and reduced social interactions (Belforte et al., 2010; Carlen et al., 2012; Duncan et al., 2004; Dzirasa et al., 2009; Gandal et al., 2012; Mohn et al., 1999). Together, these studies provide considerable support for the view that chronic global or cell-specific NMDAR dysfunction can induce attention, response inhibition, and social interaction deficits.

At present, less is known about the impact of brain regionally-specific NMDAR dysfunction on these behaviors. Although it has been shown that acute NMDAR dysfunction in the rat medial prefrontal cortex (mPFC), induced by local antagonist administration, impairs attention and response inhibition in the 5-CSRTT (Mirjana et al., 2004; Murphy et al., 2005, 2012; Pozzi et al., 2011), effects of chronic dysfunction have not yet been examined. In the present study, we begin to address whether regionally-specific, chronic dysfunction of NMDARs in discrete brain regions is sufficient to induce attention, response inhibition, and social interaction deficits. Chronic NMDA-NR1 subunit dysfunction was induced by local infusions of adeno-associated virus Cre-recombinase (AAV-Cre) into the anterior cingulate/prelimbic (AC/PL) mPFC or dorsal CA3 hippocampus of adult transgenic mice with loxP sites flanking exons 11–22 of the Grin1 gene, encoding the NR1 protein. There do not appear to be direct projections from the dorsal CA3 hippocampus to the mPFC or from the mPFC to hippocampus in the rat (Hoover and Vertes, 2007; Verwer et al., 1997); therefore, any similarities in the behavioral effects of NR1 deletions in the two brain regions are unlikely to be due to direct interactions between the regions. The AC/PL mPFC and dorsal CA3 hippocampus were chosen for analysis, in part, because results of previous studies indicate that excitotoxic lesions of these mPFC regions impair performance in the 5-CSRTT (Chudasama et al., 2003; Passetti et al., 2002) and excitotoxic lesions of both regions alter social interaction (Avale et al., 2011; Bannerman et al., 2002). In addition, dorsal CA3 NMDARs are thought to play an important role in learning and memory (Fellini et al., 2009; Kesner and Warthen, 2010; Rajji et al., 2006); although attention is fundamental to learning and memory (Muzzio et al., 2009), to our knowledge, the contribution of CA3 NMDARs to attention in the 5-CSRTT has not yet been examined.

2. Results

2.1. *Localization of NR1 deletion in mice tested in the 5-CSRTT*

Mice with bilateral mPFC or CA3 NR1-deletions ($n=9$/group) were identified based on a qualitative analysis of radiolabeled mRNA in coronal brain sections (Fig. 1). mPFC NR1-deletions were readily visualized spanning a rostrocaudal area corresponding to ~1.9 to 2.6 mm anterior to bregma, involving predominantly the AC/PL subregions of the mPFC (Paxinos and Franklin, 2001; Van De Werd and Uylings, 2014). Hippocampal NR1-deletions spanned a rostrocaudal area corresponding to ~1.7 to 2.5 mm posterior to bregma, involving predominantly the dorsal CA3 subregion of the hippocampus (Paxinos and Franklin, 2001). Only behavioral data from these mice were included in the final results.

2.2. *mPFC and CA3 NR1-deletion increased perseverative and premature responding, respectively*

Mice were trained in the 5-CSRTT to a level of ≥80% accuracy and ≤20% omissions on 3 consecutive sessions under baseline conditions of 0.8 s stimulus duration (SD) and 5 s ITI. Control, mPFC, and CA3 NR1-deleted mice attained this level within ~31 days of beginning 5-choice training sessions (31 ± 2, 32 ± 2, and 31 ± 2; $n=12$, 9, and 9, respectively), confirming that NR1 deletion did not affect acquisition of the task. Following acquisition of baseline performance, a sequence of 4 probe sessions consisting of short ITIs (SITIs), long ITIs (LITIs), reduced SDs (RSDs), and reduced stimulus intensities (RSIs) was initiated. Mice were returned to baseline conditions for 2 sessions between each probe session.

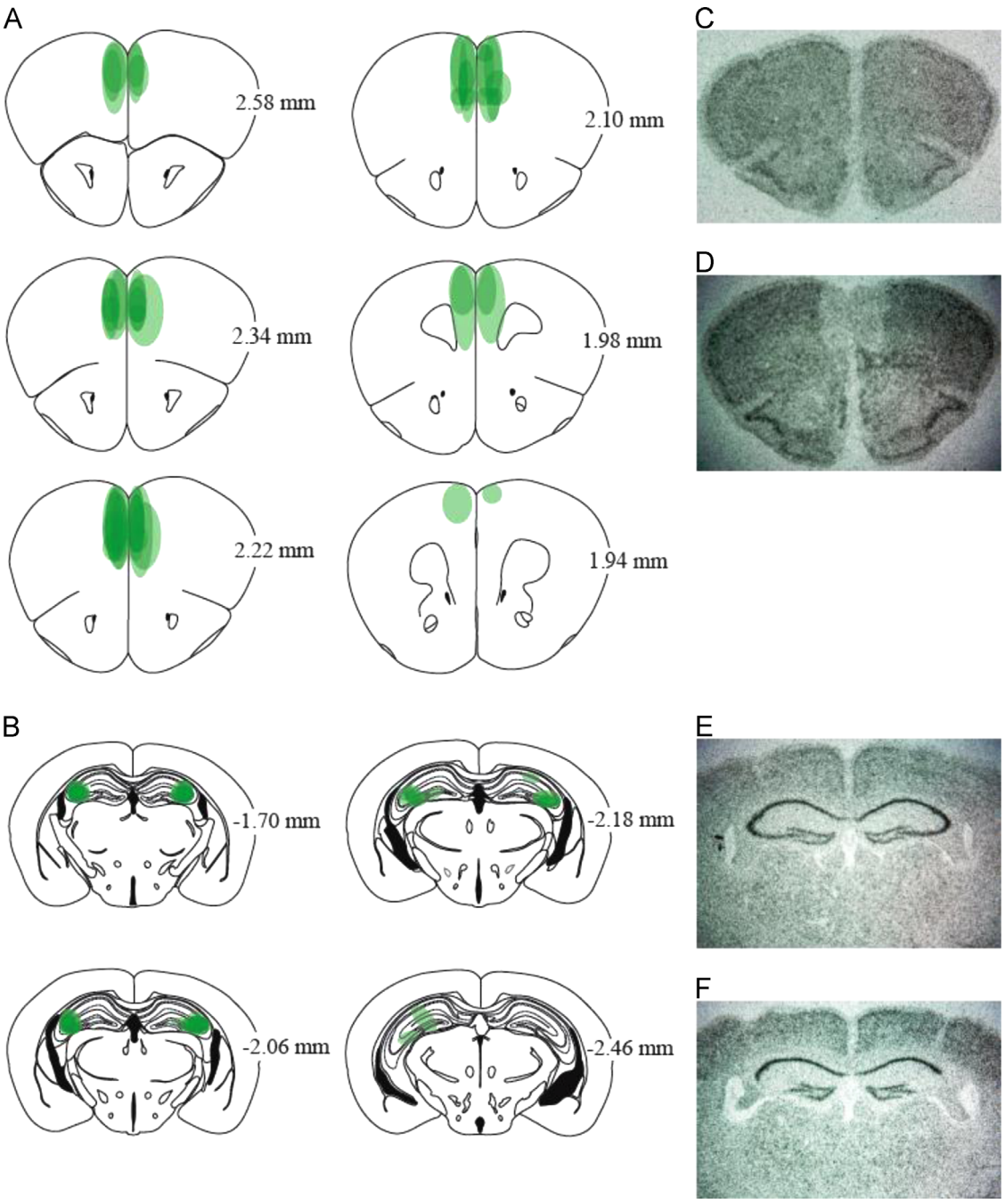

Fig. 1 – NR1 gene deletion following local administration of AAV-Cre into the mPFC or CA3 hippocampus of adult fNR1 mice. The area of NR1 gene deletion induced by bilateral mPFC or CA3 infusions of AAV-Cre (0.5 μl) was determined by qualitative analysis of coronal brain sections exposed to a radiolabeled NR1-specific mRNA probe. For each mouse, grey-shaded ovals represent the visible extent of NR1 gene deletion on corresponding brain atlas images from Paxinos and Franklin (2001). A composite of shading from all mice illustrates the overall localization of NR1 deletions in the mPFC and CA3 hippocampus (n=9/group; A, B, respectively). Numbers indicate the distance (mm) of each image from bregma. Also shown are representative radiolabeled coronal brain sections from aCSF- and LacZ-infused fNR1 control mice (C, E) and AAV-Cre-infused fNR1 mice (D, F).

