

# Expression of DISC1 binding partners is reduced in schizophrenia and associated with DISC1 SNPs

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**DISC1** has been identified as a schizophrenia susceptibility gene based on linkage and SNP association studies and clinical data suggesting that risk SNPs impact on hippocampal structure and function. In cell and animal models, C-terminus-truncated DISC1 disrupts intracellular transport, neural architecture and migration, perhaps because it fails to interact with binding partners involved in neuronal differentiation such as fasciculation and elongation protein zeta-1 (FEZ1), platelet-activating factor acetylhydrolase, isoform Ib, PFAFH1B1 or lissencephaly 1 protein (LIS1) and nuclear distribution element-like (NUDEL). We hypothesized that altered expression of DISC1 and/or its molecular partners may underlie its pathogenic role in schizophrenia and explain its genetic association. We examined the expression of DISC1 and these selected binding partners as well as reelin, a protein in a related signaling pathway, in the hippocampus and dorsolateral prefrontal cortex of postmortem human brain patients with schizophrenia and controls. We found no difference in the expression of DISC1 or reelin mRNA in schizophrenia and no association with previously identified risk DISC1 SNPs. However, the expression of NUDEL, FEZ1 and LIS1 was each significantly reduced in the brain tissue from patients with schizophrenia and expression of each showed association with high-risk DISC1 polymorphisms. Although, many other DISC1 binding partners still need to be investigated, these data implicate genetically linked abnormalities in the DISC1 molecular pathway in the pathophysiology of schizophrenia.

## INTRODUCTION

Schizophrenia is a psychiatric disorder characterized by cognitive impairment, disturbances in emotion and social functioning, and psychosis. A major genetic component to schizophrenia has long been assumed based on family and adoption studies, but this has become a reality with recent evidence for the involvement of multiple genes (1,2). However, none of the candidate genes has yet been unequivocally

confirmed and their functional roles in the pathophysiology of the disease are uncertain. Conclusive evidence of causative genes for polygenic disorders like schizophrenia will likely require demonstration that genetic variation predicts biological effects in brain that bear on the pathogenesis of the disorder. Although neuropathological and molecular findings in brains of patients with schizophrenia are subtle and often controversial, the most replicable findings implicate the hippocampal formation and the dorsolateral prefrontal cortex

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(DLPFC) (3–6). The onset of schizophrenia is in adolescence and young adulthood but early childhood is not normal in many cases, suggesting that the molecular origins of the disorder involve early neurodevelopmental processes (7–9).

There is strong evidence that *DISC1*, a gene associated with schizophrenia in several independent studies, is important for brain development and, in particular, development and function of the hippocampal formation. The disruption of the Disrupted-in-Schizophrenia 1 (*DISC1*) gene by a balanced translocation (1;11)(q42;q14) was found to strongly segregate with major mental illness in a large Scottish pedigree (10–13). Although no other families have been reported having a similar chromosomal abnormality, five of six recent studies support an association between allelic variation in the intact *DISC1* gene and risk for schizophrenia, although different polymorphisms/haplotypes in various regions of the gene have been implicated in these studies (14–19). In a family-based association study, Callicott *et al.* (18) showed significant over-transmission of a three marker haplotype containing a common non-conservative SNP in exon 11 (serine to cysteine substitution, rs821616) and two intronic SNPs in introns 9 and 10 (hCV219779 and rs821597, respectively) to the affected offspring (Fig. 1A). The high-risk polymorphisms in this sample were associated with alterations in hippocampal structure (reduced hippocampal gray matter volume) and altered hippocampal activity measured by fMRI during cognitive tests, even in normal subjects. Furthermore, recent reports have shown associations of high-risk *DISC1* polymorphisms with cognitive function and cognitive aging, suggesting that *DISC1* may contribute to schizophrenia by affecting learning and memory functions (20–23).

*DISC1* encodes a protein of 854 amino acids, which shows no homology to other known proteins and little homology between species (24–26). The amino-acid sequence of *DISC1* predicts that it may act as a scaffolding protein with multiple binding motifs, facilitating formation of protein complexes. The N-terminus (amino acid 1–347) contains nuclear localization signals, whereas the C-terminus (amino acid 348–854) appears to be important for microtubule and centrosomal targeting (27–29), although no centrosomal localization has been detected so far for the native protein. In cell culture studies, mutant C-terminus-truncated *DISC1*, a hypothetical construct not yet detected in human tissue, disrupts intracellular transport, neural architecture and migration, perhaps because it fails to interact with its binding partners.

*DISC1* is abundant in the nucleus and mitochondria (28,30). Moreover, a number of *DISC1* interactors have been identified using yeast two-hybrid assays and confirmed in follow-up cell-based studies, including fasciculation and elongation protein zeta-1 (FEZ1), platelet-activating factor acetylhydrolase, isoform Ib, PAFAH1B1 or lissencephaly 1 protein (LIS1) and nuclear distribution element-like (NUDEL) (25,27,29,31–35).

It has been speculated that mutations in the C-terminus of *DISC1*, which contains the translocation breakpoint and stretches of coiled-coil forming potential, would disrupt interactions with these other developmental proteins. Indeed, the *DISC1*-truncated form showed reduced interaction with FEZ1, a protein involved in neurite outgrowth that in the mouse mirrors the neurodevelopmental pattern of *DISC1*

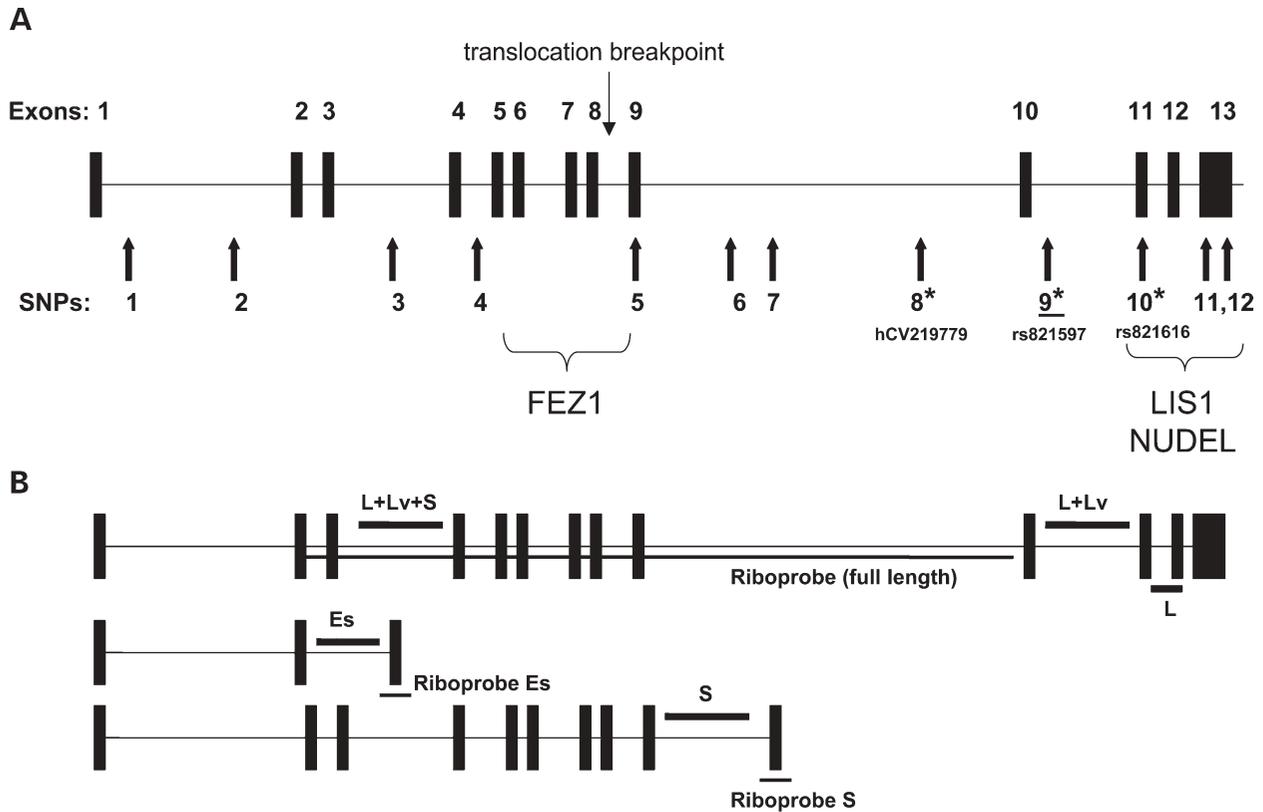
expression (32). The C-terminus of *DISC1* has been shown also to be critical in mediating interactions with NUDEL and LIS1, genes essential for neuronal migration, corticogenesis and axonal outgrowth, and binding of these proteins with *DISC1* occurs in a developmentally regulated manner (25,27,29,31). An aberrant LIS1/NUDEL/*DISC1* trimolecular complex formed as a result of mutation in *DISC1* might be predicted to affect essential developmental processes implicated in schizophrenia (31).

