

GABA-related transcripts in the dorsolateral prefrontal cortex in mood disorders



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Abstract

Reduced cortical γ -aminobutyric acid (GABA) levels and altered markers for subpopulations of GABA interneurons have been reported in major depressive disorder (MDD) by *in-vivo* brain imaging and post-mortem histological studies. Subgroups of GABA interneurons exert differential inhibitory control on principal pyramidal neurons and can be identified based on the non-overlapping expression of the calcium-binding proteins parvalbumin (PV) or calretinin (CR) or the neuropeptide somatostatin (SST). As altered markers of GABAergic functions may also be present in bipolar disorder (BPD), the specificity of particular GABA-related molecular deficits in mood disorders is not known. We used real-time quantitative polymerase chain reaction (qPCR) to assess expression levels of two GABA synthesizing enzymes (glutamate decarboxylase; GAD65 and GAD67) and of three markers of GABA neuron subpopulations (PV, CR, SST) in the dorsolateral prefrontal cortex (DLPFC; Brodmann area 9) in triads ($n=19$) of control subjects and matched subjects with BPD or MDD. BPD subjects demonstrated significantly reduced PV mRNA, trend level reduction in SST mRNA and no alterations in GAD67, GAD65, or CR mRNA levels; MDD subjects demonstrated reduced SST mRNA expression without alterations in the other transcripts. The characteristic age-related decline in SST expression was not observed in MDD, as low expression was detected across age in MDD subjects. After controlling for age, MDD subjects demonstrated significantly reduced SST mRNA expression. Decreased SST levels in MDD were confirmed at the protein precursor level. Results were not explained by other clinical, demographic or technical parameters. In summary, MDD was characterized by low DLPFC SST, whereas decreased PV mRNA appears to distinguish BPD from MDD.

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Introduction

Amino acids [glutamate and γ -aminobutyric acid (GABA)] are the most abundant neurotransmitters and are largely responsible for the excitation/inhibition balance in the brain. Their regulation by multiple neuromodulatory systems (e.g. neuropeptide and monoaminergic) make them likely candidates for integrating upstream dysfunctional molecular and cellular pathways in diseases, although primary and/or (mal)adaptive changes may be disease-specific.

In major depressive disorder (MDD), increasing evidence suggests an impaired cortical excitation/inhibition potentially mediated by decreased GABA content, as observed by proton magnetic spectroscopy in occipital and frontal cortices (Sanacora *et al.* 1999, 2004), or by transcranial magnetic stimulation paradigms (Levinson *et al.* 2010), which is reversed after antidepressant treatments (Sanacora *et al.* 2002, 2003). Large-scale gene array studies in post-mortem subjects provide additional support for altered GABAergic and glutamatergic neurotransmission in MDD and in MDD patients who died by suicide or from other causes (Klempan *et al.* 2009; Sequeira *et al.* 2007, 2009). Valentine & Sanacora (2009) have suggested a mechanism involving reduced glial-mediated amino-acid metabolism that is consistent with reports of altered

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glial content in MDD (Cotter *et al.* 2001; Ongur *et al.* 1998; Rajkowska *et al.* 1999). Moreover, morphometric studies have reported reduced density and size of cortical neurons (Rajkowska *et al.* 1999), recently attributed to interneurons in some (Maciag *et al.* 2010; Rajkowska *et al.* 2007), but not all (Cotter *et al.* 2002) studies.

The nature of the affected cells in MDD is beginning to be characterized through the use of cellular markers expressed in distinct populations of cortical interneurons. For instance, the expression patterns of the calcium-binding proteins parvalbumin (PV), calretinin (CR) and calbindin define mostly non-overlapping neuronal types (Gonzalez-Albo *et al.* 2001). The density of calbindin-immunoreactive interneurons was significantly reduced in occipital and orbitofrontal cortices of MDD subjects (Maciag *et al.* 2010; Rajkowska *et al.* 2007), but unchanged in two other studies in the cingulate cortex and dorsolateral prefrontal cortex (DLPFC) (Beasley *et al.* 2002; Cotter *et al.* 2002). Somatostatin (SST) heavily co-localizes with calbindin (DeFelipe, 1997), and was previously implicated in schizophrenia (Morris *et al.* 2008) and mood disorders (Rubinow *et al.* 1983). PV-positive interneurons were unchanged in MDD (Beasley *et al.* 2002; Rajkowska *et al.* 2007), contrasting with robust and replicated reports of down-regulated PV mRNA in schizophrenia (Hashimoto *et al.* 2003, 2008). Decreased PV mRNA levels possibly extend to bipolar disorder (BPD) subjects (Pantazopoulos *et al.* 2007), perhaps consistent with the fact that subjects with BPD, but not MDD, seem to share genetic risks with subjects with schizophrenia (Potash, 2006). A current hypothesis is that PV-related deficits contribute to the cognitive deficits that are present in schizophrenia (Lewis & Moghaddam, 2006) and to some extent in BPD, but not in MDD.

These observations suggest that distinctive patterns of GABA-related deficits may correlate with symptom dimensions, leading us to speculate that a specific pattern of GABA-related deficits may correspond with the low mood component that is common across MDD and BPD, and frequently found in schizophrenia. To begin testing this hypothesis, we investigated expression levels for markers of three subpopulations of GABA neurons: PV, as a putative discriminative marker between MDD and BPD; SST as a putative marker for altered mood regulation across conditions, due to its previously reported down-regulation in schizophrenia (Hashimoto *et al.* 2008; Morris *et al.* 2008) and mood disorders (Rubinow *et al.* 1988), and in view of its apparent anxiolytic and antidepressant-like properties in preclinical rodent models (Engin *et al.*

2008); and CR as a potential internal control expected to be unchanged across all disorders (Beasley *et al.* 2002). Finally, we also investigated expression levels for two GABA-synthesizing enzymes (glutamate decarboxylase; GAD65 and GAD67), as putative markers of the overall extent of alterations in GABA neurotransmission.