The effects of NR1 deletion on overall baseline performance were assessed by calculating the average performance across each of the 2 baseline sessions immediately preceding a probe trial (total of 8 baseline sessions; repeated measures ANOVAs confirmed that there were no significant differences among groups across baseline sessions). CA3 and mPFC NR1-deleted mice exhibited nonsignificant trends for increased premature and perseverative responding, respectively [Fig. 2A and B; $t(19)=1.5$ and 1.6, $p=0.1$, respectively]. mPFC and CA3 NR1-deletion had no effect on baseline accuracy, omissions,

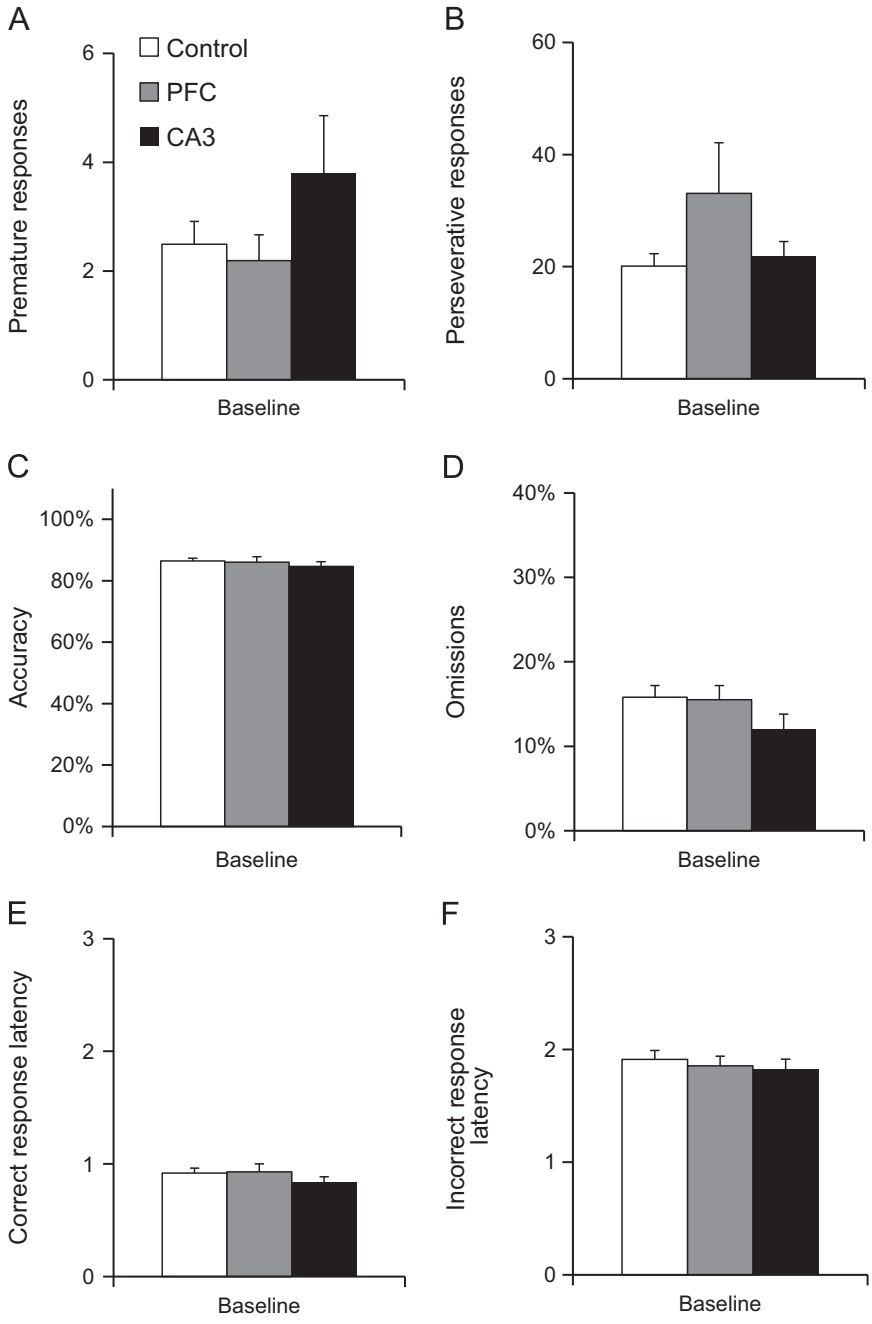

Fig. 2 – CA3 and mPFC NR1-deletion did not significantly affect baseline performance of a 5-CSRTT task. Control mice ($n=12$) and mPFC and CA3 NR1-deleted mice ($n=9$/group) were trained on a 5-CSRTT, under baseline conditions of a 5.0 s LH, ITI, and TO and a 0.8 s SD. Each bar represents a group mean ± SEM of 8 baseline sessions (2 sessions preceding each of the 5 probe trial sessions). (A, B) Relative to controls, CA3 and mPFC NR1-deleted mice exhibited a nonsignificant trend for increased premature and perseverative responding, respectively. In contrast, mPFC and CA3 NR1-deletion did not affect baseline (C) accuracy, (D) omissions, (E) correct response latencies or (F) incorrect response latencies.

correct response latency, or incorrect response latency (Fig. 2C–F). The average number of trials completed per baseline session also did not vary as a function of treatment condition (control = 95 ± 2, mPFC = 97 ± 2, and CA3 = 97 ± 2).

SITIs and LITIs were presented randomly during the first and second probe sessions, respectively. Performance under the SITI and LITI conditions was compared to that on the 2 immediately preceding baseline sessions performed under a fixed 5 s ITI. Although SITIs increased omissions and decreased premature and perseverative responding, this manipulation did not differentially affect performance of control and deleted mice (data not shown). In contrast, LITIs selectively potentiated premature responding of CA3 deleted mice [Fig. 3A; group X LITI interaction: $F(2,27)=6.8$, $p<0.01$]. Whereas all groups exhibited increased premature responding under LITI conditions, relative to baseline [control: $t(11)=4.9$,

