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Overexpression of Neuregulin 1 Type III Confers Hippocampal mRNA Alterations and Schizophrenia-Like Behaviors in Mice

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***Neuregulin 1* (*NRG1*) is a schizophrenia candidate gene whose protein product is involved in neuronal migration, survival, and synaptic plasticity via production of specific isoforms. Importantly, *NRG1 type III* (*NRG1 III*) mRNA is increased in humans inheriting a schizophrenia risk haplotype for the *NRG1* gene (Hap_{ICE}), and NRG1 protein levels can be elevated in schizophrenia. The nature by which NRG1 type III overexpression results in schizophrenia-like behavior and brain pathology remains unclear, therefore we constructed a transgenic mouse with *Nrg1 III* overexpression in forebrain neurons (CamKII kinase+). Here, we demonstrate construct validity for this mouse model, as juvenile and adult *Nrg1 III* transgenic mice exhibit an overexpression of *Nrg1 III* mRNA and Nrg1 protein in multiple brain regions. Furthermore, *Nrg1 III* transgenic mice have face validity as they exhibit schizophrenia-relevant behavioral phenotypes including deficits in social preference, impaired fear-associated memory, and reduced prepulse inhibition. Additionally, microarray assay of hippocampal mRNA uncovered transcriptional alterations downstream of *Nrg1 III* overexpression, including changes in *serotonin receptor 2C* and *angiotensin-converting enzyme*. Transgenic mice did not exhibit other schizophrenia-relevant behaviors including hyperactivity, social withdrawal, or an increased vulnerability to the effects of MK-801 malate. Our results indicate that this novel *Nrg1 III* mouse is valid for modeling potential pathological mechanisms of some schizophrenia-like behaviors, for determining what other neurobiological changes may be downstream of elevated *NRG1 III* levels and for preclinically testing therapeutic strategies that may be specifically efficacious in patients with the NRG1 (Hap_{ICE}) risk genotype.**

Key words: schizophrenia/neuregulin 1/type III/transgenic/mouse/behavior

Introduction

Neuregulin 1 (*NRG1*) has been associated with the risk of developing schizophrenia across multiple ethnic populations,[1–3] but not in all genetic studies (ie, Duan et al,[4] for a recent review, see Mostaid and colleagues[5]). Despite this, continuing to assess the role of abnormal NRG1 signaling in schizophrenia is warranted due to the heterogeneity of *NRG1* in schizophrenia[6] (which may explain the difficulties in detecting association in genetic studies), the numerous preclinical studies which illustrate the potency by which Nrg1 perturbations induce schizophrenia-like phenotypes, the studies of postmortem brain which implicated altered Nrg1/ErbB4 levels in the disease[5] and also a recent meta-analysis of NRG1 in schizophrenia which supports that a genetic association may exist in some populations.[7] The *NRG1* gene encodes the NRG1 protein, which mediates cell survival, synaptic plasticity, neuronal migration, and myelination.[8,9] Over 30 different unique isoforms of NRG1 are categorized into 6 main types (types I–VI) depending on the identity of proteins in the N-terminus.[10,11] The type III isoform (NRG1 III) is unique as it contains a cysteine-rich domain in the N-terminus and a transmembrane domain and thus, possesses 2 cleavable transmembrane regions.[10,12] NRG1 III is also the most highly expressed NRG1 isoform type in the

adult human and rodent brain suggesting that it may be the most neurobiologically significant and behaviorally potent NRG1 type.[13]

Recently, we identified a putative molecular mechanism for increasing genetic risk for schizophrenia whereby inheriting more copies of the *NRG1* schizophrenia risk haplotype (named Hap_{ICE}) increases the cortical expression of *NRG1 III* mRNA,[6] while another study has shown that the ratio of *NRG1 II* mRNA to *NRG1 III* is decreased in schizophrenia.[14] This compliments earlier studies where elevated levels of brain *NRG1 I* and *IV* mRNA,[14,15] NRG1 protein[16] and increased NRG1 signaling in postmortem tissue from schizophrenia patients have been observed.[17] Together, this evidence suggests that a gain of function of NRG1 may serve as a contributing factor to the pathophysiology of this mental disorder although there is debate about which isoform of NRG1 contributes most strongly to schizophrenia.[14,18]

Genetic disruptions that either model decreased or increased expression of particular Nrg1 isoforms can confer distinct schizophrenia-like behavioral phenotypes. A mouse model with a haploinsufficiency for *Nrg1 III* has impaired prepulse inhibition (PPI) and cognitive deficits in delayed alternation in the T-maze.[19] In gain-of-function *Nrg1* mouse models, Nrg1 type I overexpression leads to hyperlocomotion, deficits in learned fear to a context (but not to a cue)[20,21] as well as reduced cognitive performance in spatial tasks[22] social interaction, impaired social recognition memory, and PPI deficits.[21,23] The behavioral impact of expected neurobiological change resulting from inheriting the *NRG1* Hap_{ICE}, *NRG1 III* overexpression, has not yet been behaviorally characterized even though an overexpressing *Nrg1 III* transgenic mouse model was created in 2004.[24] This *Nrg1 III* mouse is not suited for assessing schizophrenia-like behaviors as the neuromuscular junction in these mice is severely compromised and exhibits a tremor.[25] Thus, we sought to construct a mouse that could be validated for both overproduction of brain *Nrg1 III* and for schizophrenia-like behavioral and brain biological phenotypes.

Materials and Methods

Generation of Transgenic Animals

Briefly, a transgenic mouse with CamKII promoter driven *Neuregulin 1 type III* was generated on a C57BL/6NTac background (for details, see supplementary methods).

Test Animals

For our behavioral studies, male heterozygous *Neuregulin 1 type III* overexpressing mice (*Nrg1 III tg*) (n = 10) and male wild type (WT) (n = 10) control littermates aged 28 (±2) weeks were used. For mRNA assays, male *Nrg1 III tg* mice and WT aged 28 days and 39.5 (±2.5) weeks were used, while mice aged 39.5 (±2.5 weeks) were used for protein western blotting. Research and animal care procedures were approved by the University of New South Wales Animal Care and Ethics Committee and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (ACEC#14/49A) (refer to supplementary methods).

