Table 3 (*Continued*)

CNVR	CNVR type	People	ProbeSetID	Psychiatric Trait locus	Permutations *p* value	CNVR_Start	CNVR_Stop	GeneSymbol
q11.21_21709612_21905954	Loss	con*	212180_at	22q11.2[4,5]	0.003267	21709612	21905954	CRKL
q11.21_21709612_21905954	Loss	con*	218492_s_at	22q11.2[4,5]	0.003155	21709612	21905954	THAP7
q11.21_21709612_21905954	Loss	con*	205881_at	22q11.2[4,5]	0.009217	21709612	21905954	ZNF74
q11.21_21709612_21905954	Loss	con*	207081_s_at	22q11.2[4,5]	3.15E-005	21709612	21905954	PI4KA
q11.21_21709612_21905954	Loss	con*	211147_s_at	22q11.2[4,5]	0.015425	21709612	21905954	P2RX6
q11.21_21709612_21905954	Loss	con*	221838_at	22q11.2[4,5]	0.016453	21709612	21905954	KLHL22
q11.21_21709612_21905954	Loss	con*	222141_at	22q11.2[4,5]	1.02E-011	21709612	21905954	KLHL22
q11.21_21709612_21905954	Loss	con*	200684_s_at	22q11.2[4,5]	0.007571	21709612	21905954	UBE2L3
q11.21_21709612_21905954	Loss	con*	205576_at	22q11.2[4,5]	0.007335	21709612	21905954	SERPIND1
q11.21_21709612_21905954	Loss	con*	212271_at	22q11.2[4,5]	0.000261	21709612	21905954	MAPK1
q11.21_21711906_21905954	Gain	bp	214406_s_at	22q11.2[4,5]	0.003467	21711906	21905954	SLC7A4
q11.21_21711906_21905954	Gain	bp	215048_at	22q11.2[4,5]	0.018037	21711906	21905954	ZNF280B
q11.21_21711906_21905954	Gain	bp	221349_at	22q11.2[4,5]	0.002569	21711906	21905954	VPREB1
q11.22_22605295_22630082	Gain	bp	211655_at	22q11.2[4,5]	0.018087	22605295	22630082	LOC100287927
q11.22_23241489_23252126	Gain	sz	215036_at	22q11.2[4,5]	0.016135	23241489	23252126	
q11.22_23242646_23248046	Gain	sz	217180_at	22q11.2[4,5]	0.017219	23242646	23248046	
q11.23_23805014_23825653	Gain	sz	203815_at	22q11.2[4,5]	0.007688	23805014	23825653	GSTT1
q11.23_24271987_24343125	Loss	sz	203815_at	22q11.2[4,5]	0.007688	24271987	24343125	GSTT1
q11.23_24278085_24341961	Loss	sz	202624_s_at	22q11.2[4,5]	0.006683	24278085	24341961	CABIN1
q11.23_24278085_24341961	Loss	sz	214623_at	22q11.2[4,5]	0.008420	24278085	24341961	FBXW4P1
q11.23_24291835_24345621	Gain	con*	204993_at	22q11.2[4,5]	5.26E-005	24291835	24345621	GNAZ
q11.23_24291835_24345621	Gain	con*	217668_at	22q11.2[4,5]	0.000836	24291835	24345621	C22orf36
q11.23_24291835_24345621	Gain	con*	215202_at	22q11.2[4,5]	0.001560	24291835	24345621	LOC91316
q11.23_24291835_24345621	Gain	con*	203878_s_at	22q11.2[4,5]	0.017484	24291835	24345621	MMP11
q11.23_24291835_24345621	Gain	con*	205582_s_at	22q11.2[4,5]	4.46E-005	24291835	24345621	GGT5
q11.23_24291835_24345621	Gain	con*	202929_s_at	22q11.2[4,5]	0.003827	24291835	24345621	DDT
q11.23_24291835_24345621	Gain	con*	211471_s_at	22q11.2[4,5]	0.000417	24291835	24345621	RAB36
q11.23_24291835_24345621	Gain	con*	217871_s_at	22q11.2[4,5]	3.15E-005	24291835	24345621	MIF
q11.23_24329367_24398674	Loss	bp	212167_s_at	22q11.2[4,5]	0.018093	24329367	24398674	SMARCB1
q11.23_24341917_24400174	Loss	bp	207215_at	22q11.2[4,5]	0.015425	24341917	24400174	GSTTP1
q11.23_24341917_24400174	Loss	bp	203877_at	22q11.2[4,5]	0.002009	24341917	24400174	MMP11
q11.23_24341917_24400174	Loss	bp	221108_at	22q11.2[4,5]	0.000987	24341917	24400174	C22orf43
q11.23_24341917_24400174	Loss	bp	206532_at	22q11.2[4,5]	2.32E-006	24341917	24400174	
q11.23_24341917_24400174	Loss	bp	215816_at	22q11.2[4,5]	3.15E-005	24341917	24400174	LOC91316
q11.23_24341917_24400174	Loss	bp	220507_s_at	22q11.2[4,5]	0.017079	24341917	24400174	UPB1
q11.23_24344364_24398674	Loss	con	211471_s_at	22q11.2[4,5]	0.016695	24344364	24398674	RAB36
q11.23_24344364_24398674	Loss	con	204993_at	22q11.2[4,5]	0.003924	24344364	24398674	GNAZ
q11.23_24344364_24398674	Loss	con	217871_s_at	22q11.2[4,5]	0.013295	24344364	24398674	MIF
q11.23_24344364_24398674	Loss	con	202315_s_at	22q11.2[4,5]	0.001560	24344364	24398674	BCR
q11.23_24344364_24398674	Loss	con	217223_s_at	22q11.2[4,5]	0.000426	24344364	24398674	BCR
q11.23_24344364_24398674	Loss	con	37652_at	22q11.2[4,5]	0.009212	24344364	24398674	CABIN1
q11.23_24356690_24369021	Gain	con*	215202_at	22q11.2[4,5]	0.001560	24356690	24369021	LOC91316
q11.23_24356690_24369021	Gain	con*	217668_at	22q11.2[4,5]	0.000836	24356690	24369021	C22orf36
q11.23_24356690_24369021	Gain	con*	204993_at	22q11.2[4,5]	5.26E-005	24356690	24369021	GNAZ
q11.23_24356690_24369021	Gain	con*	211471_s_at	22q11.2[4,5]	0.000417	24356690	24369021	RAB36
q11.23_24356690_24369021	Gain	con*	205582_s_at	22q11.2[4,5]	4.46E-005	24356690	24369021	GGT5
q11.23_24356690_24369021	Gain	con*	217871_s_at	22q11.2[4,5]	3.15E-005	24356690	24369021	MIF
q11.23_24356690_24369021	Gain	con*	202929_s_at	22q11.2[4,5]	0.003827	24356690	24369021	DDT
q11.23_24356690_24369021	Gain	con*	203878_s_at	22q11.2[4,5]	0.017484	24356690	24369021	MMP11
q11.23_25756694_25775816	Gain	bp	220507_s_at	22q11.2[4,5]	0.017079	25756694	25775816	UPB1
q11.23_25756694_25775816	Gain	bp	204183_s_at	22q11.2[4,5]	0.001594	25756694	25775816	ADRBK2
q11.23_25756694_25775816	Gain	bp	204184_s_at	22q11.2[4,5]	0.002150	25756694	25775816	ADRBK2

x = in LCLs and xxx = in brain tissue; sz = schizophrenia, bp = bipolar, con = control, con* = control outlier C_15.

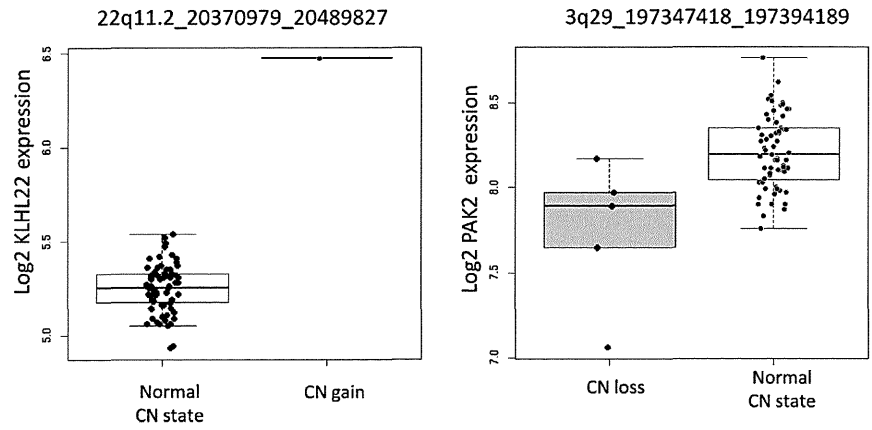

Fig. 5. Significant eCNVRs previously shown to be associated with schizophrenia or autism-spectrum disorders within candidate loci 3q29 and 22q11.2.

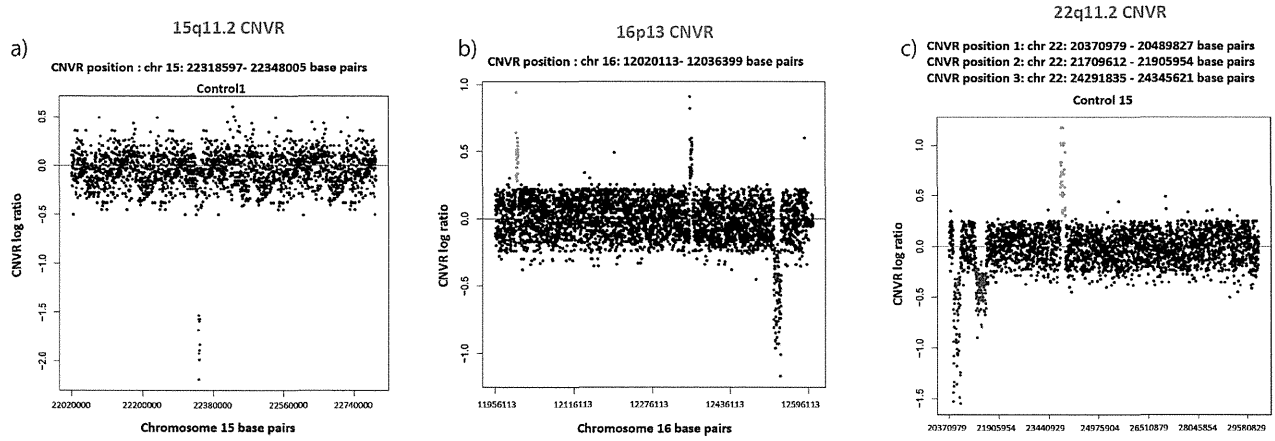

Fig. 6. Log ratio plots of 3 examples of significant eCNVRs within candidate loci 15q11.2, 16p13 and 22q11.2 previously associated with psychiatric disorders. Red dots indicate losses and green dots indicate gains. Blue dots indicate other CNVRs within this individual which were detected but these were not significantly associated with gene expression levels. (a) 15q11.2, (b) 16p13, and (c) 22q11.2.

429 transcripts were significantly associated with CNVR state after corrections for multiple testing and permutation. This corresponded to 583 CNVR-probe pairs (293 unique eCNVRs). Among the eCNVRs, a significant over-representation of rare/low frequency CNVRs ($p = 1.087 \times 10^{-10}$) and gene-harboring/genic CNVRs ($p = 1.4 \times 10^{-6}$) was observed. Overrepresentation of rare/low frequency CNVs among eCNVRs is interesting from an evolutionary point of view. A significant proportion of variance in gene expression could be explained by the eCNVR, with an average of 26% variance across the transcripts. A large proportion of negative correlations observed, demonstrated the complex relationship between CNVs and gene expression. Regulatory mechanisms such as epistasis or auto-regulatory feedback mechanisms at the level of the gene might explain the negative correlations. For instance, deletions that affect silencers or insulator elements can result in increased gene expression of the transcript (Weischenfeldt et al., 2013). Comparisons of the brain eCNVRs identified in the current study to previously reported eCNVRs yielded a 10% overlap, thereby providing a replication for these eCNVRs despite the differences in samples and study design between the studies.

