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Altered expressions of apoptotic factors and synaptic markers in postmortem brain from bipolar disorder patients

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Abstract

Bipolar disorder (BD) is a progressive psychiatric disorder characterized by recurrent changes of mood, and is associated with cognitive decline. There is evidence of excitotoxicity, neuroinflammation, upregulated arachidonic acid (AA) cascade signaling and brain atrophy in BD patients. These observations suggest that BD pathology may be associated with apoptosis as well as with disturbed synaptic function. To test this hypothesis, we measured mRNA and protein levels of the pro-apoptotic (Bax, BAD, Caspase-9 and Caspase-3) and anti-apoptotic factors (BDNF and Bcl-2), and of pre- and post-synaptic markers (synaptophysin and drebrin), in postmortem brain from 10 BD patients and 10 age-matched controls. Consistent with the hypothesis, BD brains showed significant increases in protein and mRNA levels of the pro-apoptotic factors and significant decreases of levels of the anti-apoptotic factors and the synaptic markers, synaptophysin and drebrin. These differences may contribute to brain atrophy and progressive cognitive changes in BD.

Keywords

bipolar disorder; apoptosis; caspase; BDNF; synaptophysin and drebrin

INTRODUCTION

Bipolar disorder (BD) is a prevalent, severe, and highly disabling psychiatric disease characterized by recurrent depressive and manic episodes, and is associated with increased morbidity and mortality due to general medical conditions, such as obesity, diabetes and cardiovascular disease (1).

BD has multiple risk alleles consistent with a polygenic inheritance (2), but its pathological mechanisms are not agreed on. Studies showing increased brain levels of pro-inflammatory cytokines and increased glutamatergic function suggest roles for excitotoxicity and neuroinflammation in the disease (3–6).

Studies have also reported altered apoptotic factors and their mediated responses in BD. Changes include DNA damage in peripheral blood of BD patients (7), increased pro-apoptotic

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Conflict of interest

The authors declare no conflict of interest.

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serum activity in BD patients (8) and mitochondrial dysfunction (9). Additionally, *in vivo* imaging and postmortem studies have revealed significant brain atrophy in BD patients, with a decrease in cortical thickness (10), as well as reduced numbers and/or sizes of glia and neurons in discrete brain areas (11). These studies implicate the involvement of cell death in the pathophysiology of BD.

Studies have shown that drugs used to treat BD do not induce DNA damage; rather some enhance DNA repair (12). Lithium and valproate inhibit glutamate-induced DNA fragmentation in cerebral cortical neurons (13). These drugs can suppress caspase-3 activity and stimulate B-cell lymphoma-2 (Bcl-2) expression, which render a cell less susceptible to apoptosis (14–16). Chronic administration of lithium at clinically relevant doses has been shown to enhance neurogenesis in rat hippocampus, increasing both the Bcl-2 level and the percent of new cells that display a neuronal phenotype (17,18).

Upregulated AA signaling has been associated with neuroinflammation, excitotoxicity and apoptosis (19–21). Lithium and carbamazepine when given long-term to rats to produce a therapeutically relevant plasma concentration, downregulate components of the brain arachidonic acid (AA, 20:4n-6) cascade (22), such as Ca²⁺-dependent AA-selective cytosolic phospholipase A₂ (cPLA₂) and cyclooxygenase-2 (COX-2). The postmortem BD brain demonstrates increased expression levels of cPLA₂ and COX-2 in prefrontal cortex (23). Furthermore, a recent study reported increased markers of excitotoxicity and neuroinflammation in the BD frontal cortex (24).

BD has been associated with cognitive defects (25,26), and decreased synaptic markers such as synaptophysin and drebrin, also associated with cognitive defects, have been reported in Alzheimer disease brain (27,28). Taken together, these observations suggest that BD also may be associated with altered pre- and post-synaptic brain markers.