We have explored the expression of *DISC1* and several of its molecular partners in brain tissue of patients with schizophrenia and their relationship to risk SNPs in *DISC1* that have been associated with the disease in our clinical study (18). We hypothesized that the mechanism of the genetic association of *DISC1* with schizophrenia involves disruption of a molecular pathway related to *DISC1* function. Specifically, we tested whether expression of *DISC1* binding partners is abnormal in schizophrenia, and whether schizophrenia *DISC1* risk SNPs in the region of the binding domains of these partners predict their altered expression (Fig. 1A). We inspected two brain regions strongly implicated in schizophrenia, DLPFC and the hippocampal formation, in a large collection of brains of patients with schizophrenia and normal controls. In this study, we have focused on a small set of those molecules identified through yeast two-hybrid screening and also confirmed using other methods. Our selection was based on the evidence that these proteins bind in the regions of high-risk polymorphisms identified in our clinical data set and were confirmed to play a role in neuronal migration and cellular architecture, both candidate developmental mechanisms implicated in schizophrenia. Our results support the involvement of these binding partners in the pathogenesis of schizophrenia. We have not performed an exhaustive study of all potential *DISC1* binding partners, which is important future work.

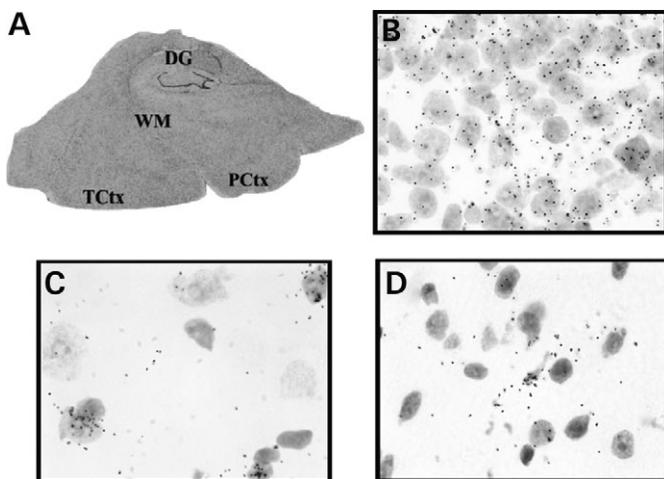
## RESULTS

### ***DISC1* mRNA is abundantly expressed in the human hippocampal formation and its expression varies across life span**

Analysis of films showed that, similar to the non-human primate (36), *DISC1* expression was most prominent in the dentate gyrus (DG) of the human hippocampus (Fig. 2) and a sense probe showed no signal above background. The strongest signal was detected in the DG throughout the life stages sampled—from the neonates to the aged individuals. In the temporal and parahippocampal cortices (PCTX), optical density was at least an order of magnitude lower than in the DG in all age groups. Silver-grain analysis revealed that a majority, but not all, granule cells in the DG express *DISC1* transcript and that the level of expression per cell was modest (Fig. 2A and B). In the cortex, *DISC1* mRNA was also present only in a subset of cells. Interestingly, some of the cortical *DISC1*-expressing cells showed even higher expression than granule cells in the DG, whereas other neighboring cells were devoid of signal (Fig. 2C). There was also specific and pronounced expression of *DISC1* over a subset of cells in the white matter (WM) (Fig. 2D).

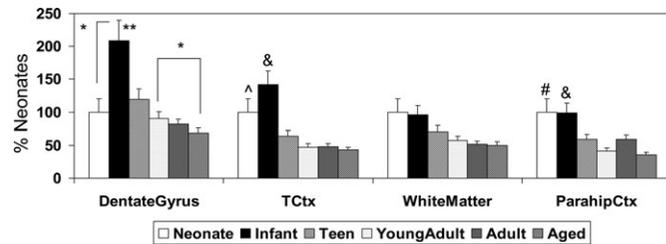


**Figure 1.** (A) A schematic of the *DISC1* gene with vertical bars representing exons (1–13) and depicting relative locations of the 12 SNPs (1–12) studied in the clinical data set (18) and in the current study. SNPs with asterisks were associated with schizophrenia in the clinical study (18). An italicized SNP9 (rs821597) most markedly predicted the hippocampal expression levels of FEZ1, LIS1 and NUDEL. A long vertical arrow indicates the location of a translocation breakpoint. Parentheses indicate approximate location of putative binding sites of FEZ1, NUDEL and LIS1 to the *DISC1* protein. (B) A schematic of the *DISC1* gene with vertical bars representing exons (1–13) and horizontal lines depicting relative locations of probes for the measurement of the expression of *DISC1* transcript levels. Probes/primers were used to measure expression of four reported isoforms of *DISC1* or their combinations by qRT-PCR (short, S; extra short, Es; long, L; Long variant, Lv) and by *in situ* hybridization histochemistry (see riboprobes S, Es and full-length).



**Figure 2.** An autoradiogram (A) and photomicrographs (B–D) of sections through the human hippocampal formation showing expression of *DISC1* mRNA. (A) Detection of *in situ* hybridization signal with a *DISC1* riboprobe in the DG, temporal cortex (TCtx), WM and PCtx; (B–D) Bright-field views of neurons after hybridization with a *DISC1*-labeled probe taken at 200 $\times$  in the DG (B), TCtx (C) and WM (D).

In the DG, PCtx and temporal cortices, *DISC1* expression significantly changed across the developmental groups: an early high rise from the neonatal period to infancy was followed by a marked reduction by teenage years and a further gradual decline with aging (Fig. 3). As *DISC1* expression in the DG significantly correlated with pH ( $r = 0.61$ ,  $P < 0.001$ ), we used pH as a co-variate in the analysis. In the DG, a one-way ANCOVA showed a significant effect of age-group on the expression of the full-length *DISC1* mRNA [ $F(5,38) = 5.08$ ;  $P = 0.002$ ]. *Post hoc* comparisons showed that *DISC1* expression was increased in the infant DG when compared with neonates (by 104%), teens (84%), young adults (103%), adults (128%) and aged adults (290%), all  $P < 0.001$ . Moreover, *DISC1* expression in the teenage group was significantly higher than in the aged adults ( $P < 0.05$ ). In the temporal and PCtx, ANCOVA also showed a significant effect of age on *DISC1* expression [ $F(5,38) = 5.25$ ;  $P = 0.001$ ;  $F(5,38) = 5.1$ ;  $P = 0.002$ , respectively]. Neonate and infant groups showed significantly more expression than teens (by 173 and 167% for temporal and PCtx, respectively), young adults (248 and 225%), adults (219 and 165%) and aged adults (310 and 276%), all



**Figure 3.** DISC1 mRNA expression across life stages in the DG, TCtx, WM and parahippocampal gyrus (ParahipCtx) expressed as a percentage of the neonatal levels. Double asterisks denote different from all other groups,  $P < 0.001$ ; asterisk denotes different from the specified group,  $P < 0.001$ ; ampersand denotes different from all other groups, except neonates,  $P < 0.001$ ; hash denotes different from all other groups except infants,  $P < 0.05$ ; hat denotes different from adults and aged adults,  $P < 0.001$ .

$P < 0.001$ . There was a significant positive correlation of DISC1 expression measured between the regions ( $r > 0.55$ ,  $P < 0.001$ ). Changes in the WM signal were not significant across age groups [ $F(5,38) = 1.3$ ;  $P > 0.2$ ]. There were no significant changes in cyclophilin expression across the age groups in the frontal cortex, whereas the changes in the hippocampal formation were moderate and showed a different pattern than DISC1 mRNA expression [data not shown here but reported in (37,38)].