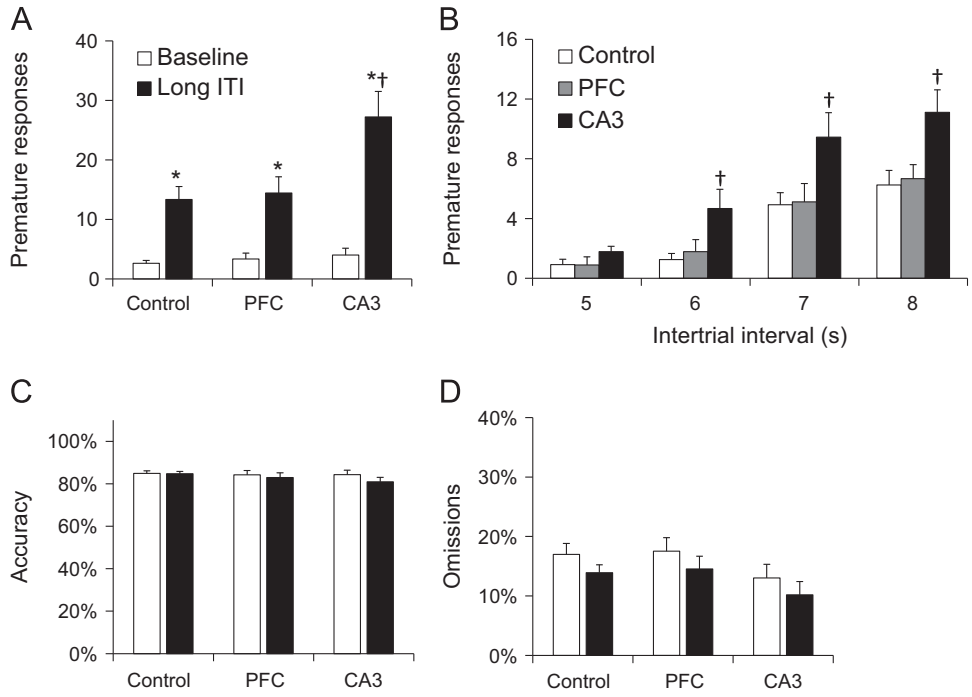

Fig. 3 – CA3 NR1-deletion selectively increased premature responding in a 5-CSRTT task, particularly under conditions of a variable LITI. Effects of mPFC and CA3 hippocampus NR1-deletion were examined under conditions of randomly presented variable ITIs of 5, 6, 7, and 8 s ($n=12$ control, 9 mPFC and 9 CA3). Each bar represents a group mean ± SEM. (A) Relative to performance on the immediately preceding 2 days of baseline testing performed under a fixed 5 s ITI, variable LITIs differentially affected premature responses as a function of treatment condition. Control, mPFC deleted and CA3 deleted mice all exhibited increased premature responding under LITI conditions, relative to baseline conditions. However, under LITI conditions CA3 deleted mice exhibited greater premature responding than control or mPFC deleted mice. (B) To further examine the effects of individual LITIs on premature responding, the LITI data from panel A are presented as a function of the randomly presented 5, 6, 7, and 8 s ITIs used during the probe trial. CA3 deleted mice exhibited greater increases in premature responding than control mice under conditions of 6, 7, and 8 s ITIs. (C) There was no effect of variable LITIs or NR1 deletion on accuracy and, (D) the LITI-induced decrease in omissions was observed across all treatment conditions. *Significantly different from baseline (within-group paired samples t-tests, $p<0.05$). †Significantly different from control and mPFC deleted mice (between-group independent samples t-tests, $p<0.05$).

mPFC: $t(8)=5.7$, and CA3: $t(8)=6.2$, respectively], this manipulation evoked a greater increase in premature responding in CA3 deleted, than control or mPFC deleted mice [$t(19)=3.1$ and $t(16)=2.5$, respectively]. To further examine the effects of individual LITIs on premature responding, the LITI data are presented as a function of the randomly presented 5, 6, 7, and 8 s ITIs used during the probe trial (Fig. 3B). CA3 deleted mice exhibited greater increases in premature responding than control mice under conditions of 6, 7, and 8 s ITIs [ITI X group interaction: $F(6,27)=2.24$, $p<0.05$; $t(19)=2.8$, 2.7, and 2.8, respectively]. In contrast, LITIs did not affect accuracy and decreased omissions to a similar extent in all groups [Fig. 3C and D; LITI omissions main effect: $F(1,27)=11.2$, $p<0.01$]. Variable LITIs did not affect perseverative responding (data not shown).

During the third and fourth probe sessions, variable reduced duration or intensity stimulus lights were randomly presented in the nosepoke apertures (RSD and RSI, respectively). Performance on these probe sessions was compared to that observed on the 2 immediately preceding baseline sessions performed under a fixed 0.8 s SD and 100% intensity. Relative to baseline performance, altering the SD or SI did not differentially affect the performance of control and deleted mice. However, during this phase of testing, mPFC deleted mice exhibited a significant overall increase in perseverative responding, relative to control and CA3 deleted mice [Fig. 4A; SD group main effect: $F(2,27)=4.46$, $p<0.05$ and SI group main effect: $F(2,27)=3.26$, $p<0.05$]. The increased perseverative responding in mPFC deleted mice appears to be persistent in that it was evident under both the reduced SD, reduced SI, and the preceding baseline conditions for both manipulations; this finding is also consistent with the trend for an overall increase in perseverative responding observed in the overall baseline performance data presented in Fig. 2B. Significant main effects of these manipulations (collapsed across treatment conditions) included decreased accuracy and increased omissions, relative to baseline responding (Fig. 4B and C; SD accuracy main effect: $F(1,27)=86.7$, $p<0.001$, SI accuracy main effect: $F(1,27)=67.3$, $p<0.001$, SD omissions main effect: $F(1,27)=5.61$, $p<0.05$, and SI omissions main effect: $F(1,27)=18.1$, $p<0.001$). As expected, reducing the SD or SI resulted in progressive decreases in accuracy and increases in omissions; however, because these manipulations did not differentially affect performance as a function of treatment condition, we have collapsed the data across the variable for presentation