Materials and methods

Human subjects

After consent was obtained from the next of kin, brain specimens were obtained during autopsies conducted at the Allegheny County Medical Examiner's Office (USA). Nineteen subject triads were used in this study, each triad consisting of one control subject, one subject with BPD, and one subject with MDD, matched for sex and as closely as possible for age (Table 1). Matching was done in order to reduce biological variance due to non-disease-related factors and to control for experimental variance. Subject groups did not differ in mean age, post-mortem interval (PMI), RNA integrity number (RIN), brain pH, or tissue storage time as determined by one-way ANOVAs (for all $F_{2,54} < 1.04$, $p > 0.36$). Consensus diagnosis and DSM-IV (APA, 1994) diagnoses for each subject were made by an independent committee of experienced research clinicians, based on medical records and results of structured interviews conducted with family members of the deceased (Glantz *et al.* 2000). All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research.

Tissue preparation

As previously described (Volk *et al.* 2000), the right hemisphere of each brain was blocked coronally, immediately frozen and stored at -80°C . Cryostat sections ($20\ \mu\text{m}$) from the anterior-posterior level corresponding to the middle portion of the superior frontal sulcus were cut serially and collected into tubes containing Trizol reagent (Invitrogen, USA) for RNA isolation, or mounted on Superfrost plus glass slides (VWR International, USA). The location of DLPFC area 9 was determined from Nissl-stained sections using cytoarchitectonic criteria as previously described (Volk *et al.* 2000). Total RNA was isolated from Trizol homogenates of sections, further purified by RNeasy columns (Qiagen, USA) and RNA integrity was assessed by measuring RIN (Imbeaud *et al.* 2005) using the Bioanalyzer 2100 (Agilent Technologies, Germany). For all subjects used in this study, RIN was ≥ 7.0 .

Real-time quantitative polymerase chain reaction (qPCR)

For the five GABA-related transcripts and three internal control transcripts, real-time qPCR analyses (Glorioso *et al.* 2006) were performed on DLPFC samples from the subject triads. Using 50 ng total RNA, cDNA synthesis by random primers and SuperScript II reverse transcriptase (Invitrogen) was conducted. The primer sets for all the GABA-related and internal control transcripts have been previously used (Hashimoto *et al.* 2008) with the exception of CR (see Supplementary Table 1). All primer pairs (see Supplementary Table 1) exhibited high amplification efficiency (>97%) in the standard curve analysis and specific single products in dissociation curve analysis. After primer validation, the comparative threshold cycle (C_t) measurement was performed for quantification using SYBR Green I Dye (Applied Biosystems, USA) and StepOne Plus Real-time PCR instrument (Applied Biosystems) according to the manufacturer's instructions. Each qPCR run included all three subjects in a triad and amplified all eight transcripts of interest in quadruplicate using a plate with 96 wells (3 subjects \times 8 transcripts \times 4 replications). Three internal control transcripts encoding for β -actin, cyclophilin A, and glyceraldehyde-3-phosphate dehydrogenase were amplified for each subject. These internal control transcripts were selected based on their stable expression across subjects with schizophrenia (Hashimoto *et al.* 2008). Furthermore, the internal control transcripts were stably expressed across our subject groups regardless of diagnosis (see Supplementary Table 2). The difference in cycle threshold for each GABA-related transcript was calculated by subtracting the mean cycle threshold for the three internal controls from the cycle threshold of each GABA-related transcript. This difference in cycle threshold (dC_t) represents the \log_2 -transformed expression ratio of each GABA-related transcript to the geometric mean of the three internal control transcripts (Vandesompele *et al.* 2002); therefore, the relative expression level of each GABA-related transcript was determined as 2^{-dC_t} .

qPCR statistical analysis

To determine the diagnosis-related expression differences of each GABA-related transcript, we utilized an analysis of covariance (ANCOVA) model with SPSS (SPSS Inc., USA). The data were averaged across the four replicates and transformed into relative expression levels (2^{-dC_t}) so that plots of data are intuitive (i.e. higher values represent greater relative

expression). The 2^{-dC_t} values were confirmed to be normally distributed within each subject group before statistical analyses were performed. To identify relevant covariate factors, Pearson correlations were performed between individual factors (age, PMI, RIN, brain pH, storage time) and expression levels. The influences of other potential confounding nominal variables (sex, drug exposure) on expression values were assessed with ANCOVA models. Results were adjusted for multiple comparisons using the Bonferroni–Holm method in which p values are ordered from the smallest ($i=1$) to the largest ($i=N$) among multiple comparisons. All together, age was retained as a significant covariate factor for SST levels and pH for GAD67, GAD65 and CR levels. As the effect of age differed between controls and psychiatric subjects (BPD and MDD) (see text), we performed two separate ANCOVAs in which controls, BPD subjects or MDD subjects were included.

Protein isolation

Acetone precipitation of proteins was carried out following RNA extraction from the TRIzol brain tissue homogenates. The lower red phenol-chloroform phase was used for protein isolation, using ethanol to precipitate DNA, and acetone to extract protein from the supernatant. Following extensive washes, the dried pellet was dissolved in $1\times$ SDS buffer. The supernatant was collected after 5 min centrifugation at 14000 rpm. An aliquot was used for protein quantification using Pierce BCA assay (Pierce, USA). Protein samples (5 μ g) were resolved by SDS–PAGE in 10% Tris/glycine gels and transferred to PVDF membrane.

Prepro-SST immunoblotting

Western blot analysis was performed using the Odyssey system (LI-COR Biosciences, USA). In brief, gel-transferred PVDF membrane were blocked in LI-COR blocking buffer and incubated with mouse anti-actin at 0.5 μ g/ml (Sigma no. A 2228) and rabbit polyclonal primary antibody for prepro-somatostatin (prepro-SST) at 0.5 μ g/ml (ab53165, Abcam). Fluorescent IR Dye 680 anti-Rabbit and fluorescent IR Dye 800 anti-mouse (LI-COR Biosciences) secondary antibodies were used in signal detection. Dual signals were detected using the LI-COR Odyssey Infrared imaging system, and prepro-SST/actin signal ratios were calculated. The specificity of the antibody was confirmed by the absence of signal on brain tissue from SST knockout compared to control mice (generously provided by Dr A. Agmon, West Virginia University). Samples were processed in matched