Functional annotation of transcripts associated with CNVRs revealed a significant enrichment of corticotrophin-releasing hormone pathway across all samples, and also upon stratification by cases and controls. However, genes overlapping CNVRs only in cases but not in controls were enriched for glutathione metabolism and oxidative stress. Glutathione is a major antioxidant in the brain and plays a crucial role in protecting against oxidative damage. It is reported that glutathione levels were decreased (Gawryluk et al., 2001) and oxidative stress is enhanced (Ng et al., 2008) in schizophrenia and bipolar disorder, and mood stabilizers increases glutathione S-transferase (Wang et al., 2004). Thus, altered glutathione and oxidative stress pathways due to CNV might be related to pathophysiology of bipolar disorder and schizophrenia.

To test whether the eCNVRs were located within psychiatric phenotype-associated loci, we performed a literature search to identify CNVRs robustly associated with psychiatric diseases and systematically checked these loci ($n = 8$ unique loci). The 293 significant eCNVRs identified in this study included 72 (24.6%) psychiatric-disorder associated eCNVRs within these 8 loci, indicating that copy number variants in these loci might be directly involved in transcriptional regulation in the brain. These eCNVRs encompassed 7 (16p11.2, 1q21.1, 22q11.2, 3q29, 15q11.2, 17q12 and 16p13.1) of the 8 tested loci. Of the 72 eCNVRs, 19 CNVRs were identified only in schizophrenia patients and 21 CNVRs were observed only in bipolar disorder patients. A total of 19 CNVRs were found only in controls of which 7 were found in a single control individual (C_15). For C_15, the CNVR burden was within the range of that detected across all other samples and by technically validating two CNVRs harbored by this individual, we excluded the possibility of sample contamination or hybridization artifacts. This control individual however due to unknown or unexplained reasons harbored several of the known bipolar disorder and schizophrenia-associated CNVRs. For the 15q13.3 region, we did not identify any CNVRs associated with gene expression levels.

Recently, Ye and colleagues identified that CNVs in 1q21.1 and 22q11.2 were significantly associated with expression levels of nearby transcripts in dorsolateral prefrontal cortex (Ye et al., 2012). We found an association between a CNVR in 1q21.1 and CHD1L as reported by Ye and colleagues and in the current study the same CNVR was also associated with gene expression levels of FMO5, PRKAB2, RNF115 and ITGA10. These CNVRs were present in 2 bipolar disorder patients and control C_15. Additionally, we identified 8 further CNVRs in 1q21.1 (6 only in bipolar patients, one in 2 bipolar patients, one control and control C_15 and one only in control C_15) significantly associated with gene expression levels of PDE4DIP, SEC22B, RBM8A, PRKAB2 and ITGA10. In line with Ye et al. (2012), we observed a significant association of a 22q11.2 CNVR in control C_15 with COMT gene expression for two separate gene expression probes. In addition, our data pointed also toward the PI4KA gene within this locus whose expression was significantly associated with 2 CNVRs (both in control C_15) in the 22q11.2 locus. The initial study by Saito and colleagues (Saito et al., 2003) identified a link between PI4KA and 22q11.2-linked psychiatric disorders. The PI4KA gene encodes a phosphatidylinositol (PI) 4-kinase which catalyses the first committed step in the biosynthesis of phosphatidylinositol 4,5-bisphosphate. Incorporating the results of all association of PI4KA with schizophrenia till date has yielded mixed results and the link between PI4KA and psychiatric disorders remains unclear (Kanahara et al., 2009; Saito et al., 2003; Vorstman et al., 2009). This is the first report highlighting a functional link between CNVRs within the 22q11.2 locus and PI4KA gene expression in the human brain, suggesting that PI4KA might indeed be related to 22q11.2-related psychiatric diseases. In summary, results of the current study replicate the findings by Ye and colleagues that 1q21.1 and 22q11.2 may be involved in pathophysiology of psychiatric disorders by affecting gene expression levels in the brain.

For an additional five candidate regions reported to be associated with schizophrenia and/or autism-spectrum disorders (3q29, 15q11.2, 16p11.2, 16p13.1 and 17q12), for the first time we

identified significant functional influence of CNVRs on prefrontal cortex gene expression, implicating that these loci confer a risk of psychiatric disorders by affecting gene expression in the brain. Of note was the finding of 6 CNVRs within the 16p11.2 locus that significantly influenced gene expression profiles of several transcripts including CORO1A, TAOK2, DOC2A, SEPHS2 and CDIPT transcripts in the human prefrontal cortex. Both deletions and duplications within the 16p11.2 region have been significantly associated with schizophrenia, autism and autism-spectrum disorders in several studies (Levinson et al., 2011; Luo et al., 2012; McCarthy et al., 2009; Weiss et al., 2008).

The current study has several strengths and limitations. On one hand, due to the small sample size, the power of this study is limited and replication of these findings in larger cohorts is warranted. Nonetheless, several of the results reported in this study overlap with previous reports, hence for these findings our study provides a replication of the previous results. Furthermore, 9.3% of autosomal CNVRs detected on the SNP array were successfully detected in the same individuals using the CNV arrays, thereby providing a technical validation of these data. Also, we acknowledge that possible confounding effects of medication or smoking or other illness-related factors are difficult to account for and might influence the gene expression profiles. To the best of our knowledge this is the most comprehensive genome-wide CNV-gene expression association analysis performed so far and the first genome-wide hypothesis-free study assessing the influence of rare/low frequency CNVs on gene expression in the human brain. Other strengths of this study include assessment of brain tissue which is more relevant for psychiatric diseases and utilization of brain samples with high pH levels to increase reliability of the data.

In conclusion, we used a hypothesis-free approach to identify brain CNVRs which significantly influence genome-wide gene expression levels of nearby transcripts. Such an integrative approach is important to prioritize functional CNVs which exhibit downstream consequences at the gene expression level over other CNVs. This study demonstrates that CNVRs influencing gene expression in the human prefrontal cortex are significantly enriched for rare/low frequency CNVs and gene harboring CNVs. Our results replicate previous findings of associations at 1q21.1 and 22q11.2 regions and suggest the possible role of candidates within the 3q29, 15q11.2, 16p11.2, 16p13.1 and 17q12 loci in schizophrenia and bipolar disorder. Future studies surveying different types of genetic variation in diverse tissues are required to fully comprehend human phenotypic diversity and disease.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neures.2013.10.009.

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ORIGINAL ARTICLE

Revisiting DARPP-32 in postmortem human brain: changes in schizophrenia and bipolar disorder and genetic associations with t-DARPP-32 expression

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Dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa (DARPP-32 or *PPP1R1B*) has been of interest in schizophrenia owing to its critical function in integrating dopaminergic and glutaminergic signaling. In a previous study, we identified single-nucleotide polymorphisms (SNPs) and a frequent haplotype associated with cognitive and imaging phenotypes that have been linked with schizophrenia, as well as with expression of prefrontal cortical DARPP-32 messenger RNA (mRNA) in a relatively small sample of postmortem brains. In this study, we examined the association of expression of two major DARPP-32 transcripts, full-length (FL-DARPP-32) and truncated (t-DARPP-32), with genetic variants of DARPP-32 in three brain regions receiving dopaminergic input and implicated in schizophrenia (the dorsolateral prefrontal cortex (DLPFC), hippocampus and caudate) in a much larger set of postmortem samples from patients with schizophrenia, bipolar disorder, major depression and normal controls (>700 subjects). We found that the expression of t-DARPP-32 was increased in the DLPFC of patients with schizophrenia and bipolar disorder, and was strongly associated with genotypes at SNPs (rs879606, rs90974 and rs3764352), as well as the previously identified 7-SNP haplotype related to cognitive functioning. The genetic variants that predicted worse cognitive performance were associated with higher t-DARPP-32 expression. Our results suggest that variation in *PPP1R1B* affects the abundance of the splice variant t-DARPP-32 mRNA and may reflect potential molecular mechanisms implicated in schizophrenia and affective disorders.

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Keywords: bipolar disorder; DARPP-32; dopamine; postmortem brain; schizophrenia; truncated

INTRODUCTION

Dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa (DARPP-32) has been implicated in the pathogenesis of schizophrenia because of its role as a molecular integrator of dopaminergic and glutaminergic signaling.[1] DARPP-32 is mainly expressed in neurons receiving dopaminergic input in a number of brain regions, particularly the caudate nucleus, putamen, nucleus accumbens and cerebellar cortex.[2–6] When DARPP-32 is phosphorylated at threonine (Thr) 34 by protein kinase A, it is converted into an inhibitor of a multifunctional serine/Thr protein phosphatase-1 (PP-1) and indirectly regulates the physiological activities of a number of downstream proteins by promoting their phosphorylation.[7] DARPP-32 contains four phosphorylation sites, Thr34, Thr75, Serine (Ser) 102 and Ser137 (Figure 1), which regulate its activity. The signaling mediated by the dopamine D_1 receptor activates protein kinase A. In contrast, D_2 receptor stimulation induces dephosphorylation of DARPP-32 at Thr34 via calcium-/calmodulin-dependent protein phosphatase 2B (PP-2B; calcineurin).

Alternative splicing modulates gene function in the majority of organisms by increasing the diversity of expressed messenger RNA (mRNA) transcripts. Human brain displays the most abundant catalog of alternative transcripts compared with any other organ[8,9] and splicing has an important role in brain development. Dysregulation of gene splicing has been implicated in a number of neurodevelopmental disorders,[10] including schizophrenia.[11] DARPP-32 has several splice variants, including one that encodes the full-length protein isoform (FL-DARPP-32) and another that encodes a truncated protein (t-DARPP-32)[12] (Figure 1). t-DARPP-32 lacks the NH_2-terminal Thr34 phosphorylation site and the PP inhibitory domain of DARPP-32,[13] which is critical for dopamine signaling and for regulating the activity of several PP-1-regulated proteins in the brain. t-DARPP-32 is frequently overexpressed in adenocarcinomas[14–18] and regulates cell growth and proliferation through the activation of the phosphoinositide-3-kinase/AKT pathway in human breast cancer cells.[19] Signaling properties of t-DARPP-32, its developmental pattern of expression in the brain and changes in psychiatric disorders have not yet been examined.

There have been a few postmortem brain studies of full-length DARPP-32 (but not t-DARPP-32) in psychiatric disorders, focused primarily on the dorsolateral prefrontal cortex (DLPFC). DARPP-32 protein levels were decreased in patients with schizophrenia and bipolar disorder[20–22] but mRNA results were inconsistent across the studies, showing no changes in schizophrenia,[23] a decrease in schizophrenia patients who died by suicide[24] and an increase in schizophrenia and bipolar disorder.[25] In other brain regions

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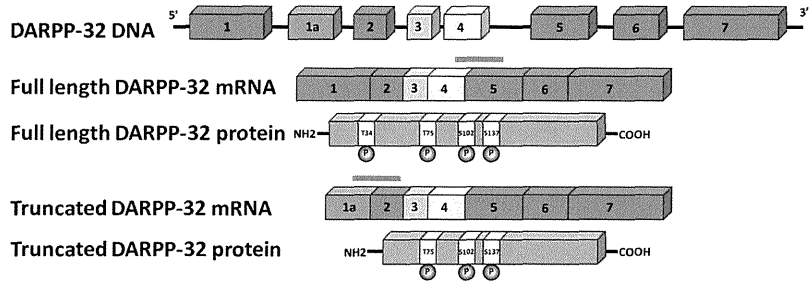

Figure 1. Genomic structure of FL-DARPP-32 and t-DARPP-32. DARPP-32 contains four phosphorylation sites (P), whereas t-DARPP-32 lacks the Thr34 phosphorylation site of DARPP-32. Thus, t-DARPP-32 is devoid of the most critical function that controls the physiological activities of several key proteins through the potent inhibition of protein phosphatase 1. Horizonal lines depict positions of Taqman assays.

changes in schizophrenia were also inconsistent,[22,26–28] perhaps because most postmortem studies are limited by relatively small sample sizes.

