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ORIGINAL ARTICLE

Molecular evidence for BDNF- and GABA-related dysfunctions in the amygdala of female subjects with major depression

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Women are twice as likely as men to develop major depressive disorder (MDD) and are more prone to recurring episodes. Hence, we tested the hypothesis that the illness may associate with robust molecular changes in female subjects, and investigated large-scale gene expression in the post-mortem brain of MDD subjects paired with matched controls ($n = 21$ pairs). We focused on the lateral/basolateral/basomedian complex of the amygdala as a neural hub of mood regulation affected in MDD. Among the most robust findings were downregulated transcripts for genes coding for γ-aminobutyric acid (GABA) interneuron-related peptides, including somatostatin (SST), tachykinin, neuropeptide Y (NPY) and cortistatin, in a pattern reminiscent to that previously reported in mice with low brain-derived neurotrophic factor (BDNF). Changes were confirmed by quantitative PCR and not explained by demographic, technical or known clinical parameters. BDNF itself was significantly downregulated at the RNA and protein levels in MDD subjects. Investigating putative mechanisms, we show that this core MDD-related gene profile (including *SST*, *NPY*, *TAC1*, *RGS4* and *CORT*) is recapitulated by complementary patterns in mice with constitutive (BDNF-heterozygous) or activity-dependent (exon IV knockout) decreases in BDNF function, with a common effect on *SST* and *NPY*. Together, these results provide both direct (low RNA/protein) and indirect (low BDNF-dependent gene pattern) evidence for reduced BDNF function in the amygdala of female subjects with MDD. Supporting studies in mutant mice models suggest a complex mechanism of low constitutive and activity-dependent BDNF function in MDD, particularly affecting SST/NPY-related GABA neurons, thus linking the neurotrophic and GABA hypotheses of depression.
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Keywords: major depression; female; amygdala; BDNF; GABA; somatostatin; neurotrophic hypothesis

Introduction

Major depression is a multifactorial psychiatric disorder that is responsible for substantial disability worldwide. Despite a high morbidity and mortality, its etiology and pathophysiology are not well defined. Notably, women are more likely to become depressed than men, display higher severity and symptom number, have frequent co-morbid anxiety symptoms, and are more prone to recurring episodes. However, the underlying biological vulnerabilities are not fully characterized yet.

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Low neurotrophic factor support has been proposed as a unifying hypothesis for reduced cell numbers in frontal cortex[1,2] and amygdala,[3,4] reduced hippocampal volume.[5,6] This interpretation is supported by indirect evidence in rodents showing that antidepressant treatments increase *BDNF* (protein) and BDNF (mRNA) levels, hippocampal *BDNF* infusion is sufficient to produce antidepressant-like effects,[7,8] and BDNF appears necessary for some behavioral responses to antidepressant treatments.[9,10] Direct evidence is more sparse and includes low circulating peripheral *BDNF* levels, which are normalized by antidepressant treatment,[11,12] and one study showing reduced pro-*BDNF*[13] and BDNF levels[14] in post-mortem hippocampal tissue of depressed patients.[15] Associations between BDNF signaling and suicide completion have been made, including reduced BDNF levels in post-mortem hippocampal or

midbrain,[16–18] decrease in TrkB.T1 expression[19] hypermethylation of BDNF promoter/exon IV[20] and an increased risk for violent suicide in subjects carrying a Met allele at the BDNF Val66Met polymorphism.[21]

Evidence in major depressive disorder (MDD) patients also suggests an impaired excitation/inhibition balance that is potentially mediated by decreased γ-aminobutyric acid (GABA) content, as observed by microarray,[22] imaging studies in occipital and frontal cortices[23,24] or by transcranial magnetic stimulation paradigms.[25] We recently reported a downregulation of the neuropeptide somatostatin (SST) in the dorsolateral prefrontal cortex[26] and subgenual cingulate cortices[27] of MDD subjects. Expression of SST is dependent on BDNF,[28] and identifies a specific GABA interneuron subtype.[29] Notably, the downregulation of SST was consistently more robust in female MDD subjects.

At the neural network level, changes in the structure, function and coordinated activity of cortical and subcortical brain regions are thought to underlie the mood regulation deficit in depression.[30] Our study focused on the amygdala as a critical component of this corticolimbic circuit of mood regulation.[31] Evidence for misregulated amygdala function includes decreased oligodendrocyte numbers and markers[3,4] and altered/sustained amygdala function in depressed subjects exposed to negative emotionally salient stimuli.[32] Here, we investigated molecular changes in the amygdala of female subjects with MDD and report robust downregulation of BDNF and of several BDNF-dependent genes. Notably, this deregulated pattern was recreated by low constitutive or reduced activity-dependent BDNF function in mutant mice and robustly affected SST expression, hence providing evidence in support of low neurotrophic and altered GABA-related functions in MDD.

Materials and methods

Human post-mortem subjects

For all subjects, consensus diagnoses of MDD following the Diagnostic and Statistical Manual of Mental Disorders (4th edition) were made by an independent committee of experienced clinical research scientists at a case conference utilizing information obtained from clinical records, toxicology exam and a standardized psychological autopsy.[33] This latter incorporates a structured interview, conducted by a licensed clinical psychologist with family members of the index subject, to assess diagnosis, psychopathology, medical, social and family histories, as well as history of substance abuse. A symptom score reflecting disease severity was calculated based on the presence at time of death of nine MDD symptoms: depressed mood, anhedonia, appetite disturbance, sleep disturbance, psychomotor change, anergia, self-recrimination, diminished ability to concentrate or make decision, and suicidality.[27,34] Each symptom is scored 0 (absence) or 1 (presence) and the symptom score is sum of the individual symptom scores.

In all, 21 pairs of subjects were analyzed (Table 1), consisting of female subjects with MDD and control subjects matched for sex, race, and as closely as possible for age, post-mortem interval and brain pH. The pairing protocol has been previously validated as reducing signal variability and inducing higher overall correlations of gene transcript levels compared with non-matched pairs.[34] All subjects died suddenly without prolonged agonal periods. All cases and controls except one pair were white Caucasian. Brains were analyzed for adequate brain pH (>6.4) and RNA integrity by optical density (optical density $\geqslant 1.6$) and Agilent bioanalyzer analysis (Agilent Technologies, Palo Alto, CA, USA; RNA integrity number expert scoring system $\geqslant 7$) as described.[34] Accordingly, subject groups did not differ in mean age, post-mortem interval (PMI), brain pH, RNA integrity number or RNA ratio ($P>0.05$). Rates of death by suicide, disease recurrence, evidence for antidepressant treatment at time of death and alcohol dependence in MDD subjects were recorded. Toxicological screens on peripheral fluids identified the presence of at least one antidepressant in 14 subjects, (Table 1), while no psychoactive substance was detected in controls.

