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

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Running Title: Dysbindin-1 isoforms in DLPFC of schizophrenia cases

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Abstract

DTNBP1 (dystrobrevin binding protein 1) remains one of the top candidate genes in schizophrenia. Reduced expression of this gene and the protein it encodes, dysbindin-1, has been reported in the dorsolateral prefrontal cortex (DLPFC) of schizophrenia cases. It has not been established, however, if all dysbindin-1 isoforms are reduced in the DLPFC or if the reduction is associated with reduced DTNBP1 gene expression. Using Western blotting of whole-tissue lysates of the DLPFC with antibodies differentially sensitive to the three major isoforms of this protein (dysbindin-1A, -1B, and -1C), we found no significant differences between our schizophrenia cases and matched controls in dysbindin-1A or -1B, but did find a mean 46% reduction in dysbindin-1C in 71% of 28 case-control pairs ($p = 0.022$). This occurred in the absence of the one DTNBP1 risk haplotype for schizophrenia reported in the US and without alteration in levels of dysbindin-1C transcripts. Conversely, the absence of changes in the dysbindin-1A and -1B isoforms was accompanied by increased levels of their transcripts. We thus found no correspondence between alterations in dysbindin-1 gene and protein expression, the latter of which might be due to posttranslational modifications such as ubiquitination. Reduced DLPFC dysbindin-1C in schizophrenia probably occurs in PSDs, where we find dysbindin-1C to be heavily concentrated in the human brain. Given known postsynaptic effects of dysbindin-1 reductions in the rodent homolog of the prefrontal cortex, these findings suggest that reduced dysbindin-1C in the DLPFC may contribute to cognitive deficits of schizophrenia by promoting NMDA receptor hypofunction.

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 reviewed by several investigators.[2-4] Indeed, DTNBP1 remains among the top candidate genes for the disorder according to recent analyses.[5,6] DTNBP1 risk SNPs and haplotypes have been associated with a diverse group of cognitive deficits, which collectively form a core feature of schizophrenia.[7-9] Even in individuals without psychiatric illness, cognitive performance is worse in carriers of DTNBP1 risk SNPs than in non-carriers.[10-12] The same is true for Sz cases carrying such SNPs. They show greater decline in general cognitive ability from a premorbid to a clinical state,[13] lower general cognitive ability in the clinical state itself,[10] lower scores on verbal, performance, and full-scale IQ tests on the Wechsler Adult Intelligence Scales-Revised,[14, 15] deficits on a spatial working memory task and a Go NoGo attentional response task,[16] and poor performance on Trail-Making Tests A and B.[17]

DTNBP1 encodes the first known member of the dysbindin protein family, dysbindin-1,[4] which is expressed in diverse neuronal populations throughout the brain.[4, 18, 19] It is found in neuronal cell bodies, as well as pre- and post-synaptic sites in areas commonly affected in schizophrenia, such as the hippocampal formation and neocortex.[4, 19] Postmortem studies on schizophrenia cases have found dysbindin-1 reductions in the hippocampal formation[19] and dorsolateral prefrontal cortex (DLPFC),[20] although the cited work on the latter area has not been fully reported.

While not yet widely appreciated, there are multiple dysbindin-1 isoforms. We have 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 three most common DTNBP1 transcripts (i.e., NM_032122, NM_183040, and NM_183041 of the National Center for Biotechnology Information [NCBI],

respectively). We began to study these isoforms to determine which account for the observed dysbindin-1 reductions in schizophrenia. This is important because the isoforms are expected to play different neuronal roles as indicated by their differential distribution in synaptic and nuclear tissue fractions.[4, 21]

We focus here on the DLPFC not only because that area is commonly affected in schizophrenia,[22, 23] but also because all the cognitive tasks on which carriers of DTNBP1 risk SNPs perform poorly (see above) are mediated in part by the DLPFC. A role for the DLPFC in these cognitive tasks is evident in studies identifying brain regions involved in general cognitive ability,[24, 25] IQ,[25-27] spatial working memory,[28-30] and mental flexibility.[31, 32]

The present study is the first full report on dysbindin-1 protein expression and transcript-specific dysbindin-1 gene expression in the same schizophrenia cases. In such cases, we specifically investigated whether dysbindin-1 protein expression in the DLPFC is altered in an isoform-specific manner and if any such alteration is correlated with transcript-specific dysbindin-1 gene expression. We also tested if any observed alterations in dysbindin-1 isoforms or their transcripts occur in the absence of the DTNBP1 risk haplotype reported in a U.S. population by Funke et al.[33]

Materials and methods

Research Design and Subjects

A matched-pairs design was used to compare fresh-frozen DLPFC tissue from 28 Caucasian schizophrenia cases with 28 non-psychiatric Caucasian controls matched for sex and age within 5 years (Table 1). With the sole exception of a schizophrenia case with a 30 h postmortem interval (PMI) retained due to good protein and RNA preservation, all the cases had low PMIs (mean ± SD = 11.9 ± 5.4 h for schizophrenia cases and 7.7 ± 3.8 h for controls). The schizophrenia cases had participated in a longitudinal study of prospectively diagnosed subjects approved by an Institutional Review Board at the University of Pennsylvania and

conducted by the University of Pennsylvania's Schizophrenia Research Center as described previoulsy.[34] Research autopsy consent was obtained from a family member or legal guardian in all cases. Psychiatric subjects met the diagnostic criteria for schizophrenia in the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV)[35] as determined in consensus conferences following review of medical records, direct clinical assessments of the patients, and interviews with care providers.

Place Table 1 about here

Dysbindin-1 antibodies

Dysbindin-1 isoforms in human DLPFC lysates were characterized using three polyclonal rabbit antibodies developed for use by our research group: Oxford PA3111A, UPenn 329, and UPenn 331 (Figure 1B). PA3111A was generated in Dr. Derek Blake's laboratory at the University of Oxford. It was raised against a long C-terminus segment of mouse dysbindin-1 (amino acids 196-352) and has been characterized in earlier reports[4, 18, 19] UPenn 329 was generated by Sigma Aldrich (St. Louis, MO, USA) against a synthetic peptide consisting of aa 313-326 in the C-terminus region of human dysbindin-1A and -1C (NCBI accession nos. NP_115498 and NP_898862, respectively). UPenn 331 was also generated by Sigma Aldrich, but against a synthetic peptide consisting of aa 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

Tissue was obtained at autopsy from the DLPFC (Brodmann areas 9 +46) and stored at -80°C (see *Supplementary Information* for description of methods of tissue collection and neuropathological assessment). Fresh frozen samples of the DLPFC were homogenized and lysed in RIPA buffer (Sigma) containing 150 mM NaCl, 1.0% IGEPAL® CA-630 surfactant, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), a protease inhibitor cocktail (Sigma Aldrich P8340),

and EDTA at a final concentration of 1mM. After centrifugation at 10000g for 10 min, the whole-cell supernatants were aliquoted and stored at -80^{o}C. Protein concentrations were determined using a bicinchoninic (BCA) protein assay (Pierce Chemical, Thermo Fisher Scientific).

