Dysbindin-1 in dorsolateral prefrontal cortex of schizophrenia cases is reduced in an isoform-specific manner unrelated to dysbindin-1 mRNA expression

Junxia Tang¹, Robert P. LeGros¹, Natalia Louneva¹, Lilly Yeh¹, Julia W. Cohen¹, Chang-Gyu Hahn¹, Derek J. Blake², Steven E. Arnold¹ and Konrad Talbot¹,*

¹Center for Neurobiology and Behavior in the Department of Psychiatry, University of Pennsylvania, Philadelphia, PA 19104-3403, USA and ²MRC Centre for Neuropsychiatric Genetics and Genomics and Department of Psychological Medicine and Neurology, Cardiff University, Cardiff, Wales CF14 4XN, UK

Received February 4, 2009; Revised July 10, 2009; Accepted July 16, 2009

INTRODUCTION

Since the initial report of Straub et al. in 2002 (1), many studies have found that genetic variation in DTNBP1 (dystrobrevin binding protein 1) is associated with schizophrenia as discussed in several reviews (2–4). Comparative analyses reported in 2008 concluded that DTNBP1 remains a top candidate for genes affecting risk of the disorder (5,6). DTNBP1 encodes the first known member of the dysbindin protein family, dysbindin-1 (4), which is ubiquitously expressed in neurons throughout the brain (4,7–9). It is concentrated in synapses of brain areas commonly affected in schizophrenia, including the striatum, hippocampal formation and neocortex (4,7,8,10). In two of those areas, the hippocampal formation (7,11) and dorsolateral prefrontal cortex (DLPFC) (9,12), schizophrenia cases have been found to display reduced dysbindin-1 gene and protein expression. It remains unknown, however, which dysbindin-1 isoform is reduced in those cases and if any dysbindin-1 isoform

DTNBP1 (dystrobrevin binding protein 1) remains a top candidate gene in schizophrenia. Reduced expression of this gene and of its encoded protein, dysbindin-1, have been reported in the brains of schizophrenia cases. It has not been established, however, if the protein reductions encompass all dysbindin-1 isoforms or if they are associated with decreased DTNBP1 gene expression. Using a matched pairs design in which each of 28 Caucasian schizophrenia cases was matched in age and sex to a normal Caucasian control, Western blotting of whole-tissue lysates of dorsolateral prefrontal cortex (DLPFC) revealed significant reductions in dysbindin-1C (but not in dysbindin-1A or -1B) in schizophrenia (P = 0.022). These reductions occurred without any significant change in levels of the encoding transcript in the same tissue samples and in the absence of the only DTNBP1 risk haplotype for schizophrenia reported in the USA. Indeed, no significant correlations were found between case–control differences in any dysbindin-1 isoform and the case–control differences in its encoding mRNA. Consequently, the mean 60% decrease in dysbindin-1C observed in 71% of our case–control pairs appears to reflect abnormalities in mRNA translation and/or processes promoting dysbindin-1C degradation (e.g. oxidative stress, phosphorylation and/or ubiquitination). Given the predominantly post-synaptic localization of dysbindin-1C and known post-synaptic effects of dysbindin-1 reductions in the rodent equivalent of the DLPFC, the present findings suggest that decreased dysbindin-1C in the DLPFC may contribute to the cognitive deficits of schizophrenia by promoting NMDA receptor hypofunction in fast-spiking interneurons.
reductions are accompanied by reductions of dysbindin-1 mRNA expression in the same tissue samples.

While not yet widely appreciated, there are multiple dysbindin-1 isoforms (4,13). We previously designated the major isoforms as dysbindin-1A, -1B and -1C in our characterization of the dysbindin protein family (4). These are the protein products of the most common DTNBP1 transcripts in humans [i.e. NM_032122, NM_183040 and NM_183041 of the National Center for Biotechnology Information (NCBI)]. These proteins are differentially distributed in human brain synaptosomes, where dysbindin-1A is associated almost exclusively with postsynaptic densities (PSDs), dysbindin-1B is associated almost exclusively with synaptic vesicles, and dysbindin-1C is associated to some extent with synaptic vesicles and to a much greater extent with PSDs (4,14). Since this indicates differential synaptic roles of dysbindin-1 isoforms, it is important to identify which of these isoforms account for observed dysbindin-1 reductions in schizophrenia. The present study is the first to address that issue and the related issue of whether or not decreased dysbindin-1 isoform(s) in schizophrenia may be due to decreased dysbindin-1 mRNA expression either in the presence or in the absence of the one DTNBP1 risk haplotype reported in schizophrenia cases residing in the USA (15).

The clinical significance of these issues is evident in studies finding that the DTNBP1 SNPs and haplotypes associated with schizophrenia are also associated with severity of certain negative (16,17) and/or positive symptoms (18,19) of the disorder. These genetic variants of DTNBP1 are, moreover, associated with diverse cognitive deficits constituting a core feature of schizophrenia (20–23), which, along with negative symptoms, contribute strongly to disability in daily functions (24). When compared with non-carriers, patients carrying DTNBP1 risk SNPs show a greater decline in general cognitive ability from the premorbid to the clinical state (25), lower general cognitive ability in the clinical state (26), lower scores on verbal, performance and full-scale IQ tests of the Wechsler Adult Intelligence Scales-Revised (27,28), deficits on a spatial working memory task and a Go NoGo attentional response task (29), and poor performance on trail-making tests A and B (30).