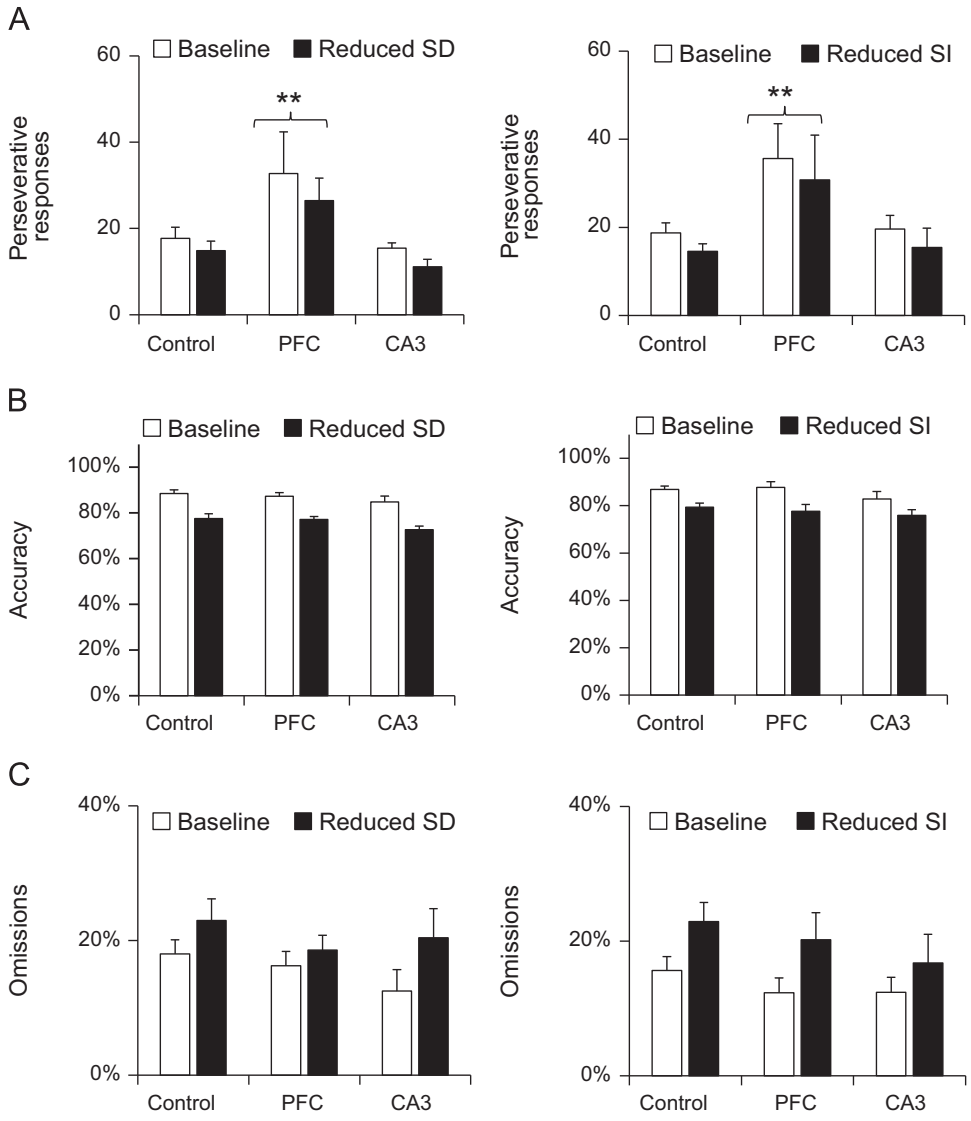

Fig. 4 – mPFC NR1-deletion increased perseverative responding in a 5-CSRTT task. Effects of mPFC and CA3 hippocampus NR1-deletion were examined in a reduced SD probe trial consisting of randomly presented SDs of 0.2, 0.4, 0.6, and 0.8 s and a reduced SI probe trial consisting of randomly presented SIs of 30%, 40%, 50%, 70%, and 100% of baseline stimulus brightness ($n=12$ control, 9 mPFC and 9 CA3). Probe trial performance was compared to that exhibited during the immediately preceding 2 days of baseline testing during which mice are tested on a fixed 0.8 s SD and 100% brightness. Each bar represents a group mean ± SEM. Relative to baseline responding, reducing the SD or SI did not differentially affect performance of control or deleted mice. (A) However, mPFC NR1-deleted mice exhibited a generalized increase in perseverative responding that was evident under baseline and manipulation conditions, this increase in reminiscent of the trend for increased perseverative responding observed under baseline conditions alone (see Fig. 2B). Reducing the SD or SI did not differentially affect accuracy (B) or omissions (C) of the deleted mice. Although overall, these manipulations decreased accuracy and increased omissions. **Significantly different from control and CA3 NR1-deleted mice (pairwise comparisons collapsed across sessions for each group; post-hoc LSD test, $p \leq 0.05$).

in Fig. 4. RSDs also significantly increased premature responding across all groups (data not shown).

2.3. *Localization of NR1 deletion in mice tested in a social interaction task*

Mice with bilateral mPFC ($n=12$) or CA3 ($n=11$) NR1-deletions were identified based on a qualitative analysis of radiolabeled mRNA in coronal sections (Fig. 5). As in our first study, visible NR1 deletions were detected within the rostrocaudal extent of the mPFC corresponding to ~1.9 to 2.6 mm anterior to bregma, with predominant involvement of the AC/PL subregions (Paxinos and Franklin, 2001; Van De Werd and Uylings, 2014), and a rostrocaudal area of hippocampus corresponding to ~1.7 to 2.5 mm posterior to bregma, with predominant involvement of the dorsal CA3 subregion (Paxinos and Franklin, 2001). Only social interaction data from mice with bilateral mPFC or CA3 NR1-deletions were included in the final results.

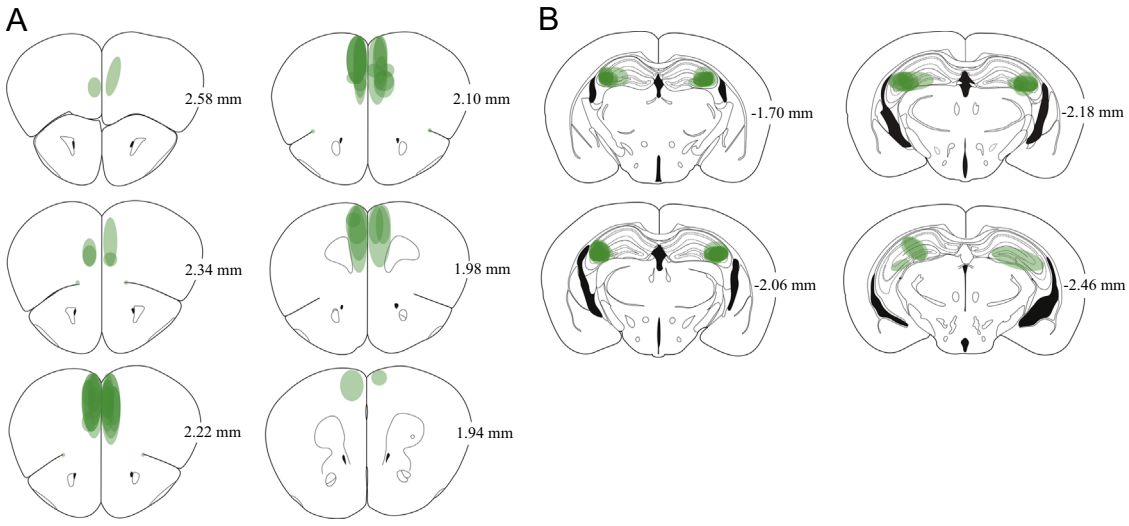

Fig. 5 – NR1 gene deletion following local administration of AAV-Cre into the mPFC or CA3 hippocampus of adult fNR1 mice. The area of NR1 gene deletion induced by bilateral mPFC and CA3 hippocampus infusions of AAV-Cre (0.5 μl) was determined by qualitative analysis of coronal brain sections exposed to a radiolabeled NR1-specific mRNA probe. For each mouse, grey-shaded ovals are used to indicate the visible extent of NR1 gene deletion on corresponding brain atlas images from Paxinos and Franklin (2001). A composite of shading from all mice illustrates the overall localization of NR1 deletions in (A) the dorsal mPFC ($n=12$) and (B) CA3 hippocampus ($n=11$). Gene deletion maps for a subset of mice ($n=2$–3/group) tested in both social interaction and 5-CSRTTs are duplicated in Fig. 1. Numbers indicate the distance (mm) of each image from bregma.

2.4. *mPFC and CA3 NR1-deletion enhanced preference for social novelty and impaired social approach, respectively*

Analysis of time spent in each chamber and in proximity to the retaining cages revealed evidence of altered sociability in CA3 deleted mice [Fig. 6A and B; treatment × chamber interaction: $F(4,72)=5.9$, $p<0.001$ and treatment × proximity zone interaction: $F(2,36)=5.6$, $p=0.008$]. Specifically, control and mPFC deleted mice spent more time in a chamber and proximity zone associated with a novel stimulus mouse than an empty retaining cage [chamber with stimulus versus empty: control group $t(15)=5.1$ and mPFC group $t(11)=2.8$; proximity zone with stimulus versus empty: control group $t(15)=5.0$ and mPFC group $t(11)=3.5$]. CA3 deleted mice did not exhibit this preference. In addition, relative to controls, CA3 deleted mice spent less time in a chamber and proximity zone with a novel stimulus mouse [$t(25)=3.8$ and 3.2, respectively] and more time in an empty chamber and proximity zone ($t(25)=2.9$ and 2.3, respectively). Relative to controls, CA3 deleted mice also took longer to enter a chamber and proximity zone associated with a stimulus mouse [Fig. 6C; treatment × chamber interaction: $F(2,36)=3.5$, $p=0.04$ and $t(25)=2.1$; treatment × proximity zone interaction: $F(2,36)=4.1$, $p=0.02$ and $t(25)=2.2$]. The number of chamber entries (Fig. 6D) and distance traveled per chamber (data not shown) did not differ as a function of treatment condition.

