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Genetic regulation of *Nrnx1* expression: an integrative cross-species analysis of schizophrenia candidate genes

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Neurexin 1 (NRXN1) is a large presynaptic transmembrane protein that has complex and variable patterns of expression in the brain. Sequence variants in *NRXN1* are associated with differences in cognition, and with schizophrenia and autism. The murine *Nrxn1* gene is also highly polymorphic and is associated with significant variation in expression that is under strong genetic control. Here, we use co-expression analysis, high coverage genomic sequence, and expression quantitative trait locus (eQTL) mapping to study the regulation of this gene in the brain. We profiled a family of 72 isogenic progeny strains of a cross between C57BL/6J and DBA/2J (the BXD family) using exon arrays and massively parallel RNA sequencing. Expression of most *Nrxn1* exons have high genetic correlation ($r > 0.6$) because of the segregation of a common *trans* eQTL on chromosome (Chr) 8 and a common *cis* eQTL on Chr 17. These two loci are also linked to murine phenotypes relevant to schizophrenia and to a novel human schizophrenia candidate gene with high neuronal expression (*Pleckstrin and Sec7 domain containing 3*). In both human and mice, *NRXN1* is co-expressed with numerous synaptic and cell signaling genes, and known schizophrenia candidates. Cross-species co-expression and protein interaction network analyses identified *glycogen synthase kinase 3 beta* (*GSK3B*) as one of the most consistent and conserved covariates of *NRXN1*. By using the Molecular Genetics of Schizophrenia data set, we were able to test and confirm that markers in *NRXN1* and *GSK3B* have epistatic interactions in human populations that can jointly modulate risk of schizophrenia.

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Introduction

Neurexins (NRXNs) are neuronal cell adhesion proteins inserted mainly into the presynaptic membrane. They serve as bridging molecules that directly bind the presynaptic neurotransmitter machinery with postsynaptic transmembrane proteins such as the neuroligins and neurotransmitter receptors.[1–6] The NRXNs have an important role in regulation of presynaptic Ca^{2+} channels and neurotransmitter release, and are also involved in the modulation of postsynaptic NMDA and γ-aminobutyric acid (A) receptor activity.[6–9]

There are three members in the NRXN gene family—*Nrxn1*, *Nrxn2* and *Nrxn3*. They are among the largest of mammalian genes, *Nrxn1* being close to 1.1 Mb and *Nrxn2* about 1.7 Mb, and all are highly conserved across vertebrates.[10,11] A fascinating aspect of the NRXNs is their enormously complex and extensive alternative splicing that can potentially produce about a thousand isoforms.[12] Each NRXN gene has two promoters and transcribes a long version (α-NRXN) and a short version (β-NRXN). Multiple isoforms of both long and short variants are produced as a result of alternative splicing from five splicing regions within each gene.[10,11] Deletion of the larger α-NRXNs leads to marked deficits in synaptic function.[13,14]

Among the NRXN genes, *NRXN1* has been implicated in neurodevelopmental and psychiatric disorders. Genome-wide association studies have consistently found links between copy number variation (primarily deletions) in *NRXN1* and autism and schizophrenia.[15–19] Associations have also been found between sequence variants in *NRXN1* and nicotine addiction and alcoholism.[20–22] In mice, deletion of *Nrxn1*α causes a decrease in excitatory synaptic transmission in the hippocampus, and behavioral changes including enhancement of motor learning and decreased prepulse inhibition (PPI) to acoustic startle—an endophenotype that models sensory-gating deficits in schizophrenia.[23]

These studies in human and mice have established an important role for *NRXN1* in the etiology of neuropsychiatric diseases. However, disorders such as schizophrenia and autism are inherently polygenic and emerge from the interplay of multiple sequence variants and environmental factors. A high-priority candidate such as *NRXN1* is bound to operate within the context of a wider network of genes and is likely to involve non-linear and epistatic interactions that contribute to disease risk. Despite the importance of epistasis (gene–gene interactions) in disease process, current large-scale genetic studies rarely go beyond linear additive models.[24] Those that do apply a network approach to genome-wide association studies[25–28] rely on known protein interactions and pathways, and are essentially constrained by prior knowledge.

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Here, we ask whether we can use gene co-expression analysis in the mouse to define and refine genetic and molecular networks for *Nrxn1* that provide insights into neuronal processes and possible disease mechanisms. To what extent can we translate from mouse to human at the level of genetic networks? Specifically, can data from a large family of mouse strains reveal novel partners for *Nrxn1* that are relevant to human disease? The *Nrxn1* locus is highly polymorphic in the BXD family and contains several single-nucleotide polymorphisms (SNPs) and insertions and deletions. Applying a reverse complex trait approach, we have treated these natural sequence variants and corresponding variations in transcript abundance as a starting point in the dissection of downstream phenotypes.[29] We used expression QTL (eQTL) analysis to evaluate the genetic regulation of *Nrxn1* and to build an interaction network around *Nrxn1* that includes other genes and also key behavioral phenotypes. Finally, we used human data extracted from the Molecular Genetics of Schizophrenia (MGS) study[30] to evaluate the translational relevance of the murine analysis and to test a specific hypothesis about an epistatic interaction between *NRXN1* and glycogen synthase kinase 3 beta (*GSK3B*).

Materials and methods

Mouse strains and resources. The BXD inbred strains were derived by crossing the parental strains C57BL/6J (B6) and DBA/2J (D2) followed by >20 generations of inbreeding.[31–33] The parental lines differ at approximately 4.8 million SNPs and at another 500 000 insertions and deletions and copy number variations and the family as a whole is highly polymorphic.[34] The BXDs are a densely phenotyped and genotyped family and have been used as a genetic reference panel for over 30 years.[35] A compendium of over 2000 phenotypes and over 45 microarray data sets for these strains is available from GeneNetwork (http://www.genenetwork.org).

For this study, transcript measurements were taken from the hippocampi of 70 BXD strains, the parental B6 and D2, and reciprocal F1 hybrids (B6D2F1 and D2B6F1). Details on the strain, sex and age of each animal can be accessed from http://www.genenetwork.org/dbdoc/UMUTAffyExon_0209_RMA.html. The mice were bred at the University of Tennessee Health Science Center or at the Jackson Laboratory, Bar Harbor, ME, USA. Hippocampal dissections were carried out at UTHSC or at the Beth Israel Deaconess Medical Center by our colleague, Dr Glenn D Rosen (details on dissection and tissue processing are in Supplementary methods). All animal procedures were approved by the Animal Care and Use Committees at UTHSC and BIDMC.

