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Neuregulin 1 Transgenic Mice Display Reduced Mismatch Negativity, Contextual Fear Conditioning and Social Interactions

Richard S. Ehrlichman[1,*], **Steven N. Luminais**[1,*], **Samantha L. White**[1], **Noam D. Rudnick**[1], **Nan Ma**[2], **Holly C. Dow**[1], **Arati S. Kreibich**[1], **Ted Abel**[2], **Edward S. Brodkin**[1], **Chang-Gyu Hahn**[1], and **Steven J. Siegel**[1,**]

[1] Department of Psychiatry, University of Pennsylvania, Philadelphia, PA, U.S.A.

[2] Department of Biology, University of Pennsylvania, Philadelphia, PA, U.S.A.

Abstract

Introduction—Neuregulin-1 (*NRG1*) is one of susceptibility genes for schizophrenia and plays critical roles in glutamatergic, dopaminergic and GABAergic signaling. Using mutant mice heterozygous for *Nrg1* (*Nrg1*$^{+/-}$) we studied the effects of Nrg1 signaling on behavioral and electrophysiological measures relevant to schizophrenia.

Experimental Procedure—Behavior of *Nrg1*$^{+/-}$ mice and their wild type littermates was evaluated using pre-pulse inhibition, contextual fear conditioning, novel object recognition, locomotor, and social choice paradigms. Event-related potentials (ERPs) were recorded to assess auditory gating and novel stimulus detection.

Results—Gating of ERPs was unaffected in *Nrg1*$^{+/-}$ mice, but mismatch negativity in response to novel stimuli was attenuated. The *Nrg1*$^{+/-}$ mice exhibited behavioral deficits in contextual fear conditioning and social interactions, while locomotor activity, pre-pulse inhibition and novel object recognition were not impaired.

Summary—*Nrg1*$^{+/-}$ mice had impairments in a subset of behavioral and electrophysiological tasks relevant to the negative/cognitive symptom domains of schizophrenia that are thought to be influenced by glutamatergic and dopaminergic neurotransmission. These mice are a valuable tool for studying endophenotypes of schizophrenia, but highlight that single genes can not account for the complex pathophysiology of the disorder.

Keywords

Neuregulin; schizophrenia; mice; animal models; event related potentials; social; behavior

1. Introduction

Schizophrenia is a devastating psychiatric illness that affects about 1% of the world's population (Lewis and Levitt, 2002). Recent molecular genetics studies have identified

**Corresponding Author Steven J. Siegel, MD, PhD Translational Research Laboratories, Rm. 2202 125 S. 31st Street Philadelphia PA 19104 Office: 215 573-0278 Fax:215 573-2041 siegels@upenn.edu.
*Denotes equal contribution in authorship

multiple candidate genes for susceptibility for schizophrenia, such as Neuregulin-1 (*NRG1*), *DISC1*, Dysbindin, *COMT*, and *GAD67* (Guidotti et al., 2000; Harrison and Weinberger, 2005; O'Tuathaigh et al., 2007a).

NRG1 is of particular interest because much of its physiological and molecular activity has been characterized previously and is relevant to the proposed pathophysiology of schizophrenia (Stefansson et al., 2002) (Wen et al., 1992; Yang et al., 2003) (Corfas et al., 2004; Dong et al., 1995; Marchionni et al., 1993; Plowman et al., 1993). NRG1 functions as a growth and differentiation factor and binds the erbB family of receptor tyrosine kinases, specifically erbB2, 3 and 4. Many isoforms of NRG1 are created by alternative splicing, but types 1, 2 and 3 constitute the major forms (Hashimoto et al., 2004). Several of these isoforms have been linked to aspects of neuronal development that are thought to contribute to the onset of schizophrenia. For instance, NRG1 type 1, also known as neu differentiation factor, modulates growth and differentiation of neural crest cells (Corfas et al., 1995; Goodearl et al., 1995). NRG1 type 2 promotes oligodendrocyte production and myelination in the CNS, while type 3 is involved in the development of Schwann cells (Edwards and Bottenstein, 2006; Marchionni et al., 1999; Meyer et al., 1997). NRG1 type 3 is thought to be the most abundant type of NRG1 found in the cortical regions of the brain. It has been shown to be important for normal sensory motor gating and memory functions as well as nicotine mediated hippocampal activity (Chen et al., 2008; Zhong et al., 2008). Of note, one line of *Nrg1* transgenic mice were found to have decreased N-methyl-D-aspartic acid (NMDA) receptor binding when compared to their wild type littermates (Stefansson et al., 2002). Additionally, NRG1 binds preferentially to the ErbB4 receptor, which can modulate the function of glutamatergic receptors, further supporting its possible role in the pathophysiology of schizophrenia (Hahn et al., 2006; Huang et al., 2000; Kwon et al., 2005; Li et al., 2007).

Changes in NRG1 expression may profoundly effect the development and function of the brain through all of the previously discussed mechanisms and, therefore, may contribute to impairments seen in schizophrenia. Individuals with schizophrenia perform poorly across a range of cognitive, behavioral, and physiological measures. Problems with working memory and social interaction can contribute to an inability to function in society (Addington and Addington, 2000; Bowen et al., 1994; Cohen et al., 2006; Corrigan et al., 1994). Additionally, deficits in gating, mismatch negativity (MMN), and pre-pulse inhibition (PPI) of startle are well described endophenotypes of schizophrenia that may underlie some of the aforementioned problems (Boutros et al., 2004; Freedman et al., 1996; Grillon et al., 1992; Javitt et al., 1998; Light et al., 2000; Parwani et al., 2000; Umbricht and Krljes, 2005). Performing similar measures in mice has added a valuable tool for researchers to model schizophrenia. Fear conditioning and novel object recognition paradigms are used to determine if mice display deficits in memory (Crawley, 1999; Hashimoto et al., 2007; Powell et al., 2007). Gating, mismatch negativity, and PPI are evaluated in animals using tasks that are very similar to those used in the human population. Studies from our group as well as others have demonstrated such schizophrenia endophenotypes in rodent models using these tasks (Ehrlichman et al., 2008; Maxwell et al., 2006; Simosky et al., 2003; Swerdlow et al., 1998; Umbricht et al., 2005).

Several groups have reported increases in NRG1 in postmortem brain studies. This may suggest that mice that over-express Nrg1, rather than $Nrg1^{+/-}$, may be a better model for schizophrenia. It is unknown, however, whether the modest increases in NRG1 previously observed are associated with enhanced NRG1 –erbB4 signaling (Chong et al., 2008; Hashimoto et al., 2004; Law et al., 2006). Our group has reported a significant enhancement in erbB4 signaling, when postmortem brain tissues were stimulated with NRG1 (Hahn et al., 2006). In our Western analysis, we failed to detect significant differences in NRG1 expression between the schizophrenia and control groups (Hahn et al., 2006). Interestingly, Nrg1 induced activation

of erbB4 in mice was found to have a trend for an increase in *Nrg1*$^{+/-}$ examined in this study (unpublished observation).