We present data here on dysbindin-1 protein and gene expression in the same DLPCF tissue samples of schizophrenia cases and matched controls. We focus on this brain area not only because it is commonly dysfunctional in schizophrenia (31,32), but because the DLPCF plays a role in all the cognitive tasks on which carriers of DTNBP1 risk SNPs perform poorly. Its role in these tasks is indicated in studies identifying brain regions involved in general cognitive ability (33,34), IQ (34,35), spatial working memory (36,37) and mental flexibility (38,39).

RESULTS

Dysbindin-1 isoforms in DLPFC

According to the Reference Sequence (RefSeq) database of NCBI, a highly curated transcript collection (40), there are three validated splice variants of DTNBP1 mRNA among the sixteen deduced in the AceView database (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html, see Fig. 1). These appear to be the most commonly expressed transcripts based on the number of cDNA clones available for their reconstruction. They encode dysbindin-1A, -1B and -1C (NCBI accession nos NP_115498, NP_898861 and NP_898862, respectively). These isoforms of dysbindin-1, along with those of dysbindin-2 and -3, constitute the dysbindin protein family, all of whose members share a still poorly understood dysbindin domain (4). The length and component parts of dysbindin-1 isoforms are indicated in Figure 2A. Unlike dysbindin-2 and -3, all the isoforms of dysbindin-1 have a coiled-coil domain (CCD) important for certain protein–protein interactions. Isoform A (351 amino acids in humans) is the full-length dysbindin-1 with a PEST domain at its C terminus. Isoform B (303 amino acids), which is not expressed in the mouse, differs from isoform A only in its C-terminus region, which is shorter and lacks a PEST domain. In contrast, isoform C (270 amino acids) differs from isoform A only in the absence of an N terminus region in front of its CCD.

Using Oxford PA3111, a validated dysbindin-1 antibody (7,8) generated against the C-terminus region of isoform A (Fig. 2B), we detected three bands in Western blots of DLPC whole-tissue lysates (Fig. 2C), as we have in synapto-somal fractions of the same tissue. We previously detected no more than two bands with PA3111 in whole-tissue lysates of human brain tissue (7). Detection of three bands in the present study may reflect use of larger protein samples (50 μg versus 20–40 μg), denser gels (12% versus 10%) and more sensitive chemiluminescence detection kits. The three bands occurred at about 48, 36 and 32 kDa (Fig. 2C). No bands were observed when the antibody had been preadsorbed with the immunogen or full-length dysbindin-1. The top band at ~48 kDa was readily identified as full-length dysbindin-1, which has been reported to run at ~50 kDa (7,8,10). This is greater than the predicted 40 kDa mass of dysbindin-1A, which is probably attributable to its highly acidic C terminus (4,10) and possibly to post-translational modifications [i.e. phosphorylation (4) and/or ubiquitination (41)]. The middle and bottom bands are almost identical to the predicted molecular masses of dysbindin-1B and -1C (35 and 30 kDa, respectively).

As shown in Figure 2C, the identity of the 48, 36 and 32 kDa bands seen with Oxford PA3111A were confirmed with other polyclonal antibodies raised against synthetically derived peptide sequences in full-length dysbindin-1. One (UPenn 329) was raised against a 14 amino acid C-terminus sequence (amino acids 313–326) that is shared by dysbindin-1A and -1C, whereas the other (UPenn 331) was raised against a 14 amino acid N terminus sequence (amino acids 24–37) that is shared by dysbindin-1A and -1B (Fig. 2B). As the location of their immunogens predicted, UPenn 329 recognized the top and bottom dysbindin-1 bands (i.e. isoforms A and C), whereas UPenn 331 recognized the top and middle dysbindin-1 bands (i.e. isoforms A and B, Fig. 2C). Even at a dilution of 1:6000, however, UPenn 331 had an unusually high affinity for dysbindin-1B with only low affinity for dysbindin-1A and no detectable affinity for dysbindin-1C (Fig. 2C). The failure of UPenn 331 to recognize dysbindin-1C is expected since its immunogen lies in the
N-terminus region of dysbindin-1A, which is absent in dysbindin-1C. These results indicate that UPenn 331 is a very sensitive and largely selective antibody for dysbindin-1B. It thus yields data complementary to Oxford PA3111, which has a high affinity for dysbindin-1A and -1C but apparently much lower affinity for dysbindin-1B judging from the weaker middle band seen in PA3111A blots (Fig. 2C). We consequently used Oxford PA3111A to detect dysbindin-1A and -1C, but UPenn 331 to detect dysbindin-1B.

Dysbindin-1 isoform expression in DLPFC of schizophrenia versus control cases

Representative results of Western blotting for dysbindin-1 isoforms in the DLPFC of schizophrenia cases and matched controls are shown in Figure 3. As detailed in Materials and Methods, such results enabled calculation of a ratio that expresses the amount of a dysbindin-1 isoform in each of the 28 schizophrenia cases compared to its matched control. A mean case–control ratio was then calculated from the results on the original and replication experiments. Log2 transformed mean ratios for all 28 pairs of schizophrenia and matched controls are graphed in Figures 4–6. The results were highly reproducible as shown by strong correlations between the ratios obtained in the original and replication experiments for all dysbindin-1 isoforms (e.g. for dysbindin-1A, \( r = 0.946, P = 9.2 \times 10^{-14} \)).

For dysbindin-1A, the direction (plus or minus) and magnitude of the case–control ratios were highly inconsistent (Fig. 4A). For dysbindin-1B, the ratios were likewise very inconsistent in direction, and their magnitudes were generally very small (Fig. 5A). Pair-wise analyses of these ratios with the Wilcoxon signed-rank test accordingly showed no significant differences between schizophrenia cases and their matched controls in levels of these two dysbindin-1 isoforms, which was the case whether raw or normalized data were used (\( n = 28, W = 78, P = 0.38 \) for dysbindin-1A; \( n = 28, W = -12, P = 0.89 \) for dysbindin-1B).

