Schizophrenia Bulletin vol. 43 no. 1 pp. 214–225, 2017
doi:10.1093/schbul/sbw120
Advance Access publication September 9, 2016

Epistatic and Independent Effects on Schizophrenia-Related Phenotypes Following Co-disruption of the Risk Factors Neuregulin-1 × DISC1

Colm M. P. O'Tuathaigh*[,1,2], Fabio Fumagalli[3], Lieve Desbonnet[4], Francesc Perez-Branguli[5,6], Gerard Moloney[7], Samim Loftus[7], Claire O'Leary[2], Emilie Petit[2], Rachel Cox[2], Orna Tighe[2], Gerard Clarke[4,8], Donna Lai[9], Richard P. Harvey[9], John F. Cryan[4,7], Kevin J. Mitchell[5], Timothy G. Dinan[4,8], Marco A. Riva[3], and John L. Waddington[2,10]

[1]School of Medicine, Brookfield Health Sciences Complex, University College Cork, Cork, Ireland; [2]Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, Dublin 2, Ireland; [3]Dipartimento di Scienze Farmacologiche e Biomolecolari, Universita` degli Studi di Milano, Milan, Italy; [4]Neurogastroenterology Laboratory, Alimentary Pharmabiotic Centre, Biosciences Institute, University College Cork, Cork, Ireland; [5]Smurfit Institute of Genetics, Trinity College Dublin, Dublin 2, Ireland; [6]IZKF Junior Research Group and BMBF Research Group Neuroscience, IZKF, Friedrich-Alexander-Universitaet Erlangen-Nuernberg, Erlangen, Germany; [7]Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland; [8]Department of Psychiatry, University College Cork, Cork, Ireland; [9]Victor Chang Cardiac Research Institute, Sydney, Australia; [10]Jiangsu Key Laboratory of Translational Research & Therapy for Neuro-Psychiatric-Disorders and Department of Pharmacology, College of Pharmaceutical Sciences, Soochow University, Suzhou, China

*To whom correspondence should be addressed; School of Medicine, Brookfield Health Sciences Complex, University College Cork, Cork T12 YN60, Ireland; tel: +353-(0)21-420-5303, fax: +353-(0)21-490-1594, e-mail: c.otuathaigh@ucc.ie

Few studies have addressed likely gene × gene (ie, epistatic) interactions in mediating risk for schizophrenia. Using a preclinical genetic approach, we investigated whether simultaneous disruption of the risk factors Neuregulin-1 (NRG1) and Disrupted-in-schizophrenia 1 (DISC1) would produce a disease-relevant phenotypic profile different from that observed following disruption to either gene alone. NRG1 heterozygotes exhibited hyperactivity and disruption to prepulse inhibition, both reversed by antipsychotic treatment, and accompanied by reduced striatal dopamine D2 receptor protein expression, impaired social cognition, and altered glutamatergic synaptic protein expression in selected brain areas. Single gene DISC1 mutants demonstrated a disruption in social cognition and nest-building, altered brain 5-hydroxytryptamine levels and hippocampal ErbB4 expression, and decreased cortical expression of the schizophrenia-associated microRNA miR-29b. Co-disruption of DISC1 and NRG1, indicative of epistasis, evoked an impairment in sociability and enhanced self-grooming, accompanied by changes in hypothalamic oxytocin/vasopressin gene expression. The findings indicate specific behavioral correlates and underlying cellular pathways downstream of main effects of DNA variation in the schizophrenia-associated genes NRG1 and DISC1.

Key words: gene × gene interaction/psychosis/genetic mouse model/endophenotypes

Introduction

Risk for psychotic illness in general, and for schizophrenia in particular, is influenced by a diversity of genetic and environmental factors.[1,2] In the face of disease complexity, current evidence suggests genetic variation in psychosis to involve the impact of rare, highly penetrant gene variants on a background of a large number of common risk genes of small effect.[1–4] While genome-wide association studies have failed to provide support for genes identified from candidate gene approaches (eg, Neuregulin-1 [NRG1] and Disrupted-in-schizophrenia 1 [DISC1]), there is evidence for association between such genes and biological pathways implicated in schizophrenia.[5,6] Our current understanding of risk loci can explain less than one-third of the heritability of psychotic illness.[7] It has been posited that such "missing heritability" can be explained by epistatic (gene × gene) interactions[8] that have received only limited empirical investigation[9] vis-à-vis well recognized gene × environment interactions.[10,11] Difficulties in investigating epistasis clinically include the considerable statistical power required for quantitative studies and lack of clarity as to the most relevant phenotype. Therefore, mutant mouse studies involving (1) simultaneous genetic disruption of 2 risk factors (NRG1, DISC1) implicated in the pathogenesis of schizophrenia and (2) phenotypes related to the psychopathology and pathobiology of psychotic illness, have the potential to inform on this challenge.

In vitro studies have demonstrated both independent and interactive effects of DISC1 and NRG1 on neuronal signaling.[12,13] Application of NRG1 to cultured immature rat neurons increased levels of an isoform of DISC1 protein, while mice with partial deletion of NRG1 or its interacting protein BACE1 (β-site amyloid precursor protein) displayed a substantial decrease in expression of the same DISC1 isoform in the cortex.[14] These findings provide cellular evidence to link NRG1 and DISC1 in a common neurodevelopmental pathway, additional data suggesting it may be mediated by ErbB receptor and PI3K/Akt signaling processes.[14,15] A recent study provided evidence for direct DISC1 involvement in the regulation of NRG1-ErbB4 signaling in adult fast-spiking neurons in a cell-autonomous manner.[16] In an in vivo study carried out in first-episode schizophrenia patients, an additive effect of variation in the NRG1 and DISC1 genes was observed for lateral ventricle enlargement, an anatomical endophenotype for schizophrenia.[17]

Here we have generated male and female mutants with simultaneous disruption of DISC1 and NRG1, by intercrossing mice with heterozygous (HET) deletion of transmembrane domain (TM)-NRG1[18] (homozygous [HOM] deletion of TM-NRG1 being lethal) and HET or HOM L100P mutation of DISC1,[19] followed by broad phenotypic evaluation of all resultant genotype-wildtype (WT) combinations. This allowed us to resolve those phenotypes subject to epistatic regulation from those for which DISC1 and NRG1 exert independent, additive, or no effects.

Materials and Methods

Mice

NRG1 × DISC1 mutant mice were generated by intercrossing the DISC1 (100P) mutant line with the HET NRG1 knockout line. Mice having HET or HOM mutation in exon 2 of mouse DISC1 (100P) were originally generated by site-directed ENU mutagenesis at the Riken Bioresource Center, Ibaraki, Japan, as described previously,[19] and were maintained on a C57BL6 background (8 backcrosses). HET NRG1 knockout mice were generated at the Victor Chang Cardiac Institute, University of New South Wales, Australia, as described previously[18] and maintained on a C57BL6 background (14 backcrosses).