Pilot studies indicated protein detection limits, the linear range of protein detection, and membrane exposure times for optimal quantification of protein concentrations. In accordance with those studies, 20 µg protein was loaded in each lane for electrophoresis. Protein samples were denatured in 5X 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^{o}C. After boiling for 5 minutes, the samples were centrifuged at 16000 g for 5 min before loading on gels. To minimize interpretive errors resulting from variable transfer efficiency and immunoreactivity,[36] 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; these 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 separated 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 the same samples were run concurrently. After rinsing with Tris-buffered saline in 0.1% Tween-20 (Sigma Aldrich), one blot was stained using the MemCode reversible protein stain kit (Pierce Chemical)[37] to assess transfer efficiency and visualize protein loading. 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 overnight at 4^{o}C with the rabbit polyclonal

antibody Oxford PA3111A 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: its top part was incubated with a mouse monoclonal β-actin antibody (Sigma Aldrich A1978) at a dilution of 1:12000, while the bottom was incubated with the rabbit polyclonal dysbindin-1 antibody UPenn 331 at a dilution of 1:6000. All the blots were reacted overnight at 4^{o}C. Like Memcode staining, immunoblotting 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 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), exposed to film (Amersham Hyperfilm ECL, GE Healthcare), and the bands 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 quantitative reverse transcriptase-polymerase chain reaction (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). Yield and purity of RNA samples were determined by spectrophotometric analysis. 260/280 nm ratios of samples were > 2.1. Genomic DNA contamination was removed by treating 2 μg of each RNA sample with 40 U deoxyribonuclease (Sigma Aldrich) for 1 h at 37^{o}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 quantitative real-time polymerase chain reactions (qPCR) with primer pairs specified in Table S1. We

measured expression of three housekeeping genes (i.e., beta-2-microglobulin [B2M], glyceraldehyde-3-phosphate dehydrogenase [GAPDH], and hypoxanthine phosphoribosyl-transferase [HPRT]) as internal controls. The primer pairs used for the housekeeping genes are also specified in Table S1.

Four primer pairs were designed to target DTNBP1 transcripts (Table S1 and Figure 4A). 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, including all those known in the DLPFC (DTNBP1 transcripts a-e, R. Straub, personal communication). The other two primer sets are selective for transcripts of either dysbindin-1B (DYS-d) or dysbindin-1C (DYS-e).

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.[38, 39] 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. Amplification of selected reverse-transcriptase-absent cDNA samples resulted in no detectable signal indicating minimal genomic DNA contamination. Once optimal reaction conditions were established, 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^{o}C for 2 min, 95^{o}C for 10 min, 40 cycles of 95^{o}C for 15 s and 60^{o}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 5' exonuclease allelic discrimination assay (Applied Biosystems), the DNA was genotyped for a SNP

(P1578 = rs1018381) tagging a 6-SNP DTNBP1 risk haplotype for schizophrenia reported in a case-control study on US Caucasians.[33]

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 discovered, however, that calibrating optical density measurements to reference samples did not yield values as reliable across blots as pair-wise ratios of each schizophrenia case to its matched control run on the same blot, as previously noted by Albert et al.[40] We thus used two approaches. For analyses which did require 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 to 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 $\log_2$ 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 ranks test for ratio data, because the log of a ratio between two values X/Y = logX - logY.

qRT-PCR. Since all samples to be compared were run at the same time in one 384-well plate, data analysis was simplified compared to that for the Western

blotting data. Real-time PCR data acquisition 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 the triplicate samples based on raw cycle threshold values. In each experiment, the standard curve had a coefficient of determination (R^2) > 0.996 and a slope between -3.32 and -3.57, which indicated an amplification efficiency of 91-100%. Negative controls comprising 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. The relative expression value of dysbindin-1 mRNA was normalized to a geometric mean of the three internal control genes performed as suggested by Vandesompele et al.[41]

Statistical analyses were performed with GraphPad Prism 3.0 software (GraphPad Software, La Jolla, CA, USA). Unpaired t-tests were used to assess statistical differences between schizophrenia cases and controls in demographic and autopsy variables (age, brain pH, and PMI). For Western blotting data, the non-parametric Wilcoxon signed-rank test was used to test if the log transformed pair-wise ratios were statistically different from the hypothetical median value of zero representing no significant differences between schizophrenia and control cases. For qRT-PCR data, the Wilcoxon signed-rank and unpaired t tests were used to test differences in PCR products between cases and matched controls. For comparison of case-control ratios of proteins and of transcripts, the Mann-Whitney U test was used. The p values reported are two-tailed with p less than 0.05 considered significant.

Results

Dysbindin-1 Isoforms in DLPFC

According to NCBI's Reference Sequence (RefSeq) database, a highly curated transcript collection,[42] there are three validated splice variants of human DTNBP1 mRNA among sixteen deduced DTNBP1 splice variants identified in the AceView database (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html). 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 numbers NP_115498, NP_898861, and NP_898862, respectively). These proteins are the known isoforms of dysbindin-1, which along with dysbindin-2 and -3, constitute the dysbindin protein family, all of whose members share a still poorly understood dysbindin domain (DD).[4] The length and component parts of dysbindin-1 isoforms are indicated in Figure 1A. All have a coiled coil domain (CCD) important for certain protein-protein interactions but absent in dysbindin-2 and -3. Isoform A (351 aa in humans) is the full-length dysbindin-1 with a PEST domain at its C-terminus. Isoform B (303 aa), 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 aa) differs from isoform A only in the absence of an N-terminus region in front of its CCD.