For dysbindin-1C, however, protein levels were reduced from 12 to 85% in the schizophrenia cases compared with matched controls in 20 of 28 pairs (71.42%, Fig. 6A). Analysis of all 28 pairs showed that dysbindin-1C was reduced in the DLPFC of the schizophrenia cases by an average of 46% compared to matched controls (\( W = 202, P = 0.0221 \)). In the 20 pairs where such reductions were seen in the schizophrenia cases, the average decrease was 59.8%. Although no significant correlations were found between dysbindin-1C levels and measures of global cognition, negative symptoms or positive symptoms (see Materials and Methods), these measures were not available on 15 of the 28 schizophrenia cases included in this study. As a result, no conclusions can be drawn about the relationship of dysbindin-1C to these variables.

Dysbindin-1 mRNA expression in DLPFC of schizophrenia versus control cases

To determine if the observed isoform-specific reduction in dysbindin-1 may be due to reductions in \( DTNBP1 \) gene expression, we measured the latter with quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) using primer pairs for \( DTNBP1 \) transcripts in general (DYS-95 and DYS-102), for \( DTNBP1 \) transcript d (DYS-d) encoding dysbindin-1B, and for \( DTNBP1 \) transcript e (DYS-e) encoding...
dysbindin-1C (see Table 2 for the primer sequences and Fig. 1 for their transcript targets). Primers selective for dysbindin-1A could not be designed since all sequences in its transcript are shared by many other DTNBP1 transcripts. Nevertheless, results with the pan DTNBP1 primers DYS-95 and -102 estimate levels of dysbindin-1A transcripts, since these are by far the most common dysbindin-1 transcripts in all tissues judging from Northern blotting results (4). This is consistent with our quantitative real-time polymerase chain reaction (qPCR) experiments, in which reactions with DYS-95 and DYS-102 had much lower cycle thresholds than reactions with primers for dysbindin-1B (DYS-d) or dysbindin-1C (DYS-e). Assuming equivalent amplification efficiency for the different primers, the cycle threshold (Ct) values for mRNA of dysbindin-1A, -1B and 1C suggest that their relative abundance in the normal DLPFC is about 64:16:1. We report data here only on data normalized to the expression of three housekeeping genes: beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyl-transferase (HPRT) as described in Materials and Methods.

In the same 28 case–control pairs in which relative dysbindin-1 isoform levels were measured, the schizophrenia cases showed evidence of significantly increased levels of dysbindin-1A mRNA judging from PCR product with DYS-95 (t = 2.72, df = 54, P = 0.009) and DYS-102 (t = 2.77, df = 54, P = 0.008). They also showed significantly greater levels of dysbindin-1B mRNA as measured with DYS-d (t = 2.34, df = 54, P = 0.023), but normal levels of dysbindin-1C transcripts measured with DYS-e (t = 0.846, df = 54, P = 0.401). These findings are illustrated in Figure 7.

We found no evidence for correlated changes in dysbindin-1 protein and gene expression within the 28 case–control pairs. As shown in Figures 4–6, neither the direction nor the magnitude of the case–control ratios for the dysbindin-1 isoforms were predicted by those of the case–control ratios for the encoding mRNAs. The apparent absence of covariance between the isoforms and their transcripts was confirmed statistically. The Pearson correlation coefficient between case–control ratios of dysbindin-1A and its mRNA was −0.008 (P = 0.65) estimated with DYS-95 and −0.090 (P = 0.80) estimated with DYS-102; that between dysbindin-1B and its mRNA was −0.015 (P = 0.941) and that between dysbindin-1C and its mRNA was −0.21 (P = 0.276).

**DTNBP1 genotyping and its relation to DTNBP1 gene and protein expression**

Only two controls and two schizophrenia cases carried the rare allele of the P1578 SNP in DTNBP1 tagging a risk haplotype for schizophrenia in the USA (15). As a result, we were unable to test the effect of this haplotype on either gene or protein expression.
expression of DTNBP1. But we were able to test if the observed differences between schizophrenia and control, cases occurred in the absence of the haplotype. That proved to be the case. Exclusion of the case–control pairs with individuals carrying the rare allele of P1578 had no effect on the outcome of statistical analyses on dysbindin-1 isoforms or transcripts.

**Consideration of potential confounding variables**

The observed variations in dysbindin-1 isoforms and their mRNAs in our schizophrenia cases were not readily attributed to non-diagnostic variables. Our matched pairs design ensured that the two diagnostic groups (schizophrenia cases and normal controls) were of the same race, had the same male/female ratio and did not differ significantly in age. Nor did the two groups differ significantly in brain tissue pH. The mean postmortem interval (PMI) was longer for the schizophrenia cases (11.9 ± 5.4 h) than for the controls (7.7 ± 3.8 h) (t = 3.46, df = 26, P = 0.0019), but PMI was not found to be significantly correlated with normalized levels of the dysbindin-1 isoforms or their mRNAs. This probably reflects the low PMIs for the two groups, both well below the time (23 h) by which appreciable degradation of many brain proteins occurs (42), especially when not stored before autopsy at the cool temperatures used in this study (43). There was also no significant difference between diagnostic groups in RNA integrity: the mean ± SD of the A260/280 nm ratio was 2.064 ± 0.02 for controls and 2.064 ± 0.04 for the schizophrenia cases, indicating that RNA quality was high in both groups. As was the case for normalized dysbindin-1 proteins, dysbindin-1 mRNAs normalized to expression of the housekeeping genes GAPDH, B2M and HPRT were not significantly correlated with PMI, age or