Experimental Groups

HET NRG1 and DISC1 mutants were crossed and offspring HET for both DISC1 and NRG1 were then used as breeding pairs to generate the following experimental genotypic groups: $NRG1^{WT}/DISC1^{WT}$, $NRG1^{WT}/DISC1^{HET}$, $NRG1^{WT}/DISC1^{HOM}$, $NRG1^{HET}/DISC1^{WT}$, $NRG1^{HET}/DISC1^{HET}$, and $NRG1^{HET}/DISC1^{HOM}$. Mice were housed in groups of 3 to 5 per cage and maintained on a standard 12/12 hour light/dark cycle. Supplementary table 1 provides a summary of group sizes and sequence of behavioral testing across several breeding cohorts. Behavioral tests were completed across 3 separate cohorts, in the following order: (1) novelty-induced hyperactivity (pre-pubertal), prepulse inhibition (PPI; adult), amphetamine-induced hyperactivity (adult); (2) PPI (pre-pubertal), novelty-induced activity (adult), sociability and social novelty (adult), dyadic social interactions (adult), clozapine effects on PPI (adult); (3) spontaneous alternation (adult), novel object recognition (adult), nest building (adult), clozapine effects on novelty-induced hyperactivity (adult). Behavioral and psychopharmacological protocols used are available in the supplementary material.

Biochemical analyses were conducted in 4 distinct, behaviorally naïve, breeding cohorts: (1) striatal dopamine (DA) D2 receptor protein expression, cortical microRNA (miRNA) profiling; (2) monoamine analyses; (3) oxytocin/vasopressin 1a mRNA expression; (4) NRG1/ErbB4 and DISC1 protein expression in the brain. Details of assays employed are available in the supplementary material. These studies were approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland.

Statistical Analysis

Repeated measures ANOVA was performed to analyze data for sociability and social novelty preference, startle habituation and %PPI for each pulse intensity (100, 110 and 120 dB). Data for all other behavioral and cellular/molecular assays were analyzed using between-subjects 2-way ANOVA with main factors of genotype, sex (where applicable), and treatment. Post hoc comparisons were carried out using independent or related *t* tests, or the Mann-Whitney U where assumptions of parametric testing were not met. Statistical significance was accepted at the .05 level of probability. All statistical analyses were carried out using the PASW software package (PASW Version 18, SPSS Inc.).

Results

Novelty- and Stimulant-Induced Hyperactivity

Hyperactivity induced by (1) a novel environment and (2) the DA-releasing, psychotomimetic agent d-amphetamine (AMPH) were evaluated as 2 models of positive, psychotic symptoms.[20,21] Male and female mice belonging to each of the 6 NRG1–DISC1 genotypes were placed in an activity box for 1 hour during early adolescence (postnatal day [PND] 32) or young adulthood (PND70). At both adolescent and adult stages, we observed hyperactivity in mice with HET knockout of NRG1 (NRG1, $F(1, 185) = 24.90$, $P < .001$), and this genotypic increase was more pronounced at PND70 vs PND32 (NRG1 × age, $F(1, 185) = 5.19$, $P = .02$; figure 1A), and in females vs

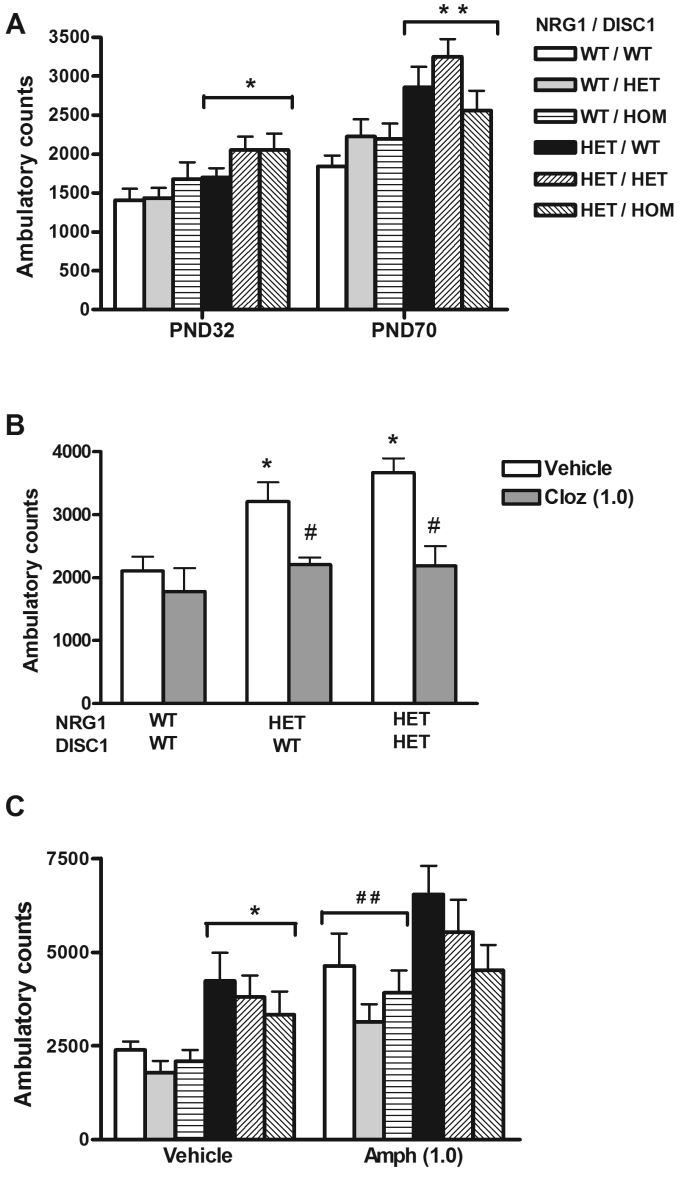

Fig. 1. (**A**) Novelty-induced hyperactivity in NRG1^{WT}/DISC^{WT}, NRG1^{WT}/DISC1^{HET}, NRG1^{WT}/DISC1^{HOM}, NRG1^{HET}/DISC1^{WT}, NRG1^{HET}/DISC1^{HET}, and NRG1^{HET}/DISC1^{HOM} at PND32 or PND70. Data are mean ambulatory counts ± SEM over the 1 h session. NRG1^{HET} show hyperactivity across both stages, and this effect is more evident at PND70. *P < .05 at PND32, **P < .01 at PND70. (**B**) Reversal of novelty-induced hyperactivity by acute clozapine (Cloz, 1.0 mg/kg) in NRG1^{HET}/DISC1^{WT} and NRG1^{HET}/DISC1^{HET}. Data are mean ambulatory counts ± SEM over the 1 h session. *P < .05, effect of NRG1 genotype independent of AMPH treatment; $^{\#}P$ < .05 vs vehicle-treated control. (**C**) Hyperactivity induced by acute amphetamine (AMPH, 1.0 mg/kg) in NRG1^{WT}/DISC^{WT}, NRG1^{HET}/ DISC1^{WT} and NRG1^{HET}/DISC1^{HET} is blunted in NRG1^{HET}. Data are mean ambulatory counts ± SEM over the 1 h session. *P < .05, Effect of NRG1 genotype independent of AMPH treatment; $^{\#\#}P$ < .01, effect of AMPH treatment vs vehicle-treated controls independent of NRG1 genotype. Abbreviations: NRG1, Neuregulin-1; DISC, Disrupted-in-schizophrenia 1; HET, heterozygous; HOM, homozygous; WT, wildtype; PND, postnatal day. Group sizes are detailed in supplementary table 1.

males (NRG1 × sex, $F(1,185) = 5.18$, $P = .02$; supplementary figure 1).