The purpose of this study is to examine the impact of Nrg1 on electrophysiological and social behaviors that have been implicated for schizophrenia. NRG1 has many isoforms and it is not entirely clear as to which isoforms are dysregulated in brains of patients with schizophrenia. Therefore, we chose to examine mice mutated for the activity of all isoforms among various mutant lines available. To accomplish this goal, we chose the mutants developed by Meyer and Birchmeier that contain mutations in EGF domain of all isoforms, which is critical for binding of Nrg1 to the receptors. In various lines of *Nrg1*$^{+/-}$ mice, recent studies showed deficits in physiology and behaviors (Chen et al., 2008; Duffy et al., 2008; O'Tuathaigh et al., 2008; Stefansson et al., 2002). We anticipated a reduction in gating of P20 and N40 event related potentials (ERPs) and an attenuation of MMN in response to novel stimuli. In addition to those electrophysiological measures, we expected the *Nrg1*$^{+/-}$ mice in this study to display impairments in PPI, memory tasks including contextual fear conditioning and novel object recognition, social interaction and increased locomotor activity.

2. Results

ERPs

A reduction in the amplitude of the second response (S2) referenced to the first (S1) was found across genotypes for both P20 and N40 ERPs (P20: $F(1,26) = 38.729$, $p<0.001$, N40: $F(1,26) = 26.34$, $p < 0.001$). There was no main effect of genotype on either P20 or N40 response ($p > 0.05$ for both). There was no stimulus by gene interaction in P20 or N40 ($p > 0.05$ for both). These data do not support the hypothesis that Nrg1 signaling modulates gating of ERPs. Wild type mice displayed a MMN from 50-75 ms ($p<0.05$). *Nrg1*$^{+/-}$ mice did not display the normal pattern of MMN. Rather, mutant mice had a positive difference wave (novel – standard) from 50-100ms (50-75 ms $p<0.05$, 75-100 ms $p<0.05$). There was a significant difference between genotypes for MMN using an independent t-test at 50-100 ms (50-75 ms $t = 2.36$, $df = 26$, $p < 0.05$; 75-100 ms $t = 2.49$, $df = 26$, $p < 0.05$) (Figure 1).

Startle and Pre-pulse inhibition

An rmANOVA showed no main effects of genotype on startle ($F(1, 26) = 0.002$, $p > 0.05$) or PPI ($F(1, 26) = 0.67$, $p > 0.05$). There was a main effect of startle intensity ($F(7, 182) = 12.8$, $p < 0.001$) but no gene by startle interaction ($F(7, 182) = 0.25$, $p > 0.05$) indicating that both wild type and *Nrg1*$^{+/-}$ mice respond similarly to startle stimuli. There was a significant effect of pre-pulse intensity ($F(2, 52) = 4.64$, $p < 0.05$), but no interaction between pre-pulse intensity and genotype ($F(2, 52) = 0.16$, $p > 0.05$) (Table 1).

In a separate cohort of mice treated with saline and amphetamine there was a significant main effect of drug ($F(1, 14) = 6.29$, $p < 0.05$) and startle intensity ($F(7, 98) = 8.13$, $p < 0.001$). There was also a significant interaction of drug and startle intensity ($F(7, 98) = 3.45$, $p < 0.005$) indicating that treatment with amphetamine reduced the level of startle in wild type and *Nrg1*$^{+/-}$ mice compared to treatment with saline. However, there was no three way interaction of gene, startle intensity, and drug ($F(7, 98)=1.05$, $p > 0.05$) showing that amphetamine affected both wild type and *Nrg1*$^{+/-}$ mice similarly in the startle experiment. When examining the pre-pulse inhibition experiment there were no significant main effects of gene ($F(1, 14) = 2.08$, $p > 0.05$), pre-pulse intensity ($F(2, 28) = 1.76$, $p > 0.05$), or drug ($F(1, 14)= 0.79$, $p > 0.05$). Additionally, there was no significant two-way interactions between drug and pre-pulse intensity ($F(2, 28) = 2.72$, $p > 0.05$) showing that amphetamine blocked the ability of wild type and *Nrg1*$^{+/-}$ mice to gate using the pre-pulses. Nor was there a three-way interaction between

drug, pre-pulse intensity, and gene ($F(2, 28) = 0.94$, $p > 0.05$) indicating that wild type and *Nrg1*$^{+/-}$ mice were not differentially affected by amphetamine (Table 2).

Locomotor activity

An rmANOVA showed that there was no main effect of genotype on activity (mean ± SEM; wild type: 1595.2 ± 129.9; *Nrg1*$^{+/-}$: 1554.3 ± 114.6) ($F(1, 22)=.06$, $p > 0.05$). There was no time by genotype interaction ($F(5,110) = 0.62$, $p > 0.05$) across the testing interval.

Contextual Fear Conditioning

There was no main effect of genotype ($p > 0.05$). There was a main effect of session ($p < 0.01$) and a session by genotype interaction ($F(2,36) = 4.56$, $p < 0.05$). A Fisher LSD post hoc test indicated no difference between genotypes for either the pre- or immediate post- shock freezing ($p > 0.05$). There was a significant reduction in freezing by the *Nrg1*$^{+/-}$ mice compared to wild type littermates during the session ($p < 0.05$) (Figure 2).

Novel Object Recognition

No difference in novel object exploration was found between wild type (mean ± SEM: 66.63% ± 1.62) and *Nrg1*$^{+/-}$ mice (mean ± SEM: 60.61% ± 3.87) ($t = 1.36$, $df = 13$, $p > 0.05$).

Social Interaction

The *Nrg1*$^{+/-}$ mice showed increased transitions relative to wild type mice during the social choice test. A rmANOVA showed a significant main effect of time ($F(1,17) = 35.274$, $p < 0.01$) and a significant main effect of genotype ($F(1,18) = 8.1$, $p < 0.05$; paired t test $p < 0.05$ between wild type and *Nrg1*$^{+/-}$ mice during habituation and social phases) on number of transitions between chambers during social testing (Figure 3A). There was no significant interaction between time by genotype ($F(1,17)=0.019$, $p > 0.05$), indicating that both wild type and *Nrg1*$^{+/-}$ mice habituated to the 3-chambered apparatus in a similar manner.

During the social phase (stimulus mouse in the “social cylinder”), the *Nrg1*$^{+/-}$ mice showed significantly lower levels of approach toward stimulus mice relative to wild type mice (significant main effect of time ($F(1,17)=21.850$; $p < 0.001$); significant interaction between time by genotype ($F(1,17)=10.329$; $p = 0.005$) (Figure 3B). In addition, *Nrg1*$^{+/-}$ mice also showed significantly lower levels of direct sniffing of the stimulus mice, relative to wild type mice (significant main effect of genotype on ($F(1,17) = 11.7$, $p = 0.003$); significant main effect of time ($F(1,17) = 92.643$, $p < 0.001$); significant interaction between time by genotype ($F(1,17) = 17.6$, $p < 0.001$) (Figure 3C)). During Phase 3 (free interaction phase) of the social choice test, none of the *Nrg1*$^{+/-}$ or wild type mice attacked (bit) a stimulus mouse, and thus there was no significant difference between mutant and wild type mice in aggressive attack behaviors.