Microarray samples

Rostral amygdala samples enriched in lateral, basolateral and basomedian nuclei were delineated as described previously,[34] dissected from frozen coronal blocks ~ 2 cm caudal to the temporal pole. Total RNA was processed for Illumina HT12 microarray analysis according to the microarray manufacturer's protocol (Illumina, San Diego, CA, USA).

Statistical analysis

Gene selection was made using a random-intercept statistical model to adjust for clinical, demographic and technical variables (details in Supplementary Information). Multiple testing was adjusted by the Benjamini–Hochberg method.[35] This resulted in a stringent selection of 116 differentially expressed genes, termed 'q-list' (adjusted P-value = Q-value <0.05; mean change $>20\%$). In addition, an exploratory list of genes was obtained using uncorrected parametric and non-parametric statistical tests (random-intercept statistical model, paired T-test and Wilcoxon test), using moderate statistical stringencies for gene selection ($P<0.05$ in at least one statistical test), as proposed previously.[34] The resulting exploratory list ($n=4131$ genes; 307 genes with fold change $>20\%$) may carry a higher rate of false positives at the single-gene level, but allowed investigation of cumulative effects over larger sets of genes and pathways (see BDNF-related genes and Ingenuity Pathway analysis).

In situ *hybridization*

Antisense and sense riboprobes for human SST mRNA were transcribed in the presence of [^{35}S]-CTP (Amersham Biosciences, Piscataway, NJ, USA) as described.[36] The sections and [^{14}C]-standards were

Table 1 Major depression and control cohorts

Pairs	*Major depression group*										*Control group*						
	Case	*Age (years)*	*Post-mortem interval (h)*	*pH*	*RNA ratio*	*RNA integrity number*	*Suicide*	*Recurrent episode*	*Antidepressant*	*Alcohol dependence at death*	*Case*	*Age (years)*	*Post-mortem interval (h)*	*pH*	*RNA ratio*	*RNA integrity number*	*Alcohol dependence at death*
1	564	56	16.8	7.02	1.94	9.2	Y	N	Flx^{T}, Dox^{T}	N	568	60	9.5	6.88	1.9	8.7	N
2	666	16	10	7.29	2	9.4	N	N	Prx^{H}	N	10 013	16	9.3	6.69	1.8	9	N
3	803	65	18	6.96	1.86	9	N	Y	Cit^{H}, $Sert^{H}$, Nor^{T}	N	1466	64	20	6.74	2.01	8.8	N
4	934	54	17.9	6.2	1.16	8.2	N	Y	$Cit^{H,T}$, $Mirt^{H,T}$, Ami^{H}, $Risp^{H}$	N	1247	58	22.7	6.37	1.28	8.4	N
5	967	40	22.2	6.56	1.64	7.4	N	Y	Flx^{H}, Dox^{H}, Li^{H}, Prx^{H}, $Sert^{H}$	Y	1282	39	24.5	6.84	1.32	7.5	N
6	986	53	11.9	6.7	1.83	8.7	N	Y	AD^{H}, Flx^{T}	N	575	55	11.3	6.81	1.79	9.6	N
7	1041	52	10.3	6.51	1.48	8.4	N	Y	Flv^{H}, $Mirt^{H}$, Nef^{H}, Ola^{H}, $Quet^{H}$, $Risp^{H}$, $Traz^{H}$, $Venl^{H}$, Zip^{H}	Y	1391	51	7.8	6.57	1.59	7.1	N
8	1157	26	13.4	6.4	1.48	7.8	Y	Y	$Cit^{H,T}$, Prx^{H}	N	1034	23	8.5	6.11	1.96	7.8	N
9	1190	47	22.3	6.55	1.64	8	Y	Y	Flx^{H}, $Traz^{H}$	Y	567	46	15	6.77	2.26	8.9	N
10	1202	39	11.2	6.39	1.79	8	N	Y	Esc^{H}, Bup^{H}	N	840	41	15.4	6.8	1.98	9.1	N
11	1221	28	24.8	6.61	1.83	7.2	N	Y	$Mirt^{H}$	N	546	37	23.5	6.74	1.95	8.6	N
12	1249	40	11.2	6.52	2.04	9	N	Y	$Flx^{H,T}$, $Traz^{H}$	N	1092	40	16.6	6.83	1.68	8	N
13	1254	39	12.8	6.37	1.92	9	Y	Y	$Mirt^{H}$, $Sert^{H}$	N	1403	45	12.3	6.67	1.8	8.2	N
14	1271	50	18.6	6.35	1.82	8.6	N	Y	$Ami^{H,T}$	Y	1318	58	18.8	6.69	1.95	7.4	N
15	1289	46	25	6.27	1.35	7.3	N	N	U	N	1280	50	23.5	6.65	1.33	7.7	N
16	1315	28	12.4	7	1.52	7.9	Y	N	Prx^{H}, Bup^{H}	Y	1099	24	9.1	6.46	1.86	8.6	N
17	1332	46	17.5	6.49	1.6	8.9	N	Y	Flx^{H}	N	627	43	14.1	7.09	1	7	N
18	1356	60	20.6	6.06	1.78	8.5	N	Y	$Cit^{H,T}$, Nor^{H}, Prx^{H}, Nef^{H}, $Traz^{H}$, Bup^{H}, $Sert^{H}$	Y	818	67	24	7.06	1.48	8.4	N
19	1360	59	18.1	6.43	1.41	7.6	Y	N	Ami^{H}, Cit^{H}, Flx^{H}, $Traz^{H}$	N	1081	57	14.9	6.78	1.8	9	N
20	1408	37	15.5	6.56	1.58	7	N	Y	$Venl^{H}$, Prx^{H}, $Sert^{H}$, Bup^{T}	Y	1196	36	14.5	6.44	1.84	8.2	Y
21	10 028	72	23.1	6.66	1.37	7	Y	N	N	N	1355	74	24.9	6.62	1.89	7	N
Mean	**46.0**	**16.2**	**6.7**	**1.67**	**8.2**							**46.8**	**16.5**	**6.6**	**1.74**	**8.2**	
s.e.m.	**3.0**	**1.3**	**0.04**	**0.05**	**0.16**							**3.2**	**1.1**	**0.06**	**0.07**	**0.16**	

Abbreviations: AD, antidepressant unspecified; Ami, amitriptyline; Bup, bupropion; Cit, citalopram; Dox, doxepin; Esc, escitalopram; Flv, fluvoxamine; Flx, fluoxetine; H, history of treatment; Li, lithium; Mirt, mirtazapine; Nef, nefazodone; N, no; Nor, nortriptyline; Ola, olanzapine; Prx, paroxetine; Quet, quetiapine; Risp, risperidone; Sert, sertraline; T, toxicological screen at time of death; Traz, trazodone; U, unknown; Venl, venlafaxine; Y, yes; Zip, ziprasidone.

exposed on the same BioMax MR film (Kodak, Rochester, NY, USA) for 3 days and analyzed with the MCID software (Cambridge, England).