Acute pre-treatment with the antipsychotic clozapine reversed novelty-induced hyperactivity in adulthood in 2 of the NRG1^{HET} genotypes that had previously exhibited hyperactivity at this stage (ie, NRG1^{HET}/DISC1^{WT}, NRG1^{HET}/DISC1^{HET}), without altering activity in NRG1^{WT}/DISC1^{WT} (NRG1, $F(2, 35) = 3.58$, $P = .04$; NRG1 × drug, $F(2, 35) = 4.31$, $P = .02$; no sex or genotype × sex interaction; figure 1B). While acute administration of AMPH increased activity across all genotypes, this increase was blunted in NRG1^{HET} in a manner unrelated to DISC1 genotype; activity levels were also higher in NRG1^{HET} in a manner unrelated to AMPH treatment (drug, $F(1, 54) = 16.91$, $P < .001$; NRG1, $F(1, 54) = 6.06$, $P = .02$; NRG1 × drug, $F(1, 54) = 7.18$, $P = .01$; no sex or genotype × sex interaction; figure 1C).

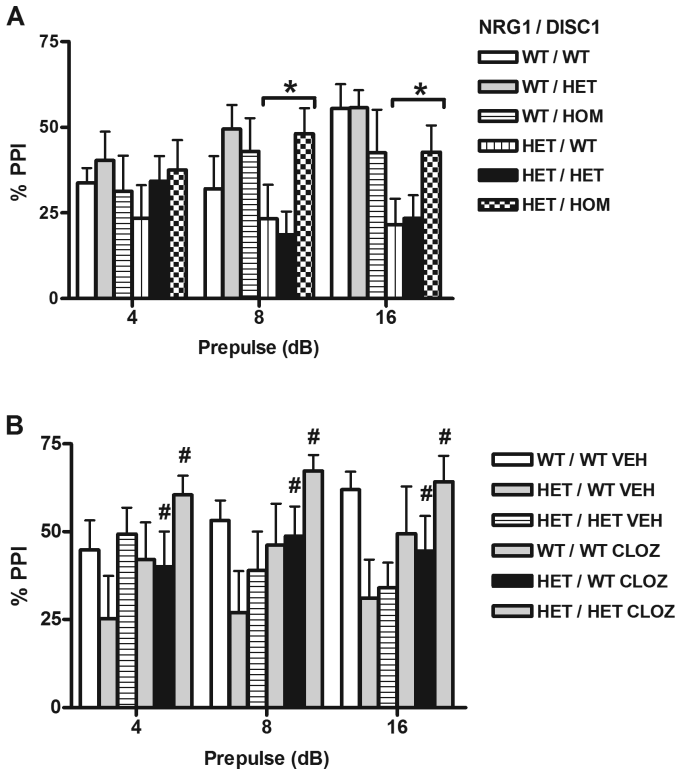

Fig. 2. Prepulse inhibition (PPI) in NRG1^{WT}/DISC^{WT}, NRG1^{WT}/DISC1^{HET}, NRG1^{WT}/DISC1^{HOM}, NRG1^{HET}/DISC1^{WT}, NRG1^{HET}/DISC1^{HET}, and NRG1^{HET}/DISC1^{HOM} at pulse intensity 120 dB. (**A**) Selective disruption of PPI at PND70 in NRG1^{HET}. Data are mean %PPI ± SEM for prepulses of 4, 8, and 16 dB. *P < .05, effect of NRG1 genotype. (**B**) PPI in NRG1^{WT}/DISC^{WT}, NRG1^{HET}/DISC1^{WT}, NRG1^{HET}/DISC1^{HET}, at pulse intensity 120 dB, following acute administration of clozapine (1.0 mg/kg) or saline. Clozapine selectively reverses PPI deficits in NRG1^{HET}. Data are mean %PPI ± SEM for prepulses of 4, 8, and 16 dB. $^{\#}P$ < .05 vs vehicle-treated controls. Abbreviations: NRG1, Neuregulin-1; DISC, Disrupted-in-schizophrenia 1; HET, heterozygous; HOM, homozygous; WT, wildtype; CLOZ, clozapine; VEH, vehicle. Group sizes are detailed in supplementary table 1.

PPI of the Acoustic Startle Response

Deficits in sensorimotor gating have been observed in schizophrenia patients in terms of disruption to PPI that is reversed by antipsychotic drugs.[22] Male and female mice belonging to each of 6 NRG1–DISC1 genotypes were assessed for PPI at PND33 (early adolescence) or PND70 (young adulthood). Male mice HOM for DISC1 showed decreased startle habituation at 110 dB relative to WTs when tested at PND70 (DISC1, $F(2, 74) = 3.10$, $P = .05$; HOM vs WT, $P < .05$). Selective disruption of PPI at 120 dB was observed in NRG1^{HET} at PND70 (NRG1, $F(1, 75) = 6.62$, $P = .01$; no effect of DISC1, sex, NRG1 × DISC1 interaction or genotype × sex interactions; figure 2A). Despite numerical attenuation of PPI deficits in NRG1^{HET} mice containing the DISC1 HOM mutation (ie, NRG1^{HET}/DISC1^{HOM}), the NRG1 × DISC1 interaction failed to attain statistical significance. Acute pre-treatment with clozapine reversed PPI deficits at pulse intensity 120 dB in NRG1^{HET} in a manner independent of DISC1^{HET} (NRG1 × drug, $F(1, 36) = 6.90$, $P = .01$; no sex or genotype × sex interaction; figure 2B), without altering baseline startle or PPI values in mice WT for both genes.

Social Interaction

Tests of sociability and social novelty preference provide robust and quantifiable measures of social withdrawal and social cognition deficits analogous to that observed in schizophrenia.[23,24] During the sociability phase, while 5 genotypes spent more time in (and made more entries into) the chamber containing the unfamiliar mouse relative to the opposite, empty chamber, this effect was absent in NRG1^{HET}/DISC1^{HOM} (NRG1 × DISC1, $F(2, 60) = 3.72$, $P < .05$; no sex or genotype × sex interaction; figure 3A). During the social novelty phase, both NRG1 heterozygosity and DISC1 heterozygosity (but not DISC1 homozygosity) were independently associated with loss of preference for spending more time in the chamber containing the new, unfamiliar mouse relative to the opposite chamber containing the previous, now familiar mouse (NRG1, $F(1, 60) = 5.93$, $P = .03$; DISC1, $F(1, 60) = 2.69$, $P = .06$; no sex or genotype × sex interaction; figure 3B).