Molecular Confirmation of Nrg1 Type III *mRNA Overexpression and Transcriptional Changes of 4 Genes from Hippocampal Microarray*

Total RNA from the olfactory bulb, prefrontal cortex, dorsal striatum, hippocampus, and cerebellum of *Nrg1 III tg* and WT mice was assayed via standardized quantitative real-time polymerase chain reaction procedure with TaqMan probes to *Nrg1 III*, and to targets identified by microarray (*RIKEN cDNA 181005G02, potassium inwardly rectifying channel member 13*, and *transthyretin*). mRNA expression was normalized to the geometric mean of reference genes (see supplementary materials for full details). Total protein was isolated from the prefrontal cortex and Nrg1 was quantified using western blotting with an antibody specific to the C-terminus of the Nrg1 protein (1:300, sc-348, Santa Cruz Biotechnology, refer to supplementary materials for details).

Microarray

Hippocampal RNA of WT (n = 10) and *Nrg1 III tg* (n = 8) mice (aged 39.5 [±2.5] weeks) were assessed using the Affymetrix GeneChip Mouse Gene 2.0 ST Array, performed by The Ramaciotti Centre for Genomics as per the manufacturer's instructions (for further details, see supplemental materials).

Behavioral Testing

Behavioral tests were performed in the following order: elevated plus maze (EPM; anxiety and exploration), social interaction (sociability), Y-maze (spatial memory), social preference task (social preference and recognition memory), prepulse inhibition (sensorimotor gating), fear conditioning (fear associated memory), and 15 min baseline open field (exploration and locomotion) with an additional 90-min period in open field after MK-801 hydrogen malate (0.25 mg/kg, [injection volume of 10 ml/kg body weight] subcutaneous injection: Sigma–Aldrich) challenge (behavioral sensitivity to N-methyl-D-aspartate [NMDA] receptor antagonism). Finally, mice underwent a comprehensive physical exam, which screened for sensory, motor function, and neurological behavioral deficits. The behavioral tasks and protocols used are described in our previous publications[26–32] (for details of all tests, see supplementary material).

Statistical Analysis

For statistical procedures, please refer to the supplementary materials.

Results

Nrg1 Type III mRNA and Protein Levels

We found a >5 times increase of normalized *Nrg1 III* mRNA in the olfactory bulb [$t(17) = 14.0$, $P < .0001$], prefrontal cortex [$t(17) = 34.3$, $P < .0001$], dorsal striatum [$t(17) = 6.8$, $P < .0001$], hippocampus [$t(17) = 13.0$, $P < .0001$] and a ~30% increase in the cerebellum of heterozygous *Nrg1 III* overexpressing (*Nrg1 III tg*) mice compared with WT mice [$t(16) = 3.0$, $P < .01$] (figure 1A) confirming that insertion of multiple copies of the transgene lead to robust forebrain *Nrg1 type III* overexpression. Supporting this, relative levels of full-length and cleaved Nrg1 protein from the prefrontal cortex across