To further clarify the possible involvement of apoptosis and synaptic loss in BD, we measured mRNA and protein levels of apoptotic factors such as Bcl-2, caspase-3/-9, Bcl-2-associated X protein (BAX), Bcl-2-associated death promoter (BAD) and brain derived neurotrophic factor (BDNF), and the protein levels of synaptophysin and drebrin, in the postmortem frontal cortex of BD patients and control subjects, matched for age, postmortem interval (PMI) and pH. We used the frontal cortex because studies have shown structural, metabolic and signaling abnormalities in this particular brain region of bipolar patients (29–34). The current study presents potential alterations in apoptotic factors with synaptic loss in postmortem brain of BD patients. An abstract of part of this work has been published elsewhere (35)

MATERIALS AND METHODS

Postmortem brain samples

This study was approved by the Institutional Review Board of McLean Hospital and the Office of Human Subjects Research (OHSR) of NIH (# 4380). Frozen postmortem human frontal cortex (Brodmann area 9) was provided by the Harvard Brain Tissue Resource Center (McLean Hospital, Belmont, MA) under Public Health Service grant number R24MH068855 (awarded to J.S. Rao.), from 10 BD patients and 10 age-matched controls. Mean age, postmortem interval (PMI) and pH of the frozen brain samples did not differ significantly between the BD and control groups: age (years, control: 43 ± 3.5 vs BD: 49 ± 7.2), PMI (hours, control: 27 ± 1.5 vs BD: 21 ± 3.0) and brain pH (control: 6.6 ± 0.16 vs BD: 6.7 ± 0.09). The pH of the frozen brain samples was measured by the method of Harrison et al. (36). The BD patients had been exposed to various psychotropic medications, as reported in an earlier publication (Table 1) (37).

Preparation of cytosolic and membrane extracts

Cytosolic and membrane extracts were prepared from postmortem frontal cortex of BD and control subjects as previously described (38). Briefly, frontal cortex tissue was homogenized in a buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EGTA, 5 mM EDTA, 1.5 mM pepstatin, 2 mM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 U/ml aprotinin and 2 mM dithiothreitol. The homogenate was centrifuged at 100,000g for 60 min at 4°C. The resulting supernatant was the cytosolic fraction, and the pellet was resuspended in the homogenizing buffer containing 0.2% (w/v) Triton X-100. The suspension was kept at 4°C for 60 min with occasional stirring and then centrifuged at 100,000g for 60 min at 4°C. The resulting supernatant was the membrane fraction. Protein concentrations of the membrane and cytosolic fractions were determined with Bio-Rad protein Reagent (Bio-Rad, Hercules, CA).

Western blot analysis

Protein (50 µg) from the cytosolic and membrane extracts was separated on 4–20% SDS-polyacrylamide gels (PAGE) (Bio-Rad). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane. Protein blots were incubated overnight in Tris-Buffered-Saline buffer, containing 5% nonfat dried milk and 0.1% Tween-20, with specific primary antibodies for BAD, Bcl-2, Bax, Caspase-3 (1:1000 dilution), Caspase-9 (1:500) (Cell Signaling, Beverly, MA), Drebrin and Synaptophysin (1:10000) (Abcam, Cambridge, MA). Protein blots were incubated with appropriate HRP-conjugated secondary antibodies (Bio-Rad) and visualized using a chemiluminescence reaction (Amersham, Piscataway, NJ) on X-ray film (Kodak, Rochester, NY). Optical densities of immunoblot bands were measured using Alpha Innotech Software (Alpha Innotech, San Leandro, CA) and were normalized to β-actin (Sigma, St. Louis, MO) to correct for unequal loading. All experiments were carried out twice with 10 controls and 10 BD postmortem brain samples. Values were expressed as percent of control.