Social approach or avoidance behaviors can also be measured by assessing social interaction between an experimental animal and an unfamiliar conspecific in

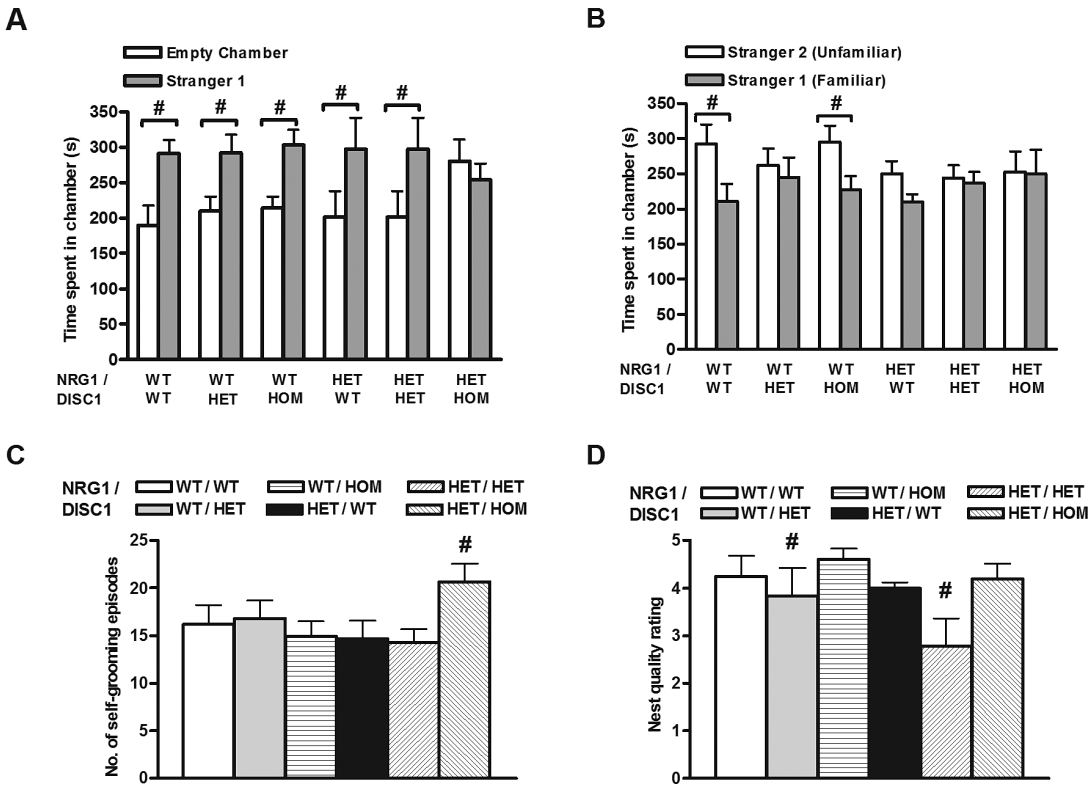

Fig. 3. Sociability and social novelty performance in NRG1^{WT}/DISC^{WT}, NRG1^{WT}/DISC1^{HET}, NRG1^{WT}/DISC1^{HOM}, NRG1^{HET}/DISC1^{WT}, NRG1^{HET}/DISC1^{HET}, and NRG1^{HET}/DISC1^{HOM}. (**A**) NRG1^{HET}/ DISC1^{HOM} show disrupted sociability. Data are mean times ± SEM spent in the empty chamber vs chamber containing *Stranger 1*. $^{\#}P < .05$ vs empty chamber. (**B**) Disruption of social novelty preference in NRG1 and DISC1 mutants. Data are mean times ± SEM spent in chamber containing *Stranger 2* vs (now familiar) *Stranger 1*. $^{\#}P < .05$ vs *Stranger 1*. (**C**) NRG1^{HET}/DISC1^{HOM} males demonstrate increased self-grooming during dyadic social interaction. Data are mean number of episodes ± SEM. $^{\#}P < .05$ vs NRG1^{WT}/DISC^{WT}. (**D**) DISC1^{HET} males demonstrate reduced quality ratings for nest building. Data are mean ratings ± SEM. $^{\#}P < .05$, effect of DISC1 genotype. Abbreviations: NRG1, Neuregulin-1; DISC, Disrupted-in-schizophrenia 1; HET, heterozygous; HOM, homozygous; WT, wildtype. Group sizes are detailed in supplementary table 1.

a novel arena.[23] Male NRG1^{HET} demonstrated a reduction in number of social investigation episodes, while the opposite effect was observed for females (NRG1 × sex, $F(1,71) = 9.72$, $P = .002$; supplementary figure 2). Male mice with simultaneous disruption to both genes (NRG1^{HET}/DISC1^{HOM}) exhibited increased frequency of self-grooming behavior (NRG1 × DISC1 × sex, $F(2,71) = 3.32$, $P = .04$; figure 3C), which is considered indicative of anxiety-related behavior in a social context.[24]

Deficits in nesting behavior have been hypothesized to relate to self-neglect and impairment in social functioning in schizophrenia.[25] Nest building behavior is assessed