![Figure 4](image-url)
brain pH. Neither diagnostic group displayed evidence of neurodegenerative processes: none of the subjects had gross neuronal loss, infarcts or abnormally high densities of amyloid plaques, neurofibrillary tangles or Lewy bodies. Finally, levels of dysbindin-1 isoforms and their mRNAs were not correlated with the antipsychotic dosage of our schizophrenia cases expressed in chlorpromazine equivalents a month prior to death, a finding in accordance with our study on dysbindin-1 protein expression in the hippocampal formation of such cases (7). This is consistent with other studies showing that chronic haloperidol administration to mice has no significant effect on dysbindin-1 gene (44) or protein expression (7). No other medications were common to a majority of schizophrenia cases studied, none of whom had a history of substance abuse.

DISCUSSION
Consistent with the preliminary report by another laboratory (12), the current study finds that dysbindin-1 in the DLPFC is reduced in schizophrenia. Of its three major isoforms, however, only dysbindin-1C was significantly altered. When compared with matched controls, ~71% of the schizophrenia cases displayed a reduction in dysbindin-1C averaging ~60%. As detailed above, these results and those discussed below on dysbindin-1 mRNA are not attributable to uncontrolled differences between cases and controls in age, sex, PMI, brain tissue pH, neurodegenerative pathology or antipsychotic medication.

The observed dysbindin-1C reduction in whole-tissue lysates of the DLPFC probably reflects a reduction of this isoform at the synaptic level. Unlike dysbindin-1A and -1B, which are concentrated in both cell nuclei (4,13) and synapses (4,7,8,14), our tissue fractionation studies on the DLPFC, anterior cingulate gyrus, superior temporal gyrus and hippocampal formation show that dysbindin-1C in the human brain is concentrated only in synapses with a small amount associated with synaptic vesicles and a large amount in PSDs (4). This is consistent with the fact that synaptic vesicles and PSDs visualized electron microscopically are labeled with the Oxford antibody PA3111, which has a high affinity for dysbindin-1C (4,8).

Our previous work provides a precedent for reduced synaptic levels of dysbindin-1 in schizophrenia. In a quantitative immunohistochemical study on the hippocampal formation using the Oxford PA3111 antibody, we discovered significant reductions in dysbindin-1 at pre-synaptic sites in 73–93% of

Figure 5. Levels of dysbindin-1B protein (A) and its mRNA levels (B) in the DLPFC of schizophrenia cases compared to matched controls (see caption to Fig. 4 for further explanation). Note the clear lack of correspondence between case–control ratios for dysbindin-1B and its transcript.
our schizophrenia cases compared with matched controls. The percent reduction depended on which case cohort and anatomical area was studied (7). Our more recent Western blotting work on synaptosomal preparations of the hippocampal formation with the antibodies used in the present study find that the pre-synaptic reduction in schizophrenia is due to decreased dysbindin-1B associated with synaptic vesicles and that this decrease is accompanied by reduced dysbindin-1C also associated with synaptic vesicles but more so with PSDs (4). Dysbindin-1A levels, as in the DLPFC, were unaltered in the hippocampal formation. There is consequently evidence that isoform-specific dysbindin-1 reductions occur in schizophrenia and that the isoforms affected vary across brain areas.

The cause of reduced dysbindin-1C in the DLPFC of schizophrenia cases is unknown. It does not require the DTNB1 risk haplotype of Funke et al. (15) since the reduction occurred in its absence. While we did not test for other DTNB1 haplotypes associated with schizophrenia, our results argue against the possibility that altered DTNB1 gene expression could account for reduced dysbindin-1 in this disorder. Such a possibility was raised by Weickert et al. (9), who found that gene expression of DTNB1 in schizophrenia was 15–20% lower in nearly all DLPFC cell layers as determined using in situ hybridization with a riboprobe that would hybridize with mRNAs of all major dysbindin-1 isoforms. Using qRT–PCR with two pan-dysbindin-1 primer sets (DYS-95 and DYS-102), however, we failed to replicate that finding in a larger case sample (of admittedly different ethnicity) with far lower PMIs [28 Caucasian schizophrenia cases with 11.9 ± 5.4 h PMIs in our study versus 14 mainly African-American schizophrenia cases with 38.0 ± 21.9 h PMIs in Weickert et al. (9)]. Moreover, despite the high quality of our RNA and protein samples, we found that the proportional amounts by which schizophrenia cases differed from their matched controls in dysbindin-1 mRNAs did not correspond to the proportional amounts by which they differed from their controls in the encoded dysbindin-1 isoforms.

The lack of correspondence between case–control variation in dysbindin-1 gene and protein expression was striking. In the schizophrenia cases, the levels of dysbindin-1A and dysbindin-1B transcripts were increased without increase in the encoded proteins. This might suggest operation of riboswitch mechanisms (45) compensating for increased dysbindin-1A and -1B protein degradation, but that possibility

Figure 6. Levels of dysbindin-1C protein (A) and its mRNA levels (B) in the DLPFC of schizophrenia cases compared to matched controls (see caption to Fig. 4 for further explanation). Note the clear lack of correspondence between case–control ratios for dysbindin-1C and its transcript.
is not consistent with the absence of any significant correlations between case-control ratios of these isoforms and their transcripts. Nor was there any indication of such a mechanism in the case of dysbindin-1C protein, levels of which were reduced without a change in levels of its transcript. These findings indicate that factors other than altered DTNBP1 mRNA expression are necessary to account for decreased dysbindin-1C in the DLPFC of schizophrenia cases. Our data do not indicate what those factors are, but they could include abnormalities in mRNA translation and in processes promoting dysbindin-1 degradation [e.g. oxidative stress, phosphorylation and/or ubiquitination (4,41)].