Total RNA isolation and real time RT-PCR

Total RNA was isolated from the frontal cortex using an RNeasy mini kit for brain and lipid tissue (Qiagen, Valencia, CA). Complementary DNA (cDNA) was prepared from total RNA using a high capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). RNA integrity number (RIN) was measured using Bioanalyzer (Agilent 2100 Bioanalyzer, Santa Clara, CA). RIN values were 6.9 ± 0.4 and 7.1 ± 0.5 (Mean \pm SEM) for control and BD samples, respectively. mRNA levels (Bcl-2, Bax, BAD, Caspase-3, Caspase-9, BDNF, Drebrin and Synaptophysin) were measured by quantitative RT-PCR, using an ABI PRISM 7000 sequence detection system (Applied Biosystems). Specific primers and probes for Bcl-2, Bax, BAD, Caspase-3, Caspase-9, BDNF, Drebrin and Synaptophysin were purchased from TaqMan^R gene expression assays (Applied Biosystems), and consisted of a 20X mix of unlabeled PCR primers and Taqman minor groove binder probe (FAM (6-carboxy-fluorescein) dye-labeled). The fold-change in gene expression was determined by the $\Delta\Delta C_T$ method (39). Data are expressed as the relative level of the target gene in the postmortem BD patients normalized to the endogenous control (β-globulin) and relative to the control (calibrator), as previously described (40). All experiments were carried out twice in triplicates, and the data were expressed relative to controls.

BDNF protein levels

BDNF protein levels were measured in brain cytosolic extracts using an ELISA kit according to the manufacturer's instructions (Chemicon International, Temecula, CA, USA). Values are expressed in pmol/mg protein.

Immunohistochemical analysis

Frozen tissue was sectioned as described earlier (41). Caspase-3 immunohistochemistry with paraffin-embedded postmortem brain sections was performed using SignalStain Cleaved Caspase-3 (Asp175) IHC detection Kit according to the manufacturer's instructions (Cell Signaling). Counterstaining was performed with methyl green (Vector Laboratories, Inc. Burlingame, CA).

Statistical analysis

Data are expressed as mean \pm SEM. Statistical significance of means was calculated using a two-tailed unpaired *t*-test. Pearson correlations were made between age, post-mortem interval and pH of the frontal cortex, and mRNA levels of tested genes in post-mortem brain from controls and BD patients combined. When three groups were compared (e.g. controls, all BD subjects and the subgroups of BD treated with lithium or controls, all BD subjects and the subgroup of BD that died by suicides), statistical significance was determined using a Bonferroni's multiple comparison test. Statistical significance was set at $p < 0.05$.

RESULTS

Increased protein and mRNA levels of BAD in frontal cortex from BD patients

Mean protein level of pro-apoptotic factor BAD was increased significantly, by 55% ($p < 0.05$), in BD compared with control frontal cortex (Figure 1a). Further, the mean mRNA level of BAD was significantly increased by 2.16 fold ($p < 0.01$) in BD compared with control brain (Figure 1b).

Decreased protein and mRNA levels of Bcl-2 in frontal cortex from BD patients

Compared with control brain, there were significant decreases in mean protein and mRNA levels of anti-apoptotic factor Bcl-2 (Figure 2a and b) by 32% ($p < 0.01$) and 0.57 fold ($p < 0.01$), respectively, in the BD brain.

Increased protein and mRNA levels of Bax and increased ratio of Bax/Bcl-2 in frontal cortex from BD patients

As illustrated in Figure 2, compared with control, protein and mRNA levels of pro-apoptotic factor Bax were elevated by 57% ($p < 0.05$) and 2.9 fold ($p < 0.01$), respectively, in BD frontal cortex (Figure 2c and d). The ratio of Bax to Bcl-2 was significantly increased in BD frontal cortex compared with control (Figure 2e) ($p < 0.01$).

Increased protein and mRNA levels of Caspase-9 and -3 in frontal cortex from BD patients

Mean protein levels of initiator Caspase-9 and effector Caspase-3 were significantly elevated by 66% and 91%, respectively, in BD brains relative to controls (Figures 3a and 3c; $p < 0.05$). Mean mRNA levels of Caspase-9 and -3 were also significantly elevated by 4.8 and 5.8 fold, respectively, in BD brains compared with controls (Figures 3b and 3d; $p < 0.001$). Increased active Caspase-3 was observed in BD compared with control brain by immunohistochemistry (Figure 3e).

Decreased protein and mRNA levels of BDNF in frontal cortex from BD patients

Compared with control brain, there were significant decreases in the mean protein ($p < 0.001$) and mRNA ($p < 0.01$) levels of BDNF (Figure 4a and 4b) in the BD brain.