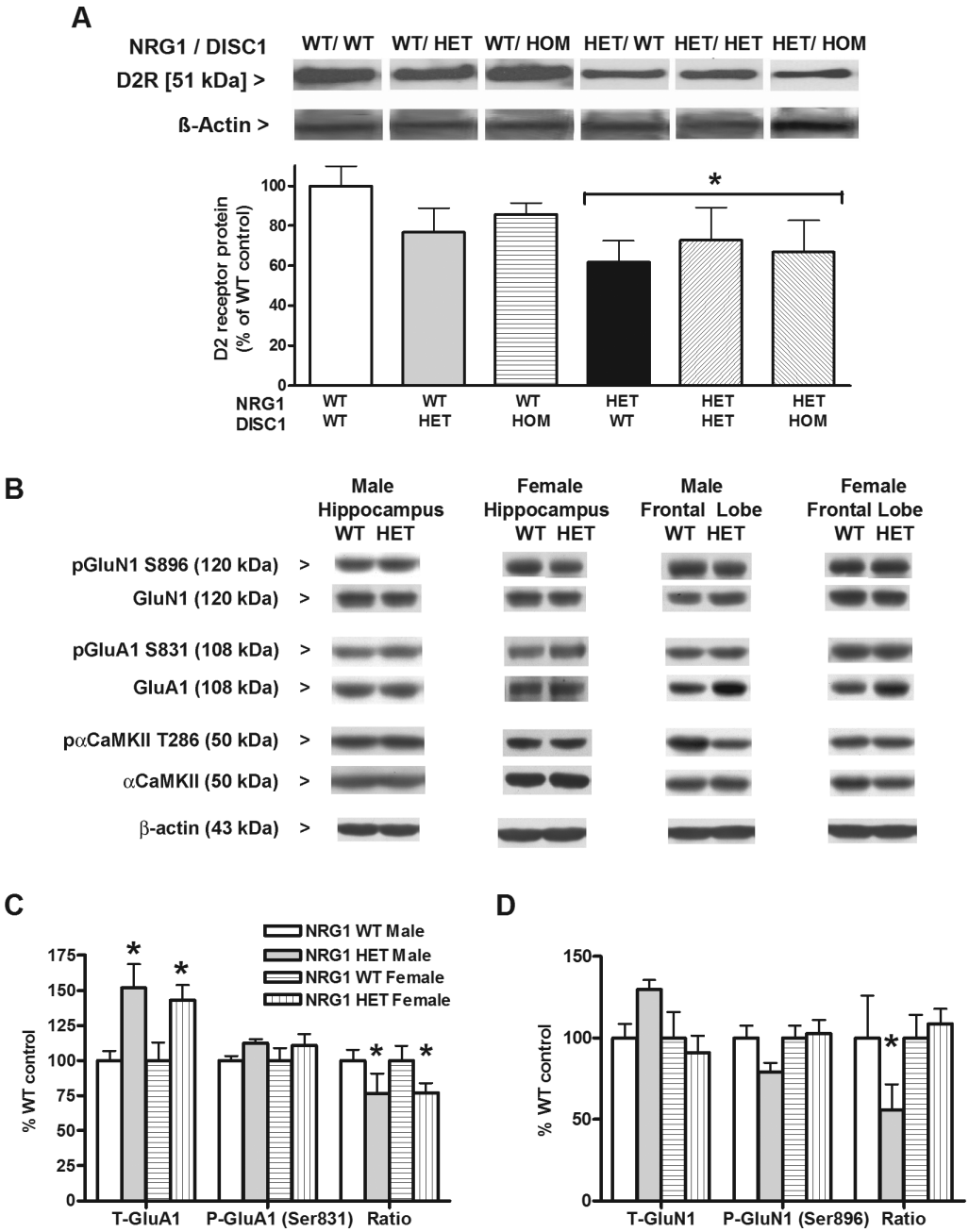

Fig. 4. Modulation of dopaminergic and glutamatergic neurotransmission in NRG1 × DISC1 and NRG1^{HET} mutants respectively. (**A**) DA D2 receptor protein levels in the striatum are reduced in NRG1^{HET} mice. Quantitative data (mean ± SEM) expressed as percentage of NRG1^{WT}/DISC^{WT} controls.*$P < .05$, effect of NRG1 genotype. (**B**) Representative immunoblots of the GluA1 (and pGluA1 S831), NMDA subunit (and pGluN1 S896), and CaMKII (and pCaMKII T286) protein levels in the frontal lobe and hippocampus in NRG1^{WT} and NRG1^{HET} mice (both sexes). (**C–D**) Quantitative data (mean ± SEM), expressed as percentage of NRG1^{WT} control, for (**C**) GluA1, and (**D**) GluN1protein levels in the frontal lobe. Increased total GluA1 and decreased total:phosphorylated GluA1 in NRG1^{HET}. Decreased ratio of total:phosphorylated GluN1 (Ser896) levels in male NRG1^{HET}. *$P < .05$, **$P < .01$, effect of NRG1 genotype. Abbreviations: NRG1, Neuregulin-1; DISC, Disrupted-in-schizophrenia 1; HET, heterozygous; WT, wildtype; DA, dopamine. Group sizes are detailed in supplementary table 1.

by singly-housing the experimental animal in a new cage containing nesting material and scoring the quality of the nest constructed 24 hours later. Male DISC1^{HET} demonstrated reduced nest weight and nest quality (DISC1 × sex, $F(2,64) = 3.43$, $P = .04$; HET vs WT, HET vs HOM, $P < .05$; figure 3D).

Spontaneous Alternation and Recognition Memory Tests

Patients with schizophrenia show deficits in a variety of cognitive domains, including spatial working memory and recognition memory processes that can be accessed in mice using maze-based or object recognition paradigms, respectively.[26,27] No significant effect of NRG1 or DISC1 genotype, nor genotype × sex interaction, was observed on spontaneous alternation or object recognition memory test performance (data available in supplementary figures 3–5).

Brain Monoamine Levels and DA D2 Receptor Protein Expression in the Striatum

Levels of monoamines and their metabolites were measured in the striatum, hippocampus, and prefrontal cortex using high-performance liquid chromatography (HPLC) with electrochemical detection (supplementary table 2). No genotype-related change in DA (or its metabolites DOPAC or HVA) or noradrenaline (NA) levels were observed in any of these structures. In the prefrontal cortex, DISC1^{HET} and DISC1^{HOM} demonstrated reduced 5-hydroxyindoleacetic acid (5-HIAA):5-hydroxytryptamine (5-HT) ratio (DISC1, $F(2,35) = 4.27$, $P = .02$; HET vs WT, HOM vs WT, $P < .05$) and decreased 5-HT levels (DISC1, $F(2, 35) = 3.35$, $P = .04$; HET vs WT, HOM vs WT, $P < .05$). In the hippocampus, NRG1^{HET} demonstrated reduced 5-HIAA:5-HT ratio (NRG1, $F(2,35) = 5.71$, $P = .01$). No sex or genotype × sex interaction was observed.

Expression levels of striatal DA D2 receptor protein were reduced in all NRG1^{HET} groups in a manner unrelated to DISC1 genotype (NRG1, $F(1, 73) = 6.47$, $P = .01$; no sex or genotype × sex interaction; figure 4A).

Glutamatergic Synaptic Protein Expression in the Brain

Studies in post-mortem brain from patients with schizophrenia indicate abnormalities of glutamate NMDA receptors in the hippocampus and prefrontal cortex,[28] and a reduced level of NMDA receptors in mice is associated with disruption to PPI.[28] Therefore, we examined the expression of essential components of glutamatergic synapses in the hippocampus and frontal lobe of NRG1^{HET} vs NRG1^{WT}. In the frontal lobe, NRG1^{HET} showed an increase in total AMPA GluA1 protein levels and a decrease in the ratio of total:phosporylated GluA1 (total protein, $F(1, 16) = 8.75$, $P = .01$; ratio, $F(1, 14) = 4.46$, $P = .04$; figures 4B and 4C).