Place Figure 1 about here

Using Oxford PA3111A, a well validated polyclonal antibody generated against the C-terminus region of dysbindin-1A (Figure 1B) ,[18,19] we consistently detected three bands in Western blots of human DLPFC lysates at a dilution of 1:1000 (Figure 1C), as we have in synaptosomal fractions of the human DLPFC, (Talbot et al., submitted). In an earlier study,[19] we detected only two bands with PA3111A in lysates of human brain tissue. Detection of three bands with that antibody in our current studies may reflect use of larger protein samples (50 µg vs. 20-40 µg), higher percentage gels (12% vs. 10%), and more sensitive chemiluminescence detection kits. The three bands lay at about 48, 36, and 32 kDa (Figure 1C). None of these bands were found when the antibody had been preadsorbed with the immunogen or with full-length dysbindin (i.e., dysbindin-1A). The heaviest band at about 48 kDa was readily identified as full-length dysbindin-1, which has been reported to run at about 50 kDa.[18,19,43] This is greater than the predicted 40 kDa mass of dysbindin-1A, probably due to its acidic nature[4,43] and post-translational modification (i.e., phosphorylation[4] and ubiquitination [Locke et

al., submitted]). The lighter bands at (36 and 32 kDa) are almost identical to the predicted molecular mass of dysbindin-1B and -1C (35 and 30 kDa, respectively).

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

Place Figure 2 about here

Dysbindin-1 Isoform Expression in DLPFC of Schizophrenia vs. Control Cases

Representative results of Western blotting for dysbindin-1 isoforms in DLPFC of schizophrenia cases and matched controls are shown in Figure 2. As detailed in the Materials and Methods, such results enabled calculation of a ratio expressing the relative amount of each dysbindin-1 isoform in each of the 28 schizophrenia

cases compared to its matched control. A mean ratio was then calculated from the results on the original and replication blots. Log_2 transformed mean ratios for all 28 pairs of schizophrenia and matched controls are graphed in Figure 3. The results were highly reproducible as indicated by strong correlations between the ratios obtained in the original and replication experiments for all dysbindin-1 isoforms (e.g. Spearman $r = 0.946$, $p = 9.2 \times 10^{-14}$ for dysbindin-1A).

For dysbindin-1A, the direction (plus or minus) and magnitude of the ratios were highly inconsistent. For dysbindin-1B, the ratios were also very inconsistent in direction, and their magnitudes were generally very small. Pair-wise analyses of each schizophrenia case to its matched control with the Wilcoxon signed-rank test accordingly showed no significant differences between schizophrenia and 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 12-85% in the schizophrenia cases compared to matched controls in 20 out of the 28 pairs (71.42%). Unlike the case with the other two isoforms, pair-wise ratio analysis showed that dysbindin-1C in the DLPFC was significantly reduced in the DLPFC of our schizophrenia cases by an average of 46% ($n = 28$, $W = 202$, $p = 0.0221$).

Place Figure 3 about here

Dysbindin-1 Gene Expression in DLPFC of Schizophrenia vs. 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 qRT-PCR using primer pairs specific for DTNBP1 transcripts in general (DYS-95 and -102), for DTNBP1 transcript d (DYS-d) encoding dysbindin-1B, and for DTNBP1 transcript e (DYS-e) encoding dysbindin-1C (see Table S1). Figure 4A shows the targets of the primers for DTNBP1 transcripts. Primers selective for dysbindin-1A

could not be designed since all sequences in that transcript are shared by many other DTNBP1 transcripts. However, results with the pan DTNBP1 primers DYS-95 and -102 provide an estimate of dysbindin-1A transcript levels, because these are by far the most common dysbindin-1 transcripts in all tissues judging from Northern blotting results.[4] Indeed, the amount of PCR product amplified with DYS-95 and -102 appeared to be far greater than that amplified with DYS-d or DYS-e as suggested by the much lower cycle threshold with DYS-95 and -102.

In the same 28 case-control pairs in which relative dysbindin-1 isoform levels were measured, the schizophrenia cases showed significant increases in overall levels of DTNBP1 transcripts as measured with DYS-95 ($t = 2.72$, $df = 54$, $p = 0.009$) or DYS-102 ($t = 2.77$, $df = 54$, $p = 0.008$) and analyzed with unpaired t-tests (Figure 4B). Such tests also showed that the schizophrenia cases had significantly greater levels of dysbindin-1B transcripts measured with DYS-d ($t = 2.34$, $df = 54$, $p = 0.023$), yet normal levels of dysbindin-1C transcripts measured with DYS-e ($t = 0.846$, $df = 54$, $p = 0.401$) (Figure 4B). The statistical results was obtained in pair-wise analyses using the Wilcoxon signed-ranks test. Comparison of case-control ratios of dysbindin-1 transcripts and dysbindin-1 isoforms with the Mann-Whitney U test revealed no significant correlations between altered gene and protein expression in the schizophrenia cases.

Place Figure 4 about here

DTNBP1 Genotyping and Its Relation to DTNBP1 Gene and Protein Expression

Only 2 controls and 2 schizophrenia cases were found to carry the rare allele of the P1578 SNP in DTNBP1 tagging a risk haplotype for schizophrenia in a US cohort.[33] As a result, we were unable to test the effect of this haplotype on either gene or protein expression of DTNBP1. However, 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 dysbindin-1C reductions in schizophrenia cases were not readily attributed to non-diagnostic variables. Our schizophrenia and control groups had the same female/male ratio and did not differ significantly in age or brain tissue pH. While mean PMI was longer in the schizophrenia cases (11.9 ± 5.4 h) than in the controls (7.7 ± 3.8 h) ($t = 3.46$, $df = 26$, $p = 0.0019$), PMI (like age and brain tissue pH) was not significantly correlated with normalized levels of dysbindin-1 proteins. Nor was there a significant correlation of normalized dysbindin-1 mRNA levels with PMI, age, or brain pH (apart from a negative correlation of dysbindin-1B transcript with brain pH). Neither diagnostic group displayed any evidence of degenerative processes; neither showed evidence of gross cell loss, infarcts, or abnormally high densities of amyloid plaques, neurofibrillary tangles, or Lewy bodies. Finally, no significant correlations were found between any dysbindin-1 isoform or transcript and the antipsychotic dosage of our schizophrenia cases in chlorpromazine equivalents one month prior to death, a finding in accordance with our study on dysbindin-1 in the hippocampal formation of such cases.[19] This is also consistent with studies showing that chronic haloperidol administration to mice has no significant effect on dysbindin-1 gene[44] or protein expression.[19]