Genetic association studies of *PPP1R1B*, the gene encoding DARPP-32 on chromosome 17q21 implicated by linkage studies in risk for schizophrenia[29] and bipolar disorder,[30] have generally not identified disease-associated single-nucleotide polymorphisms (SNPs) or haplotypes.[31–36] The exception is a study from our laboratory, which found a frequent 7-SNP *PPP1R1B* haplotype associated with the schizophrenia in a family-based association analysis, and with enhanced performance on cognitive tests in the same families that depend on frontostriatal function, and also reduced neostriatal volume and increased frontostriatal interactions in multimodal imaging study of normal subjects.[11] We reported that this same haplotype predicted lower expression of full-length DARPP-32 isoform in a relatively small sample ($n = 38$) of postmortem human DLPFC.

To extend this study to patients with schizophrenia, and examine regional and diagnostic specificity of the findings, we investigated expression of two major DARPP-32 transcripts, full-length and truncated, in the DLPFC, hippocampus and caudate of >700 subjects, including patients with schizophrenia and affective disorders, and conducted genotype expresssion analysis across the gene. We also compared temporal expression patterns of the two transcripts to gain insight into their potential roles in early brain development. Our data indicate that the expression of splice variants of DARPP-32 is altered in patients with schizophrenia and affective disorders, and shows some diagnostic specificity. The changes are transcript- and brain region-specific, and genetic variation in DARPP-32 influences alternative gene processing. These results underscore also the importance of using large sample sizes in postmortem brain studies.

MATERIALS AND METHODS

Human postmortem brain tissue collection

Postmortem brains were collected at the Clinical Brain Disorders Branch, National Institute of Mental Health (NIMH), with informed consent from the legal next-of-kin under NIMH protocol 90-M-0142 and at the Brain and Tissue Bank for Developmental Disorders of the National Institute of Child Health and Human Development under contracts NO1-HD-4-3368 and NO1-HD-4-3383. Clinical characterization, neuropathological screening, toxicological analyses, and dissections of the DLPFC and hippocampus were performed as previously described.[37] All DSM-IV Axis I lifetime psychiatric diagnoses were obtained using a combination of data from a telephone screening on the day of donation with next-of-kin, police, autopsy and toxicology reports, psychiatric records, family informant interviews with next-of-kin (NIMH psychological autopsy interview and the severe combined immunodeficiency) and/or interviews with psychiatric treatment providers. A psychiatric narrative summary was compiled from these data, which was reviewed by two board-certified psychiatrists. With respect to the major depressive disorder (MDD) cohort ($n = 138$), 64.5% of cases met criteria for MDD, recurrent, severe, without psychotic features, 21% of cases met criteria for MDD, recurrent, severe, with psychotic features, 13% met criteria for a single episode of MDD, and 1.4% of cases met criteria for dysthymic disorder. Furthermore, 63.8% of the MDD cohort died of suicide, and 10.9% of the MDD cohort died of an accidental overdose. Alcohol and/ or substance abuse/dependence comorbidity within this cohort was high (42%). The striatum including caudate, putamen and nucleus accumbens was identified on the frozen coronal slabs corresponding to the anterior one-third of the inferior temporal cortex and dissected from one hemisphere with a dental drill (Cat# UP500-UG33, Brasseler, Savannah, GA, USA). A total of 709 DLPFC samples from postmortem brains were used for this study. The set of the samples from non-psychiatric individuals consists of 326 subjects ($N = 220$ M; 106 F) ranging from fetal weeks 14–20 (43 fetal subjects; $N = 22$ M; 21 F) and early postnatal age to 85 years of age. Diagnostic analyses were carried out using 176 subjects with schizophrenia ($N = 111$ M; 65 F), 61 subjects with bipolar disorder ($N = 36$ M; 25 F), 138 subjects with MDD ($N = 79$ M; 59 F) and controls ($N = 171$ M; 73 F, >13 years). Demographic data for these samples are summarized in Table 1. To address the question of regional specificity, 466 hippocampal samples and 177 caudate samples, which overlapped with the DLPFC samples (96 and 94%, respectively), were also used in this study. Caudate samples did not include subjects with major depression.

The information about neuroleptic and antidepressant medications in subjects with psychiatric disorders was acquired from toxicology testing as previously described,[37] and lithium medication records were obtained from the subjects' medical charts. Smoking history (habits at the time of death as reported by next-of-kin) was used to establish smoking status.

DNA collection and genotyping

DNA was extracted from cerebellar tissues (Qiagen, Valencia, CA, USA). All brain samples were genotyped using Illumina Human 1M-Duo BeadChips (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. In this study, we examined 58 SNPs located in or near the *PPP1R1B* gene (Supplementary Table S1a). The genotyping of an additional four SNPs, which were not on the Illumina platform but formed a previously described haplotype, was performed using Taqman assays (Applied Biosystems, Foster City, CA, USA) (Supplementary Table S1b). To construct haplotypes for analyses of gene expression data, we used Haploview 3.2 software (http://www.broad.mit.edu/mpg/haploview/index.php) and assigned haplotypes to individuals using PHASE 2.1 (http://www.stat.washington.edu/stephens/phase/download.html), separately in Caucasian and African American populations (frequencies in Supplementary Table S2).

Quantitative real-time PCR

The expression levels of two DARPP-32 transcripts were measured in postmortem DLPFC, hippocampus and caudate samples using quantitative real-time PCR on an ABI Prism 7900 sequence detection system with a 384-well format (Applied Biosystems) by a standard curve method using Taqman assays (Hs00259967_m1, which measures both FL and t-DARPP32, and Hs00938416_g1, which is specific for t-DARPP32). As expression of t-DARPP32 is 30–60-fold lower than of FL-DARPP32 in all three brain regions examined, we will further use the symbol of FL- for the sum of FL and t-DARPP32 measured by the assay Hs00259967_m1. The expression data were normalized to a geometric mean of three housekeeping genes, beta 2-microglobulin (B2M), beta glucuronidase and beta actin expression.

Table 1. Demographic summary of postmortem brain samples

Variable	*Control*		*Schizophrenia*	*Bipolar*	*Depression*
	Fetal	*Postnatal*			
Number of samples	43	283 1–13 years; $N=39$, >13 years; $N=244$	176	61	138
Mean age (years)	14–20 GW	35.0 ± 20.8	50.0 ± 15.0	44.8 ± 14.2	45.0 ± 14.2
Sex	22 M,21 F	198 M, 85 F	111 M, 65 F	36 M, 25 F	79 M, 59 F
Race	5 CAUC, 37 AA, 1 others	132 CAUC, 139 AA, 12 others	96 CAUC, 73 AA, 7 others	51 CAUC, 6 AA, 4 others	119 CAUC, 14 AA, 5 others
Mean PMI (h)	2.6 ± 2.1	29.0 ± 14.4	38.6 ± 24.1	32.9 ± 18.4	37.9 ± 25.3
Mean brain pH	NA	6.5 ± 0.3	6.4 ± 0.3	6.4 ± 0.3	6.4 ± 0.3
Mean RIN	8.8 ± 1.3	8.2 ± 0.8	7.8 ± 1.0	8.0 ± 0.9	8.0 ± 0.9

Abbreviations: AA, African American; CAUC, Caucasian; GW, gestational week; PMI, postmortem interval; RIN, RNA integrity number; NA, not applicable.

Determination of the effects of antipsychotic drugs in rats

To test whether chronic exposure to antipsychotic drugs might contribute to changes in the expression levels observed in patients with schizophrenia, we measured the expression of FL_DARPP32 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 given intraperitoneal injections of haloperidol (0.08, 0.6 and 1 mg kg^{-1}, Research Biochemicals, Natick, MA, USA), clozapine (0.5, 5, 10 mg kg^{-1}, Sandoz Research Institute Berne, Berne, Switzerland) or vehicle (0.02% lactic acid) once daily for 28 days (8–10 rats per dose). Rats were killed 7 h after the last injection. mRNAs in the frontal cortex were measured using Taqman ABI assays (GAPDH (Rn99999916_s1), PBGD/HMBS (Rn00565886_m1), B2M (Rn00560865_m1), FL-DARPP-32: forward primer, 5′-CAGGCGGAGGTCCTGAAA-3′ and reverse primer, 5′-AACTCTGAGGACCAAGGGGAA-3′, probe 5′-GGACTGCGGGGCA-3′). The expression data of FL-DARPP32 were normalized to a geometric mean of three control genes.

Statistical analyses

Comparisons between groups were made by analysis of covariances (ANCOVAs) for each transcript with diagnosis, antemortem medication and genotype as independent variables, and sex, age at death, race, smoking status and RNA quality as covariates using STATISTICA version 7.1 (StatSoft, Tulsa, OK, USA). Fisher least significant difference *post hoc* comparisons were used to evaluate diagnostic group differences. All data were covaried by sex, age at death, race, smoking status and RNA integrity number, and Bonferroni corrections ($P<0.05$) were applied in the analysis of the SNP data.

RESULTS

Expression of DARPP-32 transcripts in the diagnostic groups

In the DLPFC, we found that the expression levels of FL-DARPP32 were significantly increased in major depression as compared with all other groups (ANCOVA: F (3584) = 5.72, $P=7.3E-4$, *post hoc* Fisher LSD: by 20%, $P=1.1E-4$ as compared with controls, $P=0.01$ as compared with schizophrenia; and $P=0.02$ as compared with bipolar disorder) (Figure 2a). The expression of t-DARPP-32 was significantly increased in schizophrenia and bipolar disorder as compared with controls (ANCOVA: F (3591) = 5.72, $P=3.1E-7$, *post hoc* Fisher LSD: by 20%, $P=3.4E-4$, by 68%, $P=9.4E-10$, respectively) (Figure 2b). The expression of t-DARPP-32 in bipolar disorder patients was also significantly higher than in patients with schizophrenia and patients with major depression (*post hoc* Fisher LSD: $P=1.5E-3$, $P=1.1E-5$, respectively) (Figure 2b).

The levels of FL- and t-DARPP32 correlated significantly in all three brain regions in all subjects (all Spearman r values were positive >0.6, P values $=<E-17$), and separately in the diagnostic groups (r values >0.4, P values $<E-05$).

As other studies[23] have shown that suicide may be a factor in the analysis of gene expression data in the prefrontal cortex, we examined the differences in t-DARPP-32 expression between schizophrenia patients who died by suicide (SZ-S) with those who died of other causes (SZ-NS). t-DARPP-32 expression was significantly lower in SZ-S as compared with SZ-NS (Mann–Whitney U-test: $P=0.014$), although there were no differences in the expression of FL-DARPP-32. In a group of MDD patients, subjects who died by suicide had significantly lower expression of FL- and t-DARPP32 than patients who died of other causes (Mann–Whitney U-test: $P=0.011$). There were no differences in the expression of the two transcripts between bipolar patients who died by suicide (BP-S) and those who died of other causes (BP-NS), Supplementary Figure S1.