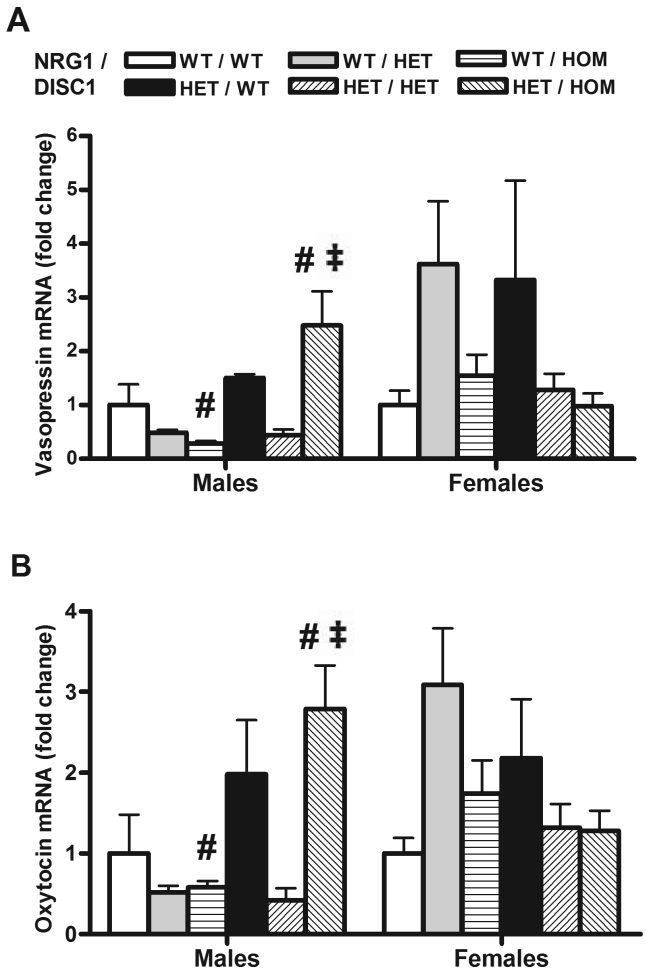

Fig. 5. Expression of oxytocin and arginine-vasopressin genes in the hypothalamus of NRG1^{WT}/DISC^{WT}, NRG1^{WT}/DISC1^{HET}, NRG1^{WT}/DISC1^{HOM}, NRG1^{HET}/DISC1^{WT}, NRG1^{HET}/DISC1^{HET}, and NRG1^{HET}/DISC1^{HOM}. (**A**) Sex-specific (males only) reduction in hypothalamic arginine-vasopressin mRNA expression ($2^{-\Delta\Delta CT}$, relative to WT) in DISC1 mutants, which is reversed in DISC1^{HOM} by heterozygous deletion of NRG1. Data are expressed as means ± SEM. $^{\#}P < .05$, DISC1 × sex interaction. $^{\ddagger}P < .05$, NRG1 × DISC1 × sex interaction. (**B**) Sex-specific (males only) reduction in hypothalamic oxytocin mRNA expression ($2^{-\Delta\Delta CT}$, relative to WT) in DISC1 mutants, which is reversed in DISC1^{HOM} by heterozygous deletion of NRG1. Data are expressed as means ± SEM. $^{\#}P < .05$, DISC1 × sex interaction. $^{\ddagger}P < .05$, NRG1 × DISC1 × sex interaction. Abbreviations: NRG1, Neuregulin-1; DISC, Disrupted-in-schizophrenia 1; HET, heterozygous; HOM, homozygous; WT, wildtype. Group sizes are detailed in supplementary table 1.

We also observed a sex-specific decrease (males only) in ratio of total:phosphorylated GluN1 (Ser896) levels in NRG1^{HET} (NRG1 × sex, $F(1, 13) = 6.14$, $P = .03$; figures 4B and 4D). Additionally, decreased total CaMKII protein levels, as well as ratio of total:phosphorylated CaMKII, was observed in the crude synaptosomal fraction of NRG1^{HET} (total protein, $F(1, 16) = 4.03$, $P = .05$; ratio, $F(1, 11) = 5.59$, $P = .04$; figure 4B, supplementary figure 6C). In the hippocampus, NRG1^{HET} showed a sex-specific increase (males only) in total:phosphorylated GluN1 (Ser896) ratio

(NRG1 × sex, $F(1, 17) = 8.43$, $P = .01$; supplementary figure 7C). NRG1^{HET} also exhibited a reduction in both total and phosphorylated CaMKII protein levels in the cytosolic fraction (total, $F(1, 13) = 4.05$, $P = .06$; phosphorylated, $F(1, 13) = 5.31$, $P = .04$; supplementary figure 7G). Total or phosphorylated NR2B, AKT or GSK3β expression levels were unaffected in either structure (supplementary figures 6 and 7).

Hypothalamic Expression of Vasopressin 1a and Oxytocin mRNA

Modification of social interaction behavior is associated with altered hypothalamic expression of the gene encoding the neuropeptide oxytocin,[29,30] whereas increased hypothalamic vasopressin expression is associated in a sex-specific manner with agonistic or affiliative behavior in male and females, respectively.[29] In males, but not in females, disruption of DISC1 reduced hypothalamic mRNA expression of both vasopressin 1a and oxytocin (vasopressin: DISC1 × sex, $F(2,71) = 3.59$, $P = .03$; HOM vs HET, HOM vs WT, $P < .05$; oxytocin: DISC1 × sex, $F(2, 71) = 3.70$, $P = .03$; HOM vs HET, HOM vs WT, $P < .05$; figures 5A and 5B). However, while disruption of NRG1 was without effect, either alone or in combination with HET disruption of DISC1, these reductions were each reversed by disruption of NRG1 with HOM co-disruption of DISC1 (vasopressin: NRG1 × DISC1 × sex, $F(2, 71) = 4.07$, $P = .02$; oxytocin: NRG1 × DISC1 × sex, $F(2, 71) = 3.63$, $P = .03$; figures 5A and 5B).

DISC1 and NRG1/ErbB4 Protein Expression in the Brain

We assessed the effects of mutation of NRG1 and/or DISC1 on NRG1 and ErbB4 protein levels in the hippocampus. An antibody that recognizes an epitope at the C-terminal region of NRG1 detected major bands of 40, 80, and 110 kDa (corresponding to NRG1 Types II, III and I isoforms, respectively). Densitometry analysis revealed a sex-specific (males only) reduction in the 40 kDa signal in NRG1^{HET} (NRG1 × sex, $F(1,59) = 6.43$, $P = .01$; figure 6A). For 110 kDa band, DISC1^{HET} and DISC1^{HOM} evidenced an increased signal, but this increase was reversed on co-disruption of NRG1