Protein isolation, prepro-SST and BDNF *immunoblotting*
Acetone precipitation of proteins was carried out following RNA extraction. Western blot analysis was performed as described.[34] Dual signals were detected using the LI-COR Odyssey Infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA), and *SST* or *BDNF* signal ratios to β-actin were calculated. Samples were processed in matched pairs on the same gel and results were replicated for a total of three different western blots.

BDNF +/− and exon-IV KO mice
Female BDNF +/+ and +/− mice (3 to 4 months old) were bred on a mixed S129/Sv × C57BL/6 genetic background. Heterozygous adult female mice with one functional BDNF allele ($BDNF^{HZ}$) exhibit 50% reduction of BDNF mRNA levels in the hippocampus.[37] Female BDNF exon IV KO ($BDNF^{IV-KO}$) mice were crossed on C57BL/6 as described.[38] Brains were rapidly removed and flash-frozen on dry ice. Caudo-rostral coronal sections were obtained on a cryostat until the caudal amygdala was observed (Figure #48 of the mouse brain atlas[39]). Brains were then flipped and cut rostro-caudally until observing the rostral amygdala (Figure #40 of the mouse brain atlas). Bi-lateral amygdalae were then micro-punctured using 0.5 mm diameter punches and stored in Trizol at −80 °C.

Real-time quantitative polymerase chain reaction (qPCR)
Small PCR products were amplified in quadruplets on a Mastercycler real-time PCR machine (Eppendorf, Hamburg, Germany), using universal PCR conditions. Results were calculated as the geometric mean of relative intensities compared with three validated internal controls (actin, glyceraldehyde-3-phosphate dehydrogenase and cyclophilin G).

See detailed methods in Supplementary Information.

Results

Altered gene expression in the amygdala of female subjects with MDD suggests a low BDNF-dependent transcriptional drive affecting SST GABA interneurons
Using a microarray approach in the amygdala of postmortem female MDD subjects compared with matched controls and taking clinical and technical parameters into account, we identified 116 gene transcripts as differentially expressed after controlling for multiple testing (q-value <0.05; effect size $>20\%$; q-list, Table 2 and Supplementary Table S1). Based on previous identification of relative glial/neuronal enrichments in gene transcript origin,[40] 23 gene transcript changes were of neuronal origin and mostly downregulated (~80%), 56 of mixed neuronal/glial origin (22 down, 34 up) and 31 of enriched glial origin and mostly upregulated (~87%). Notably, among the most downregulated genes in each subcategory were SST, neuropeptide Y (NPY), RGS4, CORT and TAC1 (Table 2), in a pattern strikingly reminiscent to that previously reported in the cortex of male mice with low BDNF,[28] thus suggesting that a deficit in BDNF function may have contributed to the gene pattern observed in MDD. We further explored this putative BDNF link by investigating a broader list of genes identified as BDNF-related in the Ingenuity Pathway database ($n=283$ genes). In all, 52 BDNF-related genes displayed significant uncorrected MDD-affected changes (Supplementary Table S2) and BDNF-related genes were significantly over-represented in the q-list of MDD-affected gene list (6% vs 1.2% in Ingenuity database; $\chi^2=17.4$, $df=1$, $P<0.0001$). Further Ingenuity-based exploratory functional analyses resulted in buildings of gene networks and canonical pathways coherent with our findings (Supplementary Figure S1).

Confirmation of altered gene expression by qPCR
Microarray results for 13 genes in the MDD q-list (*AMPH*, *CDK5RAP2*, *CORT*, *GFAP*, *KCNG1*, *MBP*, *MOBP*, *NPY*, *RGS4*, *SLC32A1*, *SST*, *SSTR1* and *TAC1*) were confirmed by qPCR (Supplementary Table S3), with a high Array/qPCR concordance (Pearson correlation $r=0.95$, $P<1e^{-6}$) and similar directionality of expression for all genes (Figure 1a).

Exploratory analysis (that is, unadjusted P-values) identified additional downregulated GABA-related genes (*GAD1*, *GAD2*, *GABRA1*, *GABRA5* and *calretinin* (*CALB2*)) and genes coding for receptors (HTR3A) found on GABAergic neurons in the amygdala,[41] but these findings were not reliably confirmed (Supplementary Table S3), potentially reflecting array false positives or a diluting effect from other unaffected GABA neuron subsets in the qPCR complementary DNA samples. For instance, parvalbumin, a marker of a different GABA neuron subset, was not affected in our cohort.

Low BDNF transcript and protein levels in the amygdala of female subjects with MDD
Owing to low detection by array, we measured BDNF by qPCR and western blotting. Results indicate significant decreases in MDD subjects of mRNA levels corresponding to the BDNF coding sequence (BDNF exon IXd; −22%; $P<0.05$, Figure 1b) and protein level, for both the precursor (pro-) and mature (m-) forms (−27% and −30%, respectively; $P<0.05$, Figures 1c–e). Low *BDNF* was observed regardless of the presence of antidepressant treatment (Figure 1d).

BDNF-IXd expression was co-regulated across all samples with the four core genes involved in the BDNF-related signature (SST, NPY, RGS4 and TAC1; average $R=0.39$, $P<0.05$), suggesting a functional link, however, the co-regulation with BDNF was lower than mutual co-regulation within those four

Table 2 Summary of the q-list core genes significantly affected in female MDD subjects