Discussion

In this first report on dysbindin-1 isoforms in schizophrenia, we have discovered that DLPFC levels of dysbindin-1C, but not those of dysbindin-1A or -1B, are significantly altered. In particular, about 71% of our cases displayed 12-85% lower levels of disbindin-1C than did their matched controls. As detailed above, these results are not attributable to uncontrolled differences between diagnostic groups. While the present findings are based on whole-tissue lysates, we have found that dysbindin-1 is highly enriched in synaptic tissue fractions of both the mouse and human brain.[4, 18, 21] While we find that some dysbindin-1 isoforms are

also enriched in cell nuclei of the brain, our studies show that dysbindin-1C in humans is concentrated primarily in postsynaptic density (PSD) fractions of the DLPFC and other brain areas studied so far (anterior cingulate gyrus, superior temporal gyrus and hippocampal formation, Talbot et al., submitted). Electron microscopic studies on tissue reacted with the Oxford antibody PA3111A have, in fact, found dysbindin-1 highly concentrated in some glutamatergic PSDs.[4, 18] It is likely, then, that the observed reduction of DLPFC dysbindin-1C in schizophrenia occurs specifically in PSDs of glutamatergic synapses.

This is the second study pointing to reduced dysbindin-1 as a synaptic abnormality in schizophrenia. Depending on the specific anatomic area studied, our previous immunohistochemical work with the Oxford PA3111A antibody indicated that presynaptic dysbindin-1 was reduced in a very similar proportion of schizophrenia cases compared to their matched controls.[19] Our more recent Western blotting work on synaptosomal preparations with the antibodies used in this study show that the presynaptic reduction in the hippocampal formation is due principally to decreased dysbindin-1B associated with synaptic vesicles and that this is accompanied by a previously undetected reduction in postsynaptic dysbindin-1 due to a decrease in dysbindin-1C (Talbot et al., submitted). As in the DLPFC, dysbindin-1A levels were unaltered in the hippocampal formation. There is thus growing evidence for isoform-specific dysbindin-1 reductions in schizophrenia that vary across brain areas.

The cause(s) of reduced dysbindin-1C in the DLPFC of schizophrenia cases is unknown. Using a riboprobe that would hybridize with mRNAs of all the dysbindin-1 isoforms studied here, Weickert et al.[45] found that DTNBP1 gene expression in schizophrenia cases was 15-20% lower in all DLPFC cell layers except layer IV. Using quantitative RT-PCR with primers specific for dysbindin-1 transcripts, however, we did not replicate that finding using a larger case sample of different ethnicity with far lower PMIs (i.e., 28 Caucasian schizophrenia cases with PMIs of 11.9 ± 5.4 h in this study versus 14 predominantly African-American

schizophrenia cases with PMIs of 38.0 ± 21.9 h in the study of Weickert et al.[45]). Moreover, we found no correspondence between schizophrenia-related changes in DTNBP1 gene and protein expression in the DLPFC, both of which occurred in the absence of the one known DTNBP1 risk haplotype[33] for schizophrenia in the US. The lack of correspondence in gene and protein expression changes was striking. In the schizophrenia cases, overall levels of dysbindin-1 transcripts were elevated for unknown reasons, yet levels of the major encoded proteins were not. Levels of transcripts specific for dysbindin-1B were increased in schizophrenia cases, but levels of the isoform itself were not. Conversely, levels of transcripts specific for dysbindin-1C were normal in the schizophrenia cases, yet levels of dysbindin-1C protein were reduced. These findings indicate that factors other than DTNBP1 gene expression account for the observed reduction in dysbindin-1C. Those factors are unknown, but may include posttranslational modifications promoting dysbindin-1C degradation such as phosphorylation and ubiquitination.

While the causes remain unknown, the consequences of lower dysbindin-1C in the DLPFC are suggested by studies on sandy mice, which have a deletion mutation in Dtnbp1 resulting in loss of dysbindin-1A and -1C.[4, 46] (Dysbindin-1B is not expressed in the mouse.[4]) As in humans, dysbindin-1A and -1C are abundant in PSD tissue fractions.[21] In the rodent homolog of human prefrontal cortex (i.e., prelimbic cortex), homozygous sandy mice display (1) reduced NMDA receptor-mediated glutamatergic input on deep layer pyramidal cells,[47] (2) reduced NMDA receptor-mediated glutamatergic input on deep layer fast-spiking interneurons with associated reduction in their spike frequency, [48] and (3) reduced amplitude and frequency of spontaneous inhibitory postsynaptic currents in deep layer pyramidal neurons.[49]These findings suggest that loss of dysbindin-1A and/or -1C in the DLPFC can promote NMDA receptor hypofunction deep layer pyramidal neurons, which are glutamatergic, and at least a subpopulation of neighboring GABAergic interneurons. Reduced DLPFC dysbindin-1C in schizophrenia may thus contribute to the NMDA receptor hypofunction suggested in that disorder by an increasingly large number of studies.[50, 51]

It has been argued that in schizophrenia NMDA hypofunction in the parvalbumin-positive, fast-spiking interneurons of the DLPFC innervating initial axon segments of pyramidal neurons in the same brain area would reduce synchronized activity of the DLPFC and thereby disrupt processes important for working memory.[52] The DLPFC is involved in several types of working memory, including spatial working memory,[28,29] deficits in which are among the most common cognitive impairments in schizophrenia.[7-9] Homozygous sandy mice, which lack dysbindin-1C, also display working memory deficits,[53-55] including those detected on a non-matching to sample task sensitive to disruption of the rodent prefrontal cortex.[53,54] It is possible, then, that reduced DLPFC dysbindin-1C in schizophrenia may contribute to spatial working memory deficits in those cases by promoting NMDA receptor hypofunction in the parvalbumin-positive interneurons of the DLPFC. Testing that possibility requires much further work, however.

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Supplementary information is available at the *Molecular Psychiatry* website.