3. Discussion

Expectations, summary of results

Multiple studies have identified *NRG1* as a candidate susceptibility gene for schizophrenia (Harrison and Weinberger, 2005; Stefansson et al., 2003; Weinberger, 2005). We expected *Nrg1*$^{+/-}$ mice to exhibit behavior consistent with mouse models that have been shown to exhibit predictive or construct validity for positive and negative symptoms as well as cognitive and electrophysiological deficits in schizophrenia (Swerdlow and Geyer, 1998). Anticipated results included reduced amplitude and gating of the P20 and N40 ERPs, disrupted mismatch negativity, reduced pre-pulse inhibition, impaired memory function in the Contextual Fear Conditioning and Novel Object Recognition tasks, decreased social interaction and increased

locomotor activity (Duffy et al., 2008; Karl et al., 2007; Moghaddam and Adams, 1998; Siegel et al., in press). We anticipated such alterations in locomotor activity in *Nrg1*$^{+/-}$ mice because of possible interactions between *NRG1* and NMDA function (Stefansson et al., 2002). It is important to note that NMDA antagonists can cause increased, decreased or no change in locomotor activity depending on dose. Therefore, the interaction between the specific *Nrg1* mutation and NMDA receptor function may result in differing locomotor profiles in the various *Nrg1* hypomorphic lines.

Nrg1$^{+/-}$ mice exhibited some, but not all, of these endophenotypes. *Nrg1*$^{+/-}$ mice had reduced freezing in the contextual fear paradigm, disrupted MMN, and reduced sociability compared to wild type controls. No differences were found in locomotor activity, P20 or N40 gating and amplitude, prepulse inhibition, or novel object recognition.

Significance as mouse model of schizophrenia

Our results differ from previous locomotor analysis of *Nrg1*-Tm$^{+/-}$ mice, suggesting that alterations in the expression of the various Nrg1 isoforms may have differential effects on task specific behaviors (Karl et al., 2007). The observation of increased transitions among *Nrg1*$^{+/-}$ mice during the social interaction task also suggests that subtle differences in the metric of activity can influence the outcome. As noted below, increased transitions can not explain the reduction in social behavior. *Nrg1*$^{+/-}$ mice exhibited impairments in experience related memory (contextual fear) but not in object related memory. Studies show that multiple brain regions are required in the creation of a conditioned response (CR), including thalamus, hippocampus and sensory cortex. LeDoux and colleagues showed that hippocampus and amygdala are important for memory consolidation and that conditioned-unconditioned stimulus convergence happens primarily in the amygdala (Davis and Shi, 2000; LeDoux, 1995; Maren, 2001). Similarly, hippocampus and amygdala both play a role in the retrieval and expression of fear memories. Novel object recognition has been shown to primarily involve the perirhinal cortex and hippocampus (Awipi and Davachi, 2008; Bachevalier and Nemanic, 2008; Buffalo et al., 2006; Cornejo et al., 2008). Thus, the hippocampus is critical for both contextual fear and novel object recognition. Alternatively, amygdalar activation is more related to contextual fear memories than novel object recognition and the perirhinal cortex is more critical for novel object recognition (Awipi and Davachi, 2008; Bachevalier and Nemanic, 2008; Buffalo et al., 2006; Cornejo et al., 2008; LeDoux, 1995; Maren, 2001). Impaired performance of *Nrg1*$^{+/-}$ mice in contextual fear testing but not novel object recognition suggests that the amygdala may be more affected by constitutive Nrg1 deficiencies than other regions of interest. The lack of significant differences in novel object recognition between *Nrg1*$^{+/-}$ mice and control mice suggest that Nrg1 deficiency does not alter the hippocampus or perirhinal cortex sufficiently to cause quantifiable changes in the novel object recognition task.

Our finding of reduced sociability in *Nrg1*$^{+/-}$ mice is somewhat different from previous reports in *Nrg1*-TM $^{+/-}$ mice. A previous study of the latter mice found no difference in sociability relative to wild type mice (no difference in social approach toward the first stimulus mouse that is introduced), but a decrease in “preference for social novelty” (decrease in approach to a stimulus mouse that is introduced second, and is relatively unfamiliar) (O'Tuathaigh et al., 2007b). Also, while our study found no difference in aggressive attack behaviors between *Nrg1*$^{+/-}$ and wild type mice, previous studies have found an increase in aggressive behaviors in *Nrg1*-TM $^{+/-}$ mice relative to wild type mice, both in a resident-intruder test and a novel environment (O'Tuathaigh et al., 2007b; O'Tuathaigh et al., 2008). The differences between our findings and those of previous studies may be due to the difference in the mutation being studied (*Nrg1*$^{+/-}$ vs. *Nrg1*-TM$^{+/-}$). Moreover, previous studies used time in the chamber as the only dependent variable for analysis in the social choice task (O'Tuathaigh et al., 2007b),

whereas we analyzed both time in chamber and direct sniffing time (sniffing of cylinder containing the stimlus mouse vs. sniffing of cylinder containing the novel object). Also, in our social choice task, we used gonadectomized A/J stimulus mice in an effort to minimize sexual and aggressive motivations of test mice. Because the previous study used intact C57BL/6J stimulus mice, it is possible that the higher level of social approach of *Nrg1*-TM$^{+/-}$ mice may have been influenced by aggressive motivations (O'Tuathaigh et al., 2007b). Future studies of *Nrg1*$^{+/-}$ mice in the social choice test using non-gonadectomized stimulus mice would be useful in order to determine the degree to which the reduced sociability phenotype that we observed is generalizable to various types of stimulus mice, and to better understand the degree to which the sociability phenotype in *Nrg1*$^{+/-}$ mice can be influenced by aggressive or sexual motivations of the test mice. Although the present study was mainly interested in the effects of genotype on the various phenotypes, future studies will include larger samples of males and females, to test the effect of sex on the phenotypes. The mouse model used to examine social interactions in this study has both face and construct validity for decreased social interactions in schizophrenia. The predictive value of these social deficits with regard to treatment of social withdrawal in schizophrenia is worthy of further study, but this is a challenge because there are not yet medications that effectively treat negative symptoms of schizophrenia (Siegel et al., in press).