Table 1. Characteristics of subjects

Triad	Control subjects								Bipolar disorder subjects								Medications ^d	
	Case	Sex/ race	Age	PMI ^a	pH	RIN	Storage time ^b	Cause of death ^c	Case	DSM-IV diagnosis	Sex/race	Age	PMI ^a	pH	RIN	Storage time ^b		Cause of death ^c
1	10 003	M/W	49	21.2	6.5	8.4	51	Trauma	1102	Bipolar disorder NOS ^{g,i,j,l}	M/W	50	12.1	6.7	8.3	61	ASCVD	AD, BV, SI
2	1374	M/W	48	21.7	6.6	7.2	23	Coronary atherosclerotic disease	1121	Bipolar I disorder ^{g,m}	M/W	40	18.5	6.4	8.3	59	Pulmonary thromboembolism	BV, AP
3	1282	F/W	39	24.5	6.8	7.5	39	ASCVD	957	Bipolar I disorder	F/W	39	22.0	6.7	8.4	83	Drowning (S)	AD, SI
4	1298	M/W	48	24.5	6.5	7.9	36	ASCVD	886	Bipolar I disorder ^h	M/W	45	27.3	7.1	8.3	99	Gunshot to chest (S)	
5	1047	M/W	43	13.8	6.6	9	70	ASCVD	1020	Bipolar I disorder ^{h,l,m}	M/W	42	6.0	6.7	8.5	74	Combined drug toxicity	AD, BV
6	795	M/W	68	12.0	6.8	8.2	115	Ruptured abdominal aortic aneurysm	1130	Bipolar I disorder ^{e,m}	M/W	65	8.9	6.7	8	59	Carcinoma of oesophagus	AD, BV, AP, SI
7	1318	F/W	58	18.8	6.6	7.4	33	ASCVD	1048	Bipolar I disorder ^m	F/W	51	21.4	6.7	7.7	69	Asphyxiation (S)	AD
8	1324	M/W	43	22.3	6.7	7.3	31	Dissection of the ascending thoracic aorta	697	Bipolar I disorder ^m	M/W	39	25.9	6.6	7.8	133	Exsanguination (S)	BV
9	1444	M/W	46	22.0	6.3	8.4	10	Pulmonary thromboembolism	1069	Bipolar I disorder ^e	M/W	48	27.2	6.9	8.1	66	ASCVD	
10	1086	M/W	51	24.2	6.6	8.1	64	ASCVD	1244	Bipolar I disorder ^{e,j,l,m}	M/W	52	23.5	6.7	8	45	Asphyxiation	AD, BV, AP
11	1391	F/W	51	7.8	6.6	7.1	20	ASCVD	10 004	Bipolar I disorder ^l	F/W	50	11.7	6.4	8.5	52	Combined drug toxicity	AP
12	1196	F/W	36	14.5	6.4	8.2	52	Positional asphyxia	1180	Bipolar I disorder ^m	F/W	28	22.3	6.3	7.5	55	Trauma (jump) (S)	AD, SI
13	1293	F/W	65	18.5	6.5	7.0	38	Trauma	10 006	Bipolar I disorder ^{g,m}	F/W	55	19.0	6.4	8.1	51	Gunshot to head (S)	
14	1153	M/W	55	28.0	6.1	8	57	Atherosclerotic and hypertensive heart disease	716	Bipolar I disorder ^{h,j}	M/W	58	27.5	6.8	8.3	130	Gunshot to head (S)	
15	789	M/W	22	20.0	6.8	7.8	117	Accidental asphyxiation	1181	Bipolar I disorder ^{i,l,m}	M/W	28	27.4	6.2	8	55	Morphine toxicity	AD, BV, SI
16	686	F/W	52	22.6	7.1	8.5	135	ASCVD	1328	Bipolar disorder NOS	F/W	49	21.5	6.7	7.5	31	Arrhythmogenic sudden cardiac arrest	AD, BV, SI
17	1247	F/W	58	22.7	6.4	8.4	45	ASCVD	1044	Bipolar I disorder ^m	F/W	56	24.5	6.1	7.1	71	Cardiac arrhythmia	AD, BV, AP, SI
18	1092	F/B	40	16.6	6.8	8.0	64	Mitral valve prolapse	984	Bipolar I disorder ^m	F/W	42	30.8	6.5	8	80	Combined drug overdose	AD, AP, SI
19	840 ^e	F/W	41	15.4	6.6	9.1	106	ASCVD	945	Bipolar I disorder ^f	F/W	43	31.8	6.7	7.2	86	Asphyxiation (S)	AD, BV, AP, SI
	Mean		48.1	19.5	6.6	8.0	58					46.3	21.5	6.6	8.0	72		
	S.D.		10.6	5.1	0.2	0.6	36					9.5	7.3	0.2	0.4	26		

Table 1 (cont.)

Major depressive disorder (MDD) subjects									
Triad	Case	DSM-IV diagnosis	Age	PMI ^a	pH	RIN	Storage time ^b	Cause of death ^c	Medications ^d
1	10 010	MDD ^{e,m}	42	14.3	6.4	7.6	48	Amitriptyline overdose (S)	AD, BV
2	1226	MDD ^{i,j,k,l,m}	44	19.3	6.5	7.5	47	ASCVD	
3	967	MDD ^h	40	22.2	6.6	7.4	81	ASCVD	
4	1053	MDD	47	24.0	6.6	8.1	68	ASCVD	
5	1215	MDD ^g	44	11.0	6.5	7.9	50	ASCVD	BV
6	698	MDD ^m	59	13.0	6.6	9.0	132	Hanging (S)	AD, AP
7	1190	MDD ^h	47	22.3	6.6	8.0	53	Asphyxiation (S)	
8	668	MDD ^{e,m}	34	24.3	6.6	8.1	137	Hanging (S)	
9	863	MDD	51	28.3	7.3	8.4	101	ASCVD	
10	1312	MDD ^{g,l}	51	24.6	6.5	8.1	33	Combined drug toxicity	
11	986	MDD	53	11.9	6.7	8.7	79	Bronchial asthma	AD, SI
12	1157	MDD	26	13.4	6.4	7.8	57	Hanging (S)	AD, SI
13	1041	MDD ^{f,l,m}	52	10.3	6.5	8.4	71	Combined drug toxicity	AD, BV, AP, SI
14	1071	MDD ^e	62	25.6	6.5	8.1	66	Gunshot to trunk (S)	
15	1131	MDD	29	26.6	6.9	8.5	59	Gunshot (S)	
16	1143	MDD ^{g,l}	49	23.4	6.4	8.1	58	Combined drug toxicity	AD, BV, SI
17	934	MDD ^m	54	17.9	6.2	8.2	89	ASCVD	AD, SI
18	1289	MDD	46	25.0	6.3	7.3	39	ASCVD	
19	1221	MDD	28	24.8	6.6	7.2	49	Pulmonary thrombosis	AD
	Mean		45.2	20.1	6.6	8.0	69		
	s.d.		10.1	6.0	0.2	0.5	29		

AD, Antidepressants; AP, antipsychotics; BV, benzodiazines/sodium valproate; RIN, RNA integrity number; SI, SSRIs; (S), indicates death by suicide.