Illumina probeset ID	*Gene title*	*Gene symbol*	*RIM P-value*	*RIM q-value*	*All MDD subjects (alr)*	*WM/GM fold change*
13 Out of the 24 neuronal genes of the full q-list						
ILMN_1812824	**Somatostatin**	**SST**	**4.09E–05**	**0.020**	**−0.84**	−1.96
ILMN_1765966	Chromogranin B (secretogranin 1)	CHGB	7.95E–04	0.034	−0.54	−3.76
ILMN_1679984	Zinc-finger, CCHC domain containing 12	ZCCHC12	1.64E–04	0.024	−0.53	−4.24
ILMN_1697512	Solute carrier family 32 (GABA vesicular transporter), member 1	SLC32A1	4.91E–04	0.030	−0.48	−7.27
ILMN_1685834	Amphiphysin, transcript variant 1	AMPH	1.63E–03	0.044	−0.39	−2.12
ILMN_2384409	**Tachykinin, precursor 1, transcript variant alpha,**	**TAC1**	**2.19E–03**	**0.048**	**−0.38**	−1.50
ILMN_1810604	ELMO/CED-12 domain containing 1	ELMOD1	2.17E–03	0.048	−0.38	−2.72
ILMN_1806147	Guanine nucleotide binding protein (G protein), gamma 3	GNG3	1.35E–04	0.023	−0.31	−1.91
ILMN_1654632	Regulator of G-protein signaling 7 binding protein	RGS7BP	2.30E–03	0.049	−0.31	−5.46
ILMN_1765966	Chromogranin B (secretogranin 1)	CHGB	7.95E–04	0.034	−0.54	−3.76
ILMN_2354547	Tumor suppressor candidate 3, transcript variant 1	TUSC3	9.76E–04	0.036	−0.34	−1.89
ILMN_1736154	ProSAPiP1 protein	ProSAPiP1	6.97E–04	0.033	0.31	−2.60
ILMN_1760798	Ryanodine receptor 2 (cardiac)	RYR2	7.82E–04	0.034	0.40	−2.65
13 Out of the 57 neuronal/glial genes of the full q-list						
ILMN_2071186	**Cortistatin**	**CORT**	**1.72E–04**	**0.025**	**−0.58**	−1.06
ILMN_1731062	**Neuropeptide Y**	**NPY**	**3.70E–04**	**0.029**	**−0.52**	−1.18
ILMN_1729165	Transcription elongation factor A (SII)-like 6	TCEAL6	8.23E–04	0.035	−0.40	1.41
ILMN_1771286	PREDICTED: similar to phosphodiesterase 4D interacting protein	LOC653513	3.22E–04	0.028	−0.39	1.17
ILMN_1690397	Dynein, cytoplasmic 1, intermediate chain 1	DYNC1I1	1.02E–03	0.037	−0.35	−1.20
ILMN_2399304	Neuron navigator 2, transcript variant 2,	NAV2	5.92E–04	0.032	0.34	1.27
ILMN_1768962	A kinase (PRKA) anchor protein 8-like	AKAP8L	4.14E–04	0.030	0.35	1.02
ILMN_1663042	Syndecan 4	SDC4	3.34E–04	0.029	0.36	−1.00
ILMN_1655611	Teashirt zinc finger homeobox 2	TSHZ2	2.02E–03	0.047	0.38	−1.04
ILMN_1696757	Tetratricopeptide repeat domain 14, transcript variant 2,	TTC14	5.92E–04	0.032	0.38	1.02
ILMN_1682775	Endothelin 1	EDN1	2.50E–04	0.027	0.42	1.26
ILMN_2078547	Hypothetical protein HSPC268	HSPC268	7.69E–04	0.034	0.42	1.02
ILMN_1744897	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	KCNN3	5.09E–04	0.030	0.45	−1.39
13 Out of the 32 glial genes of the full q-list						
ILMN_1758067	**Regulator of G-protein signalling 4**	**RGS4**	**1.04E–03**	**0.038**	**−0.47**	1.52
ILMN_2320164	Purinergic receptor P2Y, G-protein coupled 12, transcript variant 1,	P2RY12	8.38E–04	0.035	−0.46	2.49
ILMN_2402172	Septin 4, transcript variant 3,	SEPT4	8.25E–04	0.035	0.35	2.45
ILMN_1665686	Family with sequence similarity 38, member B	FAM38B	2.45E–04	0.027	0.36	5.72
ILMN_1810420	Dysferlin, limb girdle muscular dystrophy 2B	DYSF	1.75E–03	0.046	0.36	2.95
ILMN_1670881	Carbohydrate (*N*-acetylglucosamine 6-O) sulfotransferase 6	CHST6	9.30E–05	0.020	0.36	3.92
ILMN_1697176	Glial fibrillary acidic protein	GFAP	9.40E–04	0.036	0.37	3.60
ILMN_1659895	Moesin	MSN	2.53E–04	0.027	0.37	1.91
ILMN_1737631	Progestin and adipoQ receptor family member VI, transcript 1	PAQR6	7.85E–04	0.034	0.37	2.47
ILMN_2323508	Chromosome 9 open reading frame 58, transcript variant 2	C9orf58	2.20E–03	0.048	0.38	2.25
ILMN_1752668	Disheveled associated activator of morphogenesis 2	DAAM2	3.78E–04	0.029	0.42	2.25
ILMN_1750271	Myelin-associated oligodendrocyte basic protein	MOBP	4.33E–04	0.030	0.51	2.46
ILMN_2331544	Myelin basic protein transcript variant 7	MBP	6.95E–04	0.033	0.64	2.55

Abbreviations: MDD, major depressive disorder; RIM, random-intercept statistical model; SST, somatostatin.
In all, 39 out of 116 genes differently expressed in MDD (MDD) versus control are listed (full list in Supplementary Table S2).
'Neuronal', 'neuronal-glial', and 'glial' refers to enrichments of transcript origin (Supplementary Information).[40]
Bold entries refers to genes previously observed downregulated in cortex of mice with low BDNF.[28]

genes (average $R=0.57$, $P<0.01$) (Supplementary Table S4), suggesting the presence of additional regulatory events.

BDNF transcription is initiated from at least nine promoters that respond to differing stimuli and drive transcription of a short-noncoding exon spliced to a common coding exon.[42] For example, promoter IV is highly responsive to neuronal activity and induces activity-dependent BDNF expression *in vitro* and *in vivo*.[38] Using qPCR, we report no difference in expression levels for selected activity-dependent (I, IV and IXa) BDNF promoter/non-coding exons, although we note the limits of assessing short-term and activity-dependent gene activities under variable post-mortem intervals for brain collection. No changes were observed in mRNA coding for TrkB (full length or truncated forms; Supplementary Figure S2).

Could BDNF-related changes be explained by other clinical/demographic factors?