Not all of the behaviors exhibited by *Nrg1*$^{+/-}$ mice are consistent with schizophrenia. *Nrg1*$^{+/-}$ mice showed no change in either P20 or N40 amplitude or gating, suggesting that Nrg1-ErbB signaling deficits are not linked to P50 or N100 deficits seen in schizophrenia. This lack of change in P20 and N40 amplitude is consistent with a lack of change in PPI in *Nrg1*$^{+/-}$ mice in the current study. However, others have reported that *Nrg1*-Tm$^{+/-}$ mice have impaired PPI (Stefansson et al., 2002). Differences in results among these studies may be due to the specific nature of the *Nrg1* mutations in each line of mice or differences in compensatory mechanisms for deficiencies in various Nrg1 types. Alternatively, the lack of PPI impairment in *Nrg1*$^{+/-}$ mice used in this study could be related to their hybrid background strain as evidenced by the relatively low maximal PPI levels and high SEM seen in *Nrg1*$^{+/-}$ and wild type littermates. The mice used in this study had maximal PPI levels ranging from 15-30%, whereas in a comparable study with a positive PPI finding the mice had maximal levels ranging from 50 to 70% (Stefansson et al., 2002). Many tasks, including ERPs (Connolly et al., 2003; Maxwell et al., 2006; Metzger et al., 2007; Siegel et al., 2003), PPI (Bullock et al., 1997; Ralph et al., 2001; Willott et al., 2003), sociability (Brodkin, 2007; Fairless et al., 2008; Moy et al., 2004; Moy et al., 2007; Yang et al., 2007) as well as novel object recognition and fear conditioning (Orsini et al., 2004; Sik et al., 2003; Voikar et al., 2005) vary across different inbred strains. Of note, the NRG1 mutant mice are created on a 129/B6 background that would incorporate the inherent capabilities and weaknesses of these two strains.

Although *Nrg1*$^{+/-}$ mice did not show deficits in P20 amplitude or gating, they did demonstrate disrupted mismatch negativity. This MMN disruption is found in humans with schizophrenia and is correlated with functional outcome in patients (Light and Braff, 2005). Furthermore, deficits in MMN have been correlated with an impaired ability to detect and parse novel stimuli in humans, suggesting that *Nrg1*$^{+/-}$ mice may have impaired ability to differentiate novel from familiar auditory stimuli (Javitt et al., 2000). To our knowledge, other variants of *Nrg1*$^{+/-}$ mice have not been tested with auditory ERPs for comparison. Future studies analyzing ERP data from the various *Nrg1*$^{+/-}$ mouse lines may help identify which Nrg1 types are most closely associated with the electrophysiological abnormalities commonly found in schizophrenia.

Limitations

One limitation of the present study is that there may be compensatory changes in other proteins and systems in response to a reduction in Nrg1. Thus, we are unable to determine if the changes

in endophenotypes are caused directly by reduced Nrg1 expression or by changes in other proteins in response to reduced Nrg1. This is consistent with how genetic alterations would exist in humans since such allelic heterogeneity is present throughout development and maturation. Additionally, people with schizophrenia display a range of symptoms and endophenotypic deficits. Each endophenotype may present with different severities and one should not expect a single genetic mutation to recreate all possible deficits found in schizophrenia. Indeed, multiple genes and environmental factors, such as prenatal infection, urban environment, maternal stress and season of birth, have all been linked to small changes in relative risk for the disorder (Castrogiovanni et al., 1998; Izumoto et al., 1999; King et al., 2005; Pedersen and Mortensen, 2006). Additional limitations include mixed gender cohorts and a limited number of animals in several experiments.

Future Studies

As noted above, the predictive validity of reduced sociability in mice for human treatment development is difficult to establish because there are currently no medications that effectively reduce negative symptoms. Further studies aimed at pharmacologically rescuing the sociability phenotype in mouse models (e.g. using oxytocin or drugs that modulate glutamatergic signaling) may facilitate development of novel medications to treat this domain. If progress in both human and mouse studies is able to establish predictive validity of this model, studies involving all *Nrg1* hypomorphic variants may foster novel drug development to target selective types of Nrg1 found to be most closely related to specific functional deficits.

4. Experimental Procedure

Animals

Nrg1$^{+/-}$ mice were obtained from C. Birchmeier (Meyer and Birchmeier, 1995) and bred on a C57BL/6/129 hybrid background at the University of Pennsylvania. Briefly, exon 6 of the neuregulin gene is fused to beta-galactosidase, which results in partial deletion of the EGF like domains of all three major types of Nrg1. All protocols were performed in accordance with University Laboratory Animal Resources guidelines and were approved by the Institutional Animal Care and Use Committee. Mice were group housed, three to four per cage, in a light- and temperature controlled Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility. All efforts were made to minimize animal pain and discomfort. Water and standard rodent chow were available ad lib. Experiments were conducted during the light phase between 10 am and 3 pm. Each experiment utilized a separate cohort of mice and all testing times were balanced for genotype (i.e. animals from both genotypes were either tested simultaneously (ERP, PPI, locomotion) or alternating fashion (sociability).

Genotyping

DNAs were isolated from mouse tails using RED Extract-N-Amp Tissue PCR kit (Sigma). Tissue extracts were mixed with primers 1, 2 and 3 and with Extract-N-Amp PCR reaction mix. PCR was conducted by 35 cycles of amplifications with the annealing temperature of 60° C. Primer 1 (NDF-): 5'-TGC TGC TTT CTT CGC TCT TCA GAA GC -3' Primer 2 (NDF+): 5'- GAG ATG GTC ATG TCC TTG TCA CTA AC -3' Primer 3 (NDFneo): 5'- CGA ATT CGC CAA TGA CAA GAC GCT G -3'.

Electrode Implantation

Animals underwent stereotaxic implantation of electrode assemblies (PlasticsOne, Roanoke, VA) for nonanesthetized recording of auditory ERPs. Animals were anesthetized with isoflurane and unipolar recording electrodes were placed in the CA3 hippocampal region (1.4

mm posterior, 2.65 mm lateral, and 2.75 mm deep relative to the bregma) and referenced to the ipsilateral frontal sinus (Connolly et al., 2003; Connolly et al., 2004; Maxwell et al., 2004). The electroencephalogram (EEG) recorded from this configuration will strongly reflect hippocampal and frontal cortical activity due to placement of the positive and negative electrodes, respectively. Other generators will influence the EEG as an inverse function of their distance from the recording sites. ERPs recorded from this electrode configuration are characteristically similar in appearance to human recordings from the Cz scalp location as illustrated in the third figure from a prior publication by our group (Siegel et al., 2003). The electrode pedestal was secured to the skull using a methyl methacrylate-polymer compound (Ortho Jet; Lang Dental, Wheeling, IL) and ethyl cyanoacrylate (Loctite; Henkel KGaA, Düsseldorf, Germany). All animals were housed singly post electrode placement and received a minimum of 1 week recovery before ERP recordings.