#### qPCR measurement of DISC1 mRNA in the hippocampus and DLPFC

Extra short (Es) and Short (S) variants showed no amplification in the hippocampal RNA extracts and therefore could not be quantified. We measured DISC1 mRNA using ABI probe/primers (Table 1) for the following combinations of DISC1 variants: (L + Lv + S), (L + Lv) and L, all of which most likely recognized predominantly full-length DISC1 mRNA (as both S and Lv were either absent or expressed at very low levels). To assess the contribution of demographic and diagnostic variables to the expression levels of the DISC1 transcript combinations, we performed forward stepwise multiple regression analysis using diagnosis, age, sex, pH, post-mortem interval (PMI), freezer time, RNA integrity (RIN), smoking history and agonal state as independent variables in the whole cohort and then age of onset, age at hospitalization, duration of illness, daily, life and last CPZ dose and history of substance abuse in subjects with schizophrenia. The analysis revealed that the effects were largely negative. There was only a significant inverse correlation of the hippocampal expression of L transcript with PMI and a positive correlation of the expression of this transcript with an RIN,  $P < 0.05$ . Moreover, in samples from patients with schizophrenia, hippocampal expression of L transcript significantly inversely correlated with daily dosage of neuroleptics ( $P < 0.05$ ). There were no other correlations of any of the DISC1 transcript combinations normalized by PBGD, GUSB or B2M or a normalizing factor (NF, a geometric mean of these three housekeeping genes) with any other variable (data not shown).

Most importantly, in the hippocampus and in the DLPFC, DISC1 expression [as measured using probes recognizing L, (L + Lv), or (L + Lv + S) transcript combinations] was not

altered in schizophrenia, whether normalized by B2M, GUSB, PBGD or NF (geometric mean of three housekeeping genes) or without normalization, all  $F$ -values  $< 1.0$ , all  $P$ -values  $> 0.5$ , data not shown. There were no effects of diagnosis on the expression of the housekeeping genes or NF (all  $F$ -values  $< 1.5$ , all  $P$ -values  $> 0.2$ ). Moreover, there were no effects of the genotypes associated with the disease in the clinical data set: hCV219779, rs821597, rs821616 (or any other DISC1 SNPs genotyped in the post-mortem cohort) on DISC1 expression in the hippocampus or DLPFC (data not shown). Thus, expression of the most abundant DISC1 mRNA species is not altered in brain tissue from patients with schizophrenia, nor do the risk genotypes that we scored affect this measure.

#### DISC1 immunoreactivity in the hippocampus

The antibody produced one major band, which detected a 70–75 kDa protein (Fig. 4A). A similar band was shown previously by mass spectrometry to contain DISC1 sequences (39). However, the molecular identity of this band has not yet been fully elucidated. Using this antibody, we found that DISC1 immunoreactivity was significantly, albeit modestly (by ~20%), increased in the hippocampus of patients with schizophrenia [ $F(1,73) = 3.6$ ;  $P = 0.05$ ] (either expressed as percentage of an average signal of control samples per gel or as a ratio of the DISC1 signal to actin) (Fig. 4B). DISC1 protein immunoreactivity, weakly but significantly, correlated with mRNA levels measured by RT-PCR ( $r = 0.26$ ,  $P = 0.04$ ). There were no significant correlations between DISC1 immunoreactivity and any of the potentially confounding covariates analyzed in this study, including medication.

We next examined the effects of risk SNPs (hCV219779, rs821597, rs821616) genotype as independent factors. Although the groups were too small to meaningfully test for association of genotype with protein levels, *post hoc* comparisons showed that there was a significant effect of genotype in patients with schizophrenia, that is, individuals carrying the high risk G/G allele genotype at rs821597 ( $n = 3$ ) had higher DISC1 protein signal than subjects carrying a two/two allele genotype ( $P = 0.03$ ) (Fig. 4C). No other genotype showed a significant effect.

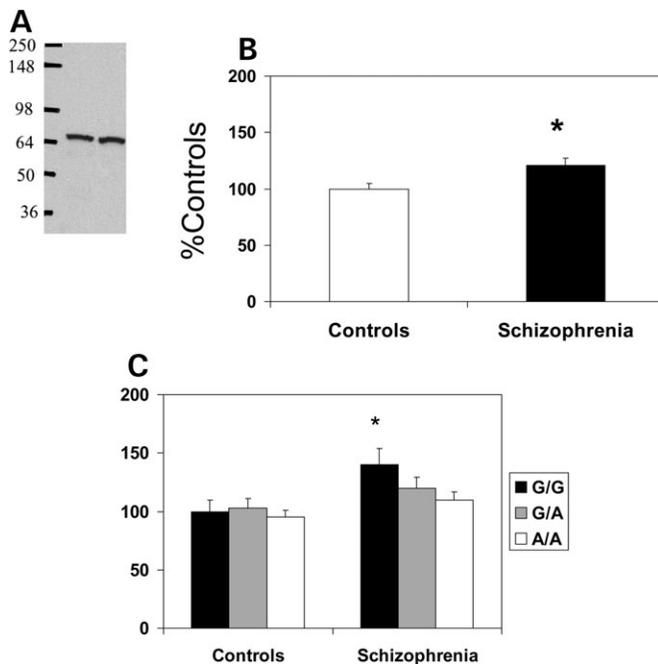
#### Expression of FEZ1, NUDEL and LIS1 mRNA in the hippocampus

The contribution of demographic and diagnostic variables to the expression levels of the DISC1-interacting molecules is presented in Tables 2 and 3. The effect of diagnosis was apparent for all three genes (Fig. 5). FEZ1 mRNA expression was significantly and markedly reduced in the hippocampus of patients with schizophrenia when normalized by any of the housekeeping genes or NF or without normalization (the geometric mean of the housekeeping genes), [ $F(1,104) = 7.2$ ;  $P = 0.008$  for the latter]. ANCOVA also showed a significant reduction when age and sex were used as covariates in the analysis [ $F(1,104) = 6.1$ ;  $P = 0.01$ ].

NUDEL mRNA expression was also significantly reduced in patients with schizophrenia with or without normalization to the housekeeping genes [ $F(1,104) = 10.2$ ;  $P = 0.001$  when normalized by NF]. This reduction remained significant

**Table 1.** Probe/primers sequences and catalog numbers of Applied Biosystems Assays-on-Demand used for gene expression by qRT-PCR

Gene/transcript	ABI-cat	Exons	Forward primer	Reverse primer	Probe
<i>DISC1/S</i>		9,10	GACTGAGAAGAGAAGTGGAGCAC	TCCAGGACTCTGCATCACAGTA	CAGGAGACTGCCTATGGATAT
<i>DISC1/Es</i>		2,3	GGACTGCCTGCTGAGAAACC	GATGTCGAGGCTTCCATGGT	TCCAGCTCCATCTGCC
<i>DISC1/L</i>	Hs00962129_m1	11,12			
<i>DISC1/L + Lv</i>	Hs00218680_m1	12,13			
<i>DISC1/L + Lv</i>	Hs00962128_m1	10,11			
<i>DISC1/L + Lv + S</i>	Hs00962133_m1	3,4			
<i>FEZ1</i>	Hs 00192714_m1	2,3			
<i>NUDEL1</i>	Hs 00229366_m1	8,9			
<i>LIS1</i>	Hs00181182_m1	6,7			
<i>reelin</i>	Hs00192449_m1	19,20			
<i>PBGD (HMBS)</i>	Hs006009297	1,2			
<i>B2M</i>	Hs99999907	2,3			
<i>GUSB</i>	Hs99999908	11,12			



**Figure 4.** *DISC1* immunoreactivity assessed using a *DISC1* antibody (1:2000) raised against peptide sequence corresponding to amino acids 601–854 of the C-terminus of *DISC1*. (A) A blot showing a major band at ~75 kDa. (B) *DISC1* immunoreactivity in the hippocampus of controls and patients with schizophrenia expressed as a percentage of controls (means  $\pm$  SD), \* $P < 0.05$ . (C) *DISC1* immunoreactivity in controls and patients with schizophrenia homozygous for G or A allele and heterozygous at SNP rs821597 expressed as a percentage of G/G controls. Asterisk denotes schizophrenia G/G carriers were significantly different from A/A carriers,  $P < 0.05$ .

when the data were co-varied by the variables showing significant correlations with *NUDEL* expression, that is, with age as a covariate [ $F(1,104) = 7.9$ ;  $P = 0.005$ ] and with age and RIN as covariates [ $F(1,104) = 7.2$ ;  $P = 0.008$ ].