In the hippocampus, the expression levels of FL-DARPP-32 were significantly increased only in bipolar disorder as compared with all other groups (ANCOVA: F (3381) = 16.59, $P=3.8E-10$, *post hoc* Fisher LSD: by 87%, $P=3.0E-9$ as compared with controls, $P=9.6E-10$ as compared with schizophrenia; and $P=7.7E-8$ as compared with major depression) (Figure 2c). The expression of t-DARPP-32 was also significantly increased only in bipolar disorder as compared with all other groups (ANCOVA: F (3382) = 2.77, $P=0.04$, *post hoc* Fisher LSD: by 101%, $P=0.02$ as compared with controls, $P=0.019$ as compared with schizophrenia; and $P=0.015$ as compared with major depression) (Figure 2d).

In the caudate, the expression levels of FL-DARPP32 were not different in patients with schizophrenia and bipolar disorder as compared with controls (ANCOVA: F (2165) = 1.42, $P=0.25$) (Figure 2e). There were also no differences in the expression of t-DARPP-32 between the groups (ANCOVA: F (2161) = 0.356, $P=0.701$) (Figure 2f).

There were no significant effects of smoking on expression levels of either transcript in the DLPFC and hippocampus, although smoking increased expression of FL-DARPP-32 and t-DARPP-32 in the caudate (ANCOVA: F (1165) = 4.42, $P=0.037$, ANCOVA: F (1161) = 5.78, $P=0.017$, respectively). There were also no significant effects of antipsychotic medication, antidepressants or lithium on the expression levels of the two transcripts in any brain region examined (Supplementary Table S3). In addition, the expression of FL-DARPP-32 was not altered in the rat frontal cortex in response to chronic treatment with the neuroleptic drugs, clozapine and haloperidol (ANOVA: F (6,55) = 0.12, $P>0.9$) (Supplementary Figure S2).

Expression of DARPP-32 transcripts during the life span

To investigate the developmental profile of expression of the two transcripts, we used samples from the DLPFC and the hippocampus of normal controls across almost the entire human life span,

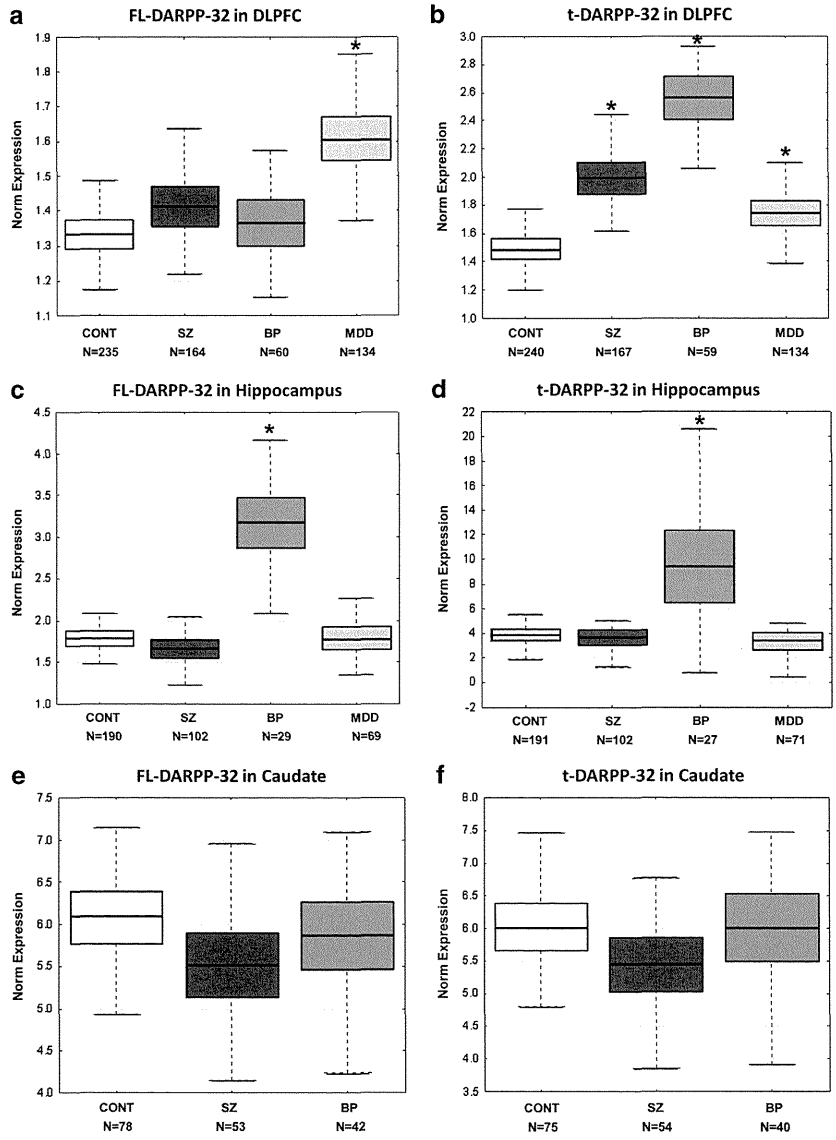

Figure 2. Diagnostic differences in the expression of DARPP-32 transcripts. Mean mRNA expression of FL-DARPP-32 (**a**, **c**, **e**) and t-DARPP-32 (**b**, **d**, **f**) in the DLPFC (**a**, **b**),hippocampus (**c**, **d**),and caudate (**e**, **f**) from control subjects (CONT), and patients with schizophrenia (SZ), bipolar disorder (BP) and major depressive disorder (MDD) measured using quantitative real-time PCR. Data are normalized to a geometric mean of three housekeeping genes, beta 2-microglobulin (B2M), beta glucuronidase (GUSB) and beta actin (ACTB) expression. The asterisks over the bars indicate the significant differences compared with controls using a *post hoc* Fisher LSD test. DLPFC, dorsolateral prefrontal cortex.

from the 14th through 20th gestational week and from birth through old age (85 years of age) (See Table 1 for subject demographics). For the caudate, samples from subjects >13 years of age were used.

In the DLPFC, the expression of FL-DARPP-32 was relatively high during the prenatal period, dropped at birth and then increased gradually throughout postnatal life until old age (Figure 3a). In contrast, t-DARPP-32 was expressed at very low levels prenatally, increased sharply from birth to pubescent ages and continued to increase, albeit at a slower pace, throughout the rest of life (Figure 3b). In contrast to high-positive correlations between the splice variants in adult control subjects and in all three diagnostic groups, there was no significant correlation between FL-DARPP32 and t-DARPP32 during development of the DLPFC (from the fetal period to 20 years of age, Spearman $r=0.12$, $P>0.1$). In the hippocampus, both transcripts were expressed at very low levels prenatally, increased gradually from birth to pubescent ages and then remained at almost constant levels (Figures 3c and d). In the caudate, which only included samples from subjects older than 13 years of age, there was no evidence for an increase in the expression of the two transcripts with aging (from 13 to 80 years of age) as in the DLPFC and to a lesser extent in the hippocampus (Figures 3e and f). Repeated measures two-way ANOVA (mixed type, factors: periods (prenatal or postnatal) and brain regions (DLPFC or hippocampus)) revealed a significant interaction effect for the FL-DARPP $=32$ expression (F (1, 236) $=10.96$; $P=1.1E-3$), but not for t-DARPP-32 (F (1, 236) $=1.81$; $P=0.18$), confirming our observation that only FL-DARPP-32 shows a different expression trajectory in the DLPFC than in the hippocampus (Supplementary Figure S4a) and that t-DARPP32 in both regions shows significantly lower expression during the prenatal period vs postnatal life (Mann–Whitney U-test: $P=6.1E-25$ in DLPFC; $P=2.0E-15$ in

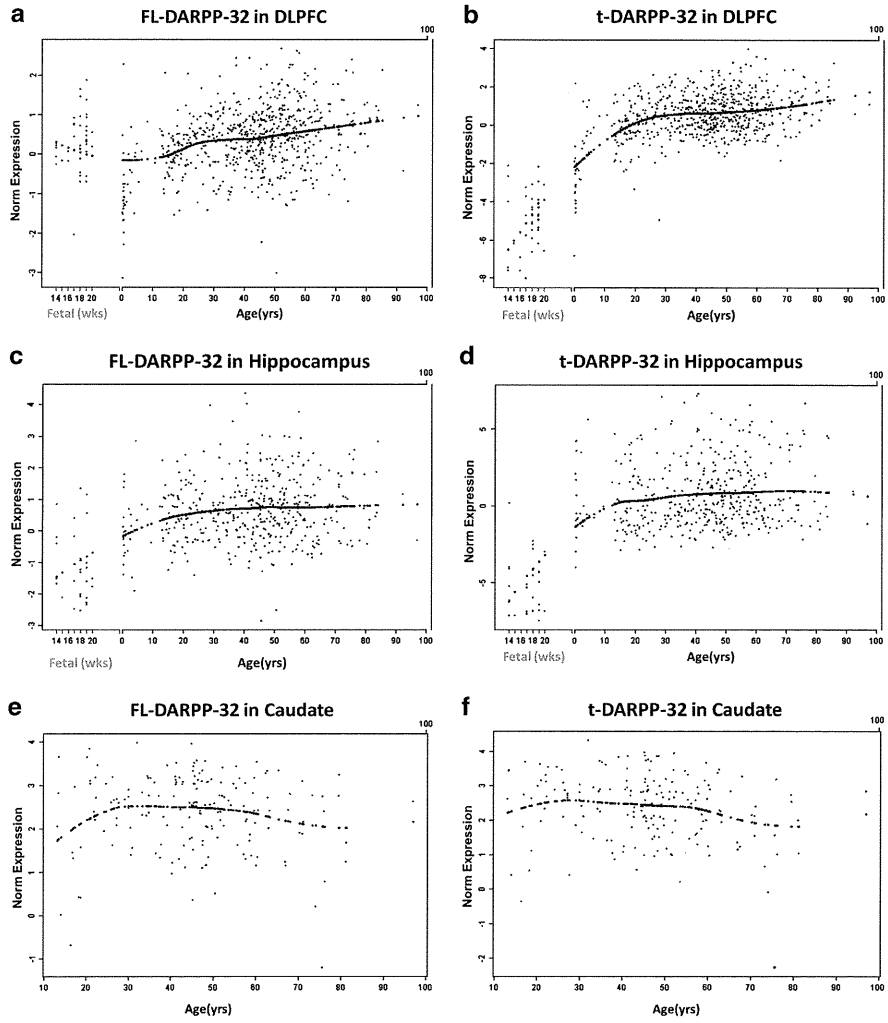

Figure 3. Developmental expression patterns of DARPP-32 transcripts in postmortem brains. Expression of FL-DARPP-32 (**a**, **c**, **e**) and t-DARPP-32 (**b**, **d**, **f**) in the DLPFC (**a**, **b**), hippocampus (**c**, **d**) and caudate (**e**, **f**) across the lifespan, from gestational week 14 through 20 and from birth (0) until old age. Gene expression is displayed as a function of age (black dots represent individual subjects). A curved line represents a LOESS fit across the lifespan. Only non-psychiatric controls are included. DLPFC, dorsolateral prefrontal cortex.

hippocampus) (Supplementary Figure S4b). These findings are summarized in a schematic overview (Figure 4).

Effects of DARPP-32 genotype on expression of DARPP-32 transcripts

We examined the effects of 58 *PPP1R1B* SNPs on the mRNA expression levels of DARPP-32 splice variants in a large cohort of patients and controls from mainly two ethnic groups, Caucasians and African Americans. Twenty five of these SNPs were highly correlated ($r^2 > 0.8$). We also examined the effects of the previously identified 7-SNP haplotype, which predicted the mRNA expression of full-length DARPP-32 isoform in a smaller sample of postmortem DLPFC obtained from Caucasian subjects,[11] and was associated with schizophrenia and with cognitive performance in a clinical study.