ERP Recording

The recording session consisted of an acclimation run immediately followed by the testing run. Each run included stimuli for a P20 and N40 amplitude and gating task immediately followed by a novelty task. Stimuli were generated and recorded by Micro1401 hardware and Spike 5 software (Cambridge Electronic Design, Cambridge, England) and were delivered through speakers attached to the cage top. Mice were tested in their home cages, which were fitted with special tops to accommodate speakers and electrode cables and placed inside a Faraday cage for a 15 min acclimation period prior to the recording session. All tones were played at 85 dB SPL compared with 70 dB background white noise. The sound pressure was calibrated inside the cage from the approximate height of the animals head with a sound level meter (Radio Shack, Cat. No. 33-2055). *Nrg1*$^{+/-}$ mice were sex and age matched to wild type controls (male n = 8, female n = 6 for each genotype). A ninth male hypomorphic mouse lost his electrode during recording; data from this subject as well as the matched wild type control was therefore discarded. An independent t-test (Statistica, Statsoft, Tulsa, OK) confirms no significant difference in age between genotypes (p=0.77). The mean age of *Nrg1*$^{+/-}$ mice was 17.82 weeks and the average age of wild type controls was 16.85 weeks at ERP testing. All ERP data were analyzed with a repeated measure ANOVA (rmANOVA) and Fisher LSD post hoc (Statistica, Statsoft, Tulsa, OK). Mice used in ERP testing were not used in other experiments.

For the P20 and N40 gating and amplitude task, eighty white-noise clicks (10 ms duration) were presented in pairs 500 ms apart with a 9-sec interpair interval at 85 dB. Waveforms were filtered between 1 and 500 Hz, baseline corrected at the average value 50 ms prior to stimulus onset, and individual sweeps were rejected for movement artifact according to a criterion of two times the root mean squared amplitude per mouse. Average waves were created from 50 ms pre-stimulus to 200 ms post-stimulus. The novelty task consisted of 24 standard tones (7 kHz) followed by a deviant tone that ranged from 5 kHz to 9 kHz in 100 Hz increments. All tones were sinusoidal, 50 ms in duration, and separated by a 500 ms inter-stimulus interval. The order of deviant tones was randomly selected so that half were higher and half lower frequency than the standard tone. Consequently, the mean frequencies of the standard and deviant tones were equal. Each novel tone was used only once for a total of 40 sets of standard/deviant trials at 85 dB. Only the 24th standard tone was used to balance the number of trials when comparing the standard and novel responses. Waveforms were filtered between 1 and 500 Hz, baseline corrected at the average value 50 ms prior to stimulus onset and individual sweeps were rejected for movement artifact based on a criteria of two times the root mean squared amplitude per mouse. No more than 5 trials were rejected from any mouse that was included in the study. Average waves were created from 25 ms prior to stimulus onset until 250 ms post stimulus. The areas under the curve of the standard and novel waveforms were calculated in 25 ms epochs for the *Nrg1*$^{+/-}$ and wild type mice. The difference in area (novel – standard) was used to evaluate the MMN (Ehrlichman et al., 2008).

Pre-pulse Inhibition

Startle responses and inhibition of startle response after presentation of a non-startling pre-pulse were registered by an accelerometer in response to acoustic stimuli delivered by a white noise generator (4–19 kHz) in a four-chamber system (San Diego Instruments, San Diego, CA). All mice were between 14 and 22 weeks of age at time of testing. After the mouse (15 *Nrg1*$^{+/-}$, 5 male, 10 female; 13 wild type, 5 male, 8 female) was placed in the test chamber, the sessions began with a 5-min acclimation interval to a background white noise of 60 dB. This was followed by a block of five 120-dB startle pulses in an effort to make the subsequent startle responses less variable. During the next 10-min block, startle responses were measured to 40-ms pulses of 0 (control), 90, 95, 100, 105, 110, 115, and 120-dB sound pressure. Each pulse was presented five times in random order with an interstimulus interval randomized from 10 to 20 s with a mean of 15 s. The startle portion of the session concluded with an additional block of five 120-dB pulses to assess potential effects of habituation. Startle trials were followed by a 10-min block of PPI trials. Each prepulse trial consisted of a 20-ms prepulse 4, 8, or 16-dB above background noise (60 dB) followed by a 40-ms pulse of 120 dB 100 ms later. Five trials of each prepulse, along with 10 startle-only trials, were presented in random order. Startle responses were collected as 60, 1-ms readings, which were averaged over the collection interval to obtain an average measure for each trial using San Diego Instruments Startle Reflex Software. For the dopamine agonist challenge mice (8 *Nrg1*$^{+/-}$, 2 male, 6 female; 8 wild type, 5 male, 3 female) were given an i.p. injection of either saline or 5 mg/kg d-amphetamine 10 minutes prior to the first tone presentation. PPI was calculated as the percent difference in startle units following the pre-pulse/startle pair as compared to the startle tone alone. *Nrg1*$^{+/-}$ and wild type mice were compared using an rmANOVA (Statistica, Statsoft, Tulsa, OK).

Contextual Fear Conditioning

The contextual fear conditioning experiments were performed in a rectangular chamber (16”L×6”W×8 3/8” H) as previously described by Graves and colleagues (Graves et al., 2003). Mice (*Nrg1*$^{+/-}$: male n = 4, female n = 7; wild type: male n = 7, female n = 2) were handled for three consecutive days before training, for 1 min each day. All mice were between 12 and 18 weeks of age at time of testing. For the training session, two 2-sec 1.5-mA scrambled footshocks were delivered at 2 min and 2.5 min after placing the mice into the conditioning chamber. Mice were removed from the chamber and returned to their home cages after 3 min. Freezing was assessed prior to and after the shock (pre- and post- shock sessions). 24 hours after training, mice were placed back into the same chamber (conditioned context) in the absence of shock for 5 min and their freezing behavior was assessed during this period (testing period). All training and testing sessions were conducted and analyzed by an individual blind to the genotypes of the animals examined. *Nrg1*$^{+/-}$ and wild type mice were compared using an rmANOVA and a Fisher LSD post hoc (Statistica, Statsoft, Tulsa, OK).

Novel Object Recognition

The novel object recognition experiment was performed as previously described by Wood and colleagues (Wood et al., 2006). All mice (*Nrg1*$^{+/-}$: male n = 4, female n = 4; wild type: male n = 5, female n = 2) were handled for 1 min a day for 2 days and then habituated to the experimental apparatus (a rectangular open field) with 5 min of exploration in the absence of objects before training. All mice were between 16 and 22 weeks of age at time of testing. During the training session, mice were placed back in the experimental apparatus for 15 min. Two identical objects were placed at specific locations in the open field for mice to freely explore. 24 hrs later, mice were placed back into the rectangular environment for 15 min for the testing session. Two objects were again present at the same location, but one of the familiar objects was replaced by a novel one. All testing and training sessions were videotaped and analyzed

by an individual blind to the genotype of the animals. Arenas were drawn using the MED Associates software (MED Associates, VT) such that each object was segmented from the background to properly allow the software to detect mouse-object interactions. Software settings were as follows: animal size (10-6000), animal color (Dark in a light background), max animal movement per frame (20 pixels), animal orientation direction by tail shape (minimum elongation of 1.5). Each mouse was scored on the percentage of time it spent on exploring each object during the training and testing session. Time spent on exploring was defined as any time the mouse approached or spent sniffing the object while oriented towards it. Scores were compared using an independent t-test (Statistica, Statsoft, Tulsa, OK).