^a PMI indicates post-mortem interval in hours; ^b Storage time (months) at -80°C ; ^c ASCVD indicates arteriosclerotic cardiovascular disease; ^d Indicates prescribed medications at time of death; ^e Alcohol abuse, in remission at time of death; ^f Alcohol abuse, current at time of death; ^g Alcohol dependence, in remission at time of death; ^h Alcohol dependence, current at time of death; ⁱ Other substance abuse, in remission at time of death; ^j Other substance abuse, current at time of death; ^k Other substance dependence, in remission at time of death; ^l Other substance dependence, current at time of death; ^m History of psychotic features.

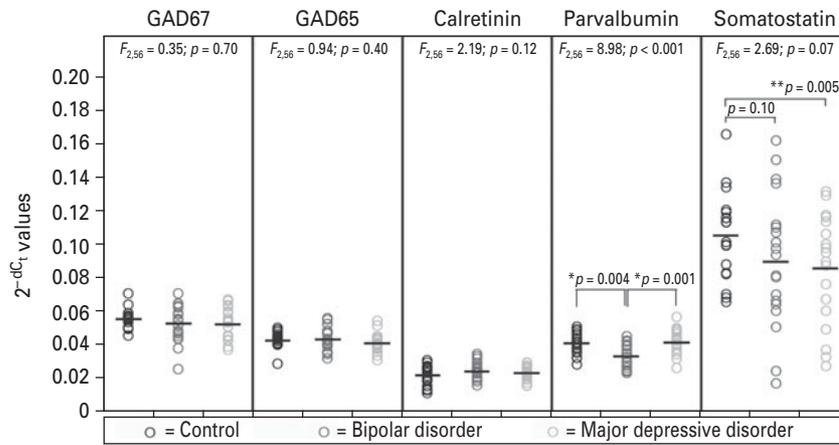


Fig. 1. GABA-related transcript expression levels across three subject groups. The 2^{-dC_t} relative expression values are shown for each subject (circles). For each transcript, the mean 2^{-dC_t} relative expression values are shown for each subject group (black hash marks). The statistics (top of graph) represent the effect of diagnosis for each GABA-related transcript. Parvalbumin transcript expression was significantly reduced in subjects with bipolar disorder. Effect of diagnosis for somatostatin transcript expression reached trend-level effect, which did not remain significant after correction for multiple comparisons. * *Post-hoc* Tukey's multiple comparison test *p* values. ** *p* values for the effect of diagnosis from one-way ANCOVAs between the subject groups indicated.

triplicates on the same gel and results were replicated three times. To control for gel-to-gel variability, two parallel statistical approaches were applied. First, paired *t* tests were run in age- and sex-matched pairs, and group results were combined across the three runs using Stouffer's approach, which considers *p* values, sample sizes, and effect directions (Whitlock, 2005). Second, an ANCOVA model with age, pair, PMI and batch processing as covariate factors was applied and group results were similarly combined across the three runs using Stouffer's approach. Significance was set at $\alpha = 0.05$.

Results

Alterations in GABA-related transcripts across subject groups

Quantification of the expression levels of GAD67, GAD65, CR, PV, and SST by real-time qPCR in 19 subject triads revealed that the rank order of the mean relative expression levels of each transcript in normal control subjects closely matched the previously reported mean expression levels of these mRNAs (Hashimoto *et al.* 2008), with SST having the highest levels of expression, CR having the lowest levels of expression, and PV, GAD65 and GAD67 having intermediate expression levels (Fig. 1).

We did not find any significant effects of diagnosis on GAD67, GAD65, or CR mRNA levels (Fig. 1). In contrast, there was a main effect of diagnosis on the

mean expression levels of PV mRNA ($F_{2,56} = 10.73$, adjusted $p < 0.001$) (Fig. 1). *Post-hoc* analyses revealed a significant reduction in mean PV mRNA expression in BPD subjects compared to normal controls (-18% , $p = 0.004$) and MDD subjects (-20% , $p = 0.001$) (Fig. 1), but no significant difference between normal controls and MDD subjects ($p = 0.88$).

Diagnosis showed a non-significant decrease in mean expression levels of SST mRNA ($F_{2,56} = 2.69$, $p = 0.07$) (Fig. 1). However, SST also displayed a 3–5 times greater variability within groups (mean coefficient of variation, $CV_{SST} = 0.18$), compared to the other four genes ($CV_{GAD65} = 0.04$, $CV_{GAD67} = 0.05$, $CV_{CR} = 0.06$, $CV_{PV} = 0.06$). Since SST transcript levels are known to markedly decrease with age (about -60% between ages 20 and 70 yr; Erraji-BenChekroun *et al.* 2005), we investigated the contribution of subjects' age as the potential source of the observed SST variability. In the ANCOVA model including all three subject groups, age was a significant determinant of expression levels ($F_{1,56} = 7.85$, $p = 0.007$). This effect appeared driven by control subjects, as they displayed significant correlation between age and SST mRNA expression ($r = -0.86$, $p = < 0.00001$) (Fig. 2). In contrast, SST transcript levels were not significantly correlated with age in either subjects with BPD ($r = -0.09$, $p = 0.72$) or with MDD ($r = -0.39$, $p = 0.10$) (Fig. 2). As a consequence, 84% of MDD subjects and 63% of BPD subjects displayed SST expression levels below the age-related trend line of control subjects (Fig. 2), consistent with a lower, age-corrected level of

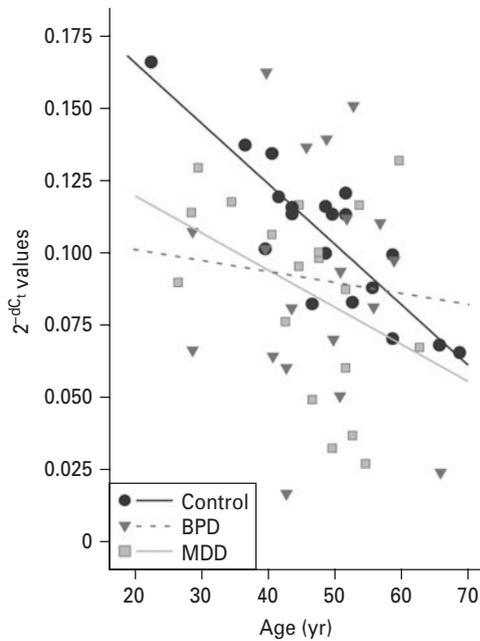


Fig. 2. The 2^{-dC_t} relative expression values of somatostatin (SST) mRNA as a function of age. Expression levels of SST mRNA are significantly correlated with age in normal control subjects ($r = -0.86$, $p < 0.00001$), but not in subjects with bipolar disorder (BPD) ($r = -0.09$, $p = 0.72$) or major depressive disorder (MDD) ($r = -0.39$, $p = 0.10$).