*LIS1* expression was similarly reduced in the hippocampus of patients with schizophrenia with or without normalization to the housekeeping genes [ $F(1,104) = 6.9$ ;  $P = 0.009$ , normalized by NF]. When co-varied by RIN or RIN and PMI (the factors that significantly correlated with *LIS1*), the

effect of diagnosis was still significant [ $F(1,104) = 4.9$ ;  $P = 0.029$ ] and [ $F(1,104) = 3.7$ ;  $P = 0.05$ , respectively] but when pH was added as a covariate, the difference between controls and patients with schizophrenia reached only trend level [ $F(1,104) = 2.97$ ;  $P = 0.08$ ]. There were no effects of diagnosis on the expression of any housekeeping genes (all  $F_s < 1.5$ ; all  $P > 0.2$ ).

Logistic regression analysis showed that *NUDEL* and *FEZ1* (but not *LIS1*) expression were useful predictors of diagnosis, together contributing 10.6% to the prediction [ $F(2,102) = 7.17$ ;  $P < 0.001$ ], with *NUDEL* contributing over 6% ( $P = 0.01$ ) and *FEZ1*, the remaining 4% ( $P = 0.05$ ).

More interestingly, *DISC1* genotypes were associated with low *FEZ1*, *NUDEL* and *LIS1* expression in the hippocampus of patients with schizophrenia and the risk alleles consistently predicted decreased expression (Fig. 6). Two-way ANOVAs with genotype and diagnosis as independent factors showed a significant effect of diagnosis, but no main effect of genotype (hCV219779, rs821597, rs821616), with the exception of genotype hCV219779 on *LIS1* expression [ $F = 2.3$ ;  $P < 0.05$ ]. Most disease by genotype interactions were also not significant, with the exception of disease by rs821597 interactions on *NUDEL* and *LIS1* expression [ $F = 2.2$ ;  $P < 0.05$ ]. However, *post hoc* testing revealed that, in all comparisons, individuals with schizophrenia homozygous for the disease-associated SNPs (C for hCV219779, G for rs821597, A for rs821616) had significantly lower gene expression of the *DISC1* interacting molecules when compared with all other groups ( $P < 0.05$ ) (Fig. 6). In the *post hoc* testing, none of the other SNPs genotyped in this cohort showed significant effects on the levels of *FEZ1*, *NUDEL* or *LIS1* (data not shown).

#### Expression of *FEZ1*, *NUDEL* and *LIS1* mRNA in the DLPFC

In the DLPFC, the contribution of demographic and diagnostic variables to the expression levels of *FEZ1*, *NUDEL* and *LIS1* are given in Tables 2 and 3. Although, age and duration of illness were highly correlated (correlation coefficient 0.90), duration of illness was a much better predictor of expression levels than age for all three genes (Table 3). There were

**Table 2.** Contribution of demographic and tissue-related variables to gene expression levels estimated using forward stepwise multiple regression analysis

	Adj $R^2$	Dx $\beta$	Age $\beta$	Sex $\beta$	RIN $\beta$	pH $\beta$	PMI $\beta$	FT $\beta$	Sm $\beta$	AS $\beta$
Hippocampus										
FEZ1	0.12***	-0.24**	-0.20*	-0.19*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NUDEL	0.30****	-0.20*	-0.34****	n.s.	0.22**	n.s.	n.s.	n.s.	n.s.	n.s.
LIS1	0.27****	-0.22*	n.s.	n.s.	0.29****	0.27**	-0.22*	n.s.	n.s.	n.s.
RLN	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DLPFC										
FEZ1	0.08**	-0.26**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NUDEL	0.11***	n.s.	-0.30**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
LIS1	0.19****	n.s.	-0.29**	n.s.	0.26**	n.s.	n.s.	n.s.	n.s.	n.s.
RLN	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Adj  $R^2$ , adjusted coefficient of determination;  $\beta$ , standardized regression coefficient; Dx, diagnosis (1-controls, 2-schizophrenia patients); Sex, 1-female, 2-male; PMI, postmortem interval; FT, freezer time; Sm, smoking history (1-No, 2-Yes); AS, agonal state (1- < 10 min; 2- > 1 h); RLN, reelin.

\* $P < 0.05$ .

\*\* $P < 0.01$ .

**Table 3.** Contribution of disease-related variables to gene expression levels estimated using forward stepwise multiple regression analysis

	Adj $R^2$	Age onset $\beta$	Age Hosp $\beta$	Duration $\beta$	Daily CPZ $\beta$	Life CPZ $\beta$	Last CPZ $\beta$	Sub Ab $\beta$
Hippocampus								
FEZ1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NUDEL	0.15*	n.s.	n.s.	-0.43*	n.s.	n.s.	n.s.	n.s.
LIS1	0.12*	n.s.	n.s.	-0.40*	n.s.	n.s.	n.s.	n.s.
RLN	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DLPFC								
FEZ1	0.37****	n.s.	n.s.	-0.49**	n.s.	n.s.	0.39*	n.s.
NUDEL	0.68****	n.s.	n.s.	-0.71****	n.s.	n.s.	n.s.	0.31*
LIS1	0.49**	n.s.	n.s.	-0.84****	n.s.	0.86*	n.s.	n.s.
RLN	0.23*	n.s.	n.s.	-0.52**	n.s.	n.s.	n.s.	n.s.

Adj  $R^2$ , adjusted coefficient of determination;  $\beta$ , standardized regression coefficient; Age onset, age of illness onset; Age Hosp, age at first hospitalization; Duration, duration of illness; Daily CPZ, average daily dose of neuroleptics in chlorpromazine (CPZ) equivalents; Life CPZ, life-time dose of neuroleptics in CPZ equivalents; Last CPZ, last recorded dose of neuroleptics in CPZ equivalents; Sub Ab, history of substance abuse (1-No, 2-Yes); RLN, reelin.

\* $P < 0.05$ .

\*\* $P < 0.01$ .

\*\*\* $P < 0.001$ .

\*\*\*\* $P < 0.0001$ .

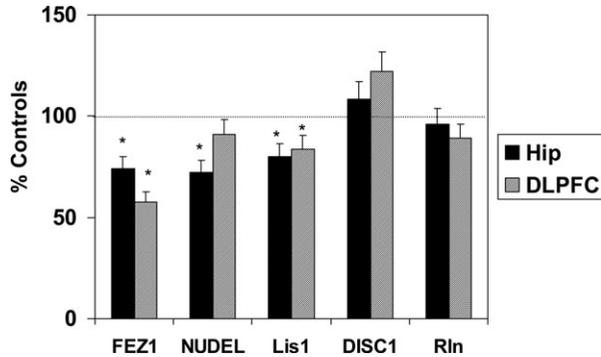
weak positive correlations between DLPFC and hippocampal expression of NUDEL ( $r = 0.31$ ;  $P = 0.003$ ), FEZ1 ( $r = 0.27$ ;  $P = 0.01$ ) and LIS1 ( $r = 0.26$ ;  $P = 0.01$ ).

In the DLPFC, all three DISC1 partners tended to show reduced expression in the cases (Fig. 5). FEZ1 expression without normalization or normalized by individual housekeeping genes or NF was significantly reduced in patients with schizophrenia either without [ $F(1,105) = 7.7$ ;  $P = 0.006$ , normalized by NF] or with age as a co-variate [ANCOVA:  $F(1,105) = 7.7$ ;  $P = 0.007$ ] (Fig. 5). LIS1 mRNA expression was also significantly reduced without or with normalization to housekeeping genes [ $F(1,105) = 5.6$ ;  $P = 0.02$ , normalized by NF] but not after co-varying for age and RIN [ $F(1,105) = 2.6$ ;  $P = 0.1$ ]. NUDEL expression was reduced without normalization [ $F(1,105) = 4.9$ ;  $P = 0.03$ ] but the effect of diagnosis did not reach significance

after normalization to housekeeping genes [ $F(1,105) = 2.7$ ;  $P = 0.1$ ]. There were no effects of diagnosis on the expression of any housekeeping genes (all  $F$ s < 1.5, all  $P$  > 0.4).

Logistic multiple regression analysis showed that of the three genes, FEZ1 and LIS1 expression were included in the model [ $F(2,101) = 5.84$ ; adjusted  $R^2 = 0.086$ ;  $P = 0.003$ ] but only FEZ1 expression made a significant contribution to the prediction of diagnosis (6.2%,  $P = 0.017$ ).