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Table 1: Demographic and Autopsy Data on Cases Studied[1]

Variable	*Control Cases*	*Schizophrenia Cases*
Number of Cases	28 Caucasians	28 Caucasians
Mean Age ± SD	83.7 ± 9.0 y	81.7 ± 7.7 y
Gender Ratio: Female/Male	17/11	17/11
Mean PMI in Hours ± SD	7.7 ± 3.8	11.9 ± 5.4
Mean Brain Tissue pH ± SD	6.19 ± 0.04	6.46 ± 0.26

[1]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 proteins measured.

Table S1: Primers Used for Quantitative RT-PCR

Primer	*Targeted mRNA (NCBI)*	*Targeted DTNBP1 Transcript (AceView)*	*Primer Sequence (Forward/Reverse)*	*Product Size (bp)*	*Protein Encoded by Targeted mRNA*
DYS-95	NM_032122	a-h + j	5'TCCCAGCTTTAATCGCAGAC3'/ 5'CAGATGCAGCAGGTTGTTCT3'	95	pan Dysbindin-1(a)[1]
DYS-102	NM_032122	a-i + k-m	5'CAGCAGGACATGGAGCAGTA3'/ 5'CATGTCCACGTTCACTTCCA3'	102	pan Dysbindin-1(b)[1]
DYS-d	NM_183040	d	5'ACGCATAAACCCCCAAGC3'/ 5'GACGGAACCACACCACTGT3'	96	Dysbindin-1B
DYS-e	NM_183041	e	5'AGTGAAAAGCAAACCCAGTTG3'/ 5'GTCCTGCCCAAAAGAAACAC3'	66	Dysbindin-1C
B2M[2]	NM_004048	—	5'TGCTGTCTCCATGTTTGATGTATCT3'/ 5'TCTCTGCTCCCCACCTCTAAGT3'	86	B2M
GAPDH[2]	NM_002046	—	5'TGCACCACCAACTGCTTAGC3'/ 5'GGCATGGACTGTGGTCATGAG3'	87	GAPDH
HPRT[2]	NM_000194	—	5'TGACACTGGCAAAACAATGCA3'/ 5'GGTCCTTTTCACCAGCAAGCT3'	94	HPRT1

[1]The two pan dysbindin-1 primers can recognize many DTNBP1 transcripts, including those for dysbindin-1A, -1B, and -1C; they differ only in recognition of minor transcripts (AceView DTNBP1 transcripts i-m) not yet detected in the DLPFC. Of the DTNBP1 transcripts currently listed on AceView, only a-e are known to be expressed in that brain area (R. Straub, personal communication).

[2]The B2M (beta-2-microtubulin), HPRT1 (hypoxanthine phosphoribosyl-transferase 1), and GAPDH (glyceraldehyde-3-phosphate dehydrogensase) primer sequences are described in Vandesompele et al.[41]

FIGURE LEGENDS

Figure 1. Major dysbindin-1 isoforms in humans and their detection with three antibodies (Oxford PA3111A, UPenn 331, and UPenn 329). **A** compares the isoforms as characterized by Talbot et al.[4] Numbers below isoforms designate amino acid (aa) sequence location beginning at the C-terminus. CCD = coiled coil domain composed of helices 1 and 2 (H1 and H2) separated by a stutter region (SR), CTR = carboxy terminus region, DD = the dysbindin domain, LZM = leucine zipper motif (LZM), NTR = amino terminus region, PD = PEST domain, and X1 and X2 are simply uncharacterized regions. **B** shows dysbindin-1A with the location of immunogens for Oxford PA3111A, UPenn 331, and UPenn 329. That for the Oxford antibody was aa 196-352 of mouse dysbindin-1A, which is 352 aa in length compared to 351 aa in humans. The UPenn antibodies were made to sequences indicated in human dysbindin-1A. **C** shows the dysbindin-1 isoforms recognized by Oxford PA3111A (1:1000), UPenn 331(1:6000), and UPenn 329 (1:40) in Western blots of whole-tissue lysates of the DLPFC lysates (50 μg per lane) from the same three normal humans as described in the Results.

Figure 2. Representative Western blots showing relative amount s of dysbindin-1 isoforms in the DLPFC of schizophrenia (S) cases compared to psychiatrically normal (N) controls matched for age and sex. Reference samples (R1 and R2) were run to assess differences across blots due to variations in experimental conditions. Numbers identify which of the 28 matched pairs tested is shown. 20 μg/lane of protein were electrophoresed, transferred to PVDF membrane, and probed with anti-dysbindin-1 antibodies with Oxford PA3111 in **A** and **C** and with UPenn 331 in **B**. Blots were processed for β-actin (**D**) or MemCode staining (**E**) to control for variations in protein levels due to sample degradation during tissue storage, lysate preparation, gel loading, and/or to efficiency in membrane transfer.

Figure 3. Levels of dysbindin-1A, -1B, and 1C in the DLPFC of schizophrenia cases compared to matched controls based on Western blotting analysis. Data are presented for all 28 case-control pairs studied along the X axis. For each of these pairs, the

amount of a dysbindin-1 isoform in the schizophrenia case to that in its matched control is expressed as a ratio shown on the Y axis. The ratio is actually the mean of ratios calculated in two separate series of Western blots on the same cases. The mean ratios were log transformed as explained in *Data Analysis*. Three bars are shown for each of the 28 case-control pairs: one for the mean ratio calculated with raw data (■), another calculated with data normalized to β-actin (⊟), and a third calculated with data normalized with MemCode results (□). The order in which case-control data is displayed from left to right varies in the three graphs; the series shown in each graph simply reflects rank ordering of the 28 pairs with increasingly positive ratios. Significant differences were found only for dysbindin-1C, in which 20 of the 28 matched pairs showed reductions in that protein ($p = 0.0221$). This finding was made with raw data and with data normalized to β-actin or MemCode staining.