Microarray data processing. To provide a comprehensive analysis of natural variation in gene expression in the mouse hippocampus, we have previously generated transcriptome profiles using conventional Affymetrix arrays for $\sim$100 strains of mice.[36] In this study, we used exon arrays (Affymetrix Exon 1.0 ST, Affymetrix, http://www.affymetrix.com) to provide a more detailed but still global analysis of the genetic control of alternative splicing in the hippocampus. The Affymetrix Exon 1.0 ST arrays contain a total of 1.2 million probe sets and uses at least four probes to interrogate an exon. Probe level intensity values were extracted from the CEL files using the GeneChip Operating Software provided by Affymetrix. Probe set level summarization and data normalization was done within GeneChip Operating Software using the Robust Multichip Averaging method. The expression values were then log-transformed and stabilized to a mean of 8 and an s.d. of 2 across the samples. The entire data set (BXD hippocampus mRNA UMUTAffy Hippocampus Exon (February 2009) RMA) can be downloaded from GeneNetwork data sharing site using the accession number GN206 (http://www.genenetwork.org/share/data/).

Array data analysis on GeneNetwork. QTL mapping was done for the probe set level data using QTL Reaper.[37,38] The results are presented as likelihood ratio statistic (LRS) scores (LOD score $=$ LRS/4.61). We used 3795 informative microsatellite and SNP markers in the BXDs for interval mapping. The BXD genotypes can be downloaded from http://www.genenetwork.org/share/data/. Up to a million permutations were performed to compute the genome-wide suggestive and significant thresholds. *Nrxn1* is located on chromosome (Chr) 17 between 90.432 and 91.492 Mb (mm9, build 37) on the minus strand and is targeted by 172 probe sets. For the probe sets that targeted exons in *Nrxn1* and mapped as *cis* eQTL, we screened the probe sequences for overlapping SNPs that may confound the QTL analysis. Although *Nrxn1* is SNP rich, most exons are devoid of SNPs. With the exception of exons 5 and 17 (each harbors a synonymous mutation), the exon targeting probe sets do not overlap SNPs and the consistent *cis* effect is reliable.

For the co-expression analysis in the mouse hippocampus, pair-wise Pearson product moment correlations were computed for all probe sets based on expression variation across the 74 lines of mice. The top 1000 probe sets correlated with *Nrxn1* were filtered by expression level. Only probe sets with expression >8 on a $\log_2$ scale, and well-annotated probe sets that map to known genes were retained for subsequent analysis. Co-expression results are presented for a representative probe set (Affy 4930721) targeting a middle exon (exon 12) that has high expression, is associated with both eQTLs on Chrs 17 and 8 (see Results section), and is well correlated with other *Nrxn1* exons. Analyses using other exons from the same correlated block of exons give similar results. We also used a publicly available human forebrain expression data (neocortex and hippocampus) generated using the Affymetrix U133 Plus 2.0 arrays (GEO accession number GSE5281).[39,40] This data set contains samples from Alzheimer's disease cohort and age-matched control subjects. We computed Pearson product correlations across 71 samples from normal subjects and retrieved the top 1000 covariates of *NRXN1* from the pool of over 54 000 probe sets.

RNA sequencing. Total RNA was isolated from the hippocampus of adult female B6 and D2 mice. Ribosomal RNA was depleted using the RiboMinus eukaryotic kit (http://www.invitrogen.com). We prepared multiplexed bar-coded

fragment libraries using the SOLiD Fragment Library Barcoding kits. Emulsion PCR was then done and libraries were amplified and sequenced on the ABI SOLiD 3 plus system (http://www.appliedbiosystems.com).

We generated a total of 77 million and 80 million 50 nucleotide RNA sequencing (RNA-seq) tags for B6 and D2, respectively. Of these, approximately 58 million and 63 million reads aligned to the mouse reference genome (NCBI37/mm9) using the ABI Bioscope software (http://www.solidsoftwaretools.com). The alignment results (BAM files) were uploaded to the Partek Genomic Suite (http://www.partek.com) and GeneNetwork's UCSC Genome Browser mirror (http://ucscbrowser.genenetwork.org/) for visualization. Number of sequence tags was counted for each exon feature within *Nrxn1*. The entire data can be downloaded from GeneNetwork's high-throughput sequence data sharing site at http://galaxy.genenetwork.org:8080/library. Additionally, read alignments can also be visualized on the GeneNetwork Genome Browser.

QTL analysis for behavioral traits. To identify behavioral phenotypes that may be downstream of *Nrxn1* we used a point-wise *P*-value of 0.01. We selected phenotypes that correlated with marker rs6313030 (located on Chr 17 at 90.460122 Mb within the *Nrxn1* gene) and then analyzed behavioral traits assayed in the BXDs using marker regression. The behavioral phenotypes with QTLs on distal Chr 17 are (1) prepulse inhibition, assayed by McCaughran *et al*.[41] in a panel of 21 BXD strains (trait ID on Genenetwork is 10396), (2) anxiety trait measure by time spent in open quadrant of zero-maze, assayed in a larger panel of 57 BXD strains[42] (trait ID 11696) and (3) handling induced convulsion as an index of ethanol withdrawal severity, measured in 25 BXD strains[43] (trait ID 10065).

Gene–gene interaction analysis. To explore epistatic associations between *NRXN1* and *GSK3B*, we first extracted all markers in *NRXN1* and *GSK3B* and in the 20 kb flanking regions from the MGS data set.[30] GAIN and non-GAIN data sets[30] were combined and markers common to both were used. Association tests and minor allele frequency (MAF) were calculated using PLINK 2.050[44] and markers with minor allele frequencies $\geqslant 0.05$ were selected (384 in *NRXN1* and 17 in *GSK3B*). Sporadic missing genotypes in these markers were imputed by MDRdt Beta V0.4.3 (http://www.epistasis.org). Multifactor dimensionality reduction (MDR) method was used to conduct gene–gene interaction analyses (MDR 2.0 beta 8.1). The details of MDR are described elsewhere.[24,45,46] We used two methods to further filter the markers. One is the odds ratio filter implemented in the MDR software, which uses the case control ratio in the data set to define high-risk and low-risk genotype combinations.[46–48] Using this approach, the top five markers from each gene were selected. The other filter was based on single marker association *P*-values in which tagged SNPs[49] with association $P \leqslant 0.15$ in both genes were selected (a total of 13 markers in the two genes). SNPs selected by both methods were used for MDR analyses and best models were generated based on a 10-fold cross-validation consistency. In this study, we limited the search to a 2-loci and 3-loci interaction model using SNPs from the first and second filters, respectively. The significance of the best model was evaluated by 10 000 permutations (MDRpt 1.0 beta 2).[50]

Additional bioinformatics tools. Gene ontology and pathway enrichment analysis was done using DAVID 6.7 (http://david.abcc.ncifcrf.gov).[51,52] Protein–protein network analysis was done using the protein interaction database and network tool available on GeneMania (http://www.genemania.org).[53] The protein network was based on known physical interactions curated from 114 sources, and the network was weighted by biological process.