Locomotor Testing

Locomotor testing was similar to previously described procedures (Halene and Siegel, 2008; Sadalge et al., 2003). Mice (*Nrg1*$^{+/-}$: male n = 6, female n = 6, wild type: male n = 6, female n = 6) were allowed to acclimate to the testing room for at least 15 min prior to recoding. All mice were between 14 and 22 weeks of age at time of testing. After acclimation, the mice were placed in a home cage environment inside an automated locomotor activity photobeam frame (30 ×24 ×8 cm) with sensors arranged in an 8-beam array strip with 1.25” spacing (MED Associates, VT). For 30 min, the distance traveled was recorded by a computer using MED PC software that was set to record the number of times the horizontal light beams were broken in 5 minute bins. All testing was conducted during the light phase and results were analyzed using an rmANOVA (Statistica, Statsoft, Tulsa, OK).

Sociability

The sociability of a separate cohort of 11 *Nrg1*$^{+/-}$ mice (7 females and 4 males) was compared to that of 8 wild type mice (2 females and 6 males). The degree of social approach for a test mouse (*Nrg1*$^{+/-}$ or wild type) towards a novel, unfamiliar stimulus mouse was measured in a social choice test in a three-chambered apparatus as previously described (Brodkin et al., 2004; Brodkin, 2007; Fairless et al., 2008; Sankoorikal et al., 2006). The three-chambered apparatus had no top or bottom and consisted of a center chamber and two end chambers, with dimensions that have been reported previously (Sankoorikal et al., 2006). Behavioral testing was videotaped with a Sony digital videocamera with NightShot (infrared) feature for recording in low light. To minimize the general stress level of the mice, the testing room was very dimly lit; the lighting within all chambers measured at 1 – 2 lux during testing. Prior to the start of the test, one end chamber was designated the “social chamber,” into which a stimulus mouse would be introduced, and the other end chamber was designated the “nonsocial chamber.” The end chamber designated as the social chamber was varied in a counterbalanced sequence among tests. Before each test, the apparatus was placed on a clean mat and clean mouse bedding. Two identical, clear, Plexiglas cylinders (each 7 cm in diameter, 12.2 cm tall) with removable, black, Plexiglas lids were placed in the apparatus, one in each end chamber. The stimulus mouse could move around easily within the cylinder. The cylinders had multiple holes (1 cm in diameter) to allow for air exchange between the inside and outside of the cylinder. Auditory, visual, and olfactory investigation between a mouse inside and a mouse outside the cylinder was thus possible. Between tests, the apparatus, cylinders, and paperweight were all washed with copious amounts of water and dried before testing the next mouse.

The following modifications of the previously described procedure were made. Because we were primarily interested in measuring nonaggressive and nonsexual affiliative social interactions of test mice, we tried to minimize aggressive and sexual motivations of the test mouse towards the stimulus mouse by pairing castrated male A/J stimulus mice with male test mice, and ovariectomized female A/J stimulus mice with female test mice. The habituation phase, (Phase 1) during which time the test mouse was allowed to initially explore the apparatus, lasted 10 minutes, which was split into two 5 min intervals for data analysis (Phase

1A and Phase 1B) and then averaged. At the beginning of the Social Phase (Phase 2), which lasted 5 minutes, a gonadectomized A/J stimulus mouse was placed in the "social cylinder" in the social side of the apparatus, and simultaneously, an inanimate object (black plastic block) was inserted into the "nonsocial cylinder". During the free interaction phase (Phase 3), which lasted 5 minutes, the cylinders were removed and the test mouse and stimulus mouse were allowed to interact freely, in order to determine whether the social approach behavior may have been influenced by aggressive motivations. Free interaction was terminated if there was more than 3 seconds of aggressive, attack behavior (biting, vigorous lunging).

The mean $^{+/-}$ standard error was calculated for the following variables for each group of mice defined by genotype: time in social and nonsocial chambers, time sniffing the social and the nonsocial cylinders, and numbers of transitions between chambers. Time sniffing the social and nonsocial cylinders was operationally defined as the time during which the test mouse made direct nose-to-cylinder contact with the social and nonsocial cylinders, respectively. These values were calculated for the Habituation Phase (Phase 1A,stimulus mouse absent, 0-5 minutes and Phase 1B, stimulus mouse absent, 5-10 minutes) as well as for the Social Phase (Phase 2 ;stimulus mouse present, 10-15 minutes). A chamber preference score was calculated for each mouse by subtracting the time spent in the nonsocial side from the time spent in the social side of the apparatus for each phase. In addition, a cylinder sniffing preference score was calculated by subtracting the time spent sniffing the nonsocial cylinder from the time spent sniffing the social cylinder for each phase. A positive score signified a predominance of approach or sniffing towards the social side, whereas a negative score implied a predominance of approach or sniffing towards the nonsocial side. For statistical analysis, the mean of the chamber preference scores for each mouse in habituation Phase 1A and Phase 1B was calculated, as was the mean of the cylinder sniffing preference score for each mouse in Habituation Phase 1A and 1B. *Nrg1*$^{+/-}$ vs. wild type chamber preference scores and cylinder sniffing preference scores during habituation (mean of Phase 1A and Phase 1B) vs. social (Phase 2) periods were compared with repeated measures ANOVAs. The number of transitions of *Nrg1*$^{+/-}$ vs. wild type mice was compared during the habituation vs. social phases, using a rmANOVA.

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Abbreviations

Nrg1, neuregulin-1; NMDA, N-methyl-D-aspartic acid; PPI, pre-pulse inhibition; MMN, mismatch negativity; ERP, event-related potentials.