Figure 4. Targets of DTNBP1 primer sequences used in this study (**A**) and relative expression levels of the targeted DTNBP1 transcripts in DLPFC of schizophrenia (Sz) cases and matched normal (N) controls (**B**) as determined with qRT-PCR using primer sequences specified in Table S1. In **A**, the locations of the targeted RNA sequences are indicated in the 2007 AceView diagram of the exonic-intronic structure of the 16 known or deduced DTNBP1 pre-mRNA transcripts. Only transcripts a-e have been found in the DLPFC, however (R. Straub, personal communication). Boxed areas are exons, and the chevrons (∧) between them are introns. Unshaded portions of exons are untranslated regions. NCBI accession numbers for mRNAs of dysbindin-1A, -1B, and -1C are given to the left of their transcripts. In **B**, relative levels of DTNBP1 transcripts are plotted for the 28 pairs of Sz and N cases in which their encoded proteins were also measured (Figure 3). For each of the four different transcripts, the data points (○) are normalized values divided by the mean expression level for that transcript on all samples (Sz and N) so that data for all four transcripts could be plotted on the same scale despite much higher levels of pan dysbindin-1 than dysbindin-1B or -1C transcripts.

Figure 1

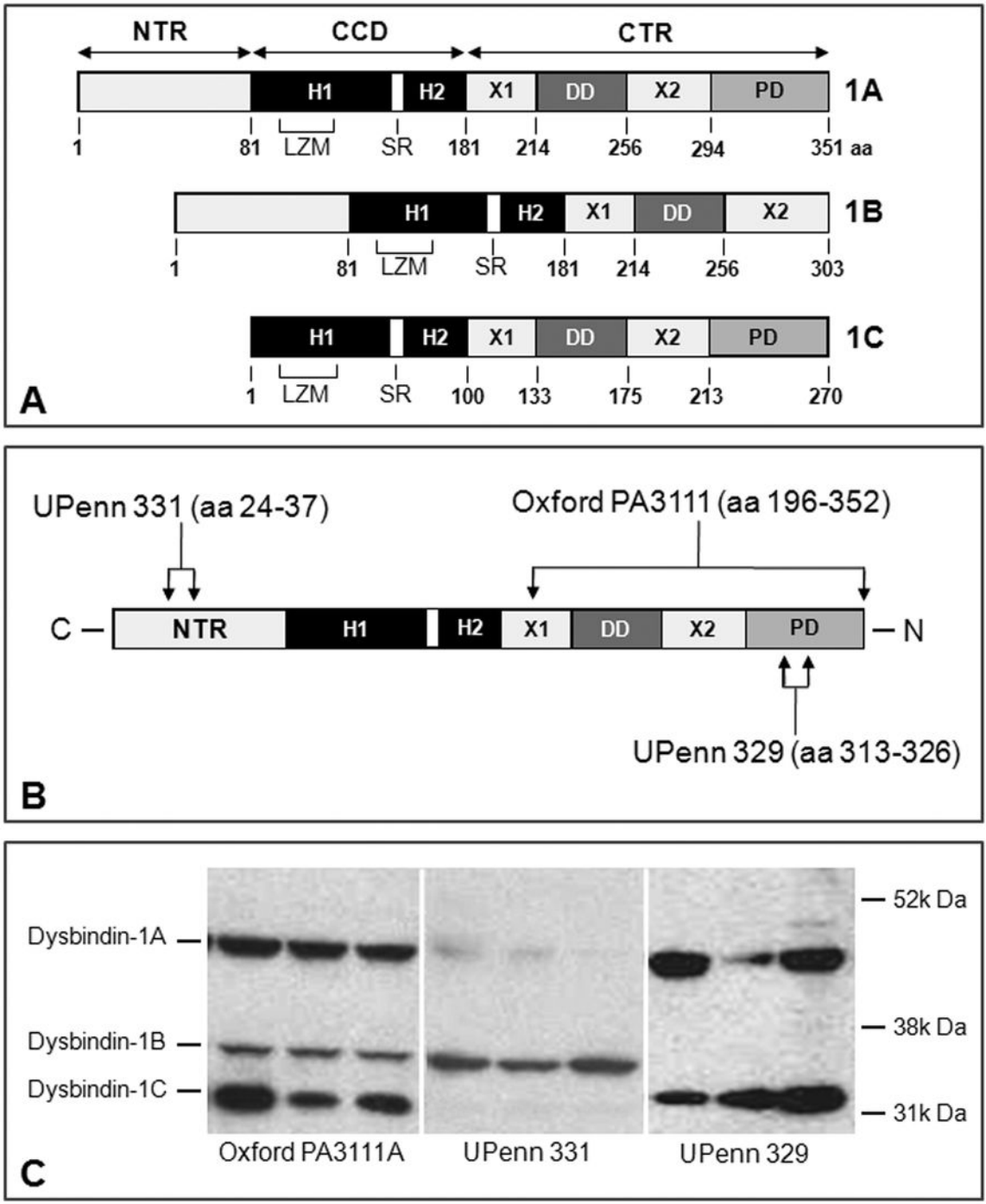

NTR
CCD
CTR
H1
H2
X1
DD
X2
PD
1A
1
81
LZM
SR
181
214
256
294
351 aa
1B
303
1C
100
133
175
213
270
A
UPenn 331 (aa 24-37)
Oxford PA3111 (aa 196-352)
C —
— N
UPenn 329 (aa 313-326)
B
Dysbindin-1A
Dysbindin-1B
Dysbindin-1C
52k Da
38k Da
31k Da
Oxford PA3111A
UPenn 331
UPenn 329
C