In the DLPFC in the whole cohort, we found that six SNPs predicted expression of t-DARPP-32, including three SNPs that were previously associated with schizophrenia and with cognitive and imaging phenotypes[11] (Figure 5a, Supplementary Figure S5). In particular, rs907094 and rs3764352, which were in complete linkage disequilibrium, showed an allele dose-dependent effect (ANCOVA: F (2563) = 8.54, P = 2.2E-4) (Figure 5b); individuals homozygous for the G allele at rs907094 and rs3764352 had significantly higher expression than heterozygous A/G individuals (P = 0.04), who had also significantly higher expression than homozygous A individuals (P = 1.1E − 3). Furthermore, we also found a significant effect of SNP rs879606 (not in linkage disequilibrium with the two SNPs described above) on the expression of t-DARPP-32 mRNA (ANCOVA: F (2562) = 7.24, P = 7.8E-4) (Figure 5c). Individuals homozygous for the A allele at rs879606 had significantly higher expression than homozygous G individuals (P = 7.4E − 4).

To address the possibility that either antipsychotics or lithium might have contributed to the observed effects, we conducted genetic association analysis in subjects who had no history of treatment with antipsychotics or lithium (all controls and major depressive patients). Similarly to the effects in all subjects, t-DARPP-32 expression in this cohort was associated with the genotypic variants at rs907094 and rs3764352 (ANCOVA: F

Transcript	Fetal vs Postnatal			Schizophrenia			Bipolar			Depression		
	PFC	HP	CAU	PFC	HP	CAU	PFC	HP	CAU	PFC	HP	CAU
FL-DARPP-32	=	↓	—	=	=	=	=	↑	=	↑	=	—
t-DARPP-32	↓	↓	—	↑	=	=	↑	↑	→	↑	=	—

Figure 4. A schematic overview of major findings in this study. The expression profile of DARPP-32 transcripts in this study are shown; an arrow pointing up indicates upregulation, an arrow pointing down indicates downregulation and '=' indicates no change. CAU, caudate; HP, hippocampus; PFC, prefrontal cortex.

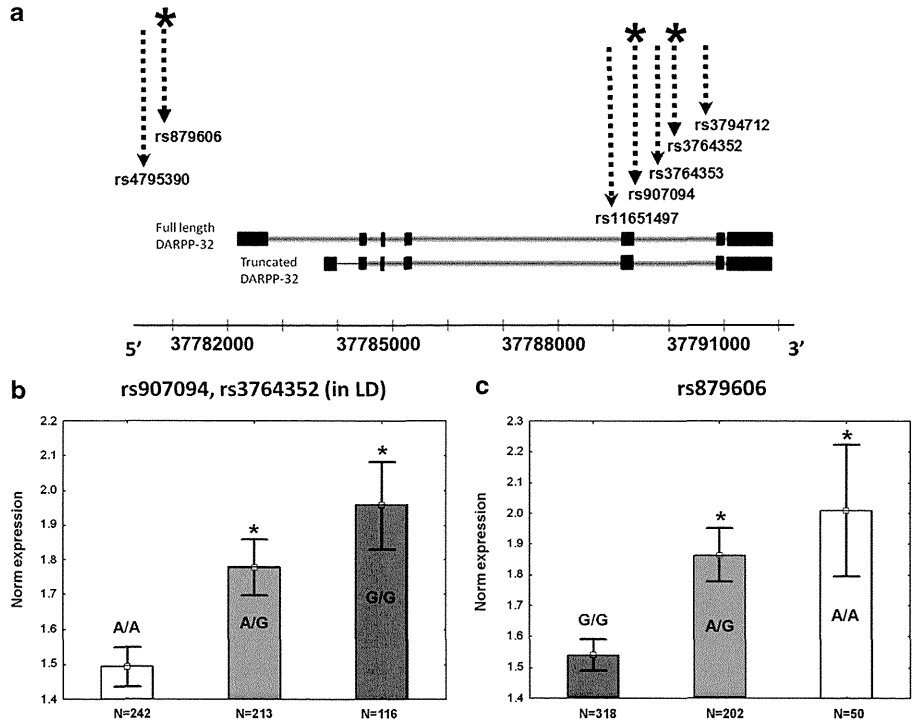

Figure 5. *PPP1R1B* SNP associations with DARPP-32 expression in DLPFC. Schematic overview of SNP positions, which were associated with DARPP-32 expression (**a**). An asterisk depicts a significant association of the SNP with t-DARPP-32. Three other SNPs, which were associated with t-DARPP-32 expression, rs8069074, rs7503377 and rs11654954, located in the far upstream region of *PPP1R1B* (Chr position: 37685401, 37708841 and 37745979, respectively) are not shown in this diagram. Seven SNPs indicated by dotted lines compose a haplotype associated with performance on cognitive tests. There was a significant association between t-DARPP-32 expression in the DLPFC and rs907094, rs3764352 (**b**) and rs879606 (**c**). Error bars in **b**, **c** are s.e.m. The asterisks over the bars indicate the significant differences compared with the major genotype using a *post hoc* Fisher LSD test. LD, linkage disequilibrium.

(2382) = 3.72, $P = 0.025$) and rs879606 (ANCOVA: F (2382) = 6.77, $P = 1.4E-3$).

Further analysis revealed a significant impact of the *PPP1R1B* haplotype, identified from clinical and cognitive associations,[11] on t-DARPP-32 but not FL-DARPP-32 expression. In accordance with the SNP analysis, expression of t-DARPP-32 was highest for carriers of the less frequent (GATGTCA) haplotype and lower for the homozygotes of the frequent (CGCACTC) haplotype and for subjects heterozygous for CGCACTC in combination with a rare haplotype (F(2270) = 3.41, $P = 0.035$, Supplementary Figure S6). There was no impact of haplotypes on FL-DARPP-32 expression in this cohort (all P-values > 0.5).

There were no significant associations (or trends) between any SNPs, genotypes or haplotypes examined in this study and the mRNA expression levels of DARPP-32 splice variants in the hippocampus and caudate in all subjects or in any of the diagnostic or race group separately.

DISCUSSION

In this study, we show that the expression of t-DARPP-32 is significantly increased in the DLPFC of patients with schizophrenia and of patients with bipolar disorder, that these alterations are region-specific and that the expression of t-DARPP-32 in the DLPFC is associated with genetic variants previously linked to schizophrenia and to cognitive functioning in an earlier study. Expression of full-length DARPP-32 transcript also showed notable regional and diagnostic specificity; FL-DARPP-32 was upregulated in the DLPFC but not hippocampus in major depression, and vice versa in bipolar disorder. This is consistent with at least some

other postmortem molecular studies, which implicate prefrontal cortex in major depression[38] and hippocampus in bipolar disorder.[39] Moreover, we show that the expression of t-DARPP-32 in the DLPFC and hippocampus increases dramatically from the fetal period to postnatal age, suggesting that t-DARPP-32 may have a role in the development of monaminergic innervation at the transition from pre- to postnatal age. It is also possible that this increase simply reflects maturation of monaminergic connections. As t-DARPP-32 appears to interfere with protein kinase A inhibition by FL-DARPP-32 in a dominant-negative fashion[40] and thus activates phosphoinositide-3-kinase/AKT pathway signaling,[20] it may promote neurogenesis in the hippocampus[41] and dendritogenesis in the neocortex.[42] Upregulation of t-DARPP-32 may also lead to attenuation of dopamine signaling because t-DARPP-32 lacks the Thr 34 phosphorylation site and PP-1 inhibitory domain, which are critical for dopamine signaling in the brain. As t-DARPP-32 and FL-DARPP-32 have antagonistic effects on responses to anticarcinogenic agents and anti-apoptotic action,[13,19,40,43] it is possible that these two transcripts have also antagonistic effects on the efficiency of dopamine system. Thus, it appears feasible that changes in t-DARPP-32 expression in the DLPFC may affect cognitive functions by altering dopamine signaling. This notion is further corroborated by the genetic findings. The minor alleles at rs907094, rs3764352 and rs879606 that have been associated with worse cognitive functioning across multiple domains[12] predict higher expression of t-DARPP-32 and their impact is specific to the DLPFC, a critical brain region for complex cognitive behaviors, personality, decision making and orchestration of thoughts and actions. Interestingly, a recent imaging report indicated that the carriers of minor alleles at rs907094, rs3764352 and rs879606 show lower connectivity within the network of brain regions (inferior frontal gyrus, parahippocampal gyrus and putamen) as compared with major allele homozygotes during associative emotional learning, posited to be dependent on the integration between glutamate and dopamine signaling.[44] Two other reports showed that genetic variation at rs907094 has been associated with processing of anger[45] and reward learning;[46] again, minor allele carriers showed worse cognitive function and learning.

There are several issues in the comparisons of the patient and control samples that require further attention. First, the confounding effects of medication and other illness-related factors on FL-DARPP-32 and t-DARPP-32 levels cannot be conclusively resolved in this study. Although we did not detect the effects of antipsychotics, antidepressants or lithium on FL-DARPP-32 and t-DARPP-32 expression levels and did not find effects of antipsychotics in the rat model, chronic administration of antidepressants and lithium (but not antipsychotics) has been reported to increase DARPP-32 immunoreactivity in the rat frontal cortex.[47] As rats also express t-DARPP-32,[48] further studies are warranted to examine the effects of chronic administration of psychotropic drugs on t-DARPP-32 expression in rat brains. It is thus possible that diagnostic differences (in particular, alterations in patients with bipolar disorder) might be owing at least in part to medication.

It should be noted that we have not measured t-DARPP-32 protein levels in this study. Our data from the subset of samples using a previously described antibody[20] that recognizes only full-length DARPP-32 protein suggest, however, that there is no relationship between DARPP-32 protein immunoreactivity and mRNA levels (Supplementary Figure 3). This may also be owing to poorly quantitative methods used to measure protein levels and/or confounding factors affecting protein stability. In general, postmortem studies in patients should be viewed with caution with regard to the difficulty to control disease-related factors that may potentially affect the analyses.

Second, our current results on the expression of DARPP-32 isoforms are somewhat inconsistent with those published in the previous study from our group.[11] We have now shown that minor alleles at three SNPs as well as the less frequent seven-SNP haplotype strongly predict an upregulation of t-DARPP-32, and that the expression FL-DARPP-32 is not significantly associated with these SNPs or with the haplotype. This is in contrast to our earlier results. One possibility is population heterogeneity or the influence of other non-genetic factors. This possibility is supported by the fact that the subset of the same Caucasian subjects/carriers of the frequent haplotype from the previous study does again show a trend for the upregulation of FL-DARPP-32 (data not shown), although the results for the whole cohort are not significant (there was a high correlation between the previous data and the current ones, $r = 0.77$). Another possibility is that our previous study might have been underpowered to detect differences with modest effect sizes.

Third, it should be noted that the associations of t-DARPP-32 expression with genotypes are tissue-specific, indicating that genetic structure, although perhaps necessary, is not a sufficient factor for regulating t-DARPP-32 expression in the brain. It is possible that epigenetic mechanisms, such as DNA methylation, are involved. We have explored this possibility in a preliminary analysis in a subset of samples, assayed with the Illumina Infinium 27 K chip. These results indicate no relationship in the DLPFC between expression levels of the two DARPP-32 transcripts and methylation status at two CpG loci in the promoter region of *PPP1RB1* that are contained on this chip (data not shown). To address the question of DNA methylation in more depth, we are currently examining a larger set of subjects using a platform that covers more CpG sites, including also intragenic loci within *PPP1RB1*.