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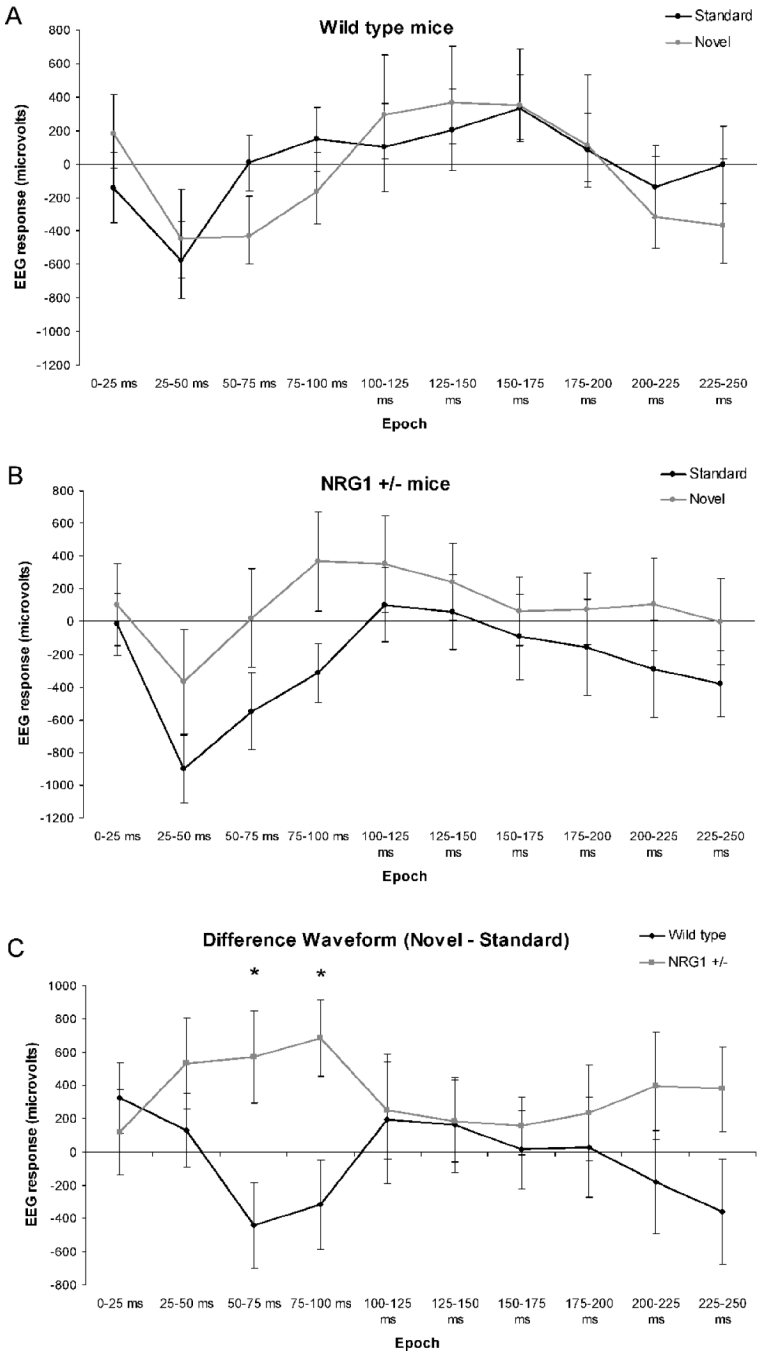

Figure 1. Mismatch Negativity

The area under the curve in response to the standard (black line) and novel (gray line) stimuli for (A) wild type and (B) *Nrg1*$^{+/-}$ mice are shown. (C) The difference in area (standard – novel) for both the wild type (black line) and *Nrg1*$^{+/-}$ (gray line) are shown. A significant difference between wild type and *Nrg1*$^{+/-}$ waveforms exists at 50-100 ms and 75-100 ms. * $p < 0.05$

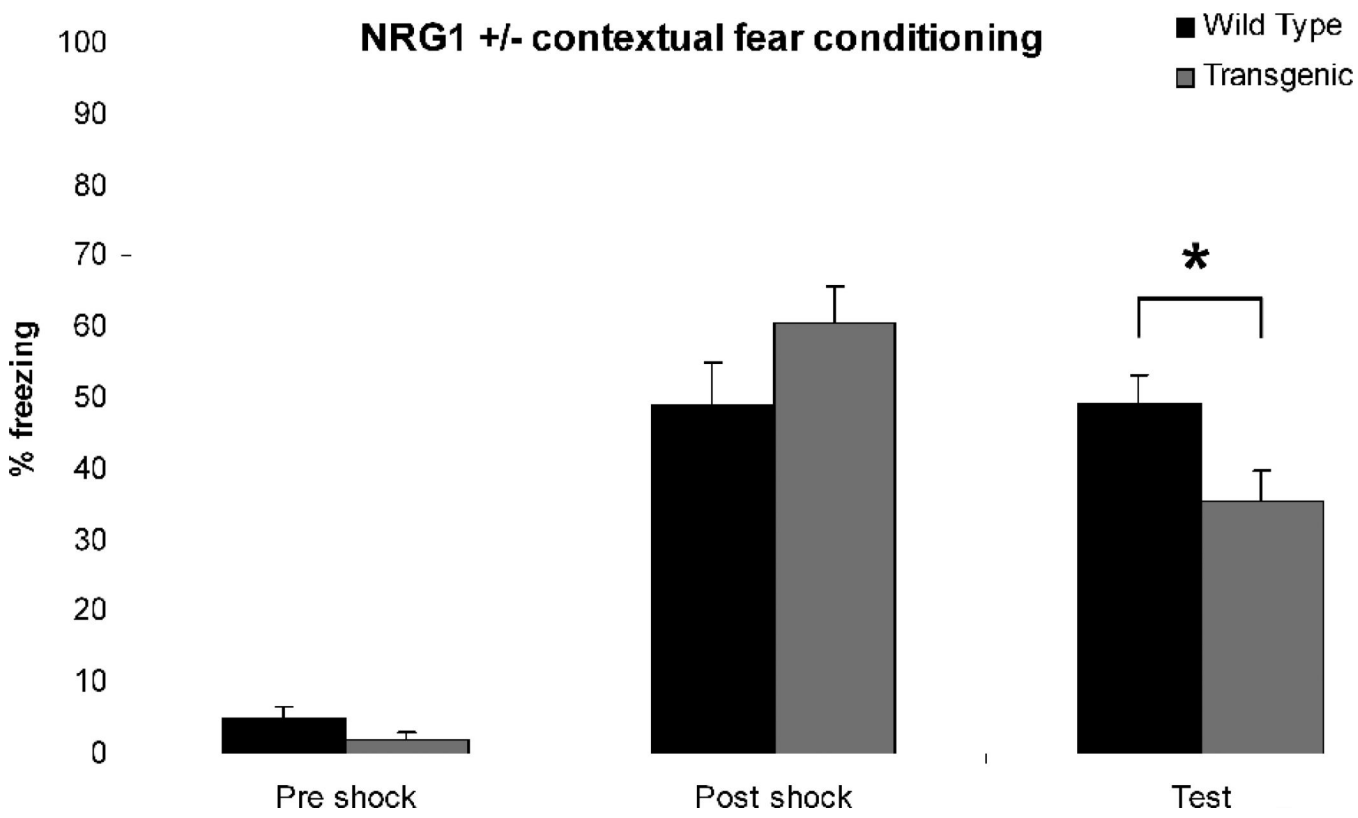

Figure 2. Contextual Fear Conditioning

Comparison of wild type (black) and *Nrg1*$^{+/-}$ (gray) mice revealed no significant difference between genotypes for the degree of pre- or immediate post-shock freezing. There was a significant decrease in freezing of *Nrg1*$^{+/-}$ mice during the test phase. * $p < 0.05$