SST mRNA expression for most MDD and BPD subjects. Due to the inability of *post-hoc* analyses to correct for covariates (i.e. age), we performed three separate one-way ANCOVA comparisons with normal controls and subjects with BPD or MDD. Results indicated a non-significant reduction in the mean expression level of SST mRNA in BPD subjects compared to normal controls (-14% ; $F_{1,37} = 2.83$, $p = 0.10$) (Fig. 1). In MDD subjects, the mean expression level of SST mRNA was significantly reduced compared to normal controls (-18% ; $F_{1,37} = 8.95$, $p = 0.005$) (Fig. 1). Mean SST mRNA expression did not differ between BPD and MDD subjects ($F_{1,37} = 0.169$, $p = 0.68$).

Exploratory analyses of potential effect of other factors on GABA-related transcript expression

Although we corrected for significant cofactors, such as age, in the results described above, there is interest in exploratory analyses of multiple co-factors, as these analyses can reveal underlying trends for further investigations in follow-up studies. Given that these assessments required subdividing the subject cohort, the resulting sample sizes may be underpowered to detect significant differences. Hence, values indicated here are not corrected for multiple testing, and should be considered exploratory.

We compared the mean expression of PV mRNA in subjects with BPD (Fig. 3a) and the mean expression of SST mRNA in subjects with BPD (Fig. 3b) or MDD (Fig. 3c) as a function of several cofactors of interest. The expression of PV mRNA in BPD subjects did not differ as a function of sex, death by suicide, antidepressant medication use at time of death (ATOD), use of benzodiazepines or sodium valproate ATOD, diagnosis of substance abuse or dependence ATOD, use of selective serotonin reuptake inhibitors (SSRIs) ATOD, or history of psychosis (all $F \leq 1.72$, $p \geq 0.21$) (Fig. 3a). Furthermore, the expression of SST mRNA in the BPD subjects did not differ as a function of sex, death by suicide, antidepressant medication use ATOD, use of benzodiazepines or sodium valproate ATOD, antipsychotic medication use ATOD, diagnosis of substance abuse or dependence ATOD, or history of psychosis (all $F \leq 2.27$, $p \geq 0.16$) (Fig. 3b). Only two subjects with BPD were on lithium at time of death and hence we did not attempt to look for an effect of lithium. Finally, the expression of SST mRNA in the MDD subjects did not differ as a function of sex, death by suicide, use of antidepressant medication ATOD, antipsychotic medication use ATOD, diagnosis of substance abuse or dependence ATOD, or history of psychosis (all $F \leq 2.03$, $p \geq 0.18$) (Fig. 3c).

Effect of antipsychotic medication use ATOD on PV mRNA expression

There was a significant effect (uncorrected p value) of antipsychotic medication use ATOD on PV mRNA expression ($F_{1,12} = 10.22$, $p = 0.01$) in the BPD subjects (Fig. 3a); however, when we restricted our examination of PV mRNA expression to BPD subjects that had no antipsychotic medication use ATOD and their matched controls ($n = 12$ pairs), there was only a trending significant reduction in PV mRNA expression in subjects with BPD (9% ; $F_{1,17} = 4.24$, $p = 0.055$) (Fig. 4a).

Effect of benzodiazepines or sodium valproate ATOD on SST mRNA expression

There was a significant effect (uncorrected p value) of benzodiazepines or sodium valproate use ATOD on SST mRNA expression ($F_{1,12} = 8.43$, $p = 0.01$) in the MDD subjects (Fig. 3c). When we restricted our examination of SST mRNA expression to MDD subjects that had no benzodiazepines or sodium valproate use ATOD and their matched controls ($n = 15$ pairs), there was a significant reduction in SST mRNA expression in subjects with MDD (13% ; $F_{1,23} = 4.52$; $p = 0.04$) (Fig. 4b).