In summary, our results show that the expression of the t-DARPP-32 transcript is altered in the postmortem brains of patients with schizophrenia and bipolar disorder, and, selectively in prefrontal cortex, is associated with genetic variation in the *PPP1R1B*. Our results emphasize the importance of using large sample sizes in postmortem brain research focused on effects of genetic variation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

YK, BKL, DRW and JEK designed the research and contributed to interpretations of all experiments and to editing of the manuscript; YK, BKL, CL, BK and DD performed the research; YK, BKL, TY and DD analyzed the data; and YK, BKL, DRW and JEK wrote the paper.

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Elevated postmortem striatal t-DARPP expression in schizophrenia and associations with *DRD2/ANKK1* polymorphism

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ABSTRACT

Background: Dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa (DARPP-32) and calcineurin (CaN) have been implicated in the pathogenesis of schizophrenia because they function as molecular integrators of dopamine and glutamate signaling. DARPP-32 and CaN are mainly expressed in the caudate nucleus and putamen; however, a few postmortem brain studies have focused on DARPP-32 expression in striatum from patients with schizophrenia.
Methods: We used immunoblotting techniques and postmortem tissue samples from patients with schizophrenia and from normal control individuals to examine the expression of two major DARPP-32 isoforms, full-length (FL-DARPP) and truncated (t-DARPP), and of CaN in the striatum. We also assessed whether there was any significant correlation between the expression levels of either protein and the A1 allele of Taq1A genotype in the dopamine D2 receptor (*DRD2*) gene/ankyrin-repeat containing kinase 1 (*ANKK1*) gene.
Results: We found that the mean t-DARPP expression level in the caudate was higher in patients with schizophrenia than in control individuals ($P < 0.05$) and the A1 allele of Taq1A genotype in *DRD2/ANKK1* was significantly associated with elevated expression of t-DARPP in the caudate. Also, the A1 allele was significantly correlated with the total score of antemortem psychiatric symptoms.
Conclusion: These results may reflect potential molecular mechanisms important to the pathogenesis of schizophrenia.

1. Introduction

Dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa (DARPP-32) may be involved in the pathophysiology of schizophrenia, because it integrates dopaminergic neurotransmission into many other neurotransmitter systems, including glutamatergic signaling (Svenningsson et al., 2004). DARPP-32 is enriched in dopamine-innervated brain regions—predominantly in the caudate nucleus, putamen, nucleus accumbens, and cerebellar cortex (Berger et al., 1990; Brene et al., 1995; Ouimet et al., 1984, 1992; Walaas and Greengard, 1984). When phosphorylated at threonine (Thr) 34, DARPP-32 is a potent inhibitor of protein phosphatase-1; consequently, phosphorylated DARPP-32 indirectly regulates the activities of protein phosphatase-1 substrates (Yger and Girault, 2011). Upon stimulation of the dopamine D_1 receptor stimulation, protein kinase A (PKA) phosphorylates Thr 34 of DARPP-32; in contrast, D_2 receptor stimulation causes cellular Ca^{2+} to increase, and this Ca^{2+} increase results in calcineurin (CaN)-mediated dephosphorylation of DARPP-32 at Thr34. CaN is the only Ca^{2+}/Calmodulin-dependent serine/threonine phosphatase currently known (Rusnak and Mertz, 2000), and it is downstream of dopaminergic and glutamatergic systems. Therefore, the molecular pathways that include DARPP-32 and calcineurin may be the key to the pathogenesis of schizophrenia. There are two major splice variants of mRNAs encoding DARPP-32: one encodes the full-length protein isoform (FL-DARPP), while the other encodes a truncated

Abbreviations: ANCOVA, analysis of covariance; ANKK1, ankyrin-repeat containing kinase 1; CaN, calcineurin; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa; DIBS, Diagnostic Instrument for Brain Science; DLPFC, dorsolateral prefrontal cortex; *DRD2*, dopamine D2 receptor gene; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders; ICD, International Statistical Classification of Diseases and Related Health Problems; PKA, protein kinase A; PMI, postmortem interval; PP-1, protein phosphatase-1; RFLP, restriction fragment length polymorphism; SDS-PAGE, sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis; Thr, threonine.

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isoform (t-DARPP) (Meyer-Lindenberg et al., 2007). Notably, t-DARPP lacks the NH_2-terminal Thr34 phosphorylation site and the protein phosphatase inhibitory domain (El-Rifai et al., 2002); therefore, it cannot transmit dopaminergic signaling or regulate the activity of protein phosphatase 1 (PP-1)-regulated proteins.

The expression of both DARPP-32 isoforms and CaN in the dorsolateral prefrontal cortex (DLPFC) from patients with schizophrenia has been examined thoroughly (Albert et al., 2002; Baracskay et al., 2006; Hakak et al., 2001; Ishikawa et al., 2007; Kozlovsky et al., 2006; Kunii et al., 2011a; Zhan et al., 2011). However, there are only a few postmortem brain studies focused on DARPP-32 or CaN expression in other brain regions (Kunii et al., 2011b; Nishiura et al., 2011) including the striatum or peripheral tissues (Torres et al., 2009). For example, our immunohistochemical findings indicated that patients with schizophrenia do not have altered DARPP-32 expression in the striatum relative to control individuals (Kunii et al., 2011a), but they do have an increased ratio of CaN immunoreactive neurons in the caudate nucleus (Wada et al., 2012); however, these immunohistochemical studies had a methodological limitation on quantitative analyses of molecular expression. Thus, detailed investigations of the expression of DARPP-32 and of CaN in the striatum of patients with schizophrenia are warranted. Here, we used immunoblotting techniques and postmortem tissue samples from patients with schizophrenia and from control individuals to investigate the expression of two major DARPP-32 isoforms—FL-DARPP, t-DARPP—and of CaN in the striatum.

Additionally, the gene for ankyrin-repeat containing kinase 1 (ANKK1) is located downstream of the dopamine D2 receptor (*DRD2*) gene. A Taq1A polymorphism that causes an amino acid substitution within the 11th of the 12 ankyrin repeats in ANKK1 (Glu713Lys of 765 residues) may influence DRD2 substrate-binding specificity, regulates the synthesis of dopamine in the brain (Neville et al., 2004) and can affect DRD2 expression (Lucht and Rosskopf, 2008). Also, this polymorphism is reportedly associated with clinical responses to antipsychotics (Kwon et al., 2008; Schafer et al., 2001) in patients with schizophrenia, although this association was not found in a recent meta-analysis (Zhang et al., 2010). There have only been a few studies that examined genetic association of Taq1A or *ANKK1* implicated by linkage studies in risk for schizophrenia (Dubertret et al., 2010; Golimbet et al., 2003; Parsons et al., 2007), both of which showed positive results. Thus, in this study we also analyzed the associations of the expression of FL-DARPP, t-DARPP, and CaN in the striatum with the Taq1A polymorphism in *DRD2/ANKK1*.

2. Methods

2.1. Human postmortem brain tissue collection

Postmortem brain tissue samples from patients with schizophrenia were obtained from the Fukushima Brain Bank at the Department of Neuropsychiatry, Fukushima Medical University. Normal adult postmortem brain tissue samples were obtained from autopsy cases at the Brain Research Institute, University of Niigata, and were used as controls. The use of postmortem human brain tissues for the present study was approved by the Ethics Committee of Fukushima Medical University and complied with the Declaration of Helsinki. All procedures were carried out with the informed written consent of the next of kin. The samples consisted of brain tissue from 12 subjects with schizophrenia and 12 control subjects. Demographic data associated with each sample are summarized in Table 1. Each patient with schizophrenia fulfilled the diagnostic criteria established by the American Psychiatric Association (Diagnostic and Statistical Manual of Mental Disorders: DSM-IV), and had no history of any other neurological disorder or substance abuse, except that some patients smoked tobacco. Our diagnoses were based on the following four data sources: psychological autopsy interviews with family informants, interviews with treating professionals, psychiatric record reviews, and semistructured tools

Table 1
Demographic summary of the subjects.

Subject	Sex	Age	PMI (h)	Cause of death	CP eq (mg)/day
Control					
1	F	35	2	Chronic myeloblastic leukemia	–
2	M	67	1	Hemophagocytosis	–
3	F	49	1	Sudden death	–
4	F	75	15	Cushing's disease	–
5	F	55	4	Adult T-cell leukemia	–
6	M	51	5.5	Acute abdomen	–
7	M	56	20	Marinesco–Sjögren syndrome	–
8	M	68	4	Myopathy	–
9	F	49	2	Crow–Fukase syndrome	–
10	M	55	4.5	Myotonic dystrophy	–
11	F	79	NA	Foix–Alajournine	–
12	M	60	2	Small infarcts	–
Total	6M/6F	58 ± 12	5.5 ± 6.1		
Schizophrenia					
1	M	70	28	Bile duct cancer	550
2	M	75	17	Pneumonia	225
3	M	64	4.5	Pneumonia	15
4	F	87	3.5	Suffocation	0
5	F	68	15	Chronic renal failure	325
6	M	60	45	Acute myocardial infarction	1650
7	F	77	25	Pneumonia	114
8	M	66	7	Pneumonia	0
9	F	65	5	Colon cancer	420
10	M	74	17.5	Pneumonia	120
11	M	71	14.5	Pneumonia	303
12	M	39	34.5	Suicide	155
Total	8M/4F	68 ± 12	18 ± 8.5		

PMI – postmortem interval; CP – chlorpromazine.

such as the Diagnostic Instrument for Brain Science (Hill et al., 2005) (DIBS) to review available clinical histories. DIBS is different from the strict criteria of DSM-IV or International Statistical Classification of Diseases and Related Health Problems (ICD)-10 and instead focuses on core diagnostic psychiatric symptoms that were present during a patient's lifetime, and may be the most effective available tool for the diagnosis of psychiatric diseases after death. The DIBS profile associated with each sample is summarized in Table 2. For each patient, the daily dosage of antipsychotics prescribed during the 3 months immediately preceding death is represented as chlorpromazine-equivalent dose (mg). Additionally, none of the individuals from whom the control samples were taken had any recorded episode of mental disorders, neurological disorders, or substance abuse, except that some of these individuals smoked tobacco. Each brain included in the present study was closely examined by neuropathologists, and no neurological disorders were found during these examinations; however, changes indicative of mild senility were evident in some brains. For each brain, the striatum included the caudate and putamen and was identified on the frozen coronal slabs corresponding to the anterior one-third of the inferior temporal cortex as previously described (Kunii et al., 2013).

2.2. DNA collection and genotyping

Genomic DNA was extracted from the brain samples and a previously described PCR-restriction fragment length polymorphism (PCR-RFLP) method was used to determine the genotype of *DRD2/ANKK1* (Taq1A) in each sample (Grandy et al., 1993; Miura et al., 2012).

2.3. Immunoblotting

Brain tissues were homogenized in lysis buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 5 mM EDTA) that contained a cocktail of protease inhibitor (Roche, Indianapolis, IN, USA). Each homogenate was subject to centrifugation at 15,000 rpm for 30 min, and the supernatant was then recovered from each sample; Micro BCA kits (Pierce Chemical,

Table 2
DIBS profile and Taq1A genotype in *DRD2* associated with each postmortem brain sample.