Results

Genetic regulation of *Nrxn1* exons. *Nrxn1* has widespread expression in the brain. We used microarrays to measure its transcript at exon level resolution in the hippocampus of 74 members of the BXD family. Additionally, we measured expression in parental strains using RNA-seq. The very deep coverage provided by the arrays and RNA-seq uncovered significant inter-exon variability in *Nrxn1* that may reflect alternative isoforms (Figure 1a). For example, low expressing exons (exons 2, 6, 11 and 19) are located within known splice sites[10,11] and the RNA-seq also showed a potentially skipped exon (exon 4 of Refseq gene NM_020252.3) (Figure 1b). To evaluate how tightly expression of exons is coupled with one another, we generated a correlation matrix of the exons and UTRs based on expression variation across the BXD family (Figure 1c). For the most part, exons form highly correlated expression blocks indicating that they are regulated as a single unit. The few exceptions are exons 1, 3, 11, 21, 22 and the UTRs, which show poor correlation with most of the other exons and may be alternatively spliced.

Nrxn1 expression is also highly variable across the strains with exons varying from 1.5-fold to 10-fold in abundance (Figure 1a). On average, the exons show a 2.5-fold variation among the BXDs. To track down genetic sources of this variation, we carried out eQTL mapping using conventional linkage analysis tools and genotypes embedded in GeneNetwork. The UTRs and canonical exons of *Nrxn1* are partly controlled by polymorphisms near the gene itself (so-called *cis* eQTL) and partly by *trans*-acting polymorphisms (Figure 1b). A tightly correlated set of exons map as a *cis* eQTL to distal Chr 17 within 0.5 Mb of *Nrxn1* itself. These exons are also strongly co-modulated by a *trans* regulatory QTL on mid-Chr 8. Several exons that are putatively subjected to splicing (for example, exons 1, 8, 11 and 21) are not modulated by either the *cis* or Chr 8 *trans* loci. Additionally, there are *trans* eQTLs on other Chrs associated with a minor set of exons (Supplementary Figure S1). For example, exons 12, 16 and 19 have *trans* eQTLs in mid-Chr 2 and exons 1, 3, 11 and 22 have *trans* eQTLs on Chr 12. These findings indicate a level of isoform specificity and joint eQTL control that corresponds to the complex splicing signatures in the exon co-expression matrix.

Sequence polymorphism in *Nrxn1* between C57BL/6J and DBA/2J. The maternal parent of the BXD strains, B6,