During the preference for social novelty phase, the familiar stimulus mouse introduced during sociability testing remained in its retaining cage and a novel stimulus mouse was placed in the previously empty cage. As illustrated in Fig. 7A, time spent in each chamber during the first 5 min of social novelty testing varied as a function of treatment condition [treatment × chamber interaction: $F(4,72)=3.0$, $p=0.025$]. Specifically, although control and mPFC and CA3 deleted mice all spent more time in a chamber with a novel mouse than a familiar mouse [$t(15)=8.0$, $t(11)=10.3$, $t(10)=6.2$, respectively], relative to controls, mPFC deleted mice spent less time in a chamber with a familiar mouse and more time in a chamber with a novel mouse [$t(26)=3.7$ and 2.7, respectively]. All groups spent more time in proximity with a novel than a familiar mouse [Fig. 7B; proximity zone main effect: $F(1,36)=149$, $p=0.03$] and this behavior did not vary as a function of treatment condition. Overall, mice were quicker to enter a chamber and proximity zone associated with a novel mouse than a familiar mouse and these latencies did not vary as a function of treatment condition [Fig. 7C; chamber and proximity zone main effects: $F(1,36)=10.6$ and 21.2, respectively]. The number of chamber entries (Fig. 7D) and distance traveled per chamber (data not shown) did not differ as a function of treatment condition.

3. Discussion

In the present study, chronic NMDAR dysfunction in the mouse AC/PL mPFC and dorsal CA3 hippocampus differentially affected complex cognitive and social behavior. Specifically, AC/PL mPFC NR1-deletion increased perseverative responding in a sustained attention task whereas dorsal CA3 NR1-deletion increased premature responding. Effects of these manipulations on inhibitory response control were observed in the absence of effects on sustained attention per se. In a test of social interaction, AC/PL mPFC NR1-deletion enhanced preference for social novelty whereas dorsal CA3 NR1-deletion decreased social approach.

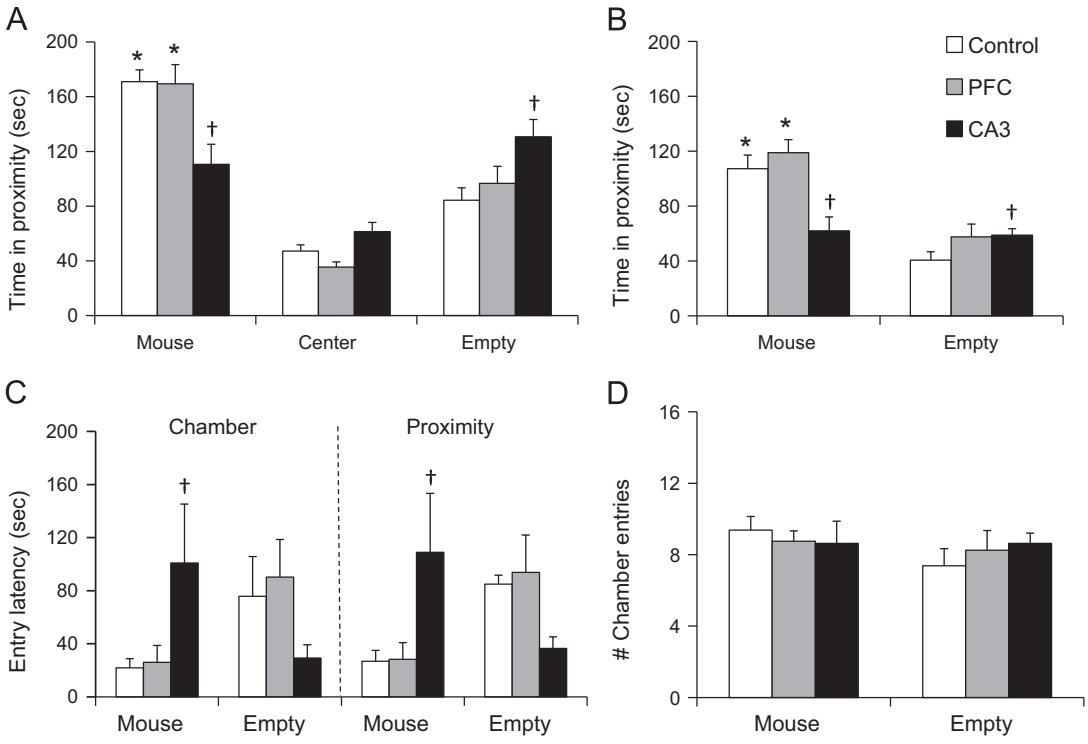

Fig. 6 – CA3 NR1 deletion selectively decreased social approach. During sociability testing, mice had free access to a 3-chambered box. One end chamber contained a wire retaining-cage occupied by a stimulus mouse and the other end chamber contained an empty retaining-cage. Each bar represents a group mean ± SEM ($n=16$ control, $n=12$ mPFC, and $n=11$ CA3). (A) CA3, but not mPFC, NR1-deletion altered the time spent in each chamber during the first 5 min of sociability testing. Control and mPFC, but not CA3, deleted mice spent more time in a chamber with a stimulus mouse than an empty chamber. Relative to controls, CA3 deleted mice spent less time in a chamber with a stimulus mouse and more time in an empty chamber. (B) CA3, but not mPFC, deletion alters the total time spent in proximity (within 3.5 cm of the outer edge of the wire retaining-cages) with a stimulus mouse and an empty cage, during the first 5 min of social approach testing. Control and mPFC, but not CA3, deleted mice spent more time in proximity with a stimulus mouse than an empty cage. Relative to controls, CA3 deleted mice spent less time in proximity with a mouse and more time in proximity with an empty cage. (C) CA3, but not mPFC, deletion altered the latency to enter a chamber and proximity zone associated with a stimulus mouse. Relative to controls, CA3 deleted mice took longer to enter a chamber and proximity zone associated with a stimulus mouse. (D) The number of chamber entries and distance traveled per chamber (data not shown) did not differ as a function of treatment condition. *Significantly different from mean time in an empty chamber or proximity zone (within-group paired samples t-tests, $p<0.05$). †Significantly different from control mean (between-group independent samples t-tests, $p<0.05$).

3.1. *mPFC and CA3 NR1-deletion induce deficits in inhibitory response control in the 5-CSRTT*

Deficits in inhibitory response control have long been identified as amongst the most robust behavioral effects induced by acute and/or repeated systemic administration of NMDAR antagonists (Amitai et al., 2007; Greco et al., 2005; Higgins et al., 2003; Jin et al., 1997; Le Pen et al., 2003; Oliver et al., 2009; Pozzi et al., 2010; Sanger, 1992; Stephens and Cole, 1996; Welzl et al., 1991). More recently, investigators have begun to examine the contribution of NMDARs in specific brain regions to these deficits. One approach to establishing regionally specific effects combines the use of local infusions of NMDAR antagonists with assessment of inhibitory response control deficits as expressed by increased premature and perseverative responding in the 5-CSRTT (Bari et al., 2008; Robbins, 2002). Using this approach, investigators have observed that acute NMDAR blockade in the rat mPFC increases premature responding (Mirjana et al., 2004; Murphy et al., 2005, 2012) and, under some circumstances, perseverative responding (Mirjana et al., 2004). The present study addresses whether the findings based on acute pharmacologic manipulations can be extended to conditions of chronic mPFC NMDAR dysfunction. Results of our study indicate that chronic NMDAR dysfunction localized to the mouse mPFC increases perseverative, but not premature, responding. Overall, both the acute pharmacologic and chronic gene deletion approaches have yielded findings consistent with a role for mPFC NMDARs in response inhibition. Inconsistent observations regarding whether mPFC NMDARs are most critical for inhibition of perseverative or premature responding may be related to species or methodological differences between the previous pharmacologic studies (performed in rats sustaining an acute manipulation following behavioral training) and the present gene deletion study (performed in mice sustaining a chronic manipulation prior to behavioral training). In addition, it is noteworthy that NR1