Decreased protein levels of synaptophysin and drebrin in frontal cortex from BD patients

Mean protein levels of Synaptophysin (Figure 4a) (38%, $p < 0.05$) and Drebrin (Figure 4c) (40%, $p < 0.01$) and mean mRNA levels of Synaptophysin and Drebrin (Figure 4b and 4d) were significantly decreased in BD compared with control brain ($p < 0.01$).

Pearson correlations with brain variables

Pearson correlations between variables (age, PMI and pH) and the mRNA levels from across all 20 brain samples (control and BD patients combined) are not statistically significant (Table 2). Using Bonferroni's multiple comparison tests between controls, all BD subjects and the subgroup of BD with lithium or controls, all BD subjects and the subgroup of BD with suicide did not show significant change in tested marker levels (mRNA and protein) between all BD subjects and the subgroup of BD with lithium or all BD subjects and the subgroup of BD died with suicide.

DISCUSSION

The present study demonstrates statistically significant decreases in protein and mRNA levels of anti-apoptotic factors (Bcl-2, BDNF) and of synaptic markers (synaptophysin and drebrin), and significant increases in pro-apoptotic factors (Bax, BAD, active Caspase- 3 and -9) in postmortem prefrontal cortex from BD compared with control subjects.

Recent brain imaging studies have revealed that the volumes of the hippocampus, amygdala, and frontal cortex are decreased in BD patients (42–44), and that numbers and sizes of glia and neurons are reduced in discrete brain areas (45,46). Several studies have also demonstrated mitochondrial dysfunction and increased pro-apoptotic activity in serum of BD patients (8,9). Although studies implicate the association of apoptosis in the pathophysiology of BD, a few studies have investigated apoptosis directly in the postmortem brain of BD patients.

Members of the Bcl-2 family play important roles in the regulation of apoptosis. The representative member of this family is Bcl-2, an inner mitochondrial membrane protein with anti-apoptotic activity (47). The Bcl-2 homologue, Bax, a monomeric cytosolic protein, displays a pro-apoptotic function. Bax can homodimerize and trigger the activation of terminal caspase by altering mitochondrial function, which results in the release of apoptosis-promoting factors into the cytoplasm. The ratio between Bax/Bcl-2 appears to be essential in deciding the life or death of a cell (48). In our current study, we showed an increased ratio of Bax/Bcl-2 and increased Caspase-3 and -9 active protein and mRNA levels. These results suggest that there might be an aberration in the apoptotic pathway of the BD brain.

We recently reported significant increases of mRNA and protein levels of calcium-dependent phospholipase A₂ (cPLA₂), which releases arachidonic acid (AA) from membrane phospholipids, in postmortem brain of BD (23). AA can bind 14-3-3 ζ protein, which has important roles in preventing apoptosis by retaining the pro-apoptotic protein BAD, and by reducing the binding of 14-3-3 ζ to phosphorylated BAD (49). Release of 14-3-3 ζ from BAD allows dephosphorylation of BAD and allows BAD to move from the cytoplasm to the mitochondria, where it can displace Bax, leading to apoptosis (50). In this study, we observed increased BAD protein and mRNA levels in postmortem brain of BD. Increased cPLA₂ expression may induce AA release which can promote early steps in the apoptotic pathway, through the dissociation of 14-3-3 ζ from phosphorylated BAD.

Serretti et al. (51) used linkage and association methods to identify genes that are involved in BD, which included the BDNF gene. BDNF is a primary neurotrophic factor, and plays important roles in cell survival, cell plasticity, and in the growth and differentiation of new neurons and synapses (52). Animal models that demonstrated upregulated AA signaling and

bipolar-like behaviors have been reported to have downregulated brain BDNF expression (53,54). Furthermore, several drugs approved for treating BD show a neuroprotective effect ascribed to increased BDNF expression (55). In our study, we demonstrated decreased BDNF mRNA and protein levels in the frontal cortex of BD. These data indicate that decreased BDNF may be part of the pathophysiology of BD.

Mood stabilizers utilized in BD, when given long-term to rats, downregulate brain expression of cPLA₂ or COX-2, which are key enzymes of AA metabolism (56). Furthermore, cPLA₂ and COX-2 are increased in