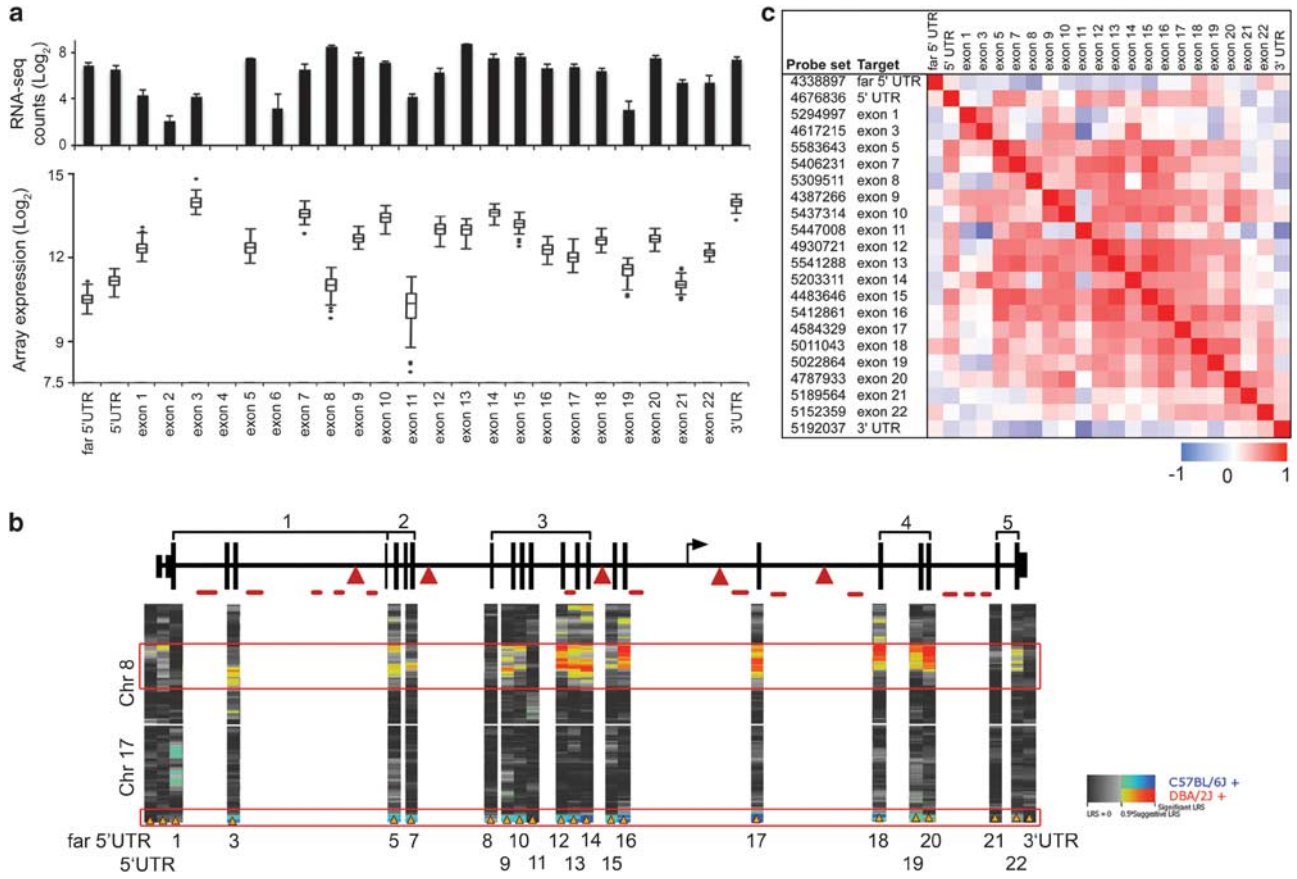

Figure 1 Expression of *neurexin 1* (*Nrxn1*) exons in mouse hippocampus. (**a**) Exon-level expression of *Nrxn1* was measured by direct sequencing of mRNA (upper bar chart; error bars are s.d.) and by using exon arrays (lower box plots). Expression values are on a $\log_2$ scale (*y* axis). There is significant variation in expression of exons among the BXDs. On average, expression varies 2.5-fold within exons. Based on RNA sequencing, exons 2 and 6 have low expression and exon 4 is skipped (there are no array probe sets for these). (**b**) The gene model depicted is based on RefSeq gene NM_020252.3. Location of the 5′ UTR for β-*Nrxn1* is shown in intron 17 (arrow) and known splice sites are indicated above (1–5). Approximate locations of insertion and deletion (indel) variants that are larger than 10 bps are shown below (lines for deletions and triangles for insertions in D2). Expression QTLs on chromosomes (Chrs) 8 (*trans*) and 17 (*cis*) are shown as heat maps for each exon and UTR. Allele from the DBA/2J parent has the positive effect on Chr 8 (red) while allele from the C57BL/6J parent has the positive effect on Chr 17 (blue). (**c**) The correlation matrix displays pair-wise correlations between the UTRs and exons in the BXDs. The lower cells are Pearson product moment and upper cells are Spearman ranked correlations. Exons 5–20 form a tightly correlated expression block (exon 11 being the exception). Correlation between the distal and proximal parts of the gene is generally low.

was the first mouse genome to be sequenced and is used as the mouse reference genome. The paternal strain, D2, has recently been resequenced at high density using next-generation systems (brief description of the 100x resequencing of the D2 genome is provided in Supplementary methods).[34] This now makes it possible to carefully review all sequence variants in and around *Nrxn1* to help identify variants that are candidates for the *cis*-effect on expression. We found numerous sequence differences between the B6 (*B*) and D2 (*D*) alleles. There are over 2900 SNPs in *Nrxn1*. Of these, only nine are in the 3′ UTR, two are synonymous mutations in exons 5 and 17, and the remaining SNPs are in introns. In all, 15 SNPs in introns have potentially high impact based on overlap with predicted functional elements in conserved regions of introns[54] (Supplementary Table S1). The introns also contain a large number of insertions and deletions (defined as insertion or deletion in *D* relative to *B*). There are 436 deletions and 340 insertions ranging in length from 1 to 2418 bp (Figure 1b and Supplementary Table S1). Although none of these overlap canonical exons, such structural variations can have significant effects on gene expression. We selected a 74-bp insertion and a 107-bp deletion located in intron 17, which harbors the alternative promoter for the β-variant[10,11] and confirmed the variants by PCR amplification (Supplementary Figure S2).

Genetic analysis of *Nrxn1* co-expression network. To elucidate a co-expression network associated with the *Nrxn1* transcript, we ranked the top 1000 correlates of *Nrxn1* expression from the pool of 1.2 million probe sets. These represent transcripts that have coordinated expression with *Nrxn1* and are potential partners in cellular functions. These top covariates (Pearson product $r > |0.6|$, $P < 3.7 \times 10^{-9}$) correspond to 572 known genes (Supplementary Material B). Consistent with the synaptic involvement of *Nrxn1*, this set of

genes is enriched in synapse-related transcripts and genes known to directly interact with *Nrxn1* (for example, *Cask* and *Nlgn1*) (Supplementary Material B). More interestingly, there is also enrichment in genes that are part of the WNT, axon guidance, mitogen-activated protein kinase and ErbB signaling pathways (for example, *Gsk3b*, *Akt1* and *Nrg3*) and this suggests that there is close interaction between *Nrxn1* and downstream cellular signaling. We also used the Genetic Association Database (http://geneticassociationdb.nih.gov) and were able to extract a set of 20 schizophrenia candidate genes (Figure 2a) in our top 572 *Nrxn1* covariates (Benjamini corrected enrichment $P<0.001$).

eQTL mapping of the co-expression network members detected regulatory loci (that is, *trans* eQTL hotspots) on (1) mid-Chr 2 (67–77 Mb), (2) proximal Chr 6 (2–20 Mb) and (3) mid-Chr 8 (50–85 Mb). The first principal component (PC1) summarization of these highly correlated transcripts, also called an eigengene,[55] retained this whole-genome eQTL pattern. This is illustrated by the eigengene for the tightly correlated schizophrenia network: the PC1 of the 20 transcripts accounts for ~55% of the total variance and maps as suggestive QTLs to Chrs 2, 6 and 8 (Figure 2b). PC1 of the *Nrxn1* exons (accounting for ~50% of the total variance) has significant QTLs on Chrs 8 and 17, and suggestive QTL on Chr 2 (Figure 2c). Although exons 7, 8, 11 and 12 of *Nrxn1* map as weak *trans* eQTL to proximal Chr 6 (Supplementary Figure S1), this association does not reach the suggestive threshold in the summarized map. Based on the comparison of eQTLs, *Nrxn1* is not likely to be a QTL hotspot for the expression traits. Instead, the co-expression network is modulated mainly by eQTL hotspots on Chrs 2, 6 and 8. A portion of the variance in *Nrxn1* comes from these *trans*-effects that connect *Nrxn1* to the network.