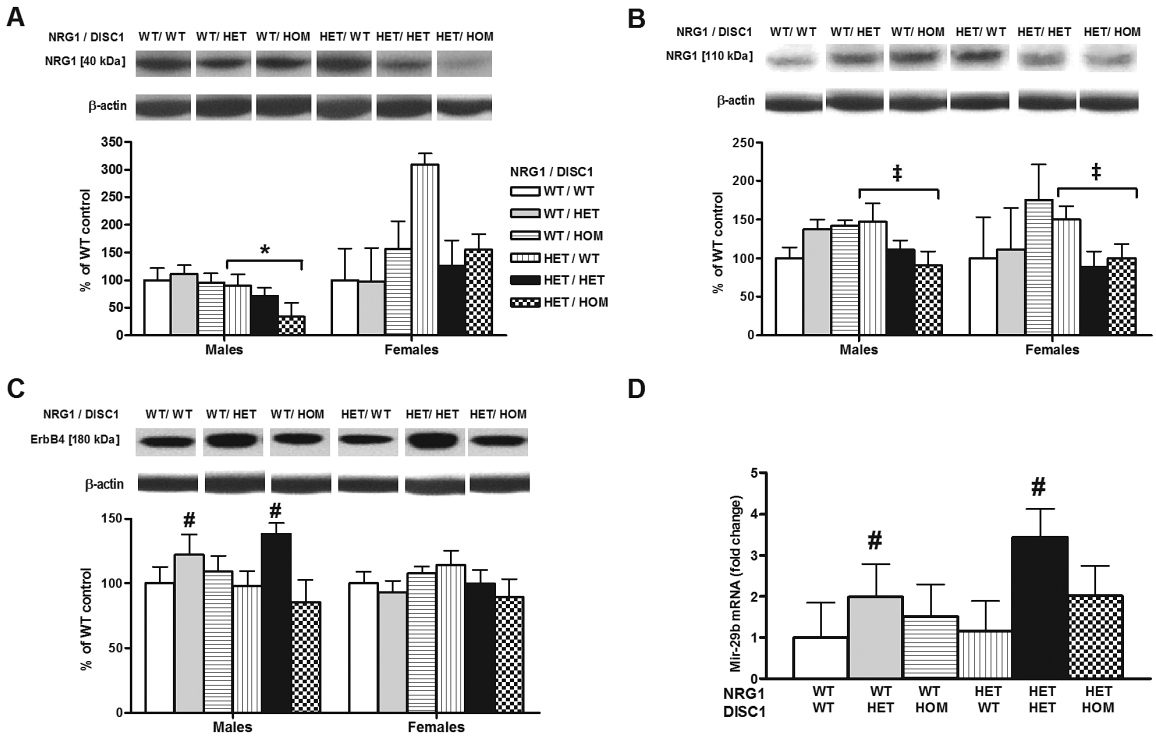

Fig. 6. Modulation of (**A**, **B**) NRG1 and (**C**) ErbB4 protein levels in the hippocampus in NRG1^{WT}/DISC^{WT}, NRG1^{WT}/DISC1^{HET}, NRG1^{WT}/DISC1^{HOM}, NRG1^{HET}/DISC1^{WT}, NRG1^{HET} /DISC1^{HET}, and NRG1^{HET}/DISC1^{HOM}. (**A**) NRG1 (40 kDa) in the hippocampus in both sexes. Decreased 40 kDa signal in male NRG1^{HET}. Data expressed as percentage of NRG1^{WT}/DISC^{WT} controls. $^{*}P < .05$, NRG1 × sex interaction. (**B**) NRG1 (110 kDa) in the hippocampus in both sexes. Increased 110 kDa signal in DISC1^{HET} and DISC1^{HOM}, marginally reversed by co-deletion of NRG1. Data expressed as percentage of NRG1^{WT}/DISC^{WT} controls. $^{‡}P < .05$, NRG1 × DISC1 interaction. (**C**) ErbB4 (180 kDa) levels in the hippocampus in both sexes. ErbB4 (180 kDa) levels in the hippocampus increased in DISC1^{HET} males. Data expressed as percentage of NRG1^{WT}/DISC^{WT} controls. $^{\#}P < .05$, DISC1 × sex interaction. (**D**) Mir-29b mRNA expression ($2^{-\Delta\Delta CT}$, relative to WT) in the prefrontal cortex increased in DISC1^{HET} relative to other genotypes. $^{\#}P < .05$, effect of DISC1 genotype. Data are expressed as means ± SEM. Abbreviations: NRG1, Neuregulin-1; DISC, Disrupted-in-schizophrenia 1; HET, heterozygous; HOM, homozygous; WT, wildtype. Group sizes are detailed in supplementary table 1.

(110 kDa: NRG1 × DISC1, $F(2,59) = 3.91$, $P = .03$; figure 6B). A sex-specific (males only) increase in ErbB4 expression levels was observed in DISC1^{HET} (DISC1 × sex, $F(2,59) = 3.10$, $P = .05$; male HET vs WT, $P < .05$; no effect of NRG1 or NRG1 × DISC1 interaction; figure 6C). It has been demonstrated previously that the DISC1-L100P mutant protein has reduced interaction with both GSK3α and β.[31] We confirm that DISC1^{HOM} display reduced binding between DISC1 and GSK3ß in the striatum (DISC1, $F(2,49) = 3.41$, $P = .04$, HOM vs WT, $P < .05$; no effect of NRG1 or NRG1 × DISC1 interaction; supplementary figure 8).

Schizophrenia-Associated miRNA Expression in the Brain

miRNAs have been shown to modulate the expression of many genes, including several that are differentially expressed in brains from patients with schizophrenia.[32,33] Expression levels in the prefrontal cortex for the following 8 selected miRNAs (distinguished by being differentially expressed in schizophrenia vs comparison samples[33]) were determined in male mice across the genotypes by quantitative RT-PCR (qRT-PCR): miR-29a, miR-29b, miR-29c, miR-34, miR-101, miR-125a-3p. DISC1^{HET} displayed elevated miR-29b relative to other genotypes (DISC1, $F_{2,37} = 4.39$, $P = .02$; HET vs HOM, HET vs WT, $P < .05$; figure 6D).

Discussion

This is the first in vivo study to describe, at a functional level, convergent and divergent phenotypes arising from individual vs co-mutation in mice of 2 risk factors, DISC1 and NRG1, that have been associated independently with the pathogenesis of schizophrenia. The model employed, whereby multiple genetic alterations are induced simultaneously, resolves aspects of the phenotype that are subject to regulation by epistasis (ie, NRG1 × DISC1 interaction) and distinguishes these from those for which DISC1 and NRG1 exert independent or no effects. Furthermore, the model identifies effects of DISC1 and NRG1 that may converge on common pathophysiological processes implicated in schizophrenia. Some of these effects were sex-specific, most commonly in males (see tables 1 and 2 for summary of phenotypes).

Disruption of NRG1, but not of DISC1, in the absence of epistasis, was associated with abnormalities, some sex-specific, in novelty and AMPH-induced hyperactivity, PPI, D2 and GluN1 / GluA1 receptor expression. We observed in NRG1^{HET} post-pubertal prominence of antipsychotic-sensitive, novelty-induced hyperactivity and PPI deficits, which may relate to emergence of the diagnostic, psychotic symptoms of schizophrenia in late adolescence/early adulthood. We also observed in NRG1^{HET} region-specific changes in GluN1-mediated signaling, together with increased GluA1 activation and decreased

Table 1. Summary of Behavioral and Psychopharmacological Phenotypes of NRG1 × DISC1 Mutants

Genotype		Cognition				Social Interaction				Psychopharmacology		
NRG1	DISC1	Novelty-Induced Activity	PPI	Y-Maze Alternation	Object Recognition Memory	Sociability	Social Novelty Preference	Dyadic Social Interaction	Nesting Behavior	Sensitivity to Psychostimulant-Induced Hyperactivity	Locomotor Responsivity to Antipsychotics	PPI Responsivity to Antipsychotics
WT	HET	=	=	=	=	=	↓	=	↓(♂ only)	=	-	=
WT	HOM	=	=	=	=	=	=	=	=	=	-	=
HET	WT	↑	↓	=	=	=	↓	[a]	=	[c]	[d]	[d]
HET	HET	↑	↓	=	=	=	↓	[a]	↓ (♂ only)	[c]	[d]	[d]
HET	HOM	↑	↓	=	=	↓	↓	[a,b]	=	[c]	-	=

Note: ↑, increased relative to NRG1^{WT}/DISC1^{WT}; ↓, decreased relative to NRG1^{WT}/DISC1^{WT}; =, no difference; -, not reported; PPI, Prepulse inhibition; NRG1, Neuregulin-1; DISC, Disrupted-in-schizophrenia 1; HOM, homozygous mutants; HET, heterozygous mutants; WT, wildtype.

[a]Sexually dimorphic change in number of social investigative episodes (decreased in males, increased in females).

[b]Sex-specific (males only) increase in self-grooming behavior.

[c]Increased relative to vehicle-treated NRG1^{WT}/DISC1^{WT}.

[d]Increased relative to vehicle-treated mice.