High-risk DISC1 genotypes had less pronounced effects on the DLPFC expression of DISC1 interacting molecules when compared with the hippocampus. For FEZ1, there appeared to be an association of the two risk SNPs (rs821597, rs821616) with the low expression levels in patients with schizophrenia, that is, *post hoc* comparisons showed that individuals with schizophrenia homozygous for the high-risk allele had significantly lower levels of FEZ1 than all other



**Figure 5.** Expression levels of FEZ1, NUDEL, LIS1, DISC1 (transcripts L + Lv + S) and reelin mRNA in the hippocampus and DLPFC of patients with schizophrenia relative to normal controls (means  $\pm$  SD). Asterisk denotes significantly different from controls,  $P < 0.05$ .

groups ( $P < 0.05$ ; Fig. 6B). However, there were no significant main effects of genotype or significant interactions in two-way ANOVAs.

#### Expression of reelin mRNA in the hippocampus and DLPFC

Reelin is an important protein in cortical development that has been implicated in schizophrenia (40,41). There is also evidence for genetic and biochemical interactions between the reelin signaling pathway and Lis1 (42). However, because it is not directly linked to DISC1 as a molecular partner, we hypothesized that it should not be affected by DISC1 SNPs. There was no significant effect of diagnosis on reelin expression in the hippocampus either without or with normalization [ $F(1,104) = 0.1$ ;  $P > 0.5$ ] or in the DLPFC [ $F(1,105) = 1.4$ ;  $P > 0.2$ ] or after co-varying the data for the contributing variables (Fig. 5). There were also no effects of DISC1 genotypes on the expression of reelin (Fig. 6E).

#### Effects of antipsychotic drugs

There were no significant effects of treatment on the expression of any gene tested [all  $F(6,62) < 1.0$ ; all  $P$ -values  $> 0.5$ , normalized to NF, a geometric mean of GAPDH, PBGD and B2M, or to the individual housekeeping genes or without normalization] (data not shown). There were also no differences between drug treatments for any of these housekeeping genes or NF (all  $F < 1.0$ ; all  $P$ -values  $> 0.5$ ).

## DISCUSSION

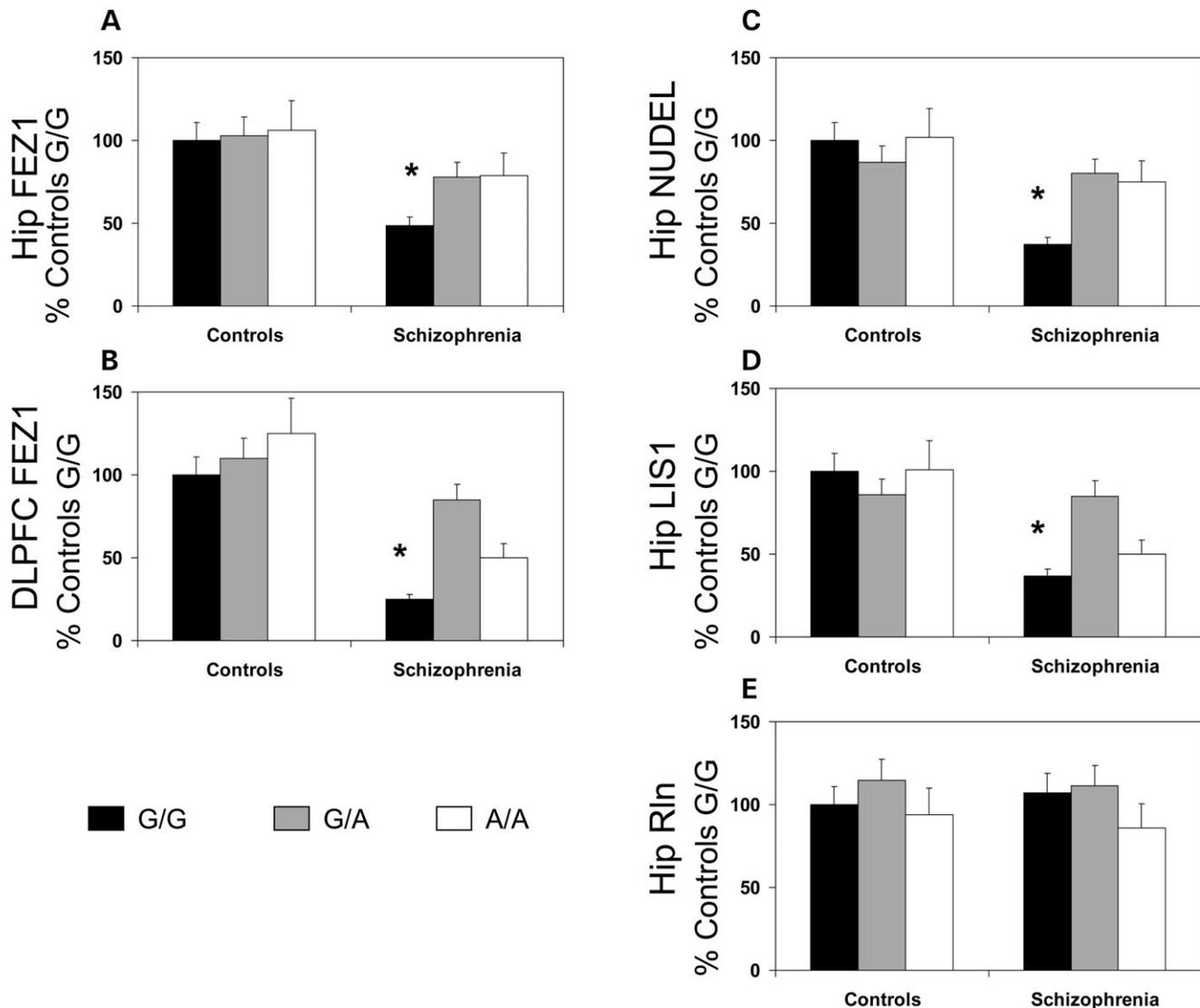
We have investigated the expression of DISC1 and three of its binding partners in the human hippocampus and DLPFC and explored their association with schizophrenia and with disease-associated DISC1 polymorphisms. We hypothesized that variations in the *DISC1* gene shown to be associated with schizophrenia would impact on the molecular phenotype of a DISC1 pathway in brain regions implicated in the disease. We report the following principal findings: (i) DISC1 expression varies during life span in the human brain; (ii) the

expression of three DISC1-interacting molecules, FEZ1, LIS1 and NUDEL, is reduced in the hippocampus and FEZ1 expression is reduced in the DLPFC of patients with schizophrenia; (iii) high-risk DISC1 genotypes predict low expression levels of FEZ1, LIS1 and NUDEL in the hippocampus, and FEZ1 in the DLPFC of patients with schizophrenia. Furthermore, in the same tissue samples, we did not observe effects of variations in the *DISC1* gene on DISC1 mRNA levels, suggesting that the mechanism of the genetic effect on expression of other genes in this pathway is not based on altered DISC1 mRNA abundance, *per se*. DISC1 immunoreactivity, however, may be enhanced in the hippocampus of patients with schizophrenia. Altogether, these results implicate genetically driven abnormalities in a DISC1 molecular pathway in the pathophysiology of schizophrenia.

#### DISC1 expression in the human brain

We found that full-length DISC1 mRNA is expressed in the human brain and that the expression of DISC1 is relatively high in a subset of granule cells in the DG as well as in a sub-population of cells in the temporal and PCtx and the WM. These data are in accordance with the report in the non-human primate brain (36). We were unable to amplify cDNA corresponding to two other transcripts of DISC1—short and extra-short—reported in the literature (26), nor were we able to detect the signal from the riboprobes designed to hybridize to these splice variants using *in situ* hybridization histochemistry. We were unable to design probes specific for Lv and thus did not measure this transcript separately. However, RT-PCR data obtained using probes recognizing combinations of transcripts L, L + Lv and L + Lv + S suggest that S and Lv are either absent or in very low abundance in our samples. Altogether, we conclude that the full-length transcript is the predominant variant of DISC1 in the adult human brain, unless our techniques failed to recognize other isoforms of DISC1.