Mouse behavioral phenotypes that map close to *Nrxn1*. We then asked whether mouse phenotypes that may be concordant with human traits are associated with the *Nrxn1* locus. GeneNetwork maintains a database of over 2000 phenotypes collected from the BXDs. We specifically searched this database for behavioral traits that have linkage with markers near *Nrxn1*. We found three such traits that have significant point-wise QTLs on distal Chr 17. First, PPI to acoustic startle [41] mapped to distal Chr 17 with a point-wise $P=0.0003$ (LRS = 16) (Figure 2d). Second, anxiety phenotype assayed on the zero-maze[42] mapped to distal Chr 17 with point-wise $P=0.005$ (LRS = 10.5). The

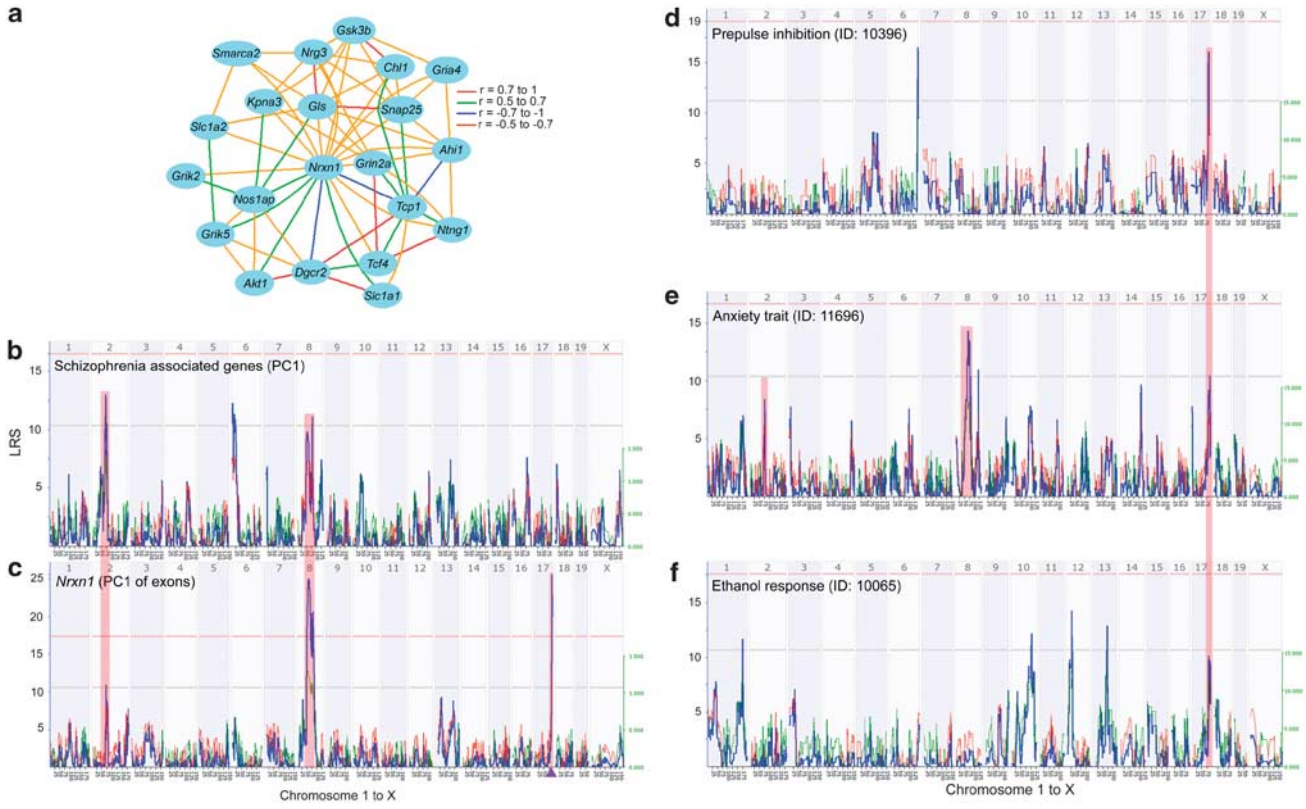

Figure 2 Overlap of co-expression and behavioral QTLs. (**a**) An enriched set of twenty schizophrenia candidate genes was extracted from the covariates of *neurexin 1* (*Nrxn1*) in the mouse hippocampus. These transcripts form a highly interconnected co-expression network with *Nrxn1*. (**b**) The co-expression network is modulated by expression QTLs (eQTLs) on chromosomes (Chrs) 2, 6 and 8. This is illustrated by the QTL map for the first principal component summarization (PC1) of the 20 schizophrenia candidates. The *x* axis represents Chrs 1 to X and the *y* axis shows the LRS score. The horizontal lines indicate the genome-wide suggestive and significant thresholds. (**c**) PC1 of the *Nrxn1* exons has significant QTLs on Chrs 8 and 17, and suggestive QTL on Chr 2. Location of *Nrxn1* in the *cis* eQTL is depicted by the triangle. Mouse behavioral traits that map close to the *Nrxn1* locus at significant point-wise *P*-values ($P<0.01$) are prepulse inhibition (**d**), anxiety trait (**e**) and ethanol response (**f**). Overlapping QTL locations are highlighted.

anxiety trait also has striking match with the *Nrxn1* exons in genome-wide QTL pattern with additional QTLs on mid-Chr 8 (LRS = 14; point-wise $P = 0.0007$) and Chr 2 (LRS of 8.4; point-wise P of 0.01) (Figure 2e). Finally, handling-induced convulsion as an index of alcohol withdrawal severity[43] mapped to distal Chr 17 with a point-wise P of 0.007 (LRS = 9.6) (Figure 2f).

We note that the behavioral traits also have QTLs that are distinct from the QTLs associated with *Nrxn1* and its co-expression network. The associations between the QTLs and multi-level phenotypes presents a complex genetic structure and the effect of *Nrxn1* on the behavior traits can either be directly mediated by the *cis* eQTL, or more likely, is transmitted via the co-expression network with major contributions by the set of *trans* eQTLs.

Cross-species analysis of the *Nrxn1* covariates. We next examined if correlations at the mRNA level are preserved across species. Our aim was to identify strong and consistent correlations that are conserved in both mice and humans and that may be novel functional and genetic associates of *Nrxn1*. We used a human forebrain expression data set[39,40] and extracted the top 1000 covariates of *NRXN1* (Pearson product $r > |0.55|$, $P < 3 \times 10^{-7}$). This corresponds to 767 unique genes (Supplementary Material B). Of these 767 genes, only 42 overlap with the 572 co-expressed genes in mice and have conserved correlations with *NRXN1* in human forebrain as well as in the mouse hippocampus (Supplementary Table S2). This includes *GSK3B* and *NTNG1*, which are both part of the *Nrxn1* primed schizophrenia network derived from the mouse covariates.[56,57]

To explore protein interactions that may be encrypted in the mRNA co-expression set, we extracted a protein–protein interaction network from the list of 767 transcript covariates (Figure 3a). In all, 261 out of the 767 query genes (34%) formed a highly interconnected protein network that incorporated most of the conserved transcript correlates of *NRXN1* including *GSK3B*, and also several other schizophrenia candidates. *GSK3B* has diverse roles in cellular signaling in the brain[57–59] and we examined the expression covariance of *GSK3B* and *NRXN1* in a number of different mouse and human brain regions and found generally strong correlations between the two (Supplementary Figure S3). The strong genetic correlation suggests interactions between *NRXN1* and *GSK3B* and places *NRXN1* as a synaptic gene that may also function as a gateway to cellular signaling cascades.