Although clinical and technical parameters were accounted for in the single-gene statistical models,

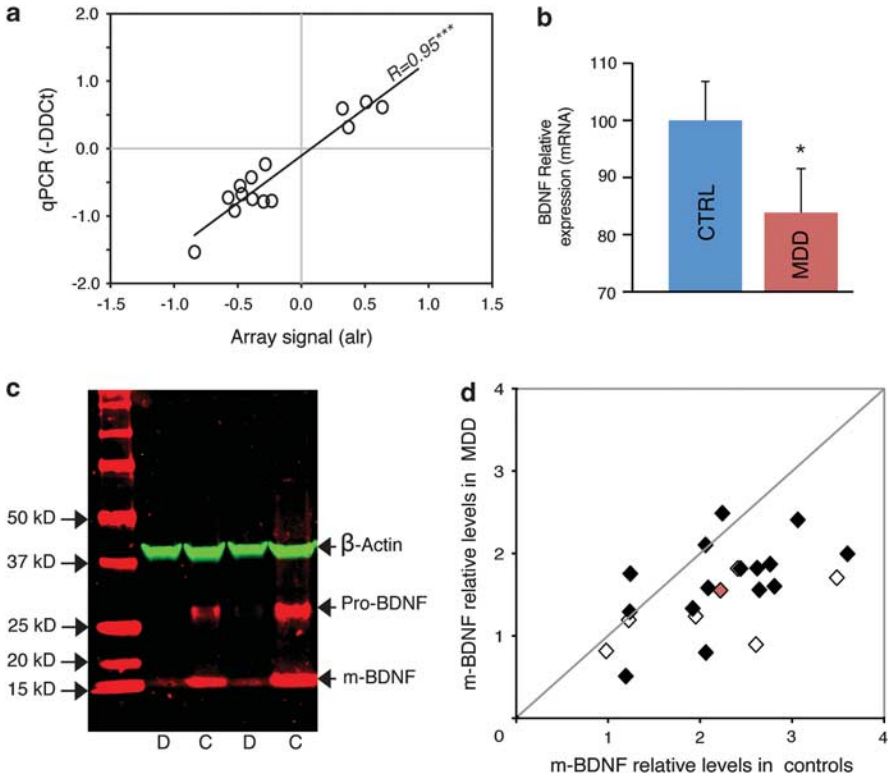

Figure 1 Quantitative polymerase chain reaction (qPCR) validation of altered gene expression and reduced BDNF transcript/protein in the amygdala of female major depressive disorder (MDD) subjects. (**a**) Confirmation of microarray results by qPCR (see also Supplementary Table S4). (**b**) BDNF-IXd coding exon transcript expression in relative expression (%) of the control group mean (*$P<0.05$). (**c**) Mature BDNF (m-BDNF) and pro-BDNF protein relative immunoreactivity migrates at the expected ~15 and 25 kD size, respectively. Examples of two sample pairs of control (C) and MDD (D) on the same gel. (**d**) Relative level of m-BDNF in MDD in function of respective paired CTRL (◆ADD-treated MDD; ◇ADD-free MDD; ◆Average of population) (*$P<0.05$).

we further investigated potential effects of selected parameters on the 52 BDNF-related genes as a whole (Supplementary Table S2).

Age. We previously reported that disease-associated genes, including BDNF, are robustly modulated by age.[43] Accordingly, we observed a correlation between age and the 52 BDNF related-gene set (averaged relative change and age; $R=0.44$, $P=0.004$; See Supplementary Figure S3); however, the effect of MDD on BDNF-related genes was still highly significant after analysis of covariance analysis with age as cofactor ($P=8.8e^{-4}$). The details of relation between age and gene expression in MDD will be discussed elsewhere.

Antidepressant treatment. Antidepressant treatment was detected in 2/3 of subjects at time of death, but data on long-term exposure and treatment efficacy was not available. BDNF levels in MDD subjects did not correspond to antidepressant treatment. Moreover, the levels of expression changes for 52 BDNF-related genes with nominal MDD *P*-values <0.05 were not different between treated and untreated MDD subjects (Supplementary Table S2; Pearson correlation, $r=0.91$, $P<1e^{-7}$). We further investigated potential antidepressant effects on orthologous genes in a mouse model of antidepressant response. In that study, 4-week chronic fluoxetine exposure reversed the high emotionality phenotype induced by chronic mild stress in mice.[44] With the exception of two genes (*ACAT2* and *RAPGEF6*) antidepressant did not affect expression of orthologous genes from the MDD-related q-list (Table 2 and Supplementary Tables S1 and S2).

Alcohol use. Alcohol use was used as a covariate in all statistical analysis performed and did not interfere with any of the positive results provided herein.

Hormonal status. The cohort encompasses a large age range and information on hormonal status and replacement therapy was largely not available. So, we hypothesized that the transcriptional activation of genes involved in regulating estrogen function (*CYP19A1*, *ESR1*, *ESR2*, *PGR* and *SULT1A1*) or for genes proposed as peripheral biomarkers of menopausal status (*AMH*),[45] may represent a useful proxy assay for hormonal status. The expression levels of these genes did not correlate with BDNF-related transcript changes (Supplementary Figure S4).

Disease severity. For each depressed subject, a symptom score reflecting the severity of the disease was calculated based on the presence at time of death of nine MDD episode symptoms (Materials and methods section).[34] Score values showed no correlation with changes in the expression of any of the BDNF-related gene, suggesting that intensity of changes were independent of disease' severity (Supplementary Table S2).

Death by suicide. The disease effect sizes on BDNF-related genes were reduced in suicide versus non-suicide MDD subjects (Supplementary Figure S5), but BDNF mRNA and pro-BDNF protein levels (but not mBDNF) were further decreased in depressed suicide victims (mRNA: $alr_{suicide/ctrl} = -0.41$ vs $alr_{non-suicide/ctrl} = -0.28$, $P = 0.34$; pro-BDNF protein: $alr_{suicide/ctrl} = -0.62$ vs $alr_{non-suicide/ctrl} = -0.47$, $P = 0.28$), although not significantly, potentially because of reduced sample size.

Translational investigation of putative mechanisms in mouse models with genetically altered changes in BDNF level or function

We next investigated whether similar changes were observed downstream from low BDNF in the amygdala of mutant mice with altered BDNF function. To differentiate the putative contribution of constitutive and activity-dependent BDNF functions, we investigated gene transcript changes in mice heterozygous for a constitutive deletion of the BDNF gene ($BDNF^{HZ}$), which display ~50% less BDNF;[37] and mice with a targeted disruption of exon IV ($BDNF^{IV-KO}$), which results in a near complete blockade of activity-dependent BDNF protein expression.[38] Using qPCR, six core genes of interest were investigated, based on confirmed changes in MDD and known BDNF-dependency, including *SNAP25*, a BDNF-regulated gene member[46] of the SNARE complex, also observed downregulated in MDD[47] (Table 3). In addition, we evaluated the expression of five other GABA-related genes displaying less robust changes (array results not qPCR-confirmed), and parvalbumin as a control GABA marker not affected in our cohort. qPCR results confirmed the BDNF-dependency for most genes in amygdala, but neither line of mutant mice mimicked the full pattern of MDD changes. Instead, gene changes in $BDNF^{HZ}$ and $BDNF^{IV-KO}$ displayed complementary profiles (Table 3), which together recreated the MDD profile, suggesting a combined low activity-dependent and constitutive BDNF function in MDD. *SST* and *NPY* were identified at the intersection of the $BDNF^{HZ}$ and $BDNF^{IV-KO}$ profiles, hence identifying SST/NPY GABA interneurons as the most vulnerable GABA interneuron subtype to low BDNF function (Table 2).