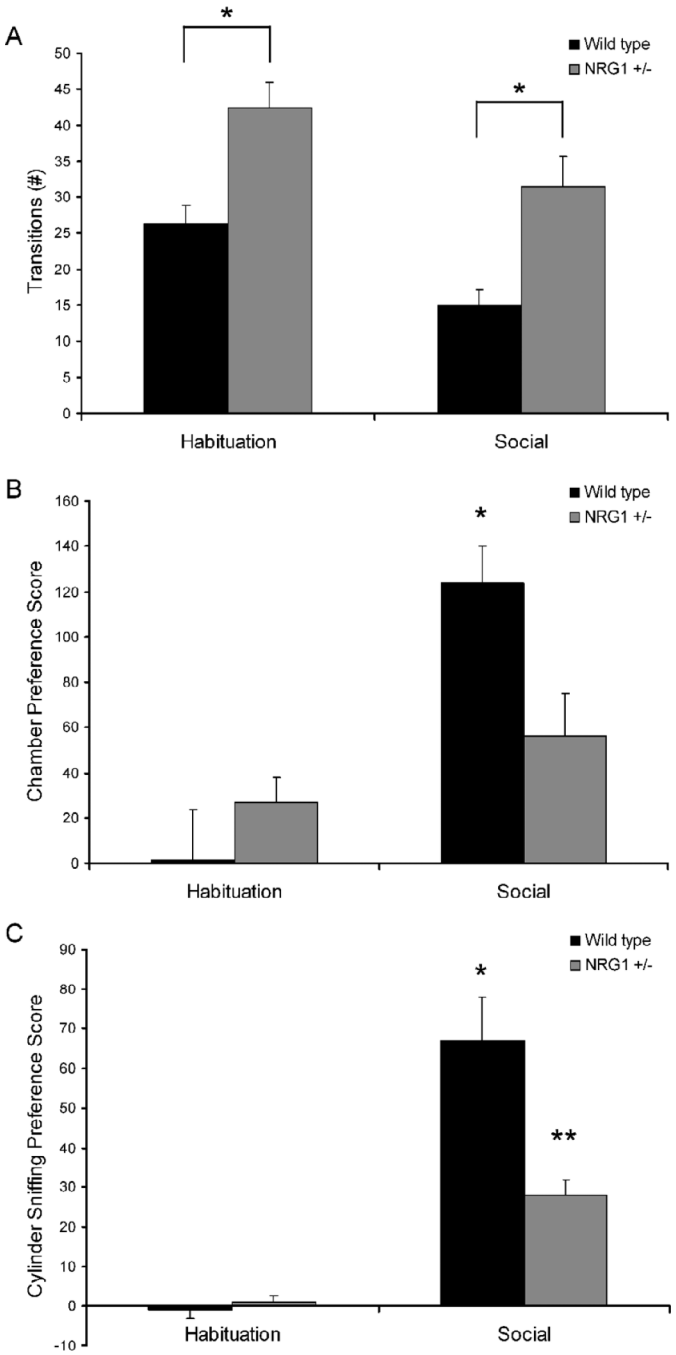

Figure 3. Social Choice Task

Habituation scores are the mean of the two 5-minute habituation periods (mean of Phase 1A and 1B), during which the cylinders were empty. Social scores are from the 5-minute Social Phase (Phase 2), during which the social cylinder contained a stimulus mouse, and the nonsocial cylinder contained a novel object. (A) *Nrg1*$^{+/-}$ mice showed increased transitions relative to wild type mice during the social choice test, suggesting that decreased social interactions were not related to a decrease in entries into the social chamber. (B) *Nrg1*$^{+/-}$ mice showed significantly lower levels of approach toward stimulus mice relative to wild type mice. (C) *Nrg1*$^{+/-}$ mice also showed significantly lower levels of direct sniffing of the stimulus mice, relative to wild type mice.

Table 1

Startle and Pre-Pulse Inhibition

Startle Intensity	WT (V ± SEM)	*Nrg1*$^{+/-}$ (V ± SEM)
0 dB	9.49 ± 0.67	9.08 ± 0.63
90 dB	10.46 ± 0.82	10.56 ± 0.77
95 dB	12.23 ± 2.12	15.55 ± 1.98
100 dB	14.2 ± 2.32	15.23 ± 2.16
105 dB	17.55 ± 2.58	19.2 ± 2.40
110 dB	24.94 ± 5.17	24.33 ± 4.81
115 dB	28.05 ± 6.79	25.92 ± 6.32
120 dB	33.78 ± 5.97	29.75 ± 5.55

PPI Intensity	WT (% inhibition ± SEM)	*Nrg1*$^{+/-}$ (% inhibition ± SEM)
+ 4 dB	−2.54 ± 10.63	10.94 ± 9.89
+ 8 dB	13.17 ± 11.27	20.18 ± 10.49
+ 16 dB	15.6 ± 10.35	27.19 ± 9.63

The mean voltages ± SEM are shown for wild type and *Nrg1*$^{+/-}$ mice for all startle intensities. The mean percent inhibition ± SEM is shown for wild type and *Nrg1*$^{+/-}$ mice for each pre-pulse intensity level (+4, +8 and +16 dB over background).

Table 2

Startle and Pre-Pulse Inhibition (Saline vs. Amphetamine)

Startle Intensity	WT (Saline)	WT (Amph)	*Nrg1*$^{+/-}$ (Saline)	*Nrg1*$^{+/-}$ (Amph)
0 dB	6.1 ± 1.47	8.63 ± 1.66	9.25 ± 1.47	7.83 ± 1.66
90 dB	8.73 ± 2.10	9.73 ± 1.87	7.9 ± 2.10	8.10 ± 1.87
95 dB	18.28 ± 3.72	15.85 ± 3.37	11.9 ± 3.72	14.45 ± 3.37
100 dB	25.98 ± 7.00	16.35 ± 3.61	18.88 ± 7.00	16.08 ± 3.61
105 dB	41.75 ± 14.38	19.30 ± 3.79	25.93 ± 14.38	18.13 ± 3.79
110 dB	58.05 ± 17.46	27.98 ± 9.75	29.3 ± 17.46	24.85 ± 9.75
115 dB	59.48 ± 18.97	30.68 ± 10.26	45.32 ± 18.97	27.53 ± 10.26
120 dB	85.95 ± 27.31	34.05 ± 7.88	42.83 ± 27.31	28.60 ± 7.88

PPI Intensity	WT (Saline)	WT (Amph)	*Nrg1*$^{+/-}$ (Saline)	*Nrg1*$^{+/-}$ (Amph)
+ 4 dB	6.54 ± 16.15	−10.81 ± 15.83	−30.41 ± 21.01	−6.54 ± 25.93
+ 8 dB	7.87 ± 13.25	−9.57 ± 13.95	−25.68 ± 18.30	−44.51 ± 23.35
+ 16 dB	29.28 ± 11.68	−8.62 ± 17.13	10.98 ± 14.07	−31.59 ± 24.95

The mean voltages ± SEM are shown for wild type and *Nrg1*$^{+/-}$ mice for all startle intensities following treatment with saline or amphetamine. The mean percent inhibition ± SEM is shown for wild type and *Nrg1*$^{+/-}$ mice for each pre-pulse intensity level (+4, +8 and +16 dB over background) following treatment with saline or amphetamine.