Test for epistasis between *NRXN1* and *GSK3B* in human schizophrenia. We hypothesize that there is close functional relation between *NRXN1* and *GSK3B*. We tested for gene–gene interactions in human using the MGS data set that has 2255 schizophrenia cases and 2641 controls.[30] We found significant 2-loci interaction between rs4563262 (*NRXN1*) and rs4340737 (*GSK3B*) with a permuted $P = 0.012$ (Figure 3b). Using a 3-loci model, there was significant interaction between rs6736816 and rs9309200 in *NRXN1*, and rs9826659 in *GSK3B* (permuted $P = 0.0373$) (Table 1). No covariate sex effect was found. Among the top five SNPs in the two genes, we also found that rs4340737 in *GSK3B* and rs9309200 in *NRXN1* are the only two SNPs that showed significant associations with schizophrenia ($P = 0.0171$ for rs4340737 and $P = 0.0293$ for rs9309200).

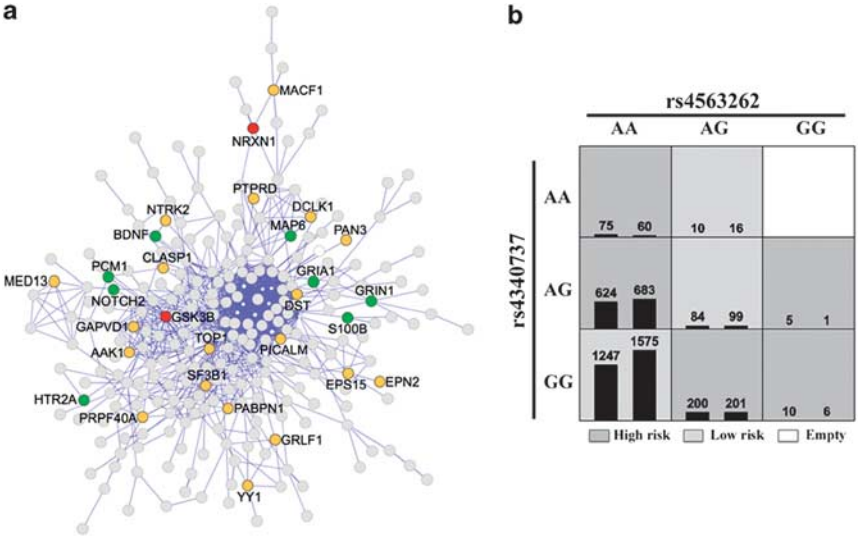

Figure 3 Deriving protein–protein and genetic interactions from co-expressed genes. (**a**) The co-expression network for *neurexin 1* (*NRXN1*) in the human brain was organized based on known protein–protein interactions. This resulted in a single large network that connected 261 out of 767 co-expressed transcripts. The network incorporates several known schizophrenia candidates (shaded green) and several conserved covariates of *NRXN1*, that is, are correlated with *NRXN1* in both human and mouse (shaded orange). *NRXN1* and *glycogen synthase kinase 3 beta* (*GSK3B*) are part of this protein network (shaded red). (**b**) Test for epistasis using the multifactor dimensionality reduction method found significant interaction between rs4563262 in *NRXN1* and rs4340737 in *GSK3B*. Distribution of cases (left bar in cell) and controls (right bar in cell) stratified by multilocus genotype. Each multifactorial cell is labeled as 'high risk' or 'low risk'. Average balanced prediction accuracy for the model was 51.85%, permuted $P = 0.0123$.

Table 1 Multilocus epistatic interaction models between *NRXN1* and *GSK3B*

No. of loci	*Best model*	*Testing balance accuracy (%)*	*Cross-validation consistency*	*10 000 permutation* P*-value*
2	rs4563262 (*NRXN1*) rs4340737 (*GSK3B*)	51.85	10/10	0.0123
3	rs6736816, rs9309200 (*NRXN1*) rs9826659 (*GSK3B*)	51.86	10/10	0.0373

Abbreviations: GSK3B, glycogen synthase kinase 3 beta; NRXN1, neurexin 1.

Evidence from mouse to prioritize potential candidates for schizophrenia. Our study demonstrates that gene co-expression analysis can effectively select related disease candidates and epistatic partners. We therefore used evidence from the co-expression analysis and mouse behavioral endophenotypes to further prioritize other potential candidates. For this, we considered genes in the MGS data set that were nominally associated with schizophrenia. The study by Shi *et al*.[30] generated a list of 1202 SNPs in the European-American cohort and a list of 997 SNPs in the African-American cohort with association $P<0.001$. We mined the SNP lists for nearby genes with mouse homologues that are among the top covariates of *Nrxn*1 in the mouse hippocampus. Additional functional evidence was gathered from knockout studies and gene ontology. On the basis of this, we identified 30 genes that are nominally associated with schizophrenia and are co-expressed with *Nrxn1*. Several of these are already known candidates for neuropsychiatric disorders including schizophrenia, autism, attention deficit hyperactivity disorder (ADHD), and bipolar disorder (Table 2).[60–73] Strikingly, 10 out of the 30 genes are also nominally associated with substance dependence and nicotine addiction.[74,75] In the mouse, deletion of six of these candidates (*CTNND2*, *DMN*, *GSK3B*, *NRCAM*, *PLCB1* and *RIMS1*) lead to behavioral changes related to PPI, social and cognitive functions, hyperactivity, fear learning and anxiety-related traits.[76–83]

Among the novel genes in Table 2, we highlight *Pleckstrin and Sec7 domain containing 3* (*PSD3*) as both a potential new candidate for schizophrenia and a candidate for the *trans*-effect associated with the co-expressed transcripts in the mouse brain. This gene has high expression in the brain and is located in mid-Chr 8 within the *trans* eQTL for the *Nrxn1* exons. Expression of *Psd3* is associated with a significant *cis* eQTL in the BXDs and is also strongly correlated with *Nrxn1* (Supplementary Figure S4).