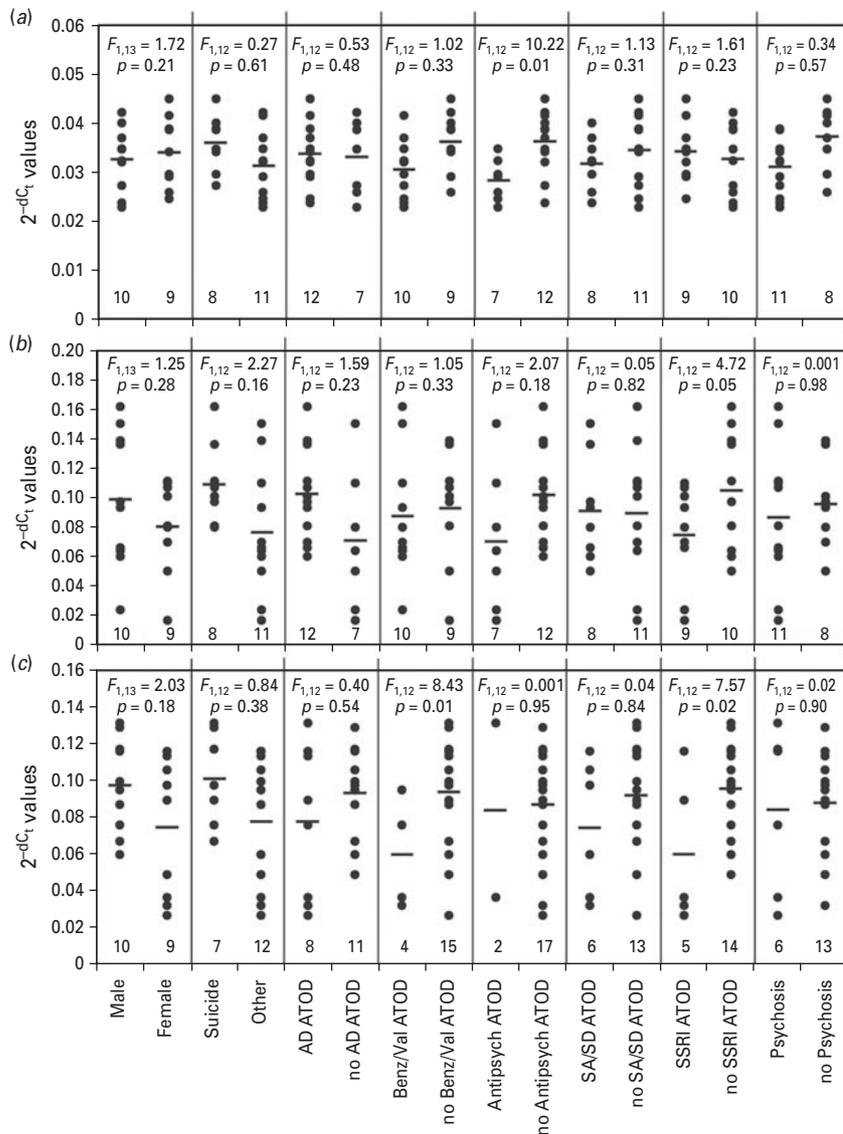


Fig. 3. The effects of confounding factors in diagnostic groups with trending or significantly altered GABA-related transcripts are evaluated for parvalbumin mRNA expression in subjects with (a) bipolar disorder (BPD), (b) somatostatin (SST) mRNA expression in subjects with BPD, and (c) SST mRNA expression in subjects with MDD. Mean (hash mark) and individual (circle) 2^{-dC_t} relative expression values are grouped by potential confounding factors. Note the varying scales for the y axis between (a) and (b) or (c). Numbers below circles indicate the number of subjects in each diagnostic group for each category. AD, Antidepressant; ATOD, at time of death; SA, substance abuse; SD, substance dependence.

Effect of SSRI use on SST mRNA expression

There was a significant effect of using SSRIs ATOD on SST mRNA expression in subjects with BPD ($F_{1,12}=4.72$, $p=0.05$) (Fig. 3b) and MDD ($F_{1,12}=7.57$, $p=0.02$) (Fig. 3c). When we restricted our examination of SST mRNA expression to BPD subjects that had no use of SSRIs ATOD and their matched controls ($n=10$

pairs), there was no significant reduction in SST mRNA expression in subjects with BPD ($F_{1,13}=0.70$, $p=0.42$) (Fig. 4c). In contrast, when we restricted our examination of SST mRNA expression to MDD subjects that had no use of SSRIs ATOD and their matched controls ($n=14$ pairs), there was a significant reduction in SST mRNA expression in subjects with MDD (12%; $F_{1,21}=4.32$, $p=0.05$) (Fig. 4d).

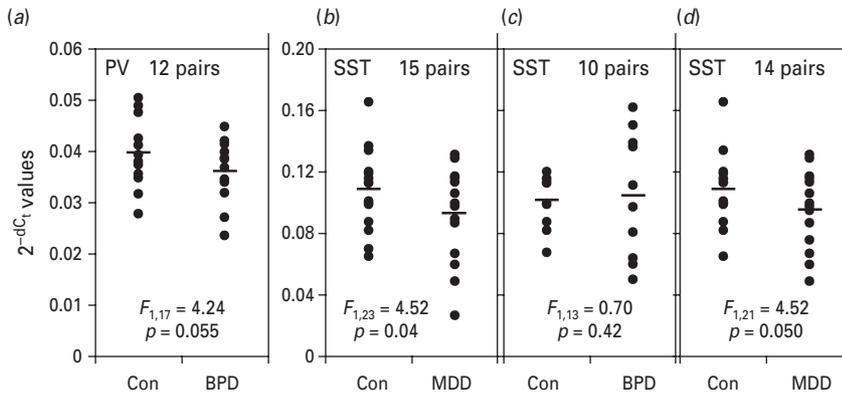


Fig. 4. GABA-related transcript expression levels after removal of subject pairs with potential confounding factors. The mean (hash mark) and individual (circle) 2^{-dC_t} relative expression values are plotted by diagnostic group for (a) parvalbumin (PV) mRNA and (b–d) somatostatin (SST) mRNA. Note the varying scales for the y axes between (a) and (b–d). Subjects with bipolar disorder (BPD) that had antipsychotic medication use at the time of death and their matched controls (Con) were removed and the subjects with BPD ($n = 12$) demonstrated a trending reduction in PV mRNA expression (a). Subjects with major depressive disorder (MDD) that were using benzodiazepines or sodium valproate at the time of death and their matched controls were removed and the subjects with MDD ($n = 15$) demonstrated a significant reduction of SST mRNA expression (b). Subjects with BPD (c) or MDD (d) that were using SSRIs at the time of death and their matched controls were removed and the subjects with MDD ($n = 14$) demonstrated a significant reduction of SST mRNA expression (d) while the subjects with BPD ($n = 10$) did not demonstrate a significant alteration in SST mRNA expression (c).

Reduced prepro-SST protein levels in MDD subjects

To confirm whether decreased SST mRNA translated to decreased protein level, we investigated tissue content level of the prepro-peptide for SST, since the active processed forms of SST rapidly degrades during the PMI (Hayes *et al.* 1991). The prepro-SST immunoreactive band (Fig. 5a) migrated at the expected size and was absent in SST-KO mice (Fig. 5b). Prepro-SST single was not correlated with PMI ($R = 0.12$, $p = 0.41$) and displayed a moderate correlation with RNA levels ($R = 0.26$, $p = 0.05$). Analyses by subgroups resulted in non-significant effects due to low sample size, but suggested a de-correlation between RNA and protein in MDD subjects (controls: $R = 0.23$, $p = 0.34$; MDD: $R = 0.03$, $p = 0.90$). A non-significant inverse correlation was observed with age ($R = -0.19$, $p = 0.15$) across all subjects and was similarly low for control and MDD subjects (controls: $R = -0.20$, $p > 0.05$; MDD: $R = -0.23$, $p > 0.05$). The SST prepro-peptide was robustly decreased by 31.5% in MDD subjects compared to controls (Stouffer's z score test on ANCOVA results, $p = 3 \times 10^{-6}$) (Fig. 5d). In contrast, no changes were observed in BPD (-2.0% , $p = 0.32$) (Fig. 5e). Exploratory analyses of co-factors in the MDD group did not reveal any effects or trends: suicide (-43%) *vs.* non-suicide (-31%); antidepressant (-39%), SSRI only (-35%) *vs.* no antidepressant (-33%); benzodiazepines or sodium valproate (-39%) *vs.* no exposure (-35%); antipsychotic (-35%) *vs.* no

exposure (-35%) (mean difference; all $F \leq 2.0$, uncorrected $p > 0.05$).