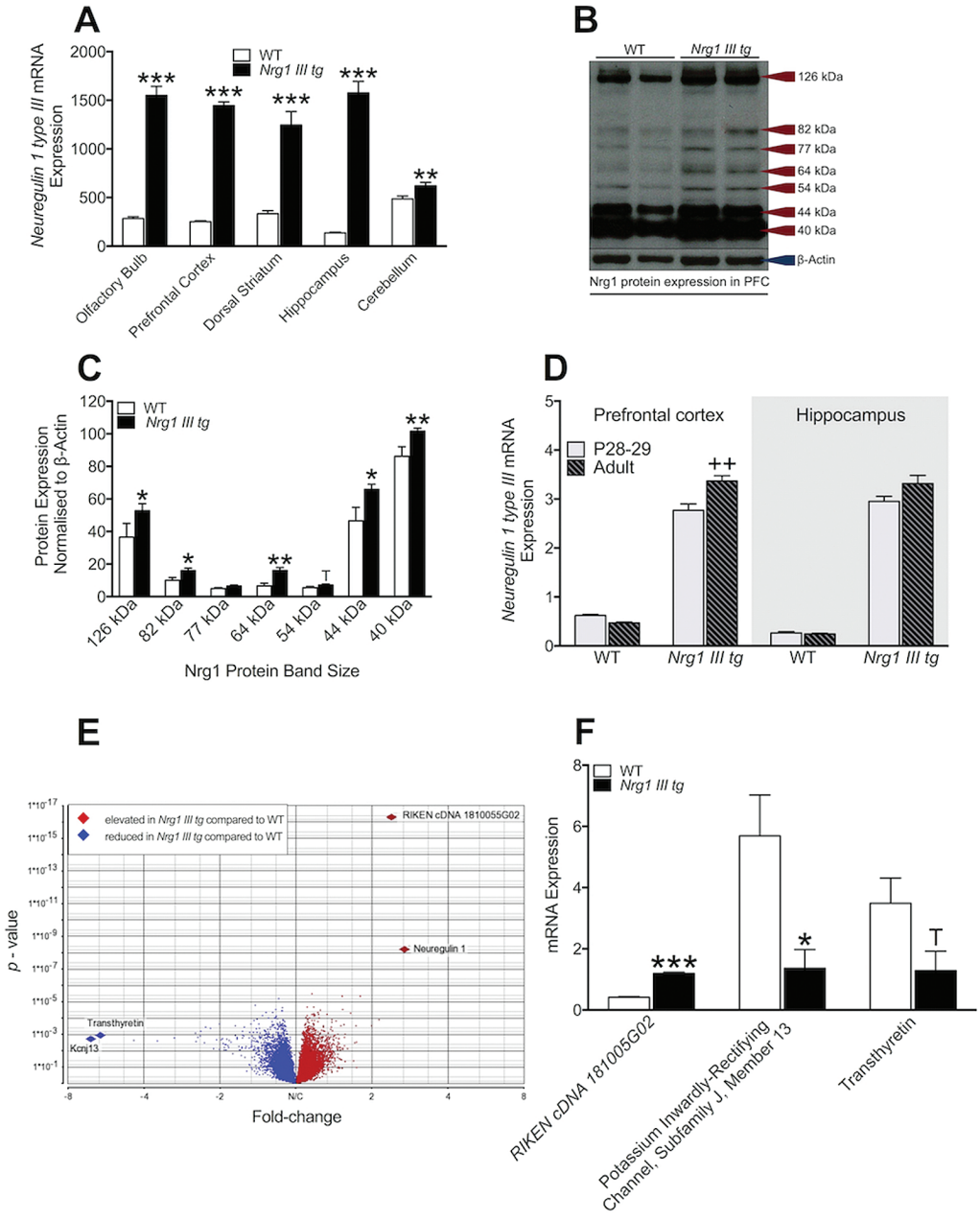

Fig. 1. *Nrg1 type III* mRNA and protein levels, hippocampal microarray, and validating qPCR in WT and *Nrg1 III* transgenic mice. (A) Normalized relative levels of *Nrg1 type III* mRNA expression in the olfactory bulb, prefrontal cortex, dorsal striatum, hippocampus, and cerebellum, (B) representative immunoreactive bands for Nrg1 protein with interpolated approximate band sizes, (C) normalized levels of Nrg1 protein levels in the prefrontal cortex, (D) normalized *Nrg1 type III* mRNA levels in the prefrontal cortex and hippocampus of postnatal day 28–29 and adult (40–42 weeks old) *Nrg1 type III* transgenic and WT mice, (E) volcano plot of hippocampal microarray with 4 representative significantly changed genes across genotype identified and (F) qPCR mRNA expression of the 3 representative genes. Data for control (WT) and *Nrg1 type III* transgenic (*Nrg1 III tg*) mice are shown as mean + SEM. Significant genotype effects vs WT are shown as "*" (*$P < .05$, **$P < .01$, and ***$P < .001$). Trend genotype effects vs WT is shown as "T" (T $P = .053$). Significant effect between postnatal day 28–29 and adult (40–42 weeks old) in *Nrg1 III tg* mice is shown as "++" (++$P < .01$).

several band sizes were elevated in transgenic mice compared with WT mice (126 kDa) [$t(14) = 2.4$, $P < .02$], (82 kDa) [$t(13) = 2.6$, $P < .02$], (77 kDa) [$t(13) = 1.7$, $P = .06$], (64 kDa) [$t(13) = 3.6$, $P < .002$], (54 kDa) [$t(13) = 1.6$, $P = .07$], (44 kDa) [$t(14) = 2.4$, $P < .02$], (40 kDa) [$t(12) = 3.1$, $P < .004$] (figures 1B and 1C).

To assess whether the transgene expression of *Nrg1 type III* mRNA varies with age, *Nrg1 type III* mRNA expression levels in *Nrg1 III tg* and WT mice were measured at 2 time points: juvenile (postnatal day 28 [P28]) and adult (39.5 [±2.5] weeks) and in 2 brain regions (prefrontal cortex and hippocampus) (figure 1E). We found a significant "genotype" by "age" interaction in the prefrontal cortex [$F(1, 25) = 11.8$, $P = .002$] whereby *Nrg1 type III* mRNA in *Nrg1 III tg* adult mice was significantly elevated compared with *Nrg1 III tg* P28 mice ($P < .001$). This effect was not seen in WT mice. For the hippocampus, we did not detect an "age" by "genotype" interaction effect for *Nrg1 type III* mRNA levels. For both ages and for both regions, *Nrg1 type III* mRNA levels were significantly increased in *Nrg1 III tg* mice compared with the respective age group of WT mice ($P < .0001$).

Hippocampal Microarray mRNA Assay Results and Confirmation via quantitative polymerase chain reaction

A total of 187 differentially expressed transcripts were detected in *Nrg1 III tg* mice compared with WT (figure 1D and supplementary materials). Of the 187 transcripts, we selected 3 (2 of those most differentially expressed and 1 at moderate level of significance) and assessed the mRNA expression of these transcripts via qPCR in order to verify the results and robustness of the microarray analysis: *RIKEN cDNA 181005G02* (upregulated compared with WT) [$t(15) = 16.9$, $P < .0001$], *Potassium inwardly rectifying channel, subfamily J member 1*3 (downregulated compared with WT) [$t(16) = 2.7$, $P = .02$] and *Transthyretin* (trend downregulated compared with WT) [$t(17) = 2.1$, $P = .053$] (figure 1F). After validating the microarray, we performed a PubMed literature search on the 187 differentially expressed transcripts and found several downregulated genes that have been implicated in schizophrenia, including *5-hydroxytryptamine (serotonin) receptor 2C*,[33] *angiotensin converting enzyme*,[34] *sulfatase 1*,[35] and *transthyretin*.[36] Additionally, we screened for immunologically associated transcripts as Nrg1 has been shown to regulate the immune response in the brain[37] and abnormal neuroinflammatory processes have been linked to schizophrenia.[38,39] In total, we found several altered immune-related transcripts including *Serpinb1b*, *Mcfd2*, *Fgl2*, *Cldn9*, *Gas6*, *Prlr*, *Gulp1*, *Cdk6*, *Spint2*, *Lbp*, *Ltc4s*, *Mir9-1*, *Sulf1*, *Ifi27*, *Kl*, *Ddr2*, *Trpm3*, *Bcl2a1a*, *C1qtnf5*, *Cldn2*, *F5*, *Lepr*, *Psmb5-ps*, *Ucp2*, *Slc39a4*, and *1500015O10Rik* (*Ecrg4*). Finally, several members of the insulin-like growth factor (Igf) pathway including *Igf2*, *Igf binding protein 2* (*Igfbp2*), and *Igfbp7* appear to be downregulated in *Nrg1 III tg* mice. These transcripts may be of interest as Igf signaling, like Nrg1 signaling, mediates neurogenesis and cell survival.[40]

Physical Exam and General EPM Behavior

Nrg 1 III tg mice did not display deficits in motor functions, neurological behaviors or in any of the sensory abilities measured in this test compared with WT mice (all *P*s > .05; table 1). Using one-way ANOVA, no differences between *Nrg1 III tg* and WT mice were observed for measures of time spent in open arms, percentage of open arm entries, overall distance travelled in the arms of the maze nor for duration or frequency of *grooming*, *head-dipping*, *rearing*, and *stretch-attend posture* (all *P*s > .05; table 1).