	DIBS total score	DIBS positive score	DIBS negative score	DIBS general score	Previous positive score	Taq1A genotype
Control						
1	–	–	–	–	–	A1/A2
2	–	–	–	–	–	A2/A2
3	–	–	–	–	–	A2/A2
4	–	–	–	–	–	A1/A2
5	–	–	–	–	–	A2/A2
6	–	–	–	–	–	A1/A2
7	–	–	–	–	–	A1/A2
8	–	–	–	–	–	A2/A2
9	–	–	–	–	–	A2/A2
10	–	–	–	–	–	A2/A2
11	–	–	–	–	–	A2/A2
12	–	–	–	–	–	A2/A2
Schizophrenia						
1	25	14	6	5	25	A2/A2
2	13	4	4	5	19	A1/A1
3	20	8	6	6	7	A1/A2
4	0	0	0	0	7	A1/A1
5	14	12	0	2	12	A2/A2
6	30	23	4	3	21	A2/A2
7	16	7	6	3	10	A1/A2
8	8	2	2	4	8	A1/A2
9	21	14	4	3	0	A2/A2
10	8	1	5	2	24	A2/A2
11	6	2	3	1	7	A1/A2
12	4	2	1	1	2	A1/A2

Rockland, IL, USA) were used to determine the protein content of each supernatant. Sample buffer [5×; 0.31 M Tris–HCl (pH 6.8), 10% SDS, 50% glycerol, 25% 2-mercaptoethanol, bromophenol blue] was added to an aliquot of each supernatant, and each mixture was boiled at 95 °C for 5 min. For each tissue sample, 20 μg of protein was subjected to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Schleicher and Schull, Dassel, Germany). Each membrane was incubated with anti-calcineurin (1:500, BD Transduction Lab, Franklin Lakes, NJ, USA) or anti-DARPP-32 antibodies (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed, and then incubated with peroxidase-conjugated anti-rabbit or peroxidase-conjugated anti-mouse Ig antibody (Jackson Immunoresearch Laboratory, West Grove, PA). A subsequent chemiluminescence reaction and X-ray film exposure (ECL kit; GE Healthcare, Little Chalfont, UK) were used to detect immunoreactivity. Image J (National Institutes of Health, USA) and densitometry were used to measure the relative amount of protein in each band.

2.4. Statistical analysis

STATISTICA version 10 (StatSoft Inc.) and analysis of covariance (ANCOVA) were used for comparisons between groups (schizophrenia versus control) for each isoform; diagnosis and Taq1A genotype were set as independent variables, and sex, age at death, and postmortem interval (PMI) were set as covariates. Fisher least significant difference post hoc comparisons were used to evaluate group differences. Also, Spearman's rank correlation coefficients were used to assess the association between expressions of each isoform and estimated total dosage of neuroleptic drugs.

3. Results

3.1. Expression of DARPP-32 isoforms and calcineurin in the diagnostic groups

The expression of t-DARPP in the caudate was significantly higher in subjects with schizophrenia than in control subjects [ANCOVA: $F(1, 18) = 4.88$, $P = 0.04$] (Fig. 1b). However, there were no significant differences with regard to the expression of FL-DARPP or of CaN in the caudate between subjects with schizophrenia and control subjects [ANCOVA: $F(1, 18) = 0.66$, $P = 0.43$, ANCOVA: $F(1, 18) = 1.44$, $P = 0.25$, respectively] (Fig. 1a, e). Similarly, expression in the putamen of each DARPP-32 isoform and of CaN did not differ significantly between subjects with schizophrenia and control subjects [ANCOVA: $F(1, 18) = 0.46$, $P = 0.51$, ANCOVA: $F(1, 18) = 2.29$, $P = 0.15$, ANCOVA: $F(1, 18) = 4.13$, $P = 0.057$, respectively] (Fig. 1c, d, f).

We also assessed whether the expression level of either DARPP-32 isoforms or CaN in patients with schizophrenia was correlated with the daily dosage of antipsychotics that was prescribed in the 3 months immediately preceding each death. There were no significant effects of antipsychotic medication on the expression level of any of these proteins in any brain region examined (all P values >0.5).

3.2. Effects of Taq1A genotype on the expression of each DARPP-32 isoform and of calcineurin

We determined the Taq1A genotype (A1/A1, A1/A2, or A2/A2) in the same subjects (Table 2). Among the 24 subjects (12 with schizophrenia and 12 control), two (8.3%) were homozygous for the A1 of *DRD2/ANKK1*, nine (37.5%) were heterozygous for the A1/A2, and 13 (54.2%) were homozygous for the A2 allele. The allele distribution was in Hardy–Weinberg equilibrium ($\chi^2 = 0.06$; df = 1; $P > 0.05$). Accordingly, we analyzed the associations of the expression of two DARPP-32 isoforms and CaN with Taq1A genotype. The expression of t-DARPP in the caudate was correlated with Taq1A genotype in *DRD2/ANKK1*; specifically, the A1 allele was associated with elevated t-DARPP expression [ANCOVA: $F(2, 17) = 6.66$, $P = 0.0073$] (Fig. 2). Individuals homozygous or heterozygous for the A1 allele (A1/A1 or A1/A2 genotype) had significantly higher t-DARPP expression in the caudate than did A2/A2 individuals ($P = 0.0036$ or $P = 0.023$, respectively). For all subjects and for each diagnostic group, there was no significant association between any Taq1A genotype and the expression of FL-DARPP or CaN in caudate or of FL-DARPP, t-DARPP, or CaN in the putamen.

Additionally, we performed Spearman's rank correlation to assess whether the A1 allele was significantly correlated with any antemortem psychiatric symptom; a postmortem DIBS analysis was used to

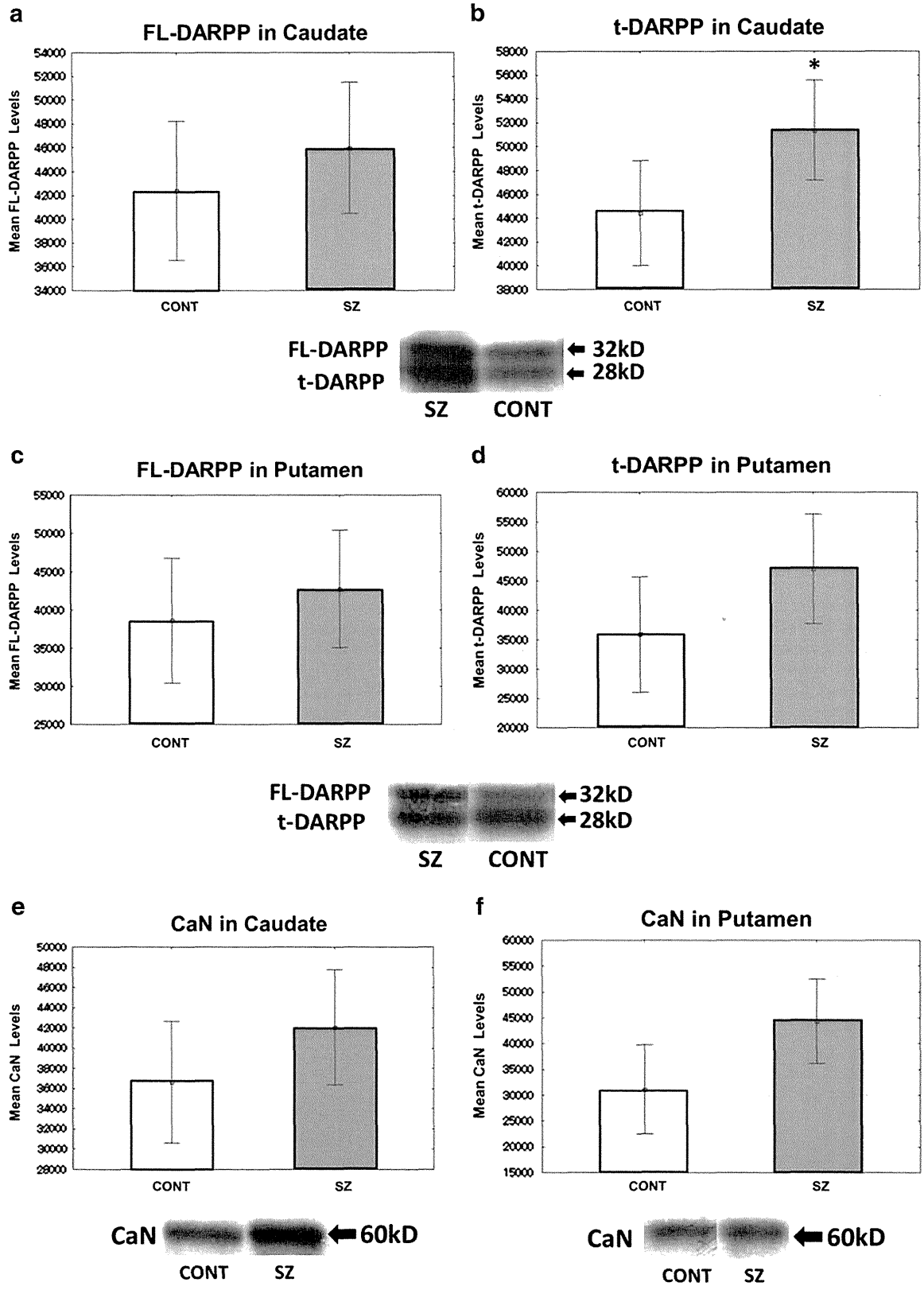

Fig. 1. The expression of each DARPP-32 isoform or calcineurin in the schizophrenia group and the control group. Mean expression of FL-DARPP (a, c), t-DARPP (b, d), or CaN (e, f) in the caudate (a, b, e) and putamen (c, d, f) of the control group (CONT) and the group of patients with schizophrenia (SZ); immunoblots were used to measure protein expression. An asterisk over a bar indicates that the difference between the SZ group and the CONT group was significant based on a post hoc Fisher LSD test.

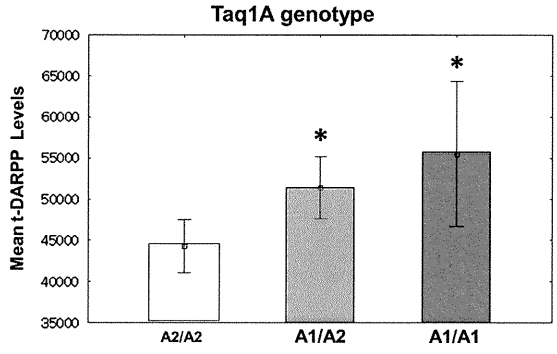

Fig. 2. Effect of Taq1A genotype in *DRD2/ANKK1* on t-DARPP expression in the caudate. There was a significant association between t-DARPP expression in the caudate and Taq1A genotype in *DRD2/ANKK1*. Error bars indicate the SE of the mean. Asterisk above a bar indicates that the difference between a minor genotype and the major genotype is significant based on a post hoc Fisher LSD test.

characterize these symptoms. The A1 allele was significantly correlated with the total score of antemortem psychiatric symptoms (R = 0.589, *P* values = 0.044) and marginally significantly correlated with the score of positive symptoms (3 months before death) (R = 0.568, *P* values = 0.054).