Low SST transcript and protein levels in the amygdala of female subjects with MDD

Low SST was confirmed by qPCR (Supplementary Table S3). Using *in situ* hybridization, SST mRNA expression was significantly decreased in the lateral ($P < 0.01$) and basomedial ($P < 0.05$) amygdaloid nuclei in MDD subjects compared with control subjects (Figures 2a–c). SST expression was overall lower in

Table 3 Expression profile of BDNF- and GABA-related genes in MDD subject compared with $BDNF^{HZ}$ and $BDNFK^{IV-KO}$ mice

	Human				Mouse			
	Arrary		qPCR		$BDNF^{HZ}$ qPCR		$BDNFK^{IV-KO}$ qPCR	
	Alr	P-*value*	Alr	P-*value*	Alr	P-*value*	Alr	P-*value*
BDNF-dependent								
TAC1	**-0.38**	**2.19E-03**	**-0.75**	**0.017**	**-1.70**	**0.010**	0.50	0.189
RGS4	**-0.47**	**1.04E-03**	**-0.68**	**0.002**	**-0.59**	**0.050**	-0.13	0.344
NPY	**-0.52**	**3.70E-04**	**-0.92**	**0.001**	**-0.46**	**0.034**	**-0.33**	**0.014**
SST	**-0.84**	**4.09E-05**	**-1.53**	**0.000**	**-0.46**	**0.099**	**-0.31**	**0.016**
CORT	**-0.58**	**1.72E-04**	**-0.73**	**0.006**	-0.08	0.320	**-0.92**	**0.001**
SNAP25	**-0.44**	**4.15E-03**	**-0.24**	**0.091**	-0.04	0.459	**-0.35**	**0.034**
Other GABA markers								
CALB2	**-0.24**	**0.031**[#]	-0.21	0.208	-0.17	0.415	-0.06	0.436
GAD1	**-0.22**	**0.004**	**-0.28**	**0.076**	**-0.45**	**0.052**	0.06	0.353
GABRA1	**-0.34**	**0.019**	0.14	0.252	-0.10	0.406	-0.18	0.182
PVALB	-0.09	0.541	0.04	0.391	0.16	0.364	-0.12	0.342
SLC6A1 (GAT-1)	0.06	0.471	-0.08	0.362	**-0.36**	**0.038**	0.08	0.443

Abbreviations: Alr, average log ratio; GABA, γ-aminobutyric acid; MDD, major depressive disorder; RIM, random-intercept statistical model.
Alr (MDD/control, $BDNF^{HZ}$/WT and $BDNF^{IV-KO}$/WT). All unadjusted *P*-values in array analyses were obtained after RIM analyses, except (#) was obtained by Wilcoxon test.
Bold entries refers to Alr values associated with a *P*-value $P < 0.1$.

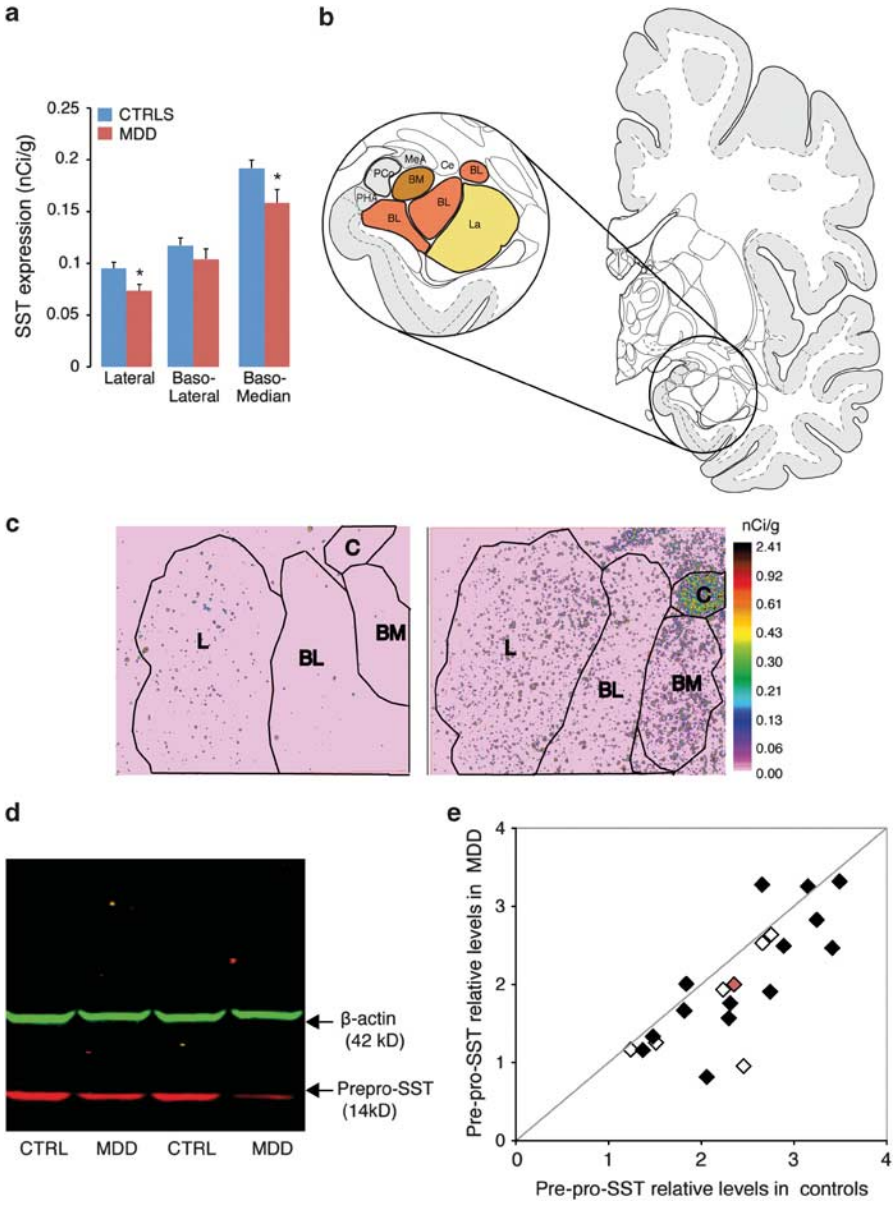

Figure 2 Reduced somatostatin (SST) mRNA and protein levels in amygdala of female major depressive disorder (MDD) subjects. (**a**) SST mRNA expression assessed by autoradiographic optical density measures in lateral/basolateral/basomedian nuclei sub-regions (*$P<0.05$). (**b**) Lateral/basolateral/basomedian nuclei details.[78] (**c**) *In situ* hybridization film autoradiograms of a MDD subject (left) displaying robust SST mRNA downregulation compared with a matched control (right). (**d**) Immunoblot of prepro-SST in CTRL and MDD subjects. (**e**) Relative prepro-SST levels in MDD in function of respective paired CTRL (◆Antidepressant-treated MDD; ◇Antidepressant-free MDD; ◆Average of population).