Discussion

Synopsis. *Nrxn1* is highly polymorphic and is associated with significant variation in expression among mice. We have exploited this variation to trace co-expression networks and eQTLs that link *Nrxn1* with other genes and also with higher order behavioral phenotypes. *GSK3B* is among the strongest and most consistent covariates of *NRXN1* in both mice and humans and we detected a significant epistatic interaction between these genes that modulates schizophrenia risk. This cross-species analysis also highlights novel candidates for schizophrenia in human, especially *PSD3*.

Sub-transcript level analysis of gene expression regulation. For a fine-scale analysis of *Nrxn1*, we have treated each exon, intron and the UTRs as discrete expression units that can be individually subjected to genetic analysis. This revealed some level of complexity in transcript regulation. For instance, exons 8, 11 and 21 show low correlation with other exons and are also associated with distinct eQTLs. Aside from these exceptions, the majority of exons have tightly correlated expression, partly due to shared modulation by two prominent eQTLs—one a *cis* eQTL at *Nrxn1*, and the other, a *trans* eQTL on mid-Chr 8.

The mid-Chr 8 *trans* eQTL also modulates several key genes correlated with *Nrxn1* including *Psd3*, *Gsk3b*, *Snap25* and *Kpna3*. This interval corresponds to human 8p21, a region that contains many genes linked to brain developmental and psychiatric disorders.[84] Polymorphisms in this region in humans may also have *trans*-regulatory effect on the expression of other schizophrenia susceptibility genes.[85] In a striking concordance between mouse and human, this part of mouse Chr 8 is also a QTL hot spot for brain structural traits in the BXDs and is linked to variations in the volume of the amygdala, hippocampus, cerebellum and striatum.[86,87] Located in this mid-Chr 8 QTL is *Psd3*, a gene that is modulated by a *cis* eQTL and is also co-expressed with *Nrxn1*. In the human MGS data set, SNPs near *PSD3* are nominally associated with schizophrenia ($P=0.00007$). Although little is currently known about the function of *PSD3*, the converging evidence from multiple sources makes this gene a high-priority candidate for the eQTL in mouse and a novel candidate for schizophrenia in human.

Variation in *Nrxn1* expression may influence schizophrenia endophenotypes. In the BXDs, PPI to acoustic startle maps to distal Chr 17 close to the *Nrxn1* locus. PPI is a classic endophenotype that models sensory gating deficits in schizophrenia patients,[88] and polymorphisms in *Nrxn1* are strong candidates with compelling face validity. Consistent with association to schizophrenia, deletion of *Nrxn1α* in mice results in decreased PPI.[23] However, in this knockout, social behavior and anxiety traits are unaffected .[23]

Unlike studies using gene knockouts, we have evaluated naturally occurring variants that will generally have more subtle effects on phenotypes. Most of the SNPs and insertions and deletions in *Nrxn1* are intronic and are unlikely to cause

Table 2 Covariates of *Nrxn1* associated with schizophrenia in the MGS data set at $P < 0.001$

Association in human				*Mouse gene and syntenic region*			*Mouse array probe ID*	*Co-expression in mouse*		*Mouse KO phenotype*	*Other human associations*	
SNP	***Locus***	P-***value***	***Gene***	***ID***	***Chr***	***Mb***		r	P(r)			***GO***
European Americans												
rs10140896	14q31.3;Intr (intronic)	9.4E–07	*EML5*	319670	12	100.08	5399699	0.65	4.7E–11			Microtubule cytoskeleton
rs7760476	6p22.3;Intr	0.00002	*ATXN1*	20238	13	45.66	4622958	−0.64	1.4E–10		Addiction; cognitive function; schizophrenia;	RNA processing
rs2725045	8p23.2;Intr	0.00004	*CSMD1*	94109	8	17.50	5613572	0.61	2.0E–09		Addiction	
rs2192420	14q24.3;Upstr	0.00006	*NRXN3*	18191	12	90.90	5145873	0.68	1.7E–12		Addiction; schizophrenia	Cell adhesion
rs1386688	8p22;Intr	0.00007	*PSD3*	234353	8	70.22	5393349	0.65	4.6E–11		Addiction	Cell signaling
rs9341356	6q13;Intr	0.00007	*RIMS1*	116837	1	22.74	5163292	0.61	2.4E–09	PPI; psychotomimetic drug response; social behavior	Cognitive performance	Neuro-transmission
rs231150	8q23.3;Dwnstr	0.0001	*TRPS1*	83925	15	50.61	4317283	0.61	2.1E–09			Transcription factor
rs17171808	5q31.2;Intr	0.0002	*JMJD1B*	277250	18	34.95	4508461	0.63	3.5E–10			Nuclear oxidoreductase
rs4822743	22q12.1;Intr	0.0002	*TPST2*	22022	5	112.74	4312446	−0.63	3.0E–10			Sulfur metabolism
rs11097407	4q22.2;Intr	0.0002	*SMARCAD1*	13990	6	65.05	5186186	0.62	7.9E–10			Chromatin modification
rs2530215	5p15.2;Intr	0.0003	*CTNND2*	18163	15	30.13	4694831	0.62	1.3E–09	Fear learning; cognitive deficits	Addiction; schizophrenia	Cell adhesion
rs11223336	11q25;Intr	0.0006	*OPCML*	330908	9	27.92	4747785	0.67	7.5E–12		Addiction	Cell adhesion, opioid receptor
rs12448737	16p13.2;Intr	0.0007	*A2BP1*	268859	16	6.81	4974807	0.62	1.5E–09		Addiction; ADHD; bipolar schizoaffective; autism	RNA processing
rs17286683	5q31.3;Upstr	0.0008	*ANKHD1*	108857	18	36.79	4412853	0.61	3.2E–09			Translation regulation
rs4624596	3q13.33;Intr	0.0009	*GSK3B*	56637	16	38.19	4314494	0.61	2.2E–09	Impaired learning; behavioral despair	Bipolar; cognitive performance	ErbB, WNT signaling
rs4860425	4q13.1;Intr	0.0009	*LPHN3*	319387	5	82.12	5370748	0.66	1.8E–11		ADHD	Neuropeptide signaling, cell adhesion
rs1536142	1p32.2;Intr	0.0009	*DAB1*	13131	4	104.29	4375891	−0.63	5.9E–10		Addiction	Cell adhesion
African Americans												
rs13065441	3q26.2;Intr	0.00002	*TNIK*	665113	3	28.56	4843478	0.68	2.6E–12		Addiction; schizophrenia	WNT, MAPK signaling
rs7125438	11q25;Intr	0.00002	*OPCML*	330908	9	27.92	4747785	0.67	7.5E–12		Addiction	Cell adhesion, opioid receptor
rs409797	7q31.1;Intr	0.00008	*NRCAM*	319504	12	45.67	5569519	0.64	1.4E–10	PPI; social, cognitive functions	Autism	Cell adhesion
rs6963574	7q32.3;Intr	0.00008	*MKLN1*	27418	6	31.44	4419118	0.71	8.4E–14			Cytoskeletal response
rs596929	11q24.2;Intr	0.0001	*KIRREL3*	67703	9	34.32	5276457	0.61	2.9E–09		ADHD; response to antipsychotics	Cell adhesion, synapse formation
rs2826771	21q21.1;Intr	0.0002	*NCAM2*	17968	16	81.51	5268421	0.63	4.3E–10		Psychiatric disorders	Cell adhesion
rs1351569	8p23.2;Intr	0.0002	*CSMD1*	94109	8	17.50	5613572	0.61	2.0E–09		Addiction	
rs17446308	20p12.3;Intr	0.0002	*PLCB1*	18795	2	134.68	5117797	0.66	2.2E–11	PPI; social behavior; hyperactivity; cognitive impairment	Cognitive performance	Phospholipid metabolism