4. Discussion

Here, we showed that the expression of t-DARPP was significantly increased in the caudate of patients with schizophrenia, and that the expression of t-DARPP in the caudate is associated with Taq1A polymorphism in *DRD2/ANKK1*. To the best of our knowledge, this is the first study to show that 1) t-DARPP expression was altered in postmortem samples of striatum from patients with schizophrenia, and 2) t-DARPP expression in the striatum was associated with a genetic polymorphism linked to clinical responses to antipsychotics. Previous postmortem study shows that 1) the expression of t-DARPP transcript is significantly upregulated in the DLPFC of patients with schizophrenia and those with bipolar disorder (Kunii et al., 2013), and 2) the expression of t-DARPP transcript in the DLPFC is associated with genetic variants linked to schizophrenia and to cognitive functioning (Kunii et al., 2013). Notably, A1 allele of Taq1A polymorphism in *DRD2/ANKK1* carriers expressed more t-DARPP than A1 allele noncarriers in the caudate. The A1 allele of Taq1A polymorphism in *DRD2/ANKK1* is known to be related to the elevated activity of striatal L-amino acid decarboxylase (Laakso et al., 2005), which synthesizes dopamine; therefore, neurotransmission via dopamine is increased in the striatum of subjects that carry A1. Consequently, the increased dopaminergic signaling in the striatum of patients with schizophrenia and A1 may lead to more severe positive symptoms. This hypothesis is partially substantiated by our finding that patients with schizophrenia and A1 had marginally significantly higher scores with the DIBS assessment of antemortem positive symptoms. Interestingly, findings from one clinical psychopharmacological study indicate that the percentage improvement in positive symptoms was significantly higher for patients with A1 than for those without A1 (Suzuki et al., 2000). These findings could be interpreted to mean that 1) A1-carrying patients express fewer DRD2 molecules than A1-free patients (Jönsson et al., 1999), 2) antipsychotics may occupy DRD2 more promptly and interrupt dopamine neurotransmission via DRD2 more effectively in A1 patients, and 3) the antipsychotics may therefore be able to improve positive symptoms more effectively in A1 patients than in A1-free patients.

Alternatively, upregulated t-DARPP may lead to a reduction of dopamine signaling in the brain because t-DARPP lacks the Thr 34 phosphorylation site and PP-1 inhibitory domain that are essential for dopamine signaling in the brain. FL-DARPP and t-DARPP probably function as antagonists with regard to the efficiency of dopaminergic systems, because these two isoforms have antagonistic effects on anti-apoptotic actions and on clinical reactions to carcinostatic drugs (Belkhiri et al., 2008; El-Rifai et al., 2002; Gu et al., 2009; Vangamudi et al., 2010). Thus, it seems reasonable that alterations in t-DARPP expression in the caudate may affect decision-making that occurs via the striatum by changing dopamine signaling and therefore lead to cognitive impairment or negative symptoms. Taken together, these findings indicated that the positive symptoms and cognitive impairment/negative symptoms may be more severe for patients with schizophrenia and the A1 allele of *DRD2/ANKK1* than for A1-free patients. This hypothesis is supported by our finding that the total score for antemortem psychiatric symptoms was significantly higher for patients with schizophrenia and A1 than for those without A1.

There are several limitations in the present study that require further attention. Postmortem studies that use samples from patients generally should be evaluated with special caution because it is difficult to control disease-related factors that may have effects on the results. First, confounding effects of medication, other disease-related factors, or both may have affected the expression of DARPP-32, CaN, or both. Although we did not detect any effects of antipsychotic drugs on DARPP-32 or CaN expression levels in this postmortem study, further studies that use animal models of the mammalian brain are warranted to examine the effects of chronic administration of antipsychotics on the expression of these proteins in the striatum. Second, our study population was small and, subjects with controls were elder and included more females than that with schizophrenia. In addition, PMI of schizophrenia group was longer than that of control group. We detected changes in some brain tissues that are usually associated with mild senility, but it was hard to determine whether these changes were equally distributed among the schizophrenia group and the control group. These aging, PMI, or sex-related modifications of the brain may have had some influence on the expression of either DARPP-32 isoform, CaN, or some combination thereof although we found that there is no correlation between either age or PMI and the t-DARPP expression in the caudate using Spearman's rank correlation. The findings must be confirmed via postmortem examination of brains from younger, larger and more matched cohorts. Finally, we did not examine the nucleus accumbens during this study even though the mesolimbic dopaminergic pathway may play a critical role in the pathophysiology of schizophrenia. Further studies are also needed to examine the expression of these proteins in postmortem nucleus accumbens.

5. Conclusion

In summary, our results show that the expression of the t-DARPP isoform was higher in postmortem brains from patients with schizophrenia than those from control subjects, and, selectively in the caudate, is associated with genetic variation in the *DRD2/ANKK1*. Our results may reflect important molecular mechanisms involved in the pathogenesis of schizophrenia.

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CORRESPONDENCE

3R and 4R tau isoforms in paired helical filaments in Alzheimer's disease

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Isoform-specific tau antibodies RD3 and RD4 are useful tools for investigating expression and localization of three-repeat (3R) and four-repeat (4R) tau isoforms. Recently, transition from 3R to 4R tau in Alzheimer's disease (AD) was proposed based on immunohistochemical studies with RD3 and RD4 [3]. Here, we show that two factors influence immunoreactivity to these antibodies. First, deamidation at the RD4 epitope abrogates immunoreactivity to RD4, and second, presentation of RD3 and RD4 epitopes is reciprocally affected by protease. Asparagine at position 279 in the RD4 epitope is predominantly deamidated to aspartic acid in pathological tau in AD brains [2, 4]. Consequently, the presence of 4R tau in AD pathologies may be underestimated when RD4 is used. However, anti-4R (available from Cosmo Bio Co., Ltd.) raised against RD4 peptide with N279D substitution stained both wild-type and deamidated 4R tau, and strongly stained RD3+/RD4− tangles and smearing tau fragments in Sarkosyl-insoluble fraction of AD brain [2].

It was reported that RD3 stained abundant ghost tangles in entorhinal cortex and tangles in CA1, but failed to stain fine processes of tangles and threads [3], while RD4 failed to detect ghost tangles in entorhinal cortex [3]. To understand these findings, we examined the influence of protease on immunoreactivity. Paraffin sections of AD brains were treated with 10 μg/mL Proteinase K (Pro-K) for 30 min after autoclaving (Ac) and formic acid (FA) treatment. RD3 staining was strongly enhanced (Fig. 1a, b). Conversely, RD4 immunoreactivity almost completely disappeared after Pro-K treatment (Fig. 1c, d). Not only ghost tangles but also RD3−/RD4+ tangles and their processes became RD3-positive after Pro-K treatment (Fig. 1a, b), strongly suggesting that the RD3 epitope was buried in tau filaments of intracellular tangles and threads, and was exposed by Pro-K treatment. Contrary to expectation, anti-4R staining was also enhanced by Pro-K treatment (Fig. 1e, f). It is possible that the recognition site of anti-4R is distinct from that of RD4 and is exposed by Pro-K treatment of sections. Anti-4R antibody may recognize the carboxyl-half of the antigen peptide, while RD4 recognizes the amino-terminal half around N279. Pro-K treatment was also effective in immunostaining of free-floating AD sections with a lower concentration.

To confirm these findings biochemically, Sarkosyl-insoluble fractions from two AD brains were treated with trypsin or Pro-K, then immunoblotted with RD3, RD4, anti-4R and anti-pS396 (Fig. 1g–j). RD3 strongly stained many bands and smears, as seen with pS396 (Fig. 1g, j),

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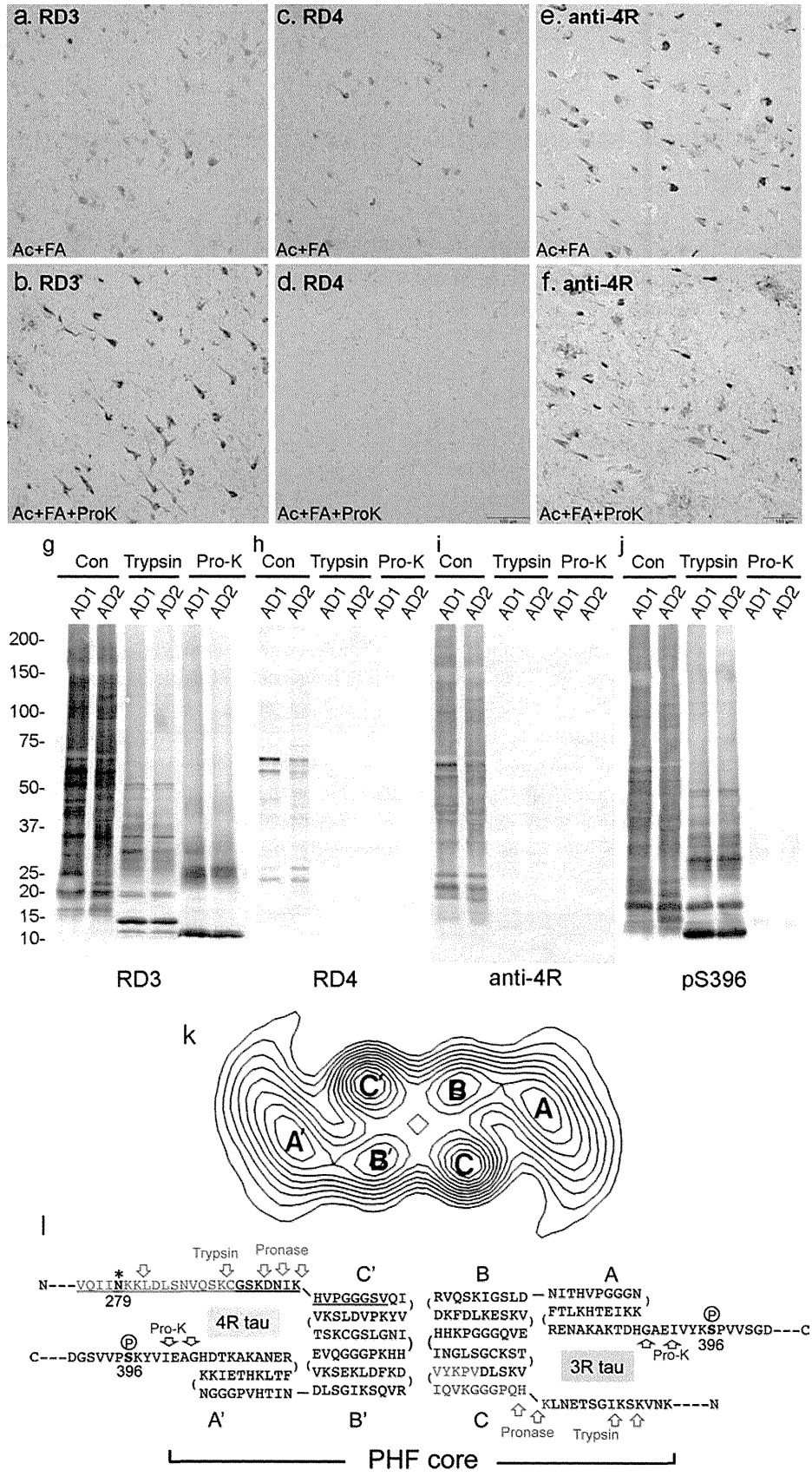

Fig. 1 **a–f** Immunostaining of AD sections after Ac and FA treatment before (**a**, **c**, **e**) and after (**b**, **d**, **f**) Pro-K treatment, using RD3 (**a**, **b**), RD4 (**c**, **d**) and anti-4R (**e**, **f**). *Bar* 100 μm. **g–j** Immunoblots of Sarkosyl-insoluble tau from two AD brains, before (*Con*) and after treatments with trypsin or Pro-K, using RD3 (**g**), RD4 (**h**), anti-4R (**i**) and pS396 (**j**). **k–l** Computed cross-section through a paired helical filament (**k**) [reproduced from Ref. [1], with permission of the publisher], a predicted folding model of 3R and 4R tau in PHF (**l**). RD3 and RD4 epitopes are indicated by *blue* and *red*, respectively. 4R tau specific insertion is indicated by *underlining*. The deamidation site N279 is indicated by *asterisks*. Phosphorylation of Ser396 is indicated. Possible trypsin, pronase and Pro-K cleavage sites are indicated in *green*, *purple* and *dark blue arrows*, respectively. The protease-resistant domain of PHF is indicated as *PHF-core*