the basolateral nucleus and not significantly different between control and MDD subjects ($P=0.146$). Low *SST* was also observed at the precursor protein level by quantitative western blot analysis (Figures 2d and e).[48] Finally, the correlation between mRNA levels of BDNF with SST levels ($r=0.56$, $P<0.0001$) was also observed at the protein level ($r=0.59$, $P<0.0001$).

Discussion

We report robust gene transcript changes in the post-mortem amygdala of female subjects with MDD. The pattern of altered transcripts in the most significantly dysregulated genes was reminiscent of changes observed in mice with low BDNF. Accordingly, we detected in human MDD subjects a significant downregulation of BDNF at the RNA and protein levels, confirmed the presence of a broader profile of low BDNF-related changes, and independently verified results by qPCR for several genes, including *SST* and *NPY*, two markers for a specific subset of GABA interneurons. We show that the core profile of BDNF-dependent gene dysregulation is recreated by the union of complementary patterns of gene transcript changes downstream from either

low constitutive or activity-dependent BDNF function in mice. This suggests a model where parsimonious BDNF deficits may result in distinct molecular profiles, cellular deficits including increased vulnerability of SST-bearing GABA neurons, leading to potentially combinatorial pathological phenotypes. The robust findings observed here in human and rodent cohorts, in concert with supporting SST-related female-biased findings in subgenual cingulate cortex and dorsolateral prefrontal cortex,[26,27] suggest that female-specific factors (such as organizational or activational hormonal effects) contribute an added biological vulnerability to MDD. In summary, these results provide both direct (low BDNF) and indirect (low-BDNF-dependent gene profile) evidence in support of the low neurotrophin hypothesis of MDD, while also linking it to the low GABA hypothesis of depression.

Low BDNF as an intermediate molecular phenotype in complex brain illnesses

Multiple known etiological factors affect BDNF, including, genetic, naturalistic (aging,[49] exercise[50] and stress[51]) and biochemical (serotonin, insulin-like growth factor-1)[52] factors. Hence, low BDNF activity is not specific to MDD, and also observed in schizophrenia,[53] dementia and neurodegenerative disorders.[54] So, low BDNF and associated downstream gene changes can be considered a true intermediate molecular phenotype, which is associated with specific upstream etiological factors and putative downstream molecular/cellular effects (Figure 3). Here, our studies in MDD suggest an origin/cause that includes a combination of activity-dependent and constitutive deficits in BDNF, while the downstream proximal effects include molecular (altered GABA markers) and potentially pathophysiological effects (altered GABA-related microcircuitry function).

This model can account for how the low function of a common and widely distributed neurotrophic factor such as BDNF can be implicated in multiple disorders, and yet may still lead to disease-specific outcomes. It also provides a perspective on why low BDNF may not be causative of a particular psychiatric diagnosis, but instead contributes one set of changes to complex molecular pathologies of those illnesses, where 'complexity' reflects an array of parallel and interacting biological pathologies (defined as pathogenic modules in Figure 3), each with their own etiological factors and mediating pathways. In MDD, such patterns may result from individual genetic liability, prior biological insults (that is, infection, inflammation), altered developmental trajectories, or biochemical exposure (that is, interferon or glucocorticoids) for instance.

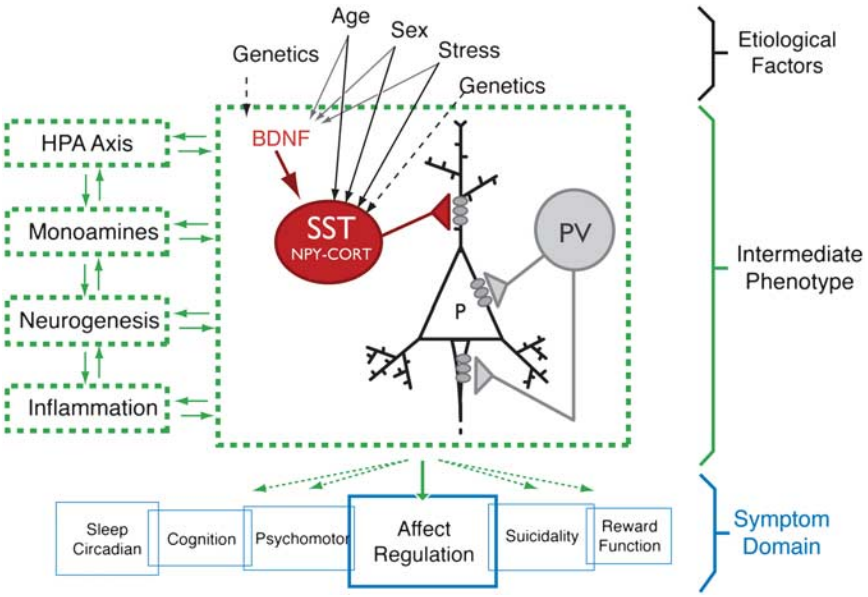

Figure 3 A proposed model of a BDNF–somatostatin (SST)–γ-aminobutyric acid (GABA) molecular intermediate phenotype within the complex multi-module pathophysiology of major depressive disorder (MDD). Our results suggest that the frequent observation of low SST in MDD is linked to altered BDNF function. The two components of this 'molecular intermediate phenotype' (BDNF and SST) are known to be controlled by sets of unique and/or shared upstream etiological factors. Their downstream impact on integrated GABA function will be determined by the extent of SST-expressing cell dysregulation and by the degree of disease effects on other components of the GABA microcircuitry (for example, parvalbumin (PV), not affected here). So, altered GABA circuitry may be considered a one-scale-higher 'cellular intermediate phenotype'. The outcome of dysregulated BDNF–SST–GABA function in the amygdala and in an extended corticolimbic neural network is hypothesized to alter mood regulation and contribute to the 'affect dysregulation' symptom domain of the illness. In this model, sets of additional molecular/cellular intermediate phenotype (or modules) are under the control of their respective etiological factors, display multiple levels of interactions, but potentially impinge on different neural networks, which in turn mediate distinct symptom domains.