Although the causes of the observed dysbindin-1C reductions are unknown, their synaptic consequences are suggested by studies on sandy mice. These animals have a deletion mutation in the mouse ortholog of DTNBP1 (46) leading to reductions in the two major dysbindin-1 isoforms expressed in mice (i.e. dysbindin-1A and -1C) (4,46). The reduction in these isoforms is total in the homozygous sandy mice (4,46). In the putative rodent equivalent of the human DLPFC (i.e. prelimbic cortex), homozygous sandy mice display synaptic abnormalities in pyramidal cells and the fast-spiking GABAergic interneurons innervating those pyramidal cells. These parvalbumin-containing interneurons are driven via NMDA receptor-mediated input (47) and in turn inhibit pyramidal cell output in a manner generating gamma frequency oscillations in the cerebral cortex enhancing cognition (48,49). In the presumptive DLPFC of homozygous sandy mice, pyramidal cells show both evidence of afferent and efferent impairments, specifically reductions in NMDA receptor-mediated glutamatergic currents and stimulus-induced glutamate release (50). In the same brain area, fast-spiking interneurons of homozygous sandy mice likewise show evidence of afferent and efferent impairments, namely decreased NMDA receptor-mediated glutamatergic input from the pyramidal cells and decreased release of GABA onto pyramidal cells (51). The afferent abnormalities just noted are postsynaptic phenomena and are consistent with view that reduction in postsynaptically localized dysbindin-1A and/or -1C may promote hypofunction of NMDA channels in both pyramidal neurons and fast-spiking interneurons in the DLPFC. A greater impact is expected on interneurons, however, because their excitability (unlike that of pyramidal cells) is driven mainly by NMDA channels (47,52). Fast-spiking interneurons in the putative rodent DLPFC are, in fact, less excitable in sandy than in wild-type mice (53).

The observed reduction of dysbindin-1C may thus promote dysfunction in the DLPFC by inducing NMDA receptor hypofunction in fast-spiking interneurons and consequently impairing generation of gamma frequency oscillations, which facilitate cognitive processes as noted above (48,49). An increasing number of studies indicate that NMDA receptor hypofunction (47,54) and related deficits in generating gamma oscillations (54–56) are basic features of schizophrenia that can contribute to its cognitive deficits (52,55,57,58). The DLPFC is known to be involved in several types of working memory, including spatial working memory (36,37), deficits in which are common in schizophrenia (20–22). Homozygous sandy mice also display working memory deficits (59–61), including those detected on a non-matching to sample task sensitive to disruption of the rodent prefrontal cortex (59,60). It is possible, then, that reduced dysbindin-1C in the DLPFC of schizophrenia cases contributes to spatial working memory deficits in that disorder. This merits further investigation given that the continuing absence of highly effective treatments for the debilitating cognitive deficits in schizophrenia.

**MATERIALS AND METHODS**

**Research design, subjects and clinical assessment**

A matched-pairs design was used to compare fresh-frozen DLPFC tissue samples from 28 schizophrenia cases with 28 non-psychiatric controls matched for sex and for age within 5 years (Table 1). All subjects were Caucasians who
Table 1. Demographic and autopsy data on cases studied

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<tr>
<th>Variable</th>
<th>Control cases</th>
<th>Schizophrenia cases</th>
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<tbody>
<tr>
<td>Number of cases</td>
<td>28 Caucasians</td>
<td>28 Caucasians</td>
</tr>
<tr>
<td>Mean age ± SD</td>
<td>83.7 ± 9.0 years</td>
<td>81.7 ± 7.7 years</td>
</tr>
<tr>
<td>Gender ratio: male/female</td>
<td>11/17</td>
<td>11/17</td>
</tr>
<tr>
<td>Mean PMI ± SD</td>
<td>7.7 ± 3.8 h</td>
<td>11.9 ± 5.4 h</td>
</tr>
<tr>
<td>Mean brain tissue pH ± SD</td>
<td>6.19 ± 0.04</td>
<td>6.46 ± 0.26</td>
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There were no significant differences between control and schizophrenia cases on any variable listed except PMI. As with age and pH, however, PMI was not significantly correlated with levels of the normalized proteins or mRNA measured.

had been living in eastern or central Pennsylvania, USA. Clinical records either indicated or suggested that all were of western European ancestry. The schizophrenia cases participated in a longitudinal study of prospectively diagnosed subjects approved by an Institutional Review Board at the University of Pennsylvania and conducted by the Schizophrenia Research Center at that university (62). They met the diagnostic criteria for schizophrenia in DSM-IV (63) as determined in consensus conferences after review of medical records, direct clinical assessments, and interviews with care providers. None had alternate or ambiguous DSM-IV diagnoses nor a history of substance abuse, neurological disorders (e.g. Alzheimer’s disease, epilepsy, Parkinson’s disease) or acute neurological insults (anoxia, strokes, traumatic brain injury). None of the subjects (controls or schizophrenia cases) had been on ventilators near the time of death.