Our immunoblotting with an anti-DISC1 antibody directed against the C-terminus of the protein detected one major band. However, its size does not correspond to the predicted 98 kDa full-length DISC1 protein and cannot be the product of known DISC1 variants, as S and Es transcripts (predicted sizes: 74 and 39 kDa, respectively) miss C-termini and thus would not be recognized by this antibody. Moreover, these mRNA isoforms were not detected in our samples. It has previously been shown that the 70–75 kDa band contains DISC1 sequences, and thus it was speculated that it may correspond to a post-translational modification form of the full-length protein, perhaps arising by N-terminal processing (39). This pattern of immunoblotting is similar to blots reported by others in cells, and in animal and human protein extracts with various C-terminus-directed anti-DISC1 antibodies (25,30,32,39,42). Given the uncertainty of the function of this putative DISC1-related protein or the mechanism by which it is generated, we are unable to comment further on the evidence for its apparent increased immunoreactivity in brain tissue from patients with schizophrenia. It also should be noted that an earlier study in another sample of brains of patients with schizophrenia using the same antibody found no evidence of increased immunoreactivity of this putative



**Figure 6.** Association of DISC1 genotype (rs821597) with low FEZ1, NUDEL and LIS1 expression in patients with schizophrenia ('controls G/G'  $n = 25$ , 'controls G/A'  $n = 33$ , 'controls A/A'  $n = 15$ , 'patients with schizophrenia G/G'  $n = 11$ , 'patients with schizophrenia G/A'  $n = 14$ , 'patients with schizophrenia A/A'  $n = 7$ ). Individuals carrying a high-risk G-allele of SNP rs821597 show low levels of expression of FEZ1 in the hippocampus (A), DLPFC (B), NUDEL (C), LIS1 (D) but not reelin (E) in the hippocampus. Asterisk denotes different from A-carriers,  $P < 0.05$ .

DISC1 species (though that study was relatively underpowered when compared with ours) (39).

We also report that DISC1 mRNA is developmentally variable in the human brain, showing high levels of expression in early childhood (neonatal period, infancy) and then declining rapidly by teenage years and into adulthood. This developmental pattern differs somewhat from observations in the rodent brain, although different life stages were investigated in the rodent and human (i.e. we did not explore embryonic expression and are missing subjects between infant and teenage years) and the correspondence between rodent and human brain development has not been fully elucidated. Nevertheless, in accordance with our data, others reported higher expression of DISC1 mRNA in the hippocampus of the mouse during periods of active neurogenesis and during puberty [at postnatal (P) day 7 (32), at embryonic day (E)

13.5, P35 (43) and at P21 (44)]. In the mouse, DISC1 mRNA was also seen in the developing cerebral cortex, with the highest levels of expression observed at E14, E16 and E18, the stages at which neuronal birth and migration are active. We report here high expression of DISC1 in the human neonatal period and infancy, that is, times of exuberant changes in cortical neural architecture and synapse formation. Together, these data suggest that the overall pattern of DISC1 expression is similar across species and underscore its potential role in early human brain development, including the development of the hippocampal formation and the neocortex.

#### DISC1 mRNA expression in schizophrenia

We did not detect changes in DISC1 mRNA expression levels in the hippocampus or DLPFC of patients with schizophrenia

nor did we observe the effects of our high-risk variations in the *DISC1* gene on its transcriptional activity. This may not be surprising given the fact that the risk alleles identified in our clinical study (18) are common and that they are located far from the transcriptional start sites of the *DISC1* variants, although it is conceivable that SNPs in non-coding regions or at some distance from 5' or 3' regulatory regions may be involved in regulating transcription, splicing or mRNA stability. Moreover, we have not scored other SNPs in the gene that have been associated with schizophrenia by other clinical groups (see introduction), some of which may be closer to regulatory elements in the gene. Importantly, our failure to find differences in *DISC1* mRNA expression (or in *reelin* mRNA expression) in the same samples, in which reduced expression of *DISC1* partners is found, argues that our results are not caused by a non-specific reduction in transcriptional activity in this tissue.

### Expression of genes in the *DISC1* molecular pathway

We found that the expression of *NUDEL*, *FEZ1* and *LIS1* (but not *reelin* or any of the housekeeping genes) was significantly reduced in the hippocampus of patients with schizophrenia and associated with high-risk *DISC1* polymorphisms. The findings in the DLPFC implicated *FEZ1* and *LIS1* but not *NUDEL* (though all transcripts tended in the same direction). We propose that the expression of these molecules is altered in schizophrenia at least in part as a result of aberrant interactions with putative mutated forms of *DISC1* protein or because of altered processing of *DISC1* mRNA. These changes may have important implications for the development and function of the human nervous system, initiating a cascade of events leading to psychiatric illness during adult life. Although we are uncertain as to why the results appear to be stronger in the patient sample (though absent significant interactions in a majority of comparisons, this is not clearly the case), it is not far-fetched to expect that a risk SNP would show greater biologic effects in the ill sample, having other risk factors (e.g. other risk genes) that exaggerate the biologic impact of another biologic risk factor.

*FEZ1* (or *UNC-76* in *Caenorhabditis elegans*) is involved in axonal outgrowth and fasciculation. Its expression levels and anatomical localization in development curiously mirror those reported for *DISC1* in the rodent (45). The *FEZ1*/*DISC1* interaction occurs through the conserved region of *FEZ1* protein (amino acid 247–392) and the region of *DISC1*, which covers a stretch well preceding the translocation breakpoint and immediately following it (amino acid 446–633) (32). Accordingly, it was shown that a truncated *DISC1* protein lacking a whole region beyond the translocation breakpoint (amino acid 597) was still able to form weak interactions with *FEZ1*. In light of these findings, it is unclear how high-risk *DISC1* mutations in the region even further downstream from the critical binding site, even if functional, would affect interactions with *FEZ1*, unless the variations mark other polymorphisms in the gene that impair *FEZ1*/*DISC1* binding. On the other hand, polymorphisms in the introns can affect gene function by affecting regulatory motifs within introns or splicing.

In contrast to *FEZ1*, both *NUDEL* and *LIS1* bind to an outmost C-terminal region in *DISC1* (amino acid 727–854) that is close to our high-risk polymorphisms (Fig. 1A) and lost in a putative protein truncated by the translocation. The *DISC1*/*NUDEL* complex peaks in late embryonic/early postnatal life (31). Both *NUDEL* and *LIS1*, and perhaps also the trimolecular complex with *DISC1*, are important for various developmental processes, including neuronal migration (46). One of the recently emphasized subcellular targets of *DISC1*/*NUDEL* complex is mitochondria; in this regard, two motifs in *DISC1* were highlighted as critical for mitochondria targeting and stabilization: amino acid 440–597 (a binding site for *FEZ1*) and 598–854 (a site for *NUDEL* and *LIS1*) (29). Thus, mutations in *DISC1* in regions close to the putative binding sites (Fig. 1A) and ensuing defective interactions with the binding proteins may have deleterious consequences for mitochondrial function contributing to the pathophysiology of schizophrenia. No other *DISC1* polymorphisms examined in this study had any effect on the expression of the interacting molecules and there was no effect on *reelin*, a molecule in a related but distinctly separate pathway.

### Limitations

An obvious limitation of our study is the use of human post-mortem brain tissue for quantification of gene expression that can be complicated by an array of confounding factors. Although we have collected extensive information about the subjects and the samples, assessed the effect of multiple pre- and postmortem factors on RNA by multivariate analysis and used a large number of samples from controls and patients with schizophrenia, it is still possible that some of these factors caused bias in our data.

It also should be noted that we have performed many statistical tests on the same tissue samples, and some of the significant findings would not survive arbitrary correction for the multiple tests performed. We believe that with our approach, which underscores testing biologically meaningful relationships predicted on the basis of previous basic and clinical studies and our prior evidence of clinical association with specific SNPs and not others, supports the validity of our results. Moreover, corrections for multiple testing of SNPs and molecules that are not strictly independent variables is not straightforward and how best to do this is not obvious. For example, arbitrary correction could lead to increased type two error. Rather than losing power in an already low power (relatively small sample size) analysis and risking not detecting a true difference using Bonferroni's corrections, an ultimate test should be an independent replication of the data using a separate collection. Nevertheless, after correcting for five tests, the diagnostic differences in hippocampal *NUDEL* mRNA and *FEZ1* mRNA expression and prefrontal *FEZ* mRNA expression would still remain significant.

There are also many unanswered questions that require further research. The most important one is related to the identification of the putative altered *DISC1* protein or processing of a *DISC1* transcript and clarification of its role in the molecular pathway. It will be of interest to test whether mutations in the interacting molecules predispose to schizophrenia [there has been recently one report suggesting weak

association of FEZ1 variants with schizophrenia (47) and whether epistatic effects between these genes exist]. It is also plausible that a balanced translocation disrupts other genes as suggested by a recent report that the gene encoding phosphodiesterase 4B (*PDE4B*) is disrupted in a subject diagnosed with schizophrenia and a relative with chronic psychiatric illness (48). Moreover, the role of DISC1-interacting genes in human brain development is unknown. This may shed light on the importance of DISC1 pathway in neurodevelopmental disorders, such as schizophrenia, and perhaps explain regional differences in the expression of these genes in the disorder. It is also of interest whether our findings extend to other patient cohorts and to other major mental illnesses. We are currently investigating these issues.