Figure 2

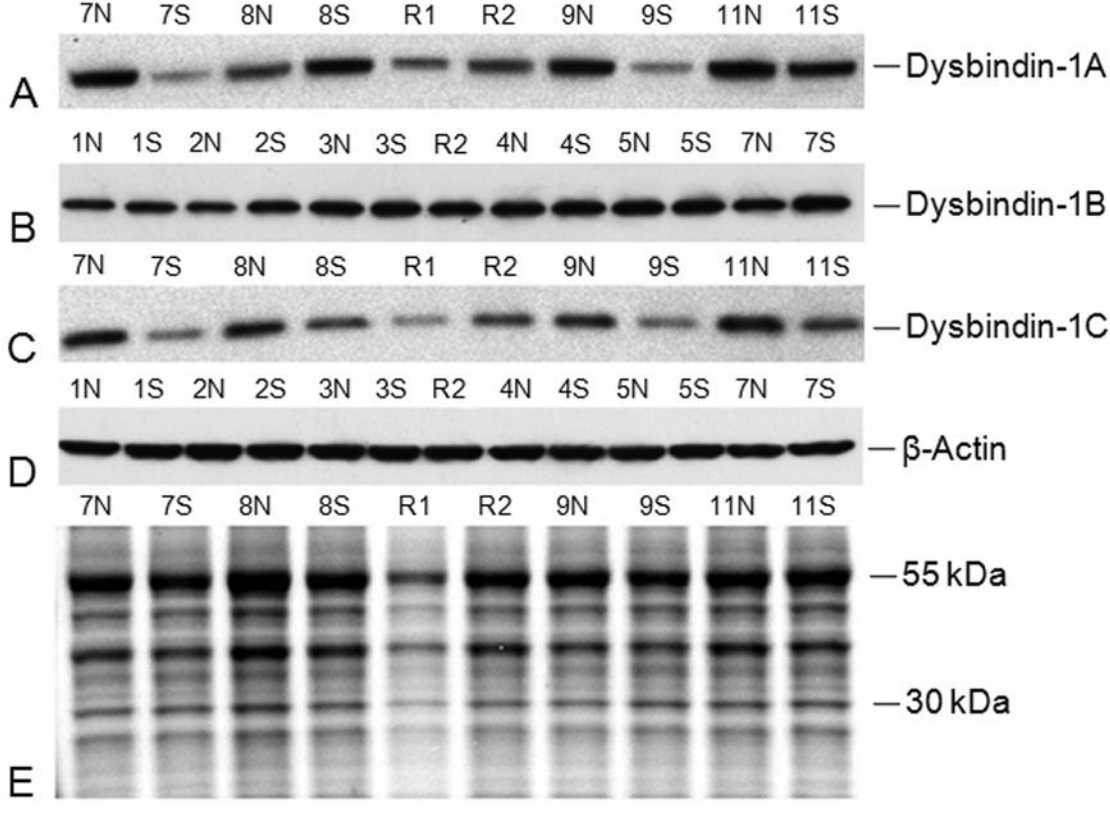

7N 7S 8N 8S R1 R2 9N 9S 11N 11S
A
Dysbindin-1A
1N 1S 2N 2S 3N 3S R2 4N 4S 5N 5S 7N 7S
B
Dysbindin-1B
7N 7S 8N 8S R1 R2 9N 9S 11N 11S
C
Dysbindin-1C
1N 1S 2N 2S 3N 3S R2 4N 4S 5N 5S 7N 7S
D
β-Actin
7N 7S 8N 8S R1 R2 9N 9S 11N 11S
55 kDa
30 kDa
E