Discussion

Results from this study suggest that mood disorders may not be associated with deficits in GABA synthesis, as transcript levels for GAD65 and GAD67 were unchanged in both BPD and MDD subject groups. However, disease-related alterations in translation, protein stability or enzyme activity cannot be excluded. Our results do suggest disease-specific patterns in the expression of transcripts found in distinct populations of GABA neurons such that PV mRNA expression was selectively decreased in BPD subjects, whereas SST displayed a significant down-regulation in MDD subjects. The robust and well-characterized down-regulation of SST levels with increasing age (Erraji-BenChekroun *et al.* 2005; Morris *et al.* 2008) may have complicated the analysis of disease effects and is discussed below. Hence we confirmed the decreased SST phenotype at the protein level, and report robust decreases in MDD and no changes in BPD. The expression of CR mRNA, which is present in $\sim 45\%$ of GABA neurons in the primate DLPFC (Gabbott & Bacon, 1996), was not significantly altered in either BPD or MDD subjects. In contrast to these results, previous studies demonstrated a distinctly different profile of GABA-related alterations in DLPFC of

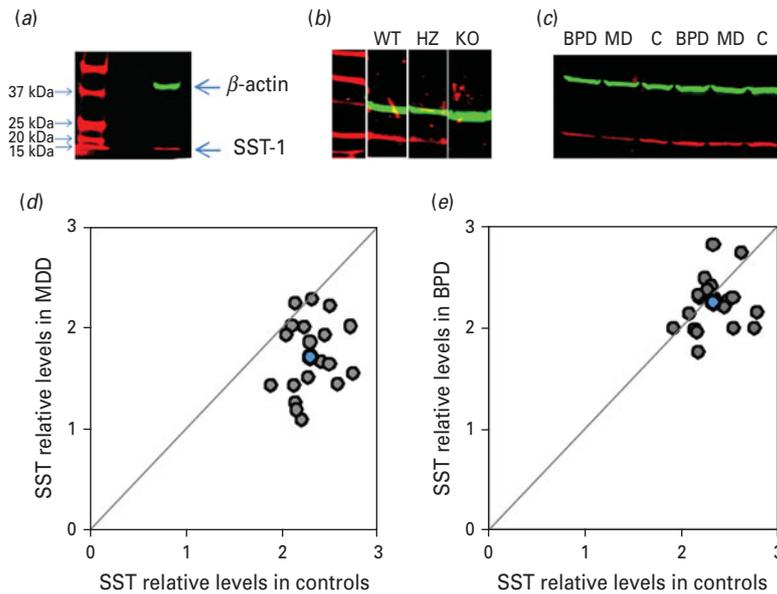


Fig. 5. Reduced somatostatin (SST) precursor protein levels in major depressive disorder (MDD) and no changes in bipolar disorder (BPD). (a) SST precursor protein relative immunoreactivity migrated at the expected ~ 14 kDa size, and (b) displayed a dose-dependent decrease between WT, SST-HZ and SST-KO mice. (c) Non-related lanes were removed for direct comparison. Examples of control (C) and MDD paired samples run on the same gel. Relative levels (compared to actin) are presented in control *vs.* (d) MDD and (e) BPD subjects. Data points below the no-change line represent low expression in the disease cohort. Blue dots indicate the mean group differences. Quantitative results and statistics are presented in the text.

subjects with schizophrenia with robust reductions in GAD67, PV, and SST mRNAs and a small reduction in GAD65 mRNA (Akbarian & Huang, 2006; Guidotti *et al.* 2000; Hashimoto *et al.* 2008; Lisman *et al.* 2008; Mellios *et al.* 2009). The presence of the disease-specific patterns of altered GABA-related markers has implications for diagnostic classifications, and for the interpretation of disease mechanisms and associated functional changes.

Decreased PV expression in BPD

Low PV mRNA expression in BPD was not associated with any demographic or clinical cofactors, including antipsychotic exposure. Consistently, PV mRNA expression was not altered in the PFC of monkeys with long-term exposure to high plasma levels of haloperidol, which produced marked extrapyramidal symptoms and required treatment with benztropine mesylate (Hashimoto *et al.* 2003); furthermore, PV expression was reported to be increased (Scruggs & Deutch, 1999) or not altered (Cahir *et al.* 2005) in the frontal cortex of mice chronically treated with either haloperidol or clozapine. Together, these findings suggest that the reduction in PV mRNA is associated with the disease process of BPD.

In contrast to previous studies demonstrating reduced GAD67 protein (Guidotti *et al.* 2000) and

reduced GAD67 mRNA + neuron density (Woo *et al.* 2008), we did not observe GAD67 mRNA changes in the DLPFC of BPD subjects. In both previous studies, the reduction in GAD67 was found only in BPD subjects that had a history of psychosis, which was not detected here, although our analysis may have been limited by small sample size (BPD with psychosis, $n = 11$) and associated reduced analytical power; thus, we cannot exclude the possibility of psychosis driving the effect. However, the literature base is still relatively small to draw any firm conclusions. Moreover, given that on other measures mediated by the PFC, such as cognition, BPD shows some similarity but less severe impairments than subjects with schizophrenia, it may be that a GAD deficit will be inconsistently detectable. Finally, contrasting with the notion of a common factor inducing both a decrease in PV and GAD67 mRNAs as suggested in schizophrenia (Hashimoto *et al.* 2005), the selective PV reduction observed in BPD may reflect alterations in transcriptional regulation secondary to the common genetic liability for both illnesses. For example, the PV gene lies in the vicinity of the marker D22S278 (GenBank NT_011520), a putative susceptibility gene for BPD as well as schizophrenia (Schwab & Wildenauer, 2000). Importantly, results of low PV mRNA in BPD will have to be extended to protein levels. In schizophrenia, PV mRNA levels

were shown to be lower per cell, but the numbers of PV mRNA-positive and PV-immunoreactive cells are unchanged (Hashimoto *et al.* 2003). Thus, until we are able to quantify PV cellular protein levels (as a measure of immune-fluorescence for instance) it will be difficult to assess whether PV protein levels are changed.