### Summary

Susceptibility genes for psychiatric disorders represent basic mechanisms of disease and identify pathogenic molecular pathways. *DISC1* has been a promising candidate susceptibility gene for schizophrenia and affective psychosis with molecular effects presumably related to development and neuroplasticity. Our results suggest that a putative abnormality in *DISC1* biology in individuals carrying high-risk mutations, impacts on the regulation of specific binding partner genes and causes abnormalities in a DISC molecular pathway involved in cortical development, mitochondrial transport and synaptic development and plasticity (49). These subtle molecular events may represent a pathogenic pathway related to schizophrenia.

## MATERIALS AND METHODS

### Human postmortem tissue

Postmortem hippocampal and DLPFC tissue samples were collected at the Clinical Brain Disorders Branch, NIMH from 79 normal controls and 43 patients with schizophrenia. The hippocampus was identified on frozen coronal slabs from the medial temporal lobe and dissected using a dental drill (Cat UP500-UG33, Brasseler, USA). The lateral ventricle and the fimbria fornix were used as the medio-dorsal boundary and the subiculum and underlying WM as the ventral boundary. The adjacent PCtx was not included in the dissection. For the DLPFC, gray matter tissue from the middle frontal gyrus was obtained from a coronal slab corresponding to the middle one-third immediately anterior to the genu of the corpus callosum. WM was trimmed off.

On the basis of the assessment of the regional RIN, two subcohorts were selected for comparisons of mRNA expression levels in the hippocampus and DLPFC (Table 4). There were 68 overlapping controls and 25 subjects with schizophrenia in the hippocampal and DLPFC cohorts. A smaller subgroup was selected from the initial cohort for protein experiments (Table 4). Expression of *DISC1* mRNA was also examined at several stages of brain development and aging, in tissue slices through the posterior hippocampus from 38 normal controls aged 5 weeks through 86 years (Table 5).

All brain tissue used in this study was obtained with informed consent from the legal next of kin under NIMH

protocol 90-M-0142. Diagnoses were determined by independent reviews of clinical records by two Board Certified psychiatrists using DSM-IV criteria. Normal subjects have been screened with a psychological autopsy (at least 30 items) by interviews with the next of kin by our staff in addition to being interviewed by medical examiner investigators. Moreover, all brain tissue from normal controls has been screened by macro and microscopic neuropathological examinations and toxicology screening.

Total daily, lifetime and last dose of neuroleptic medication was calculated for each patient with schizophrenia and converted to chlorpromazine equivalents (CPZ; mean daily dose  $480 \pm 267$  mg/day; mean lifetime CPZ equivalent  $3.20 \text{ mg} \times 10^6 \pm 0.5 \times 10^6$ ; mean last recorded CPZ dose  $468 \pm 81$  mg/day). Smoking and substance abuse history was also recorded. When information was missing, smoking was verified through toxicological analysis of nicotine and cotinine levels in blood or brain. Toxicological analysis was conducted for every case by a forensic toxicologist. Non-psychiatric cases with toxicology screenings positive for ethanol above the American Medical Laboratory Inc. (0.05 g/dL) or positive for any medication above therapeutic levels or any illicit drugs were excluded from the control group. Positive toxicology was not an exclusion criterion for cases with schizophrenia.

### RNA extraction and reverse transcription

Tissue was pulverized and stored at  $-80^\circ\text{C}$ . Total RNA was extracted from 300 mg of tissue using the TRIZOL Reagent (Life Technologies Inc., Grand Island, NY, USA). The yield of total RNA was determined by absorbance at 260 nm. RNA quality was assessed with a high-resolution capillary electrophoresis (Agilent Technologies, Palo Alto, CA, USA) and samples showing clearly defined, sharp 18S and 28S ribosomal peaks, 28S/18S ratios  $>1.2$  and RIN  $\geq 3.8$  were included. Total RNA ( $4 \mu\text{g}$ ) was used in  $50 \mu\text{l}$  of reverse transcriptase reaction to synthesize cDNA, using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA).

### Oligonucleotide and primers design

Commercial Taqman probes/primer sets were used for *DISC1* (Applied Biosystems, Foster City, CA, USA Assays-on-Demand), as well as for *NUDEL*, *FEZ1*, *Reelin* and three endogenous control genes: porphobilinogen deaminase (*PBGD*),  $\beta$ -2-microglobulin (*B2M*) and  $\beta$ -glucuronidase (*GUSB*) (Table 1). In particular, the sequence of the *NUDEL* probe was specific for *NUDEL* and not for *Nde1*, a structurally and functionally similar protein, or any other sequence. In addition, custom-made primers/probes were designed using PRIMER EXPRESS software, to amplify specific transcripts based on the unique exon structure of each *DISC1* isoform (25) (Fig. 1A).

### Quantitative real-time PCR

Expression levels of mRNAs were measured by real-time quantitative (RT-PCR), using ABI Prism 7900 sequence

**Table 4.** Characteristics of subject cohorts used for mRNA expression and immunoreactivity measurements

	<i>n</i>	Race	Sex	Age	PMI	pH	RIN
Hippocampus- mRNA							
Controls	73	44AA/2A/3H/24C	19F/54M	41.6 ± 14.8	31.4 ± 14.4	6.61 ± 0.26	5.7 ± 1.0
Schizophrenia patients	32	17AA/1H/14C	9F/23M	46.3 ± 15.2	35.2 ± 15.2	6.53 ± 0.27	5.4 ± 1.1
DLPFC- mRNA							
Controls	70	41AA/3A/4H/22C	19F/51M	41.7 ± 15.2	32.6 ± 14.1	6.61 ± 0.24	6.7 ± 1.3
Schizophrenia patients	36	17AA/3H/16C	15F/21M	49.8 ± 18.3	36.1 ± 16.8	6.51 ± 0.27	6.5 ± 1.6
Hippocampus- protein							
Controls	53	31AA/1A/3H/18C	16F/37M	41.6 ± 16.3	30.4 ± 14.3	6.61 ± 0.24	5.7 ± 0.4
Schizophrenia patients	22	10AA/12C	5F/17M	42.6 ± 15.9	36.6 ± 14.7	6.52 ± 0.26	5.5 ± 0.6

AA, African American; A, Asian; H, Hispanic; C, Caucasian; F, female; M, male; PMI, postmortem interval (h); RIN, Agilent RNA integrity number (on a scale 1–10).

**Table 5.** Characteristics of subjects used for mRNA expression measurement across life stages

Group	<i>n</i>	Race	Sex	Age	PMI	pH
Neonates	5	AA	5F/0M	2.65 ± 1.35 (m)	39.9 ± 21.0	6.46 ± 0.27
Infants	5	AA	2F/3M	7.4 ± 3.0 (m)	45.3 ± 13.4	6.53 ± 0.18
Teens	9	AA	0F/9M	16.8 ± 1.68 (y)	32.2 ± 22.9	6.39 ± 0.33
Young adults	5	AA	0F/5M	22.3 ± 1.63 (y)	30.1 ± 10.1	6.35 ± 0.43
Adults	9	AA	0F/9M	41.4 ± 6.5 (y)	32.5 ± 12.9	6.38 ± 0.28
Aged	5	AA	0F/5M	76.7 ± 6.9 (y)	45.8 ± 15.1	6.32 ± 0.26

Age of neonates and infants given in months (m), other groups in years (y). AA, African American; F, female; M, male; PMI, postmortem interval (h).

detection system with 384-well format (Applied Biosystems). Each 10–20 µl reaction contained 900 nM of primer, 250 nM of probe and Taqman Universal PCR Mastermix (Applied Biosystems) containing Hot Goldstar DNA Polymerase, dNTPs with dUTP, uracil-*N*-glycosylase, passive reference and 100–200 ng of cDNA template. PCR cycle parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 59°C or 60°C for 1 min. PCR data were acquired from the Sequence Detector Software (SDS version 2.0, Applied Biosystems) and quantified by a standard curve method using serial dilutions of pooled cDNA derived from RNA obtained from hippocampi or DLPFC of 10–12 normal control subjects. In each experiment the  $R^2$ -value of the curve was more than 0.99, the slope was between –3.2 and –3.5 (amplification efficiency 96–101%) and controls comprising no-template cDNA resulted in no detectable signal. All samples were measured in a single plate for each gene, and their cycles at threshold ( $C_t$ ) values were in the linear range of the standard curve. All measurements were performed in triplicates. The data were normalized to control genes and a geometric mean of these genes (50).