Low SST as an obligate and/or critical downstream phenotype of mental illnesses?

Similar remarks could apply to SST downregulation, which has been reported in schizophrenia,[36] bipolar disorder,[26] Alzheimer's,[55] during normal aging[49] and across brain regions in MDD.[26,27] Here, the rodent findings convincingly suggest that low SST represents a common phenotype downstream of altered BDNF function (Table 3), and could thus be considered an obligate molecular/cellular consequence across BDNF-linked illnesses. In that sense, low SST could be viewed as a consequence of disease-related events, but its contribution (that is, cause), in the amygdala or other regions, to clinical phenotypes is not known. SST is expressed in a subgroup of ~20% of GABA interneurons that provide delayed and sustained inhibition onto principal pyramidal neurons through dense projections onto dendritic arbors, and to some extent may provide unspecific normalizing inhibition (at least in mouse cortex).[56] Hence, low SST is unlikely to remain biologically silent at the GABA microcircuitry level. However, this will depend on (1) whether all SST neurons are affected or whether a portion of SST cells are missing, (2) whether GABA function itself is affected in SST neurons or (3) if deficits are specific to altered neuromodulatory peptides within those cell population, as SST, NPY and CORT (all reported low here) share similarities in cellular origins and may be implicated in the disease process on their own.

Moreover, the impact of low SST is expected to vary based on the broader GABA microcircuitry pathological context. For instance, changes in parvalbumin-expressing GABA neurons, which directly target the cell soma and axon (Figure 3) (or other subtypes), are poised to interact with SST neuron-mediated inhibition, and may accordingly translate into distinct pyramidal cell regulation, pathophysiological network activity and potentially symptom-related behavioral outcomes such as schizophrenia and bipolar disorder.[26,57] Upstream factors affecting SST (for example, aging, stress and glutamate) also affect additional biological modules (Figure 3). Together, it is expected that the combinatorial recruitment of different modules through various (and potentially interacting) etiological pathways will together result in complex disease-related patterns. We propose that BDNF, BDNF-dependent SST and other related gene changes, represent one of those complex biological modules, with its own risk factors and modulators.

Finally, brain region-specificities in terms of cellular composition and intrinsic vulnerabilities may further determine pathophysiological outcomes. We speculate that the changes observed here in the amygdala, an area specialized in detecting and assessing emotional salience of incoming stimuli, will contribute to altered stimulus integration, and in turn to increased and sustained reactivity of the amygdala in MDD patients,[58] although this hypothesis will need to be tested in genetic rodent systems.

Expected partial cues from BDNF- or SST-related genetic mouse models

Post-mortem analyses do not allow discriminating if observed alterations are causative and/or consequential to mental illness, but dissecting disease pathways is possible in mouse models. However, the prediction of the model described above (Figure 3) is that current genetic mutations in mice do not recapitulate the full context and complexity of molecular changes observed in illness and so that they will only partially test causal link with altered behavior. For instance, decreased BDNF has been observed in rodents after chronic stress,[59] but not consistently,[60] and low BDNF by itself is not sufficient to generate an anxious/depressive-like phenotype,[61,62] and in some regions can exert antidepressant-like effects.[63] On the other hand, disruption of BDNF (global or forebrain-specific)[64,65] leads to higher emotionality when combined with exposure to chronic stress in female mice,[62,66] while male $BDNF^{HET}$ were as vulnerable as WT to chronic stress.[68] Dipping further in BDNF specificity, mice lacking the activity-dependent BDNF promoter-IV appear more prone to develop increased emotionality.[67] Similarly, SST gene disruption in mice does not induce behavioral alterations other than motor impairment,[68] suggesting that low SST by itself is not sufficient to induce anxious/depressive-like states. However, the behavioral studies in SST^{KO} mutant mice may not have been optimized to detect subtle baseline/trait changes, were performed in compromised mice with complete loss of SST function, and did not assess increased vulnerability to develop higher emotionality, such as after inducing stress protocols for instance.

Comments and limitations

SST and co-expressed genes

SST and co-expressed genes (*NPY*, *CORT* and *TAC1*) were used as surrogates for GABA function, but it remains to be determined whether markers for GABA function within SST neurons or only neuropeptides are affected. Indeed, SST itself displays antidepressant activity that is GABA-independent.[69]

Sex and species differences

A direct comparison of female and male cohorts will be needed to fully assess the higher vulnerability of females in developing BDNF-related profiles. The goal of this study was to use the increased disease prevalence in female subjects as an enrichment strategy for a potential more homogeneous underlying molecular pathology. Our prior study in the amygdala of male subjects[33] suggests that the phenotype may not be as robust in male subjects, although we cannot at this point determine whether this was due to cohort heterogeneity or to smaller sample size. Nevertheless, neuroimaging reports on the impact of altered BDNF function on amygdala response to stressful stimulation, and pathways to depression and anxiety[70,71] suggest that the uncovered changes in BDNF pathway

may be equally relevant to male and female subjects. Increased molecular profiles in female subjects are supported by our prior studies of SST in cingulate and dorsolateral prefrontal cortex,[26,27] and BDNF-related profiles were not observed in the amygdala of male depressed subjects.[34] Biological differences in disease processes or clinical specificities of that cohort (familial MDD) may underlie these results. Finally, it should be noted that numerous other genes were observed differentially expressed in this study (Supplementary Table S2), including metabolic-, energetic-, vascular- and glial-related changes, which will need to be further investigated. We have focused on the impact of low BDNF on GABA markers, but our microarray study also confirmed genes previously reported affected in the context of stress regulation and/or mood disorders, such as *NPY*, *TAC1* and *RGS4*. Indeed, individuals with low NPY display an increase in neural responsivity to negative stimuli and appear overrepresented in subjects with MDD.[72,73] *TAC1* has been associated with bipolar mood disorders,[74] and the tachykininergic system may be a target for new therapeutic opportunities. Decreased *RGS4* dorsolateral prefrontal cortex levels have been reported in schizophrenia,[75] and *RGS4* polymorphisms appear associated with depression factors in this illness.[76] Finally, while expression of *SNAP25* seems higher in prefrontal cortex in schizophrenia,[77] alterations in its levels have been reported in ventral hippocampus of depressed subjects,[47] suggesting a disease-differential BDNF-activity regulation of this SNARE complex member.

Conflict of interest

David A Lewis currently receives investigator-initiated research support from the BMS Foundation, Bristol-Myers Squibb, Curridium and Pfizer and in 2008–2010 served as a consultant in the areas of target identification and validation and new compound development to AstraZeneca, BioLine RX, Bristol-Myers Squibb, Merck, Neurogen and SK Life Science.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)