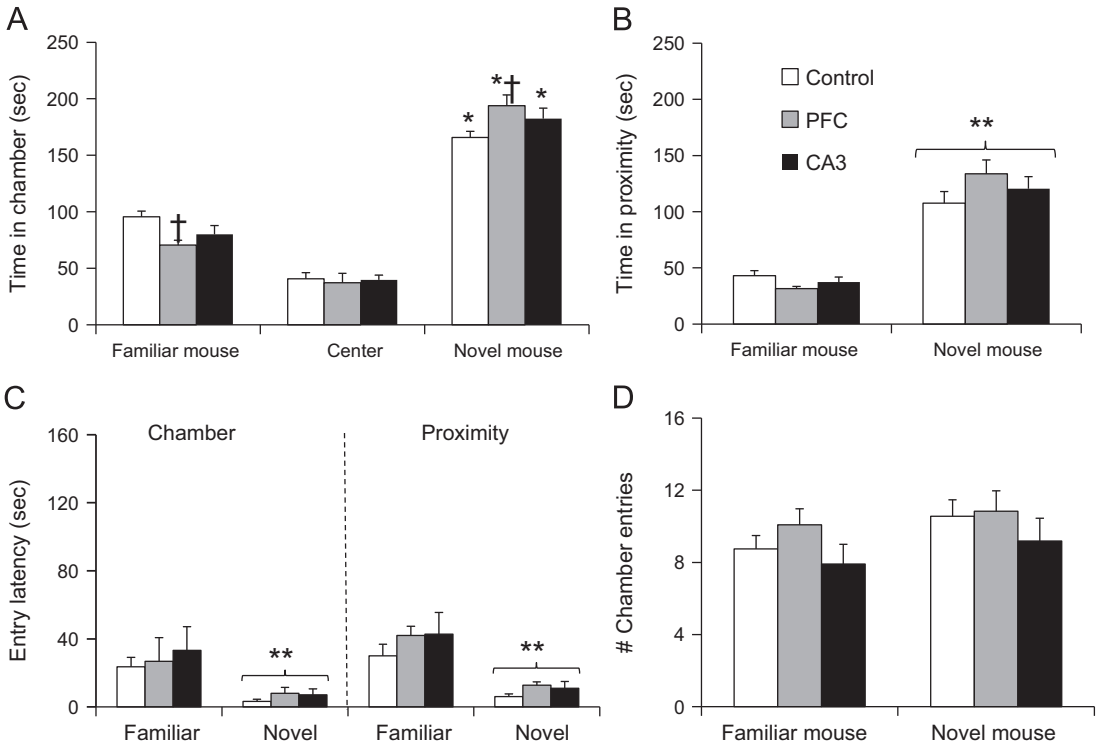

Fig. 7 – mPFC NR1-deletion selectively increased preference for social novelty. Immediately following sociability testing, preference for social novelty was assessed. During social novelty testing, the "familiar" stimulus mouse introduced during sociability testing remained in the retaining-cage and a "novel" stimulus mouse was placed in the previously empty retaining-cage. Each bar represents a group mean ± SEM ($n=16$ control, $n=12$ mPFC, and $n=11$ CA3). (A) mPFC, but not CA3, NR1-deletion altered the time spent in each chamber during the first 5 min of social novelty testing. Control mice and mPFC and CA3 deleted mice spent more time in a chamber with a novel mouse than a familiar mouse. Relative to controls, mPFC NR1-deleted mice spent less time in a chamber with a familiar mouse and more time in a chamber with a novel mouse. (B) Time spent in proximity (within 3.5 cm of the outer edge of the wire retaining-cages) with a familiar versus novel mouse did not vary as a function of treatment condition. Overall, mice spent more time in proximity with a novel than a familiar mouse. (C) Latency to enter a chamber or proximity zone associated with a familiar versus novel mouse did not vary as a function of treatment condition. All groups entered the chamber and proximity zone associated with the new mouse more quickly than that associated with the familiar mouse. (D) The number of chamber entries and distance traveled per chamber (data not shown) did not differ as a function of treatment condition. *Significantly different from mean time spent in a chamber with a familiar mouse (within-group paired samples t-tests, $p<0.05$). †Significantly different from control mean (between-group independent samples t-tests, $p<0.05$). **Significantly different from familiar mouse, collapsed across treatment condition (ANOVA main effect, $p<0.05$).

dysfunction in the present study was localized to the AC/PL subregions of the mouse mPFC. Previously, it has been suggested that antagonism of NMDARs in the infralimbic, but not PL, subregion of the rat mPFC potentiates premature responding (Murphy et al., 2005). Studies are currently underway in our lab to assess whether infralimbic NR1 deletions potentiate premature responding.

We are not aware of published research examining the effects of discrete hippocampal NMDAR dysfunction on inhibitory control in the 5-CSRTT. In our study, a trend for increased premature responding in dorsal CA3 NR1-deleted mice under baseline conditions (constant 5 s ITI) was exacerbated when mice were tested under conditions of variable LITIs (5, 6, 7, or 8 s). Increased premature responding may be related to deficits in inhibitory control in the CA3 NR1-deleted mice, with progressively longer ITIs allowing for progressively more premature responding. It may also be that impaired timing abilities in the CA3 NR1-deleted mice are expressed as a greater tendency to exhibit premature responses, as may be expected if the mice are unable to judge whether sufficient time has passed to expect the onset of another trial. The latter interpretation is consistent with (1) an emerging behavioral literature indicating that the dorsal hippocampus plays a role in response timing that is independent of a role in response inhibition (Tam and Bonardi, 2012a, 2012b; Tam et al., 2013) and (2) research indicating that systemic NMDAR antagonists affect both response inhibition and timing in rats performing a DRL task (Welzl et al., 1991). Our data are not consistent with recent studies in which excitotoxic lesions of the dorsal hippocampus failed to affect premature responding in rats previously trained on the 5-CSRTT (Abela et al., 2013; Chudasama et al., 2012). Again, the apparent inconsistencies may be due to significant methodological differences related to the use of global excitotoxic

lesions of the dorsal hippocampus (Abela et al., 2013; Chudasama et al., 2012) versus localized dorsal CA3 NR1 deletion (present study) and behavioral effects being assessed in subjects pretrained on the task (Abela et al., 2013; Chudasama et al., 2012) versus naïve to the task (present study).

3.2. *mPFC and CA3 NR1 deletion-induced deficits in response inhibition occur in the absence of attention deficits in the 5-CSRTT*

In our study, persistent disruption in NMDAR function in the mouse AC/PL mPFC or dorsal CA3 hippocampus did not affect attention, as assessed by analysis of accuracy and omissions in the 5-CSRTT. A lack of effect of our dorsal CA3 NR1-deletion on attention is consistent with the observation that excitotoxic lesions of the rat dorsal hippocampus do not affect attention as assessed in the 5-CSRTT (Abela et al., 2013). However, the absence of attention deficits following AC/PL mPFC NR1-deletion in our mouse model was unexpected. Previous investigators reported attention deficits in the 5-CSRTT following local application of NMDA antagonists directly into the mPFC (Mirjana et al., 2004; Murphy et al., 2005, 2012; Pozzi et al., 2011) and results of excitotoxic lesion studies have been interpreted to suggest that the rodent AC subregion of the rat mPFC plays a predominant role in attention (Chudasama et al., 2003; Muir et al., 1996; Passetti et al., 2002). Again, it is noteworthy that our protocol was designed to assess performance in rodents acquiring the 5-CSRTT following NMDAR manipulation whereas all previous studies trained subjects on the task prior to the experimental manipulation. Additional studies are required to address whether mPFC NR1-deletion impairs attention in subjects already familiar with the task. Alternatively, it may be that specific dysfunction of NMDARs in the AC mPFC is not sufficient to impair sustained attention, and that factors other than NMDAR blockade contribute to the effects observed following acute local application of NMDA antagonists.