### DISC1 mRNA *in situ* hybridization in the human hippocampus

To assess the relative temporal, regional and cellular distribution of the DISC1 signal in the human hippocampal formation and the overlaying neocortex, we examined

normal control individuals from a separate brain collection using riboprobe *in situ* hybridization. Coronal sections (two anatomically matched slices per brain) were cut from blocks dissected from the caudal third of the medial temporal lobe. A riboprobe recognized a 1172 bp fragment of human *DISC1* (accession no. NM\_018662) corresponding to nucleotides 915–2087 (exons 2–10) (36) (Fig. 1B). A cDNA template was generated by subcloning the DISC1 insert into a pCRII vector (Invitrogen). Sense and antisense riboprobes were then synthesized using a T7 promoter and a *Hind*III linearized vector (sense strand) and SP6 promoter with the *Not*I linearized vector for the antisense riboprobe. <sup>35</sup>S-UTP-labeled probes for DISC1 with specific activity of  $7.4 \times 10^8$  cpm/µg were synthesized and purified by ethanol precipitation. Hybridization was performed overnight at 55°C using a hybridization buffer (200 µl) containing DISC1 riboprobe (5 ng/ml). Slices and the <sup>14</sup>C standards were exposed to Kodak BioMax film for 8 weeks and analyzed using NIH Image. Slides were then dipped in emulsion, exposed for additional 12 weeks, counterstained with Nissl substance and used to evaluate silver grain distribution. In addition, to assess transcript-specific expression of Es and S variants, T7/T3 promoter-tagged DISC1 riboprobe templates were designed by PCR as described previously (51). Outer/inner primers for two rounds of amplification were chosen in exon 3 for the transcript Es (outer: 5' GGAATCATTTTCCCCTT GGT 3' and 5' GGCAGTGGGCACAATAAACT 3', inner: 5' ccaagccttcattaaccctcactaaaggaga AAAGTCGGCCCTC CATATCT 3' and 5' cagagatgcataatacgaactactataggaga TTCTGGAAGCAAGGGAAGAA 3') and in exon 10' for the transcript S (outer: 5' ACTTCTCTGTACTACAGTGT TAT 3' and 5' ATATAAGTACTGTGATGCAGAGT 3', inner: 5' ccaagccttcattaaccctcactaaaggagaCCTGGACACA GAGAAGTCAGC 3' and 5' cagagatgcataatacgaactactata gggaga TGTCTCTTCAATGCCCTTTCA 3') (Fig. 1B). However, in pilot studies no signal was detected in slices by ISHH using Es or S probes (data not shown).

### Immunoblotting

Human hippocampal tissue samples (100–130 mg; 1 g tissue: 10 ml buffer) were homogenized in a protease inhibitor–Tris-glycerol extraction buffer (AEBSF 0.024%, aprotinin 0.005%,

leupeptin 0.001%, pepstatin A 0.001%, glycerol 50%, Tris 0.6%). Protein concentration was determined using the Bradford assay. Homogenates (4 µg of total protein/10 µl) were diluted with water, XT sample buffer and 1X antioxidant (Bio-Rad Laboratories Inc., Hercules, CA), and heated to 80°C for 5 min. Ten microlitres of sample was loaded onto precast 10% Bis-Tris polyacrylamide gels (Bio-Rad Laboratories Inc.), and proteins were separated by electrophoresis at 200 V for 1 h. Each gel contained a molecular weight marker ladder SeeBlue Plus 2 (Invitrogen), a pooled sample from 10 normal controls at three concentrations (2, 4 and 6 µg of total protein content per 10 µl), and 20 samples from patients with schizophrenia and controls. A total of four separate gels were used in one experiment ( $n = 75$  samples). Gels were transferred onto nitrocellulose membranes at 85 V for 30 min, membranes blocked for 1 h in 10% goat serum in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T), and incubated with an anti-DISC1 antibody (1:2000) raised against peptide sequence corresponding to amino acid 601–854 of the C-terminus of DISC1 (25,39) and anti-actin (1:3000 dilution) antibody. Blots were rinsed in TBS-T, incubated in a peroxidase-conjugated rabbit or mouse (actin) secondary antibody (1:10 000 dilution, Chemicon, Temecula, CA) for 2 h in 5% normal goat serum in TBS-T, rinsed in TBS-T and developed in ECL-plus (Amersham/GE Healthcare, Buckinghamshire, UK). The values were expressed as the percentage of a mean of controls on the same gel and as a ratio to actin.

#### DISC1 genotype determination

DNA was extracted from cerebellar brain tissue of subjects used in the RT-PCR and immunoblotting experiments ( $n = 122$ ) using a protocol by PUREGENE (Gentra Systems, MN, USA). Twelve SNPs were genotyped: (1) rs751229, (2) rs1572899, (3) rs1934909, (4) rs1538976, (5) hCV1650667, (6) rs4079841, (7) rs999710, (8) hCV219779, (9) rs821597, (10) rs821616, (11) and (12) rs1411776, based on an earlier report from our laboratory (18) (Fig. 1B). Three of these SNPs, (8) hCV219779, (9) rs821597, (10) rs821616, showed evidence of association with schizophrenia in our earlier report (18), and one (10) rs821616, a non-synonymous SNP in exon 11, was particularly strongly associated. Thus, we made an *a priori* prediction that these three SNPs, and not the others, would show an effect on gene expression. Genotyping was performed using the Taqman 5'-exonuclease allelic discrimination assay (details available on request). Genotype reproducibility was routinely assessed by re-genotyping all samples for selected SNPs and was generally >99%.

#### Determination of the effects of antipsychotic drugs in rats

To test whether chronic exposure to antipsychotic drugs might have contributed to changes in the expression levels observed in patients with schizophrenia, we measured the expression of NUDEL, FEZ1 and DISC1 mRNA in the frontal cortex of rats treated chronically with clozapine and haloperidol. All procedures were performed in accordance with the National Institutes of Health Guidelines for use and care of laboratory

animals. Male Sprague–Dawley rats (weight 250 g) were on a 12-h light/dark cycle (lights on/off 6:00 AM/6:00 PM) in a temperature-controlled environment and with *ad lib* access to food and water. Rats were randomly assigned to drug treatment groups (8–10 per dose) and given intraperitoneal injections of haloperidol (0.08, 0.6 and 1 mg/kg), clozapine (0.5, 5, 10 mg/kg) or vehicle (0.02% lactic acid) once daily for 28 days. Haloperidol (Research Biochemicals Inc., Natick, MA, USA) (20 mg/ml) was prepared in 1% lactic acid, then further diluted with water and 1 M NaOH added to obtain pH 5.3. Clozapine (a gift from Sandoz Research Institute Berne Ltd., Berne, Switzerland) (100 mg) was dissolved in 0.1 M HCl, diluted with water and neutralized to pH 5.2. Rats were killed 7 h after the last injection. Frontal cortex without WM was dissected and frozen at –80°C. RNA was extracted as described earlier for human tissue and mRNA measured by RT-PCR using the following Taqman ABI assays: for FEZ1 (Rn00579248), DISC1 (Rn00598264\_m1), NUDEL (Rn00502723\_m1), GAPDH (Rn99999916\_s1), PBGD/HMBS (Rn00565886\_m1), B2M (Rn00560865\_m1).

Taqman assays for LIS1 or reelin were not available, so we did not quantify mRNA of these genes in the rat tissue. The expression data of target genes were normalized to a geometric mean of the three previously described control genes.

#### Statistical analyses

Statistical analyses were conducted using Statistica [StatSoft Inc., 2005, STATISTICA (data analysis software system)] version 7.1., www.statsoft.com. Multiple regression analyses were used for determining the contribution of demographic, tissue- and disease-related variables to the gene expression levels and logistic regression for determining the diagnostic prediction. Comparisons between diagnostic groups were made using ANCOVA for each mRNA with diagnosis as the independent variable and continuous variables as covariates. Covariates were those variables, which were significantly impacting the expression of a particular gene. Thus, not surprisingly, covariates were not identical for each gene assay. Effects of genotype variation on gene expression were examined using ANOVA with genotype and diagnosis as independent variables. A co-variate analysis was used in case of significant contributions of the variable to the gene expression levels.

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