Decreased SST in MDD

Several lines of evidence suggest that the significant reduction in the expression of SST mRNA and of precursor protein levels in subjects with MDD may be related to the disease process. Low SST levels were not explained by any potential confounds, including antidepressant treatment. In fact, antidepressant exposure in rodent models results in either increase (Pallis *et al.* 2009) or no change (Surget *et al.* 2009) in SST mRNA levels, suggesting that the reduction in SST mRNA expression is associated with the disease process of MDD. Here the trend level of decreased expression in subjects with BPD (-14% , $p=0.10$) may need to be further investigated in independent cohorts of BPD and control subjects, especially in view of the lack of age-related correlation for SST transcripts in this disease group (see next). However, the lack of differences in SST precursor peptide levels in that group strongly supports the absence of alterations in SST expression in the DLPFC of BPD.

SST in age and disease

Down-regulated SST transcript levels with increasing age is a robust finding that has been confirmed across studies in the rat (Vela *et al.* 2003), monkey (Hayashi *et al.* 1997), and humans (Erraji-BenChekroun *et al.* 2005; Morris *et al.* 2008), including in this study (Fig. 2). Interestingly, a correlation between age and SST transcript levels was only observed here in the control group, as SST mRNA levels were low at all ages in most subjects with MDD or BPD. This pronounced age effect in controls resulted in the appearance of increased between-subject variability for RNA measures (Fig. 1). Age-detrending the data was not possible here, as the slopes of age-related effects differ between control and disease cohorts (Fig. 2). In contrast, we observed a weak negative correlation of SST precursor peptide levels with increasing age. This may reflect additional levels of regulation in translation and post-translational processing of SST to its mature form, as reflected by the overall moderate correlation between RNA and protein levels and absence of correlation in MDD, and/or the limitation of measuring precursor protein levels, rather than the active processed form.

Thus, the true respective contribution of age may not have been fully assessed.

A first interpretation of these findings is that decreased SST levels *per se* are not a core feature of the disease phenotype in schizophrenia and MDD, as older control subjects eventually reach SST levels at, or below, those observed in these diseases at younger ages. Alternatively, lower than optimal SST function during developmental periods of neural and synaptic plasticity might interfere with the normal trajectory leading to adult neural network organization, leaving the DLPFC vulnerable to the pathogenetic processes underlying these psychiatric disorders. In this model, lower than normal SST expression induces alterations in biological pathways that are typically regulated by higher SST function. As developmental windows of plasticity close, these maladaptive changes become fixed and SST-independent. A similar mechanism has been proposed, for instance, for altered serotonin function during development supporting changes in adult emotionality, in concert with pharmacological blockade in rodents (Ansorge *et al.* 2004) or genetic variants in humans (Sibille & Lewis, 2006). This hypothesis is consistent here with the cross-sectional evidence of low SST levels in subjects with MDD, although the exact trajectory of SST level within individuals is not known. A third and related possibility, is that low MDD-related SST levels in the adult brain (as measured here) occur in the context of broader changes in the local microcircuitry, so that converging molecular, cellular and signaling changes (of which low SST is only one) are manifest as altered functional homeostasis, leading to disease symptoms. Importantly, the degree of biological vulnerability to any of these putative SST-related disease mechanisms may be further moderated by genetic liability and adverse environmental events.

Any of these pathways would also be consistent with the speculation that brain functions downstream from low SST are involved in altered mood regulation in MDD and some individuals with schizophrenia. SST, also known as somatotropin-release inhibiting factor (SRIF), belongs to a family of neuropeptides widely distributed in the brain and periphery, where it exerts potent inhibitory effects on various neuroendocrine functions (Weckbecker *et al.* 2003). Previous reports suggested low SST in cerebrospinal fluid of depressed subjects (Rubinow *et al.* 1988), is potentially related to dysregulated corticosteroid function (Kling *et al.* 1993). In the cerebral cortex, SST co-localizes with GABA and has similar inhibitory function on post-synaptic target neurons, and summates or synergizes with GABA function. A potential link between low

SST and altered mood regulation in psychiatric disorders is also consistent with the anxiolytic and antidepressant-like effects of intracerebroventricular injection of SST in rats (Engin *et al.* 2008). Moreover, as brain-derived neurotrophic factor (BDNF) is required for normal SST expression (Glorioso *et al.* 2006), the observed or suggested decreased BDNF expression in schizophrenia, MDD and BPD (Hashimoto *et al.* 2005; Nestler *et al.* 2002; Weickert *et al.* 2003) may represent an upstream mechanism for low SST in psychiatric disorders.

Summary and limitations

The distinct GABA-related transcript abnormalities reported here in subjects with MDD and BPD, and observed elsewhere in schizophrenia (Hashimoto *et al.* 2008), suggest that distinct patterns of altered markers of GABAergic function are present across different sets of neuropsychiatric disorders, potentially in correlation with symptom dimensions. Specifically, decreased PV in schizophrenia and BPD correlate with cognitive dysfunction, and may distinguish BPD and schizophrenia from MDD. Mood disorders on the other hand are not associated with widespread deficits in GABAergic gene transcript levels in DLPFC, but with more restricted decreased SST levels. The fact that no changes were observed in the BPD group suggests that low SST may not correspond with the low mood component present across these neuropsychiatric disorders. Alternatively, low SST was reported in the hippocampus of BPD subjects (Konradi *et al.* 2004), so disease-specific patterns of regional changes may still contribute to the mood emotion dysregulation and low mood symptoms in BPD. Nevertheless, interpreting the findings from these studies as defining differences between BPD and MDD requires replication studies. Moreover, the small samples sizes preclude definitive interpretations regarding the potential influence of the effects of medications, death by suicide and other factors on the findings. Future studies will need to focus on the actual role of the SST abnormalities in the disease process, as observed changes could represent a cause, consequence or compensation of the underlying disease process (Lewis & Gonzalez-Burgos, 2008), in order to determine its potential as a target for therapeutic interventions.

Note

Supplementary material accompanies this paper on the Journal's website (<http://journals.cambridge.org/pnp>).

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Statement of Interest

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