3.3. *Effects of mPFC and CA3 NR1-deletion on social interaction*

Systemic NMDAR antagonists and global 90–95% knockdown of the NR1 subunit induce a behavioral phenotype in mice that is consistent with increased social withdrawal (Corbett et al., 1995; Duncan et al., 2004; Gandal et al., 2012; Mohn et al., 1999; Sams-Dodd, 1996, 1998). In our study, localized dysfunction of NMDARs in the mPFC and CA3 hippocampus differentially affected social behavior. Dorsal CA3 NR1-deletion decreased social approach but did not affect preference for social novelty, whereas AC/PL mPFC NR1-deletion increased preference for social novelty but did not affect social approach. Dissociable effects of NMDAR dysfunction on social approach and preference for social novelty have been reported previously. Transgenic mice with global reductions in affinity for the NMDAR coagonist glycine exhibit impaired social approach and normal preference for social novelty (Labrie et al., 2008). Our findings indicate that NMDAR dysfunction in the dorsal CA3 hippocampus is sufficient to impair social approach, suggesting that this region normally facilitates social approach behavior. In contrast, results of a recent study indicate that excitotoxic lesions of the mouse mPFC increase social interaction (Avale et al., 2011), suggesting that this region normally inhibits social interaction. Our observation that AC/PL mPFC NR1-deletion increased preference for social novelty is consistent with this hypothesis and furthermore, suggests that mPFC NMDARs play a primary role in this function. It will be important to reconcile why mPFC NR1-deletion failed to affect social approach and yet increased preference for social novelty in our paradigm. One explanation is that during phase 1 of testing, behavior was motivated by competing interests of engaging in social approach and exploring a novel environment. As a result, a deletion-induced increase in social approach may be masked by a competing interest to explore the novel environment. In phase 2 of testing, the mouse is now familiar with the environment and the preference for social interaction emerges in the form of a preference for social novelty.

3.4. *Conclusion*

In the present studies, we used targeted knock-in mice with loxP sites flanking exons 11–22 of the NR1 gene combined with local AAV-Cre infusions to chronically disrupt NMDAR function in the mPFC and CA3 hippocampus. We observed that impaired NMDAR function in either region results in deficits in response inhibition, with AC/PL mPFC and dorsal CA3 NR1-deletion leading to increased perseverative (compulsive) and premature (impulsive) responding, respectively. The latter findings are consistent with emerging evidence that compulsive and impulsive behaviors are mediated by distinct, albeit overlapping, neural circuits (Bari and Robbins, 2013; Fineberg et al., 2010). Social interaction was also differentially affected by AC/PL mPFC and dorsal CA3 NR1-deletion. NMDAR dysfunction in these regions increased preference for social novelty and decreased social approach, respectively. Together, results of the present studies suggest that impaired NMDAR dysfunction in the AC/PL mPFC and dorsal CA3 hippocampus may both contribute to impaired response inhibition associated with psychiatric illness, whereas social withdrawal may be more closely aligned with NMDAR dysfunction in the dorsal CA3 hippocampus and social intrusiveness may be more closely aligned with NMDAR dysfunction in the AC/PL mPFC.

4. Experimental procedure

4.1. *Animals*

Breeding pairs of homozygous floxed NR1 (fNR1; developed on a C57BL/6N genetic background) mice with loxP sites flanking exons 11–22 of the NR1 gene (Tsien et al., 1996) were obtained from the Greene Lab. DNA was extracted (Fermentas, Vilnius, Lithuania; Fast Tissue-to-PCR Kit) from ear-notch samples collected on postnatal day (PN) 10–20. The fNR1 genotype was confirmed using polymerase chain reaction and gel electrophoresis to identify the presence of a neomycin phosphotransferase sequence and absence of a GRIN1

sequence (Life Technologies, Carlsbad, CA; custom DNA primers). Female mice were removed from the litter at birth. The male mice were weaned on PN21 and individually housed on PN50. Male subjects from 8 litters were distributed as evenly as possible across the treatment conditions. The vivarium was temperature (20–26° C) and humidity (30–70%) controlled. All treatments were conducted during the light phase (7 am to 7 pm). Rodent chow and water were available ad libitum, except during 5-CSRTT training and testing. All protocols were approved by Western Washington University Animal Care and Use Committee using criterion established by the U.S. Animal Welfare Act and the National Research Council *Guide for the Care and Use of Laboratory Animals, 8th Edition*.

4.2. *NR1 deletion*

Male mice (PN70–90) were anesthetized with isoflurane (Henry Schein Animal Health, Tualatin, OR; NDC 11695-6775-2) and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). Anesthesia was maintained using isoflurane/oxygen vapor (Dräger Medical Inc., Telford, PA). The eyes were protected with ophthalmic ointment (Akorn Pharmaceuticals, Lake Forest, IL; NDC 17478-235-35). The surgical site was shaved, disinfected, and locally anesthetized (0.25% Bupivacaine; Hospira Inc., Lake Forest, IL; NDC 0409-1159-01). The scalp was incised and holes were drilled in the skull above the injection site. Bilateral infusions (0.5 μl) of AAV-Cre, AAV-β-galactosidase (AAV-LacZ), or artificial cerebrospinal fluid (aCSF) were delivered by pressure ejection (AAV-Cre and -LacZ were obtained from the Harvard Institute of Human Genetics, Boston, MA). With the skull flat, injection coordinates for the mPFC were +1.6 AP and ±0.4 ML from bregma, and −1.5 DV from dura and the CA3 hippocampus were −1.9 AP and ±2.0 ML from bregma, and −1.8 DV from dura. A glass micropipette (tip O.D. ~80 μm) was lowered to the injection site and remained in place for 5 min before the infusion began. Infusions were delivered over 10 min using a picopump (World Precision Instruments, Sarasota, FL). The micropipette was left in place for 5 min, removed, and the infusion was repeated in the contralateral hemisphere. Skull holes were filled with bone wax (Surgical Specialties Corporation, Reading, PA), the incision was closed using polypropylene sutures (Ethicon Endo-Surgery Inc., Cincinnati, OH), 0.25% Bupivacaine was applied topically to the incision, and 0.25 ml of 0.9% sterile saline was administered subcutaneously. Mice remained undisturbed in the vivarium for ~1 month prior to initiation of behavioral testing. All behavioral testing was performed by investigators blind to the treatment conditions.

4.3. *5-CSRTT*

Prior to initiation of training in the 5-CSRTT, mice were placed on food restriction and trained to retrieve food pellets (14 mg chocolate pellets; TestDiet, St. Louis, MO) from the food receptacles of 5/9-hole test chambers equipped with ABET II interface and software (Chamber Model 80610A-CL; Lafayette Instrument Company, Lafayette, IN). Free-feeding weights were assessed daily for 3 days. Food restriction was then initiated and for 1 week mice were provided with sufficient food to reduce their mean free-feeding body weights by ~2% daily. During the first week of training in the task, body weights were maintained at ~85%. Each week thereafter, body weights of food-restricted mice were allowed to increase by ~1%, consistent with the weight gain of age-matched free-feeding fNR1 male mice. During the first 8 sessions of training in the 5/9-hole test chambers, all nosepoke apertures were closed and training was designed to associate head entry responses into the food receptacle with acquisition of a pellet. Specifically, during sessions 1–3, 1 pellet was delivered into the food receptacle every 40 s for 20 min. A clear-acrylic food receptacle door was pinned open and the receptacle light was continuously on. During sessions 4–6, 1 pellet was delivered into the food receptacle every 60 s for 20 min. From session 4 on, the food receptacle door was released. The receptacle light was illuminated when a pellet was delivered and remained illuminated until the pellet was retrieved. During sessions 7–8, pellet retrieval resulted in delivery of another pellet 10 s later; this sequence was repeated for a maximum of 20 min or 100 trials.