Figure 3

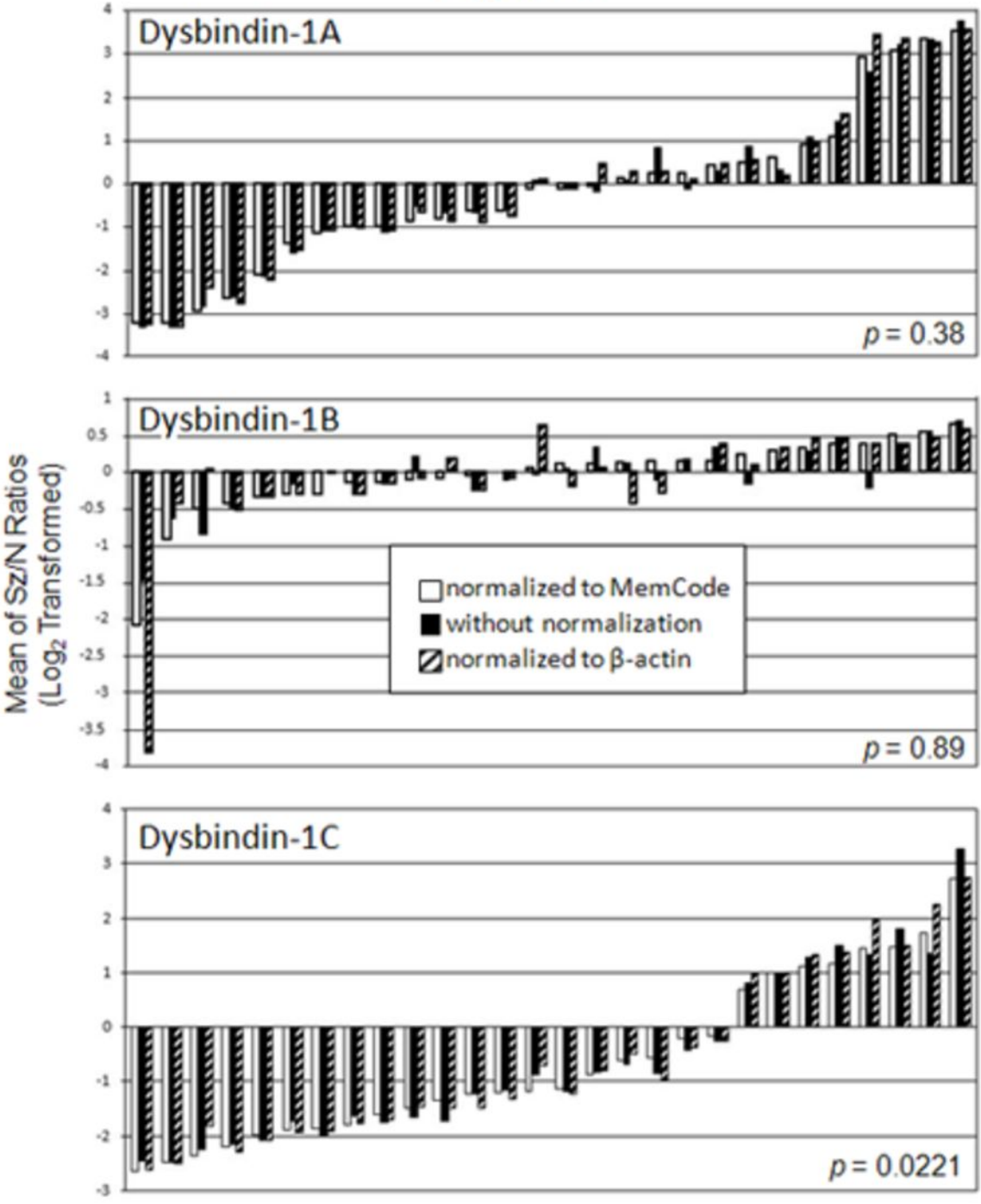
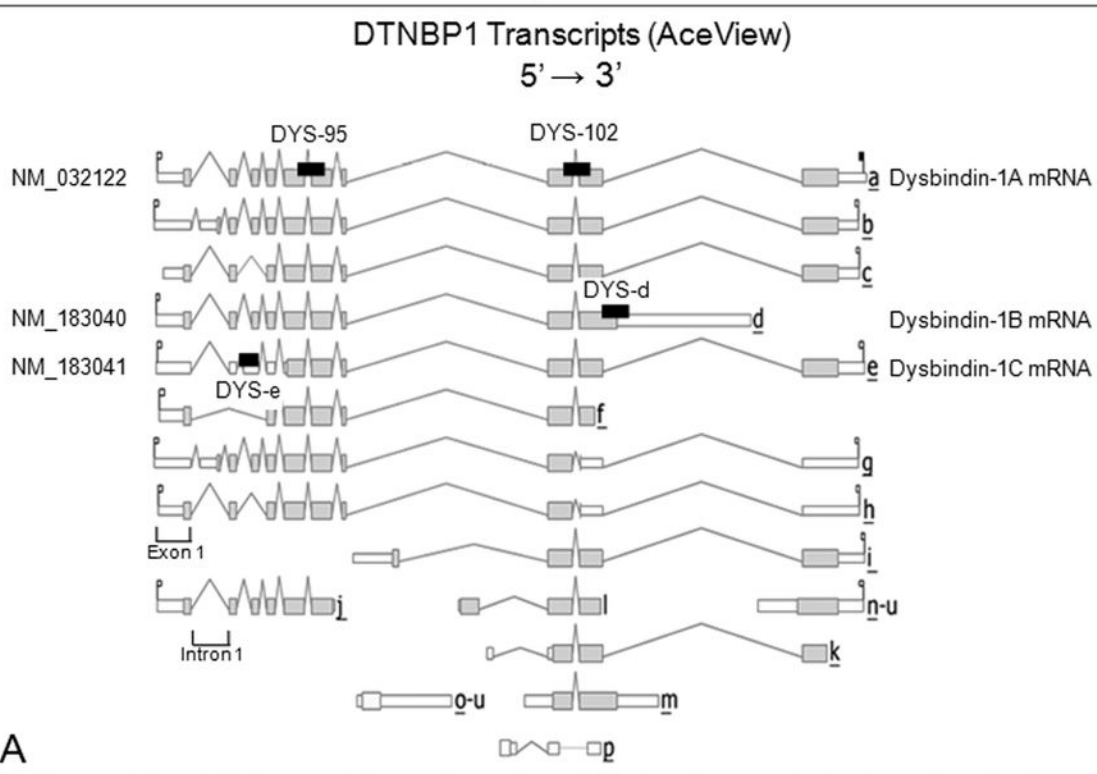
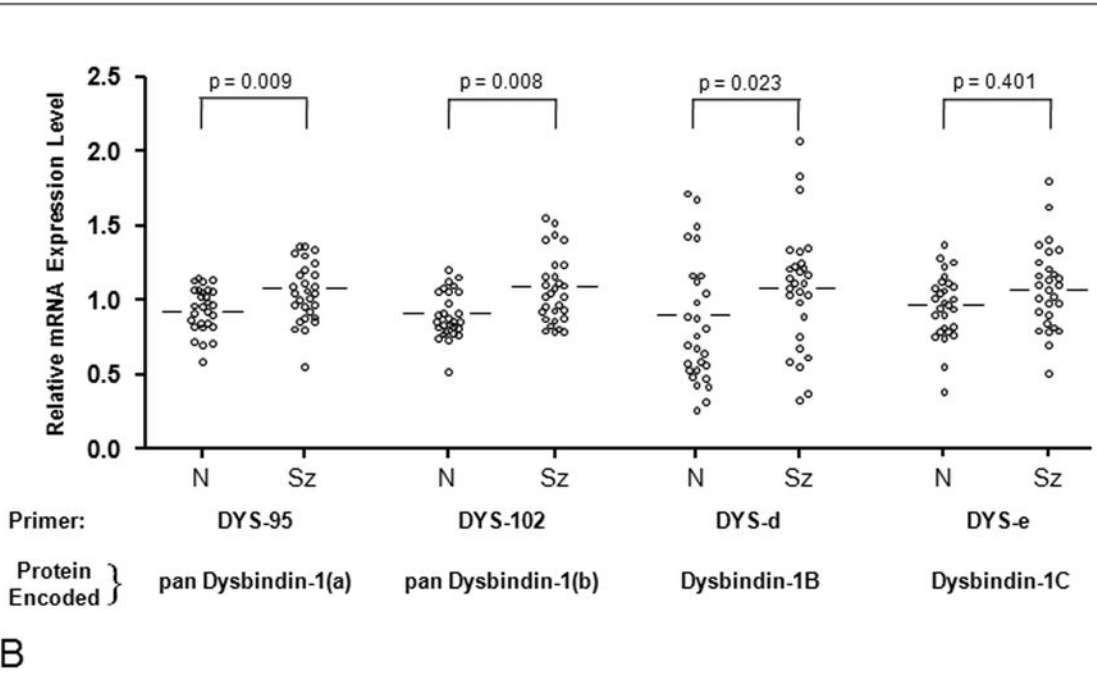

Dysbindin-1A
p = 0.38
Dysbindin-1B
normalized to MemCode
without normalization
normalized to β-actin
p = 0.89
Dysbindin-1C
p = 0.0221
Mean of Sz/N Ratios (Log2 Transformed)
Case-Control Pairs (Sz vs. N Control)

Figure 4


DTNBP1 Transcripts (AceView)
5' → 3'
DYS-95
DYS-102
NM_032122
a Dysbindin-1A mRNA
b
c
DYS-d
NM_183040
d
Dysbindin-1B mRNA
NM_183041
e Dysbindin-1C mRNA
DYS-e
f
g
h
Exon 1
i
j
l
n-u
Intron 1
k
o-u
m
p
A


Relative mRNA Expression Level
2.5
2.0
1.5
1.0
0.5
0.0
p = 0.009
p = 0.008
p = 0.023
p = 0.401
N
Sz
N
Sz
N
Sz
N
Sz
Primer:
DYS-95
DYS-102
DYS-d
DYS-e
Protein Encoded
pan Dysbindin-1(a)
pan Dysbindin-1(b)
Dysbindin-1B
Dysbindin-1C
B