All schizophrenia cases had at least one antemortem clinical assessment in which attempts were made to evaluate patients on the Mini-Mental State examination (MMSE, a test of global cognition), the Brief Psychiatric Rating Scale, the Scales for Assessment of Negative (SANS) and Positive Symptoms (SAPS), the Abnormal Involuntary Movement Scale (AIMS) and a physical dependency rating scale. Of the 28 schizophrenia cases included in this study, only 13 had been sufficiently cooperative to complete MMSE, SANS and SAPS testing. Other schizophrenia cases could not be substituted for these because they did not meet one or more of the inclusion criteria (i.e. matching available controls on demographic and autopsy variables, absence of vascular and neurodegenerative dementia and availability of fresh frozen DLPFC tissue).

Tissue collection and neuropathological assessment

Autopsy consent was obtained from next-of-kin or legal guardian in all cases. After death, cases were stored at 2–4°C until transport to the University of Pennsylvania, where all autopsies were performed. With the sole exception of a schizophrenia case with a 30 h PMI retained due to its well-preserved protein and RNA, all the cases had low PMIs (mean ± SD = 11.9 ± 5.4 h for schizophrenia cases and 7.7 ± 3.8 h for controls).

After sagittal bisection of the brain, one hemisphere was sectioned into coronal slabs, which were frozen overnight at −80°C and then sealed in plastic bags for storage at that temperature. DLPFC (Brodmann areas 9 + 46) was later identified in the frozen coronal slabs, from which samples including all six layers were dissected for the present study. The same tissue samples were used for measurement of dysbindin-1 isoforms and their mRNAs. For neuropathological assessment, DLPFC tissue was dissected from the non-frozen hemisphere at autopsy. This tissue was fixed in neutral-buffered formalin for 12–24 h, embedded in paraffin and sectioned coronally at 6 μm. Some of the sections were stained with hematoxylin and eosin to detect gross cell loss and infarcts; others were immunohistochemically reacted with antibodies to β-amyloid 1–42 (BA2332) (64), hyperphosphorylated tau (AT8, Thermo Scientific, Rockford, IL, USA) or either ubiquitin (Ub-1, Zymed Laboratories, South San Francisco, CA, USA) or α-synuclein (LB509) (65) to detect amyloid plaques, neurofibrillary tangles or Lewy bodies, respectively.

No cases were included in this study which met clinical and neuropathological criteria for any form of vascular or neurodegenerative dementia. All the cases included were thus free of gross neurodegeneration and did not have abnormal levels of senile plaques, neurofibrillary tangles, or Lewy bodies.

Dysbindin-1 antibodies

Dysbindin-1 isoforms in human DLPFC lysates were detected with three polyclonal rabbit antibodies developed for use by our research group: Oxford PA3111 (0.1 mg/ml), UPenn 329 (0.2 mg/ml) and UPenn 331 (1.4 mg/ml). PA3111 was raised against a long C-terminus segment of mouse dysbindin-1A (amino acids 196–352) as described earlier (4,7,8). UPenn 329 was generated for the senior investigators of the current report by Sigma Aldrich (St Louis, MO, USA) against a synthetic peptide consisting of amino acids 313–326 in the C-terminus region of human dysbindin-1A and -1C (i.e. NCBI accession nos. NP_115498 and NP_898862, respectively). UPenn 331 was also generated by Sigma Aldrich, but against a synthetic peptide consisting of amino acids 24–37 in the N-terminus region of human dysbindin-1A and -1B (NCBI proteins NP_115498 and NP_898861, respectively).

Quantification of DTNBP1 isoforms by western immunoblotting

Fresh frozen samples of the DLPFC were thawed, homogenized and lysed in RIPA buffer (Sigma Aldrich R0278) with a protease inhibitor cocktail (Sigma Aldrich P8340) and EDTA at a final concentration of 1 mm. After centrifugation at 10 000g for 10 min, the whole-cell supernatants were aliquoted and stored at −80°C. Protein concentrations were assayed using a bicinchoninic (BCA) protein assay (Pierce Chemical, Thermo Fisher Scientific).

Pilot studies indicated protein detection limits, linear range of protein detection, and membrane exposure times for optimal quantification of protein concentrations. We accordingly loaded 20 μg protein in each lane for electrophoresis. Protein samples were denatured in 5× Laemmli sample loading buffer (0.3 M Tris, 100 mg/ml SDS, 0.1 ml/ml 2-mercaptoethanol, 0.5 ml/ml glycerol, 0.05 mg/ml bromophenol blue, pH 6.8) at 95°C. After boiling for 5 min, samples were centrifuged at 16 000g for 5 min before loading on gels. To
Table 2. Primers used for quantitative RT–PCR

<table>
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<th>Primer</th>
<th>Targeted mRNA (NCBI)</th>
<th>Targeted DTNBP1 transcript (AceView)</th>
<th>Primer sequence (forward/reverse)</th>
<th>Product size (bp)</th>
<th>Protein encoded by targeted mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DYS-95</td>
<td>NM_032122</td>
<td>a–i+a</td>
<td>5'TCCAGCCTTAATCCGAGAC3'</td>
<td>95</td>
<td>pan Dysbindin-1(a)*</td>
</tr>
<tr>
<td>DYS-102</td>
<td>NM_032122</td>
<td>a–i+k+g–m</td>
<td>5'CAGAGACATGAGCGCTGTC3'</td>
<td>102</td>
<td>pan Dysbindin-1(b)*</td>
</tr>
<tr>
<td>DYS-d</td>
<td>NM_183040</td>
<td>d</td>
<td>5'ACGATCAAAACACCCAAGC3'</td>
<td>96</td>
<td>Dysbindin-1B</td>
</tr>
<tr>
<td>DYS-e</td>
<td>NM_183041</td>
<td>e</td>
<td>5'AGGAAAACACCCACACCTG3'</td>
<td>66</td>
<td>Dysbindin-1C</td>
</tr>
<tr>
<td>B2M†</td>
<td>NM_000408</td>
<td></td>
<td>5'TCTGTCATTCATTGAGTAATC3'</td>
<td>86</td>
<td>B2M</td>
</tr>
<tr>
<td>GAPDH‡</td>
<td>NM_002046</td>
<td>—</td>
<td>5'TGCACACACACCTGTTAGC3'</td>
<td>87</td>
<td>GAPDH</td>
</tr>
<tr>
<td>HPRT†</td>
<td>NM_000194</td>
<td>—</td>
<td>5'GGCATGGACTGTGGTCATGAG3'</td>
<td>94</td>
<td>HPRT</td>
</tr>
</tbody>
</table>