Table 2 *(Continued)*

Association in human			Mouse gene and syntenic region			Mouse array probe ID	Co-expression in mouse		Mouse KO phenotype	Other human associations	GO
SNP	**Locus**	**P-value**	**Gene**	**ID**	**Chr**	**Mb**		r	P(r)			
rs11955233	5q14.1;Dwnstr	0.0003	*JMY*	57748	13	94.22	5373932	0.64	1.1E−10			Cytoskeleton, transcription regulation
rs2167566	2p15;Intr	0.0004	*USP34*	17847	11	23.27	5362770	0.62	1.5E−09			Protein metabolism
rs1456729	Xp21.1;Intr	0.0005	*DMD*	13405	X	81.15	4416174	0.62	8.7E−10	Anxiety related	Muscular dystrophy	
rs13250856	8q11.23;Intr	0.0005	*RB1CC1*	12421	1	6.24	4969100	0.64	1.2E−10			
rs12928607	16p13.2;Intr	0.0006	*A2BP1*	268859	16	6.81	4974807	0.62	1.5E−09		Addiction; ADHD; bipolar schizoaffective; autism	RNA processing
rs10249419	7q31.2;Intr	0.0007	*CTTNBP2*	30785	6	18.46	5198380	−0.67	1.2E−11			Cytoskeleton
rs2256900	10q23.1;Intr	0.0009	*NRG3*	18183	14	39.81	4390722	0.62	1.3E−09		Addiction; schizophrenia	ErbB signaling
rs6460537	7q11.22;Intr	0.0009	*AUTS2*	319974	5	132.93	5208797	0.65	5.6E−11		Bipolar schizoaffective	

Abbreviations: GO, gene ontology; KO, knockout; MAPK, mitogen-activated protein kinase; NRXN1, neurexin 1; PPI, prepulse inhibition; SNP, single-nucleotide polymorphism.

gene disruption. However, these more moderate variants model the type of variation seen in human populations. A detailed analysis of structural variations in the NRXN genes in human has found a range of deletions and duplications.[89] The deletions in *NRXN1*, particularly those that span exons, are strongly associated with schizophrenia. Additionally, there are possible associations with other psychiatric conditions and alcoholism that indicate pleiotropic effects of *NRXN1*.[89] This is consistent with our findings in mouse in which QTLs for ethanol response and anxiety map near *Nrxn1*.

Potential functional and epistatic partners: *Nrxn1* and *Gsk3b*. Hundreds of susceptibility variants have been identified for schizophrenia in recent large-scale genomic studies. Associated genes fall into diverse functional categories and this supports the impressive genetic heterogeneity of complex neuropsychiatric diseases.[90,91] The approach we have taken here is to use a population model and exploit endogenous variations in a high-priority candidate as a starting point for a systematic gene-to-phenotype analysis. *NRXN1* has been repeatedly linked to schizophrenia and autism[15–19] and as we show, a co-expression network primed by this gene captures other genes that work closely with *Nrxn1* and are involved in disease pathway.

Co-expression and eQTL analyses led us to genes already linked to *Nrxn1* (for example, *Cask*, *Nlgn1*, γ-aminobutyric acid and glutamate receptors), and also led us to novel genes that have less obvious, but potentially interesting links to *Nrxn1*. *Gsk3b* is one such gene that we detected and then validated as a novel genetic partner of *Nrxn1*. GSK3B has diverse functions in the cell, including synaptic and intracellular signal transduction[57–59] and could therefore be a conduit between the synaptic role of NRXN1 and cell signaling. Supporting this hypothesis, we found significant genetic interactions between SNPs in *NRXN1* and *GSK3B* that indicate that susceptibility to schizophrenia can be modified to an extent by the combination of alleles in the two genes. For example, in the 2-loci model, the *AA* genotype at rs4563262 (*NRXN1*) and the *AG* and GG genotypes at rs4340737 (*GSK3B*) interact to increase or decrease vulnerability.

Despite the genetic interactions, to our knowledge, no study has yet demonstrated direct physical link between NRXN1 and GSK3B. However, the presynaptic region is a potential locus of cellular interaction between the two proteins. GSK3B, in addition to its many functions in cell signaling, also modulates presynaptic vesicle release by phosphorylating the voltage-dependent calcium channels and reducing intracellular Ca^{2+} levels.[59] It also regulates the development of the active zone—the site of vesicle fusion and neurotransmitter release.[92] NRXN1, a predominantly presynaptic protein, modulates release by selectively acting on voltage-dependent calcium channel (P/Q and N type), possibly by modifying the coupling of Ca^{2+} channels to release-ready vesicles.[13] Variants of NRXN1 and GSK3B may therefore have a collective influence on the synaptic environment and consequently on disease susceptibility. Both the genetic interaction and a potential molecular interaction are consistent with a model in which neurotransmission and intracellular signaling

are coupled in the etiology of schizophrenia and other psychiatric disorders.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)