Social Behaviors

No differences were detected in the social interaction test between *Nrg1 III tg* and WT mice for duration and frequency of social behaviors or for overall active social interaction time (all *P*s > .05; table 1). When analyzing the sociability trial of the social preference test, we found that both WT and *Nrg1 III tg* mice spent more time in the chamber containing a mouse than the empty chamber ["chamber" $F(1, 18) = 9.3$, $P = .007$; no "chamber" by "genotype" interaction] (figure 2A) indicating normal preference for social stimuli, supporting our findings in the social interaction test. However, in the social recognition/preference memory test, we found a significant "chamber" by "genotype" interaction effect [$F(1, 18) = 4.7$, $P = .04$] where WT mice spent a significantly higher percentage of time in the chamber containing the novel mouse as compared with the familiar mouse [$P = .03$] whereas the *Nrg1 III tg* mice did not demonstrate this preference for the novel mouse [$P = .5$]. In agreement with this, WT mice spent a significantly higher percentage of time in the chamber containing the novel mouse than by chance [58.97%, $t(18) = 2.2$, $P = .04$], whilst the time spent by *Nrg1 III tg* mice in the chamber containing the novel mouse was lower and was not significantly different than chance [47.20%, $t(18) = .8$, $P = .44$].

Cognitive Behaviors

For the Y-maze, we did not detect a "genotype" effect on percentage distance travelled in the novel arm, which was above chance (33%) for both genotypes [*t*-test: WT: 42.3%, $t(18) = 5.0$, $P < .0001$; *Nrg1 III tg:* 41.4%, $t(18) = 4.2$, $P < .0001$; and both genotypes performed similarly for percentage novel arm entries and for time spent in novel arm: table 1]. For fear-associated learning, *Nrg1 III tg* mice had WT levels of baseline *freezing* in the first 2 min of the conditioning trial [$F(1, 17) = 0.4$, $P = .5$] and in the first 2 min of the cue trial, prior to cue presentation [$F(1, 17) = 1.2$, $P = .3$]. In contrast, *Nrg1 III tg* mice displayed reduced *freezing* levels compared with WT

Table 1. Results for Behavioral Measures from the Elevated Plus Maze (EPM), Social Interaction Test (SI), Y-Maze (YM), Open Field (OF), Prepulse Inhibition (PPI), Accelerod, and Beam Walking Task

Behavioral Test	Parameter	WT	*Nrg1 III tg*	*F* (*df*)	Sig.
EPM	Time in open arms [s]	9.4 ± 6.9	20.8 ± 6.2	1.5 (1, 18)	*P* = .2
	Time in open arms [%]	4.2 ± 3.2	9.4 ± 2.9	1.5 (1, 18)	*P* = .2
	Overall distance travelled [m] (meters)	10.5 ± 0.8	10.4 ± 0.9	0.002 (1, 18)	*P* = 1
	Stretch-attend posture [n]	27.3 ± 2.3	23.7 ± 2.2	1.3 (1, 19)	*P* = .3
	Stretch-attend posture [s] (seconds)	34.6 ± 3.3	28.9 ± 3.0	1.6 (1, 18)	*P* = .2
	Head-dipping [n]	17.2 ± 2.4	14.3 ± 2.4	0.8 (1, 18)	*P* = .4
	Head-dipping [s]	12.3 ± 1.7	10.3 ± 1.6	0.7 (1, 18)	*P* = .4
	Rearing [n]	13.5 ± 2.5	14.8 ± 2.4	0.1 (1, 18)	*P* = .7
	Rearing [s]	9.3 ± 1.7	10.9 ± 1.7	0.4 (1, 18)	*P* = .5
	Grooming [n]	8.2 ± 1.3	7.2 ± 1.2	0.3 (1, 18)	*P* = .6
	Grooming [s]	17.2 ± 2.9	12.0 ± 2.8	1.7 (1, 18)	*P* = .2
SI	Total social interaction time [s]	83.7 ± 10.3	87.3 ± 10.8	0.1 (1, 19)	*P* = .8
	Nosing/sniffing [n]	55.6 ± 3.8	57.5 ± 4.0	0.1 (1, 19)	*P* = .7
	Nosing/sniffing [s]	54.7 ± 7.4	58.7 ± 7.8	0.1 (1, 19)	*P* = .7
	Anogenital sniffing [n]	19.6 ± 2.7	19.3 ± 2.8	0.01 (1, 19)	*P* = .9
	Anogenital sniffing [s]	17.5 ± 2.5	18.4 ± 2.6	0.1 (1, 19)	*P* = .8
	Crawling over [n]	9.1 ± 1.4	8.2 ± 1.4	0.2 (1, 18)	*P* = .6
	Crawling over [s]	8.1 ± 1.8	6.2 ± 1.9	0.5 (1, 19)	*P* = .5
	Following [n]	4.3 ± 1.1	5.2 ± 1.2	0.4 (1, 19)	*P* = .6
	Following [s]	3.4 ± 0.8	3.0 ± 0.9	0.1 (1, 18)	*P* = .7
YM	Time in novel arm [%]	32.1 ± 7.7	35.3 ± 6.2	1.0 (1, 18)	*P* = .3
	Distance in novel arm [%]	42.3 ± 5.0	41.4 ± 5.2	0.1 (1, 18)	*P* = .7
	Time in entries in novel arm [%]	37.3 ± 5.2	39.7 ± 4.8	1.2 (1, 18)	*P* = .3
OF	Distance travelled at baseline [cm]	1678.9 ± 503.1	1758.0 ± 258.9	0.2 (1, 19)	*P* = .7
PPI	Overall average PPI [%]	50.0 ± 4.4	32.6 ± 4.4	5.3 (1, 18)	*P* = .03
Accelerod	Latency to fall [s]	158.7 ± 48.3	199.4 ± 60.4	2.7 (1, 16)	*P* = .1
Beam walking task	Latency to reach platform [s]	40.2 ± 25.1	26.4 ± 17.9	1.6 (1, 16)	*P* = .2

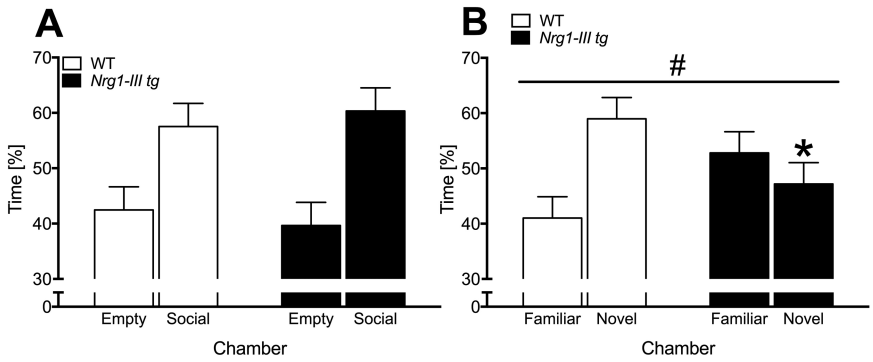

Fig. 2. Social preference testing. (A) Sociability (ie, preference for spending time in social chamber/empty chamber) (%) and (B) social recognition memory (ie, preference for spending time with a novel mouse/familiar mouse) (%), where significant genotype effects vs WT results are shown as "*" (*P < .04), while "#" above line indicates "chamber" by "genotype" interaction (*P < .04). Data for control (WT) and *Nrg1 type III* transgenic (*Nrg1 III tg*) mice are shown as mean + SEM.

mice during the first 2 min of the context trial [$F(1, 17)$ = 6.4, P = .02] (figure 3A). Additionally, *Nrg1 III tg* mice exhibited an overall reduction in total time spent *freezing* to the context compared with WT mice [$F(1, 17)$ = 4.7, P < .05] indicating a deficit in contextual fear conditioning (figure 3B). In the cue test, there was no difference in total *freezing* duration between genotypes during cue presentation [$F(1, 15)$ = 1.8, P = .2]. There was a significant "genotype" by "time" interaction across 1 min blocks during cue presentation [$F(4, 15)$ = 3.4, P = .02]; however, we did not find any significant differences across genotype at individual time-points by post hoc analysis (figure 3C). Furthermore, *freezing* duration prior to cue presentation (ie, first 2 min) and during cue presentation (ie, third to seventh min) was not different between *Nrg1 III tg* mice and WT.

Prepulse Inhibition

For PPI testing, we found that all mice exhibited an increasing startle response to increasing startle stimuli [$F(1, 18)$ = 40.6, P < .001; "startle" by "genotype":

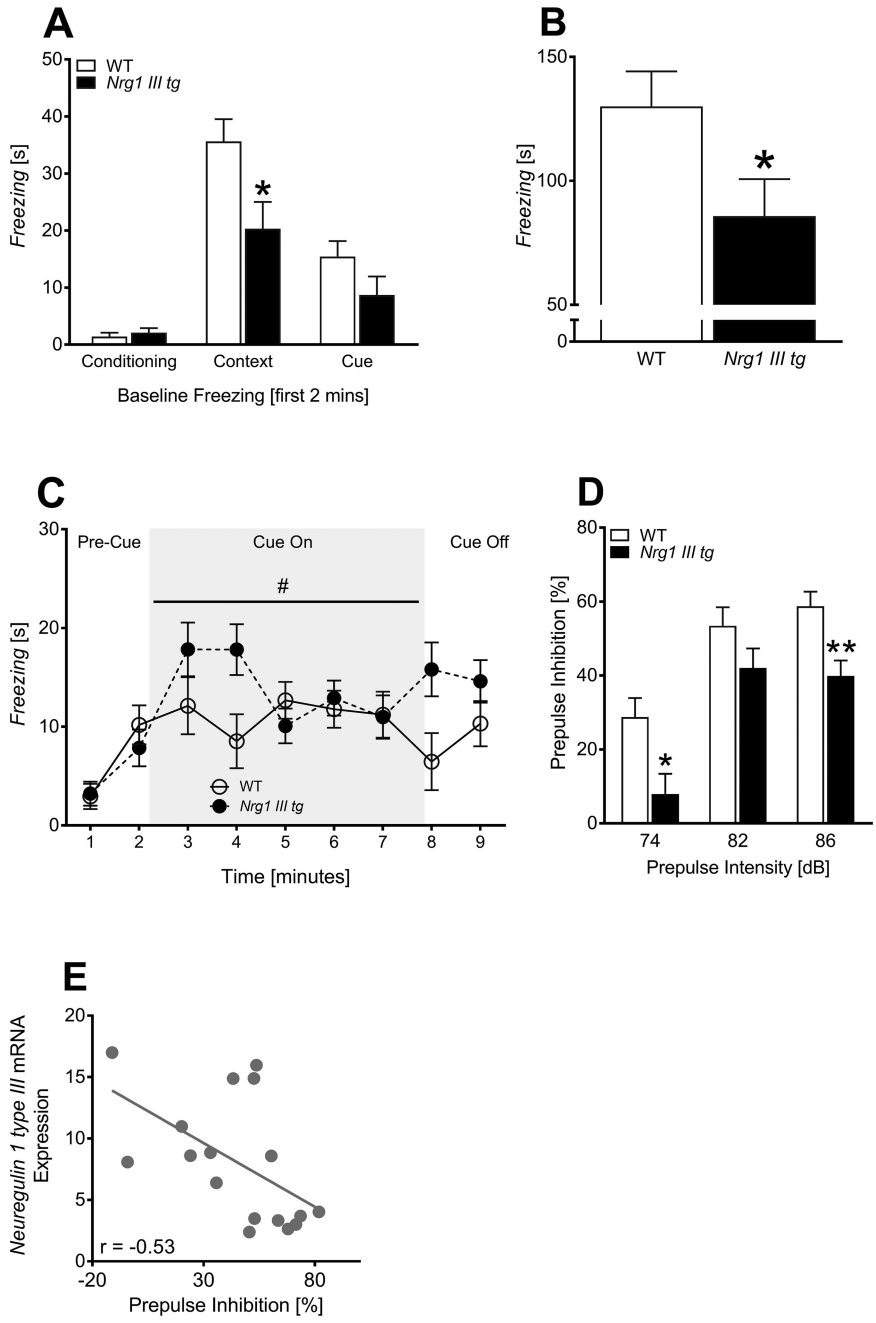

Fig. 3. Fear-associated memory, sensorimotor gating (ie, prepulse inhibition) and correlation analysis: *Nrg1 type III* mRNA levels with percentage prepulse inhibition. (A) Time spent *freezing* (s) in the first 2 min of conditioning trial, context trial and cue trial, (B) total *freezing* time (s) during context test, (C) time spent *freezing* (s) during cue trial, shown in 1-min blocks, (D) percent prepulse inhibition (%) at 3 different prepulse intensities, and (E) inverse correlation of prefrontal cortex *Nrg1 type III* expression with percentage prepulse inhibition performance (r = Pearson's correlation co-efficient). Significant genotype effects vs WT results are shown as "*" and "**" (*P < .05, **P < .001), while "#" above line indicates "time" by "genotype" interaction (*P < .05). Data for control (WT) and *Nrg1 type III* transgenic (*Nrg1 III tg*) mice are shown as mean + SEM.

$F(2, 18) = 0.8$, $P = .4$] and increased PPI with increasing prepulse intensities [$F(1, 18) = 75.4$, $P < .001$] (figure 3D). A PPI deficit was detected as the %PPI of *Nrg1 III tg* mice was significantly reduced at prepulse intensities of 74 [$F(1, 18) = 5$, $P = .04$] and 86 dB [$F(1, 18) = 7.6$, $P = .01$], but not at 82 dB [$F(1, 18) = 1.7$, $P = .2$] (figure 3D). In line with this finding, *Nrg1 III tg* mice exhibited significantly lower PPI averaged across prepulse intensities compared with WT mice [$F(1, 18) = 5.3$, $P = .03$] (table 1). We also assessed whether the level of *Nrg1 type III* mRNA overexpression negatively correlated with PPI performance by correlating *Nrg1 type III* mRNA levels in the prefrontal cortex of behaviorally tested *Nrg1 III tg* and WT mice with their %PPI. We found that increasing

Nrg1 type III mRNA levels correlated with impaired PPI performance [$r = -.53$, $P = .03$] (figure 3E).

Baseline and MK-801-Induced Open Field Behavior

We detected a significant effect of "time" for distance travelled during the first 15 min of the open field test indicating intact habituation [$F(14, 16) = 3.3$, $P = .007$]; however, we did not detect a significant "time" by "genotype" interaction effect (figure 4). Furthermore, overall baseline locomotion during these 15 min was almost identical between genotypes [$F(1, 19) = .2$, $P > .7$] (table 1). Comparing baseline locomotion to the first 15 min following drug treatment showed a significant effect of "time" for total distance travelled [$F(1, 16) = 29.2$, $P < .001$], while no "time" by "genotype" interaction was found [$F(1, 16) = .1$, $P = .7$]. Locomotion post drug treatment across 5-min blocks was increased as a result of the MK-801 treatment [RM ANOVA for "drug": $F(1, 17) = 37.6$, $P < .001$]; however, no "genotype" or "drug" by "genotype" interaction effect was detected (figure 4B).

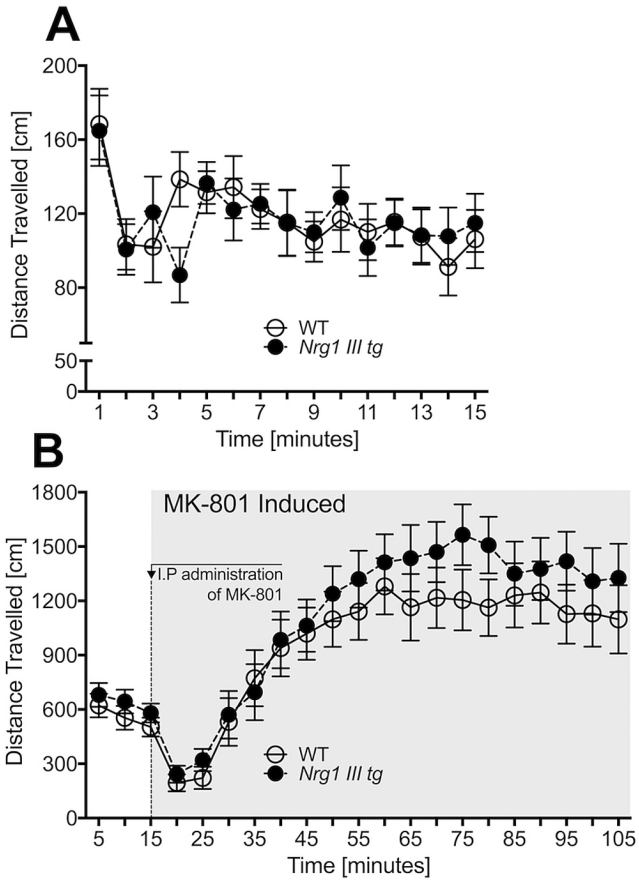

Fig. 4. Baseline and MK-801 induced locomotion in the open field test. (A) Distance travelled (cm) across 1-min blocks in the first 15 min (drug-free) and (B) distance travelled (cm) across 5-min blocks during the 105-min test period. A single, intraperitoneal injection (IP) of 0.25 mg/kg of MK-801 was given after 15 min of baseline testing. Data are shown for control (WT) *Nrg1 type III* transgenic (*Nrg1 III tg*) and mice are presented as mean + SEM.

Discussion

Here, we created a transgenic mouse that mimics the overexpression of *Nrg1 type III* in the brain that is linked to increased genetic risk of developing schizophrenia. We find that this mouse can serve as a model of aspects of schizophrenia-like behavior (ie, endophenotypes) as deficits in social recognition memory, contextual fear conditioning, and sensorimotor gating were detected. However, more general social behaviors, baseline locomotion and exploration, locomotor response to MK-801, and anxiety did not appear to be altered. Additionally, we have uncovered schizophrenia-related transcriptional changes in the hippocampus of *Nrg1 III tg* mice.

Nrg1 III tg mice did not possess sensory, neurological reflex, or motor deficits in the domains measured; nor do they have a generalized anxiety phenotype. This suggests that effects of *Nrg1 III* overexpression on schizophrenia-like behavior is a consequence of impaired higher order processing of information, and not of an underlying physical impairment or stress response-related differences.

Assessing social interaction in our mice was of interest as schizophrenia patients have difficulty in forming and maintaining meaningful social relationships.[41] We found that *Nrg1 III tg* mice preferred the presence of another mouse over an empty chamber. However, *Nrg1 III tg* mice failed to exhibit a normal preference toward a novel mouse, suggesting that an overexpression of *Nrg1 type III* might contribute toward an aversion or lack of preference toward social novelty. Similarly, *Nrg1* transmembrane domain heterozygous mice have normal levels of sociability in social preference task but have reduced social preference for the novel mouse.[42] Two separate mouse lines with CamKIIα promoter driven overproduction of Nrg1 type I exhibit both social withdrawal and a loss of preference for the novel mouse.[21,43] These results, in tandem with ours, highlight the importance of isoform specific levels of Nrg1 in relation to each other in mediating social deficits that may resemble the negative symptoms of schizophrenia.

Cognitive symptoms in schizophrenia patients correlate with structural and functional abnormalities in brain regions including the hippocampus (HPC).[44] Fear conditioning in rodents requires highly integrated brain circuitry which includes the HPC.[45] We found impoverished fear-associated memory to context in the *Nrg1 III tg* mouse, a result which occurs across numerous other *Nrg1* perturbations.[25,43,46,47] We found alterations in the hippocampal transcriptome of *Nrg1 III tg* mice that may provide clues in uncovering the mechanisms behind this behavioral phenotype. One downregulated transcript

relating to the serotonin pathway, *5-hydroxytryptamine (serotonin) receptor 2C*, has been shown to be reduced in schizophrenia patients[33] and has also been shown to modulate conditioned fear to context in rodents.[48] Due to our findings, we intend to undertake further investigations into the signaling pathway of a few of these candidate genes and assess whether *Nrg1 type III* upregulation may disrupt these pathways in the hippocampus and cause deficits in fear conditioning in *Nrg1 III tg* mice.

In addition to altered *5-hydroxytryptamine (serotonin) receptor 2C*, our microarray revealed other altered mRNA transcripts in the hippocampus of *Nrg1 III tg* mice which bear relevance to schizophrenia including changes in *angiotensin converting enzyme*,[34] *sulfatase 1*,[35] and *transthyretin*.[36] Interestingly, the *DiGeorge critical region gene 8* haploinsufficiency mouse ($Dgcr8^{+/-}$) also shares many of the same hippocampal transcriptional alterations as the *Nrg1 III tg* mouse, suggesting that alterations to either *Nrg1 III* and *Dgcr8* may impact on similar transcriptional processes in the hippocampus. The $Dgcr8^{+/-}$ mouse possesses compromised hippocampal neurogenesis and cognitive deficits, both of which are reversed with the infusion of hippocampal Igf2.[49] Given the similarity in the hippocampal transcriptomic profile between the $Dgcr8^{+/-}$ and Nrg*1 III tg* mice, and the role of Nrg1 in modulating hippocampal neurogenesis,[50,51] we suggest that investigating the role of *Nrg1 III* overexpression in neurogenic regions in the *Nrg1 III tg* mouse, and whether any abnormality in this domain implicates altered Igf signaling will be of interest for further studies.

Interestingly, we also found alterations in 26 immunologically associated transcripts in the hippocampus of *Nrg1 III tg* mice. Several of these transcripts including *Fgl2*, *Gas6*, *Ltc4s*, *Ifi27*, *Kl*, *Cldn2*, *Lepr*, *Ucp2*, and *1500015O10Rik* (*Ecrg4*) are of particular interest as they have demonstrated the capacity to modify immune responses within the brain or in neuronal cell culture.[52–60] Given the link between inflammation and schizophrenia,[38,39] and also emerging evidence illustrating that Nrg1 signaling may be able to influence the inflammatory-like pathways in the brain,[37] it may be of value to continue to assess the role of Nrg 1 type III overexpression on brain inflammation within the context of schizophrenia-like neuropathology.