Table 2. Summary of Biochemical Phenotypes of NRG1 × DISC1 Mutants

Phenotype	Genotype				
	NRG1	WT	WT	HET	HET	HET
	DISC1	HET	HOM	WT	HET	HOM
Glutamate						
Frontal lobe expression of AMPA GluA1 protein		-	-	↑	-	-
Frontal lobe ratio of total:phosphorylated GluA1 protein		-	-	↓	-	-
Frontal lobe ratio of total:phosphorylated GluN1 protein		-	-	↓ (♂ only)	-	-
Frontal lobe expression CaMKII protein and total: phosphorylated CaMKII ratio		-	-	↓	-	-
Hippocampus ratio of total:phosphorylated GluN1 protein		-	-	↑ (♂ only)	-	-
Hippocampus expression of total and phosphorylated CaMKII protein		-	-	↓	-	-
Frontal lobe & Hippocampus total/phosphorylated NR2B, AKT, or GSK3β protein		-	-	=	-	-
Dopamine						
Striatal dopamine receptor D2 protein expression		=	=	↓	↓	↓
Brain monoamines						
PFC & hippocampus NA		=	=	=	=	=
PFC & hippocampus DA		=	=	=	=	=
PFC & hippocampus HVA & HVA:DA		=	=	=	=	=
PFC & hippocampus DOPAC & DOPAC:DA		=	=	=	=	=
PFC 5-HT and 5-HIAA:5-HT		↓	↓	=	↓	↓
Hippocampus 5-HT		=	=	=	=	=
Hippocampus 5-HIAA:5-HT		=	=	↓	↓	↓
Neuropeptides						
Hypothalamic expression of oxytocin mRNA		=	↓ (♂ only)	=	=	↑ (♂ only)[a]
Hypothalamic expression of vasopressin 1a mRNA		=	↓ (♂ only)	=	=	↑ (♂ only)[a]
DISC1 and NRG1/ErbB4						
Hippocampus expression of NRG1 protein		=	=	↓	↓	↓
Hippocampus expression of ErbB4 protein		↑ (♂ only)	=	=	↑ (♂ only)	=
Striatal binding of DISC1 and GSK3ß proteins		=	↓	=	=	=
Schizophrenia-associated miRNAs						
Brain expression of miR29b mRNA		↑	=	=	↑	=
Brain expression of miR-29a, miR-29c, miR-34, miR-101, miR-125a-3p mRNA		=	=	=	=	=

Note: ↑, increased relative to $NRG1^{WT}/DISC1^{WT}$; ↓, decreased relative to $NRG1^{WT}/DISC1^{WT}$; =, no difference; -, not reported; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CaMKII, Ca2+ /calmodulin-dependent protein kinase II; AKT, protein kinase B; GSK3β, glycogen synthase kinase 3 beta; PFC, prefrontal cortex; NA, noradrenaline; DA, dopamine; HVA, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-Hydroxyindoleacetic acid; miRNA, MicroRNA.
[a]Increased expression relative to $NRG1^{WT}/DISC1^{HOM}$.

CaMKII activity in the frontal lobe. Post-mortem studies have shown decreased GluN1 protein expression in the prefrontal cortex of schizophrenia cases,[34] and dysregulation of NMDA receptor formation and function in the hippocampus during development has been linked with the pathogenesis of schizophrenia.[35] Antipsychotic-sensitive hyperactivity and PPI deficits have also been observed in mice having reduced expression of GluN1.[36] Emerging evidence suggests inhibition of NMDAR activity reduces activity in the PI3/AKT pathway, and that decreased NRG1/ErbB4 signaling may contribute to schizophrenia via modulation of this pathway.[37]

Disruption of DISC1, but not of NRG1, in the absence of epistasis, was associated with abnormalities in nest building and social investigation, DISC1-GSK3β interaction, and ErbB4 and miR-29b expression. In agreement with a recent report,[38] we failed to observe hyperactivity or PPI deficits in DISC1 L100P mutants; these data are in direct contrast with earlier findings.[19] However, we identified specific cellular phenotypes (notably the reduced interaction with GSK3ß), social behavior deficits (disruption of social novelty preference, nesting deficits) in this model.

Increased expression of miR-29b was observed in the prefrontal cortex of HET DISC1 mutants. Previous studies have shown that miR-29b is differentially expressed in the prefrontal cortex of schizophrenia patients.[39] Among the gene targets identified for miR-29b are the schizophrenia-associated genes ZDHHC8 and BACE1 (which plays a role in proteolysis of NRG1), as well as DNA methyltransferases DNMT3A and DNMT3B.[40]

Strikingly, co-disruption of both NRG1 and DISC1 revealed "pure" epistasis (ie, NRG1 × DISC1 interaction in the absence of any effect of disruption of either NRG1 alone or DISC1 alone) in terms of decreased sociability and increase in self-grooming. This profile of epistasis involves a shift from socially-directed to self-directed behavior in a manner similar to the negative symptoms of schizophrenia, together with disruption in hypothalamic expression of the oxytocin and/or vasopressin genes. Both the animal and human literature has linked oxytocin and vasopressin with social affiliative and agonistic behavior,[41] and intranasal oxytocin administration has been shown to improve negative symptoms in schizophrenia patients.[42]

The present study demonstrates that, in general, male mutants were more affected than females by mutation of NRG1, DISC1 or NRG1 × DISC1. Sexually dimorphic effects in genetic mouse models of psychosis are commonly observed.[43,44] These differences mirror gender effects reported for schizophrenia patients, where males demonstrate show lower premorbid functioning, earlier age of onset, more severe cognitive deficits, and poorer prognosis.[45,46] Both NRG1 and ErbB1-4 are highly expressed in the hypothalamus[47] and ErbB3/4 signaling in hypothalamic cells plays a critical role in female sexual development.[48] Mutant mice containing dominant-negative ErbB4 receptors demonstrate impaired reproductive capacity and altered sexual maturation due to disrupted responding of hypothalamic astrocytes to NRG1.[49]

Susceptibility factors associated with risk for schizophrenia display notable promiscuity with other neurodevelopmental and psychiatric disorders, providing a molecular genetic basis for clinical comorbidity and common domains of psychopathology.[50] We report here separate and interactive effects of NRG1 and DISC1 on various behaviors associated with positive and negative symptoms, but not with cognitive impairment. As our current understanding of risk loci can explain less than one-third of the heritability of psychotic illness,[3,7] it has been posited that such "missing heritability" can be explained by epistatic interactions between genes implicated in the pathogenesis of the disorder.[8] The present model system shows that co-disruption of 2 genes associated with the pathophysiological basis of schizophrenia, here NRG1 and DISC1, can reveal epistasis in terms of (1) modifying the phenotypic effect(s) of one gene or the other, and (2) creating phenotypic effects not manifested following disruption of either gene alone, in individual aspects of function, here social behavior and putative molecular correlates. These findings provide a conceptual and empirical basis for the systematic, clinical investigation of epistasis to inform on the nature of genetic risk for schizophrenia in relation to specific domains of the psychosis phenotype.

Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

Funding

This work was supported by Science Foundation Ireland (SFI) Principal Investigator Grant 07/IN.1/B960 (J.L.W.) and Health Research Board of Ireland Postdoctoral Fellowship PD/2007/20 (C.M.P.O'T). The Alimentary Pharmabiotic Centre is a research centre funded by SFI, through the Irish Government's National Development Plan. G.C., J.F.C., and T.G.D. were supported by SFI (grant numbers SFI/12/RC/2273, 02/CE/B124 and 07/CE/B1368) and by the Health Research Board (HRB) through Health Research Awards (grant no HRA_POR/2011/23, T.G.D., J.F.C. and G.C.; HRA_POR/2012/32, J.F.C., T.G.D.; and HRA-POR-2014–647: G.C., T.G.D.). J.F.C. is also funded by the European Community's Seventh Framework Programme (FP7/2007–2013, grant agreement 201 714) as is T.G.D. (grant number KBBE.2013.2.2-02). G.C. is supported by a NARSAD Young Investigator Grant from the Brain and Behavior Research Foundation (grant number 20771). T.G.D. and C.M.P.O'T are also funded by the University College Cork Translational Research Access Programme. DISC1 (100P) mutants were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan.

Acknowledgment

The authors declare no competing financial interests.

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