*The two pan dysbindin-1 primers can recognize many DTNBP1 transcripts, including those for dysbindin-1A, -1B and -1C, but transcripts for dysbindin-1A appear to far exceed those for other dysbindin-1 isoforms as explained in Results. DYS-95 and -102 differ only in recognition of minor transcripts (i–m) not yet detected in the DLPFC. Of the DTNBP1 transcripts listed on AceView, only a–e are known to be expressed in the DLPFC (R. Straub, personal communication).
†The B2M (beta-2-microglobulin), HPRT1 (hypoxanthine phosphoribosyl-transferase 1) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primer sequences are described by Vandesompele et al. (71).

minimize variability due to variable transfer efficiency and immunoreactivity (66), samples from a schizophrenia case and its matched control were always run side-by-side on the same gel. To test reproducibility across blots, one or two reference samples were loaded on every gel. Reference samples were simply DLPFC lysates of a normal human case that yielded strong signals for dysbindin-1 isoforms in tests. Proteins were separated by SDS–polyacrylamide gel electrophoresis on precast 12% Tris–glycine 1.5 mm gels (Invitrogen Novex, Carlsbad, CA, USA). The proteins were then electroblotted onto a polyvinylidene difluoride (PVDF) transfer membrane (Bio-Rad, Hercules, CA, USA) using a Novex XCell II Blot Module at 30 V for 2 h.

Two blots with samples of the same lysates were run concurrently. After rinsing with Tris–buffered saline in 0.1% Tween-20 (Sigma Aldrich), one blot was stained using the MemCode reversible stain kit (Pierce Chemical) (67) to assess transfer efficiency and visualize protein loading. The relative amounts of total protein between 55 and 30 kDa were determined by optical density measurement in a GS-800 Bio-Rad calibrated densitometer. After de-staining and blocking with 5% wt/vol non-fat dry milk in PBST (phosphate buffered saline with 0.1% Tween-20) for 1 h, this first blot was incubated at 4°C overnight with the rabbit polyclonal antibody Oxford PA3111 at a dilution of 1:1000 in TBS (with 5% milk and 0.1% Tween-20). The other blot was cut horizontally at the level of the 38 kDa marker: the top part was incubated with a mouse monoclonal β-actin antibody (Sigma Aldrich A1978) at a dilution of 1:12 000, and the bottom was incubated with the rabbit polyclonal dysbindin-1 antibody UPenn 331 at a dilution of 1:6000. Both of these blots were reacted overnight at 4°C. Like MemCode staining, immuno-blotting of β-actin served as a control for variable protein levels due to tissue storage time, sample preparation, protein loading and transfer efficiency. After washing, immunoblots were incubated for 1 h at room temperature with a horseradish-peroxidase-linked secondary anti-rabbit or anti-mouse antibodies (Amersham Pharmacia/GE Healthcare), developed via a chemiluminescence reaction (ECL or ECL plus kit, Amersham Biosciences) and exposed to film (Amer- sham Hyperfilm ECL, GE Healthcare). The bands were quantified in a Bio-Rad densitometer. The immunoblotting experiments were repeated according to strictly standardized procedures to minimize variability in results across blots.

Quantification of mRNA levels of DTNBP1 transcripts by qRT–PCR

Total RNA was obtained from the same fresh frozen blocks of DLPFC tissue sampled for protein measurements. RNA was extracted using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). A pilot test on 12 randomly selected samples using the Agilent 2100 Bioanalyzer indicated a high degree of RNA integrity (28 s/18 s rRNA ratios of at least 1.8). Yield and purity of all RNA samples studied were determined by spectrophotometric analysis. The mean ± SD of the 260/280 nm ratios was 2.06 ± 0.04 and 2.06 ± 0.04 for controls and schizophrenia cases. Contamination by genomic DNA was removed by treating 2 μg of each RNA sample with 40 U deoxyribonuclease (Sigma Aldrich D7691) for 1 h at 37°C. An Applied Biosystems (Foster City, CA, USA) high-capacity kit was used to synthesize cDNA from 20 μl of RNA from each sample by means of reverse transcriptase reactions. RNA extraction, DNA removal and reverse transcriptase reactions were performed concurrently on all samples to be compared.