PPI is commonly impaired in schizophrenia patients[61] and the measurement of PPI in humans and mice is almost identical.[30] We found that male *Nrg1 III tg* mice exhibited a PPI deficit which is comparable to the PPI deficit found in *Nrg1 type III* haploinsufficient mice,[19] whereas a complete knockout of *Nrg1 III* postnatally does not induce a PPI deficit.[25] Additionally, we found that increasing *Nrg1 type III* mRNA levels predicted worse PPI performance. Thus, the type of Nrg1 isoform, magnitude of expression, location of transgene expression and timing of isoform change may mediate changes in sensorimotor gating. If normal adult PPI performance is contingent on the timing of the expression of certain *Nrg1* isoforms and optimal levels of *Nrg1*, assessing *Nrg1 III* overexpression within the context of pre and perinatal development might provide insight into the neurobiological development of this schizophrenia-like behavioral phenotype. Our result supports that, in our transgenic mice, the impact of overexpression of *Nrg1 type III* could be mediated before adulthood when PPI was assessed, as we found increased expression of *Nrg1 III* mRNA in the juvenile brain prior to adolescence.

The overall conditioned fear response to a discreet cue in the *Nrg1 III tg* mouse was at the level of WT mice. The subtle "genotype" by "time" interaction during cue presentation suggests that fear response to the conditioned cue over an extended period of exposure may be altered and future work on the extinction of a learned fear might be warranted. At present, only one study has assessed the role *Nrg1* on extinction fear memory, wherein hypomorphic *Nrg1 II* rats froze less during extinction trials to a discreet cue 96 h after conditioning.[62] Similarly to our finding, both full-length and N-terminal fragment overexpression of *Nrg1* does not appear to elicit a deficit in learned fear to cue,[20,43] suggesting that an upregulation of Nrg1 may not play a role in disrupting basic learned fear to a discreet cue.

Our results suggest that short-term spatial memory is intact with forebrain *Nrg1 type III* overexpression as *Nrg1 III tg* mice exhibited WT levels of exploration of the novel arm on the Y-maze. Other Nrg1 mouse models, however, exhibit impairments in short-term/working spatial memory in a variety of cognitive tasks. The heterozygous *Nrg1 type III* knockout mouse has deficits in 60 s delayed spontaneous alternation[19] while an *Nrg1 type I* overexpressing mouse has decreased performance in several spatial memory tasks, which is rescued when transgene expression is shut-off in adulthood.[21] Together, these results suggest that short-term spatial memory deficits in *Nrg1* mutant mice might be isoform dependent and/or vulnerable to changes in the specific concentration of these Nrg1 isoforms relative to one another.

Our *Nrg1 III tg* mouse failed to have the expected baseline or drug-induced increases in locomotion. Baseline ambulatory distance travelled in an open field arena did not appear to be impacted by *Nrg1 type III* overexpression. This is in alignment with the heterozygous *Nrg1 type III* knockout mouse that also does not exhibit hyperlocomotion.[19] Furthermore, several other Nrg1 I overexpressing mice only show a locomotor phenotype under certain conditions (eg, dependent on age or drug treatment).[22,63] We did not observe a genotype difference in mice exposed to MK-801, suggesting that *Nrg1 type III* overexpression does not alter the response to NMDA receptor perturbation known to induce hyper-locomotion.

Our study possesses limitations, including the use of only male mice. It is important to interrogate the molecular and behavioral phenotypes in both male and female

mice in order to assess how abnormal Nrg1 signaling may be contributing to the schizophrenia-like symptoms in both males and females. Thus, using the effect sizes from this study, we intend to follow-up with comprehensive characterization of female *Nrg1 III tg* mice. Another limitation is that we only used one transgenic line and thus cannot rule out the impact of transgene insertion on the phenotypes observed in the *Nrg1 III tg* mouse. Additionally, the overexpression of forebrain *Nrg1 type III* mRNA in *Nrg1 III tg* mice was >5 times higher than WT mice while schizophrenia patients carrying *NRG1* Hap_{ICE} alleles only have a ~30% elevation in *Nrg1 type III* mRNA compared with non *NRG1* Hap_{ICE} allele carriers.[6] However, it is worth mentioning that several Nrg1 type I transgenic mouse models also overexpress Nrg1 by a magnitude of >4 times.[20,21,23,43] Another limitation of the study is that we did not assess whether antipsychotics are capable of reversing the schizophrenia-like behavioral deficits exhibited by *Nrg1 III tg* mice (ie, test predictive validity of model). Future studies may consider including an antipsychotic treatment in this transgenic mouse model.

In sum, the results of this study indicate that forebrain driven overexpression of *Nrg1 III* in a novel mouse model causes schizophrenia-like deficits across several behavioral domains and supports the candidacy of this transgenic mouse model for future studies. Given that a subset of schizophrenia patients carry copies of the *NRG1* Hap_{ICE} gene, which appears to upregulate *NRG1 III* in their forebrain, evidence from the current study gives weight toward the role of forebrain *NRG1 III* overexpression contributing toward some of the clinical symptoms of the disease, at least in a subset of individuals. Additionally, these findings also highlight the use of this *Nrg1 III* overexpressing model to assess the biological consequences of the upregulation of this isoform type. Future research will need to be conducted in order to extend our understanding of forebrain driven *Nrg1 type III* overexpression on schizophrenia related phenotypes. For example, it will be relevant to assess if NRG1 type III overexpression (eg, in PFC) is linked to increased ErbB4 activity. In line with our previous work, we will also consider sex effects and gene–environment interactions in our new model.[64–66]

Supplementary Material

Supplementary data are available at *Schizophrenia Bulletin* online.

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Contributions

T.K. and C.S.W. with J.C.O. designed the study and wrote the protocol. C.L.M. and M.M. constructed and generated the mouse. J.C.O. performed the experiments. J.C.O. wrote the first draft. J.C.O., T.K., D.S., and M.K. were responsible for the data analysis. J.C.O., T.K., C.L.M., M.M., D.S., M.K., and C.S.W. reviewed and revised the manuscript draft. Each of the authors has reviewed the manuscript and has approved the final manuscript.

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