During 5-CSRTT nosepoke training sessions, 1 of 5 open nosepoke apertures was randomly illuminated and a nosepoke into the aperture within the SD (initially set at 32 s) or during a limited hold (LH; 5 s) immediately following the SD was reinforced by delivery of a pellet into the food tray. Retrieval of the pellet initiated a 5 s ITI. A timeout (TO; 5 s), signaled by illumination of the houselight, occurred if a mouse made a response during the ITI or failed to make a correct response. Sessions were terminated after 20 min or 100 trials. When a mouse reached performance of ≥80% accuracy (correct responses/correct+incorrect responses) and ≤20% omissions (failure to exhibit a response/total number of trials), the SD was decreased in the next session, proceeding through a self-paced series of daily sessions (32, 16, 8, 4, 2, 1.8, 1.6, 1.4, 1.2, 1.0, and 0.8 s SDs).

Probe sessions began when a mouse attained ≥80% accuracy and ≤20% omissions under the 0.8 s SD condition for 3 consecutive days. Probe sessions consisted of variable SITIs (2, 3, 4, and 5 s), LITIs (5, 6, 7, and 8 s), RSDs (0.2, 0.4, 0.6, and 0.8 s), and RSIs (30%, 40%, 50%, 70%, and 100%). Probe sessions were performed in the order listed above and mice were returned to baseline parameters (5 s ITI, LH, and TO; 0.8 s SD) for 2 sessions between each probe session. Accuracy, omissions, premature responses (nosepokes during an ITI and punished with a TO), perseverative responses (repetitive nosepokes into an illuminated aperture during a SD or LH; these responses are not punished), correct response latency (latency between onset of a stimulus light and a correct nosepoke response), reward collection latency (latency to retrieve a food pellet), and total number of trials completed were recorded.

4.4. *Social interaction*

The apparatus and method for assessing social interaction were based on previous research (Nadler et al., 2004). Briefly, an acrylic box ($L \times W \times H = 63 \times 42 \times 22$ cm) was separated into three equally-sized chambers by black partitions (Fig. 8). Partitions had a single passage ($L \times W \times H = 11 \times 0.5 \times 6$ cm)

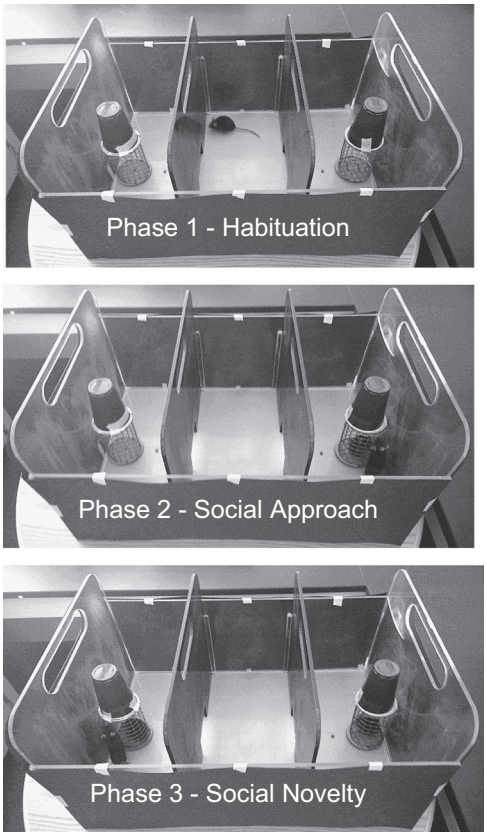

Fig. 8 – Social behavior was assessed using an apparatus and method based on previous research (Nadler et al., 2004). A single-trial test consisted of three phases. During the habituation phase (Phase 1), a test mouse was placed in and retained in the center chamber for 5 min; the end chambers were empty and not accessible to the mouse. A stimulus mouse was then placed in a retaining cage located in one of the end chambers; the empty retaining cage was also manipulated at this time but remained empty. To initiate the social approach phase (Phase 2), the guillotine doors were raised simultaneously and the test mouse was free to explore all chambers for 10 min. The test mouse was then coaxed back into the center chamber and the guillotine doors are lowered. Preference for social novelty (Phase 3) was then assessed by placing a second stimulus mouse in the previously empty retaining cage. The guillotine doors were raised and the test mouse again had free access to all chambers for 10 min.

that could be opened and closed. Circular wire-mesh retaining cages ($D \times H = 8 \times 10$ cm), positioned adjacent to each end wall, were used for presentation of stimulus mice. Naive adult male fNR1 mice were used as stimulus mice. For 3 days immediately prior to testing, stimulus mice were habituated to the apparatus by placing them in a retaining cage within one end chamber for 5 min daily.

For 1 week prior to testing, control and deleted fNR1 test mice were handled daily for ~5 min. Social behavior was assessed in a single trial. During habituation, a test mouse was retained in the center chamber for 5 min; the end chambers were empty and not accessible to the mouse (Fig. 8: Phase 1 – habituation). Following habituation, a stimulus mouse was placed in one of the wire-mesh retaining cages and the other retaining cage was manipulated and returned to the chamber empty; the stimulus mouse location was pseudorandomly determined such that the assignment of end chambers was equally distributed across treatment conditions. The inter-compartment passages were opened simultaneously, initiating a 10-min social approach phase (Fig. 8: Phase 2 – social approach). Following social approach testing, the test mouse was coaxed into the center chamber and the passages were closed. Preference for social novelty was assessed by placing a second stimulus mouse in the previously empty retaining cage. The passages were opened and the test mouse was free to explore all chambers for 10 min (Fig. 8: Phase 3 – social novelty). Time spent in the three chambers and proximity zones surrounding the two retaining cages was determined by analysis of videorecordings using Ethovision XT (Noldus Information Technology, Leesburg, VA). Proximity zones were defined as an area within 3.5 cm of the outer edge of the retaining cage. A mouse was considered to have entered a chamber or proximity zone when the head and center points of the body were within the region.

4.5. *In situ hybridization analysis of NR1 deletion*

Mice were anesthetized with Sleepaway (0.04 ml; Fort Dodge Animal Health, Fort Dodge IA; NDC 0856-0471-01) and perfused transcardially with phosphate buffered saline (PBS) for 10 min and then 4% paraformaldehyde (pH 7.4) for 20 min at a rate of 6 ml/min. Brains were postfixed in 4% paraformaldehyde overnight and then stored in 30% sucrose in PBS for ~24 h. All solutions were prepared using diethylpyrocarbonate-treated Type 1 water. 15 μm coronal sections were cut using a cryostat (Leica, Buffalo Grove, IL). Every fourth section between 1.34 and 2.8 mm anterior to bregma (PFC infused mice) or 1.46 and 2.46 mm posterior to bregma (CA3 infused mice) was mounted on glass slides (Paxinos and Franklin, 2001). Sections were processed for in situ hybridization, as previously described (Rajji et al., 2006). The resulting images were used to map the area of gene deletion in each subject, as defined by the region of visibly reduced NR1-specific mRNA probe, relative to adjacent non-deleted tissue. Maps from individual mice, determined to have accurately placed and bilateral deletions, were superimposed and placed on brain atlas plates (Paxinos and Franklin, 2001) to create a composite image.

4.6. *Statistical analyses*

All statistical analyses were performed using PASW Statistics 18 (IBM SPSS Incorporated, Armonk, NY). Data were subjected to ANOVA followed by pairwise comparisons to determine the contribution of individual means to significant interactions and main effects. The level of significance for all analyses was maintained at $p \leq 0.05$. Because control infusions into the PFC and CA3 hippocampus did not differentially affect behavior, data from these mice is combined into single control condition for further statistical analysis and graphing.

Author contributions

JMF: designed and supervised studies and finalized figures, data analysis, and manuscript. GAD, AMI, CJN, TVN, PCR, IS: contributed to behavioral testing, data analysis, and manuscript draft. SAP: performed AAV infusions, analysis of gene deletion region, and contributed to manuscript draft. RWG: provided fNR1 breeding pairs, performed in situ hybridization, and contributed to manuscript draft.

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