DTNBP1 mRNA levels were measured using qPCR with primer pairs specified in Table 2. As internal controls, expression of three housekeeping genes, namely B2M, GAPDH, and HPRT. The primer pairs used for the housekeeping genes are also specified in Table 2.
Four primer pairs were designed to target *DTNBP1* transscripts (Table 2 and Fig. 1). Two of these, DYS-95 and DYS-102 (named for the size of the PCR product they yield), targeted most of the 16 *DTNBP1* transcripts currently listed by AceView (2007), including all known to be expressed in the DLPFC (*DTNBP1* transcripts g–g, R. Straub, personal communication). The other two primer sets used are selective for transcripts of either dysbindin-1B (DYS-d) or dysbindin-1C (DYS-e). The Ct values for DYS-95 and DYS-102 were almost the same, whereas those for DYS-d were about four times longer and those for DYS-e about six times longer.

qPCR was performed with an Applied Biosystems Prism 7900HT sequence detector system. A standard curve method was used to determine relative levels of gene expression (68,69). Amplification of cDNA was performed on a pooled cDNA sample from human controls at different concentrations to define a linear range for all genes. Predicted sizes of PCR amplicons were verified by agarose gel electrophoresis. No detectable signal was found upon amplification of selected reverse transcriptase-absent cDNA samples, indicating minimal genomic DNA contamination. Once optimal reaction conditions were determined, PCR amplification was performed in a total reaction volume of 20 μl with 2 μl cDNA (50 ng samples), 0.5 μM of each primer and 10 μl of SYBR Green Master Mix (Applied Biosystems). The PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 10 min. All samples for a target transcript were measured in a single 384-well plate. All measurements were performed in triplicate.

*DTNBP1* genotyping

DNA was obtained from fresh frozen brain tissue using the Gentra PUREGene cell kit (Qiagen, Valencia, CA, USA). Using a TaqMan S’ exonuclease allelic discrimination assay (Applied Biosystems), the DNA was genotyped for a SNP (P1578→rs1018381) tagging a six-SNP *DTNBP1* risk haplotype for schizophrenia reported in a case–control study on US Caucasians (15).

Data analysis

**Western blotting.** Comparisons of dysbindin-1 levels were naturally complicated by the fact that the number of samples exceeded the loading lanes of a single gel. We initially tried solving this problem by expressing optical density (OD) of bands as a percentage of one or two reference samples run on all blots. We found, however, that calibrating OD measurements to reference samples did not yield values as reliable across blots as did pair-wise ratios of each schizophrenia case to its matched control run on the same blot, as earlier recognized by Albert et al. (70). We thus used two approaches. For analyses requiring comparison across blots (i.e. correlation of age, brain pH and PMI with dysbindin-1 isoform levels in all samples), the OD of each band in a western blot was divided by that of the reference sample (or samples) run on the same blot after normalizing for loading variations using β-actin levels. Averages of these values on the original and replication blots were used in correlational analyses. For analyses which did not require comparison across blots (i.e. Wilcoxon tests of differences within case–control pairs run on the same blot), we determined the OD for a dysbindin-1 isoform in each schizophrenia case compared with its matched control run in an adjacent lane of the same blot using (a) raw OD data, (b) OD data normalized to β-actin and (c) OD data normalized to density of MemCode staining. These OD ratios obtained from data on the original and replication blots were averaged and then log₂ transformed such that a zero ratio reflected no difference between a schizophrenia case and its matched control, a negative ratio reflected a decrease in the schizophrenia case, and a positive ratio reflected an increase in that case. The log transformation permits use of the Wilcoxon signed-rank test for ratio data, because the log of a ratio between two values \(X/Y = \log X - \log Y\).

**qRT–PCR.** Since all samples to be compared were run at the same time in one 384-well plate, data analysis was simplified compared with that for western blotting data. Real-time PCR data accrual and analysis were performed using SDs version 2.0 software (Applied Biosystems). Data points were omitted if they varied more than 1% from the mean of triplicate samples based on raw cycle threshold values. The standard curve had a coefficient of determination (\(R^2\)) > 0.996 and a slope between \(-3.32\) and \(-3.57\) in each experiment, which indicated an amplification efficiency of 91–100%. Negative controls (i.e. no-template cDNA) yielded no detectable signal. An expression value for each reaction was quantified against a standard curve constructed from serial dilutions of the pooled cDNA. Without normalization, there were significant correlations between tissue pH and all *DTNBP1* transscripts ([3.2], P < 0.008) except that for dysbindin-1C (r = 0.19, P = 0.12). There were no significant correlations with tissue pH, however, when the expression values of dysbindin-1 mRNAs were normalized to a geometric mean of the mRNA expression values for the three internal control genes (*B2M, GAPDH* and *HPRT*) following the method of Vandesompele et al. (71). Consequently, only mRNA expression values normalized in this manner were used in final analyses.

**Statistical analyses.** These were performed with GraphPad Prism 3.0 (GraphPad Software, La Jolla, CA, USA). Unpaired t-tests were used to test statistical differences between schizophrenia cases and controls on demographic and autopsy variables (age, brain pH and PMI). The non-parametric Wilcoxon signed-rank test was used to test if log transformed pair-wise ratios derived from Western blotting data were statistically different from the hypothetical median value of zero reflecting no significant differences between schizophrenia and control cases. For qRT–PCR data, unpaired t-tests were used to evaluate differences in PCR products between the two diagnostic groups. For comparison of case–control ratios of proteins and transcripts, Pearson correlational analyses were used. The \(P\)-values reported are two-tailed with those less than 0.05 considered significant.

**ACKNOWLEDGEMENTS**

We thank the clinical staff and residents of the Schizophrenia Center and the Division of Medical Pathology at the
University of Pennsylvania for assessment and autopsy of the subjects. We are grateful for the collaboration of state hospitals in the Commonwealth of Pennsylvania and above all for the generous participation of the patients and their families.

Conflict of Interest statement. None declared.

FUNDING
This work was supported by US National Institutes of Health grants [MH072880, MH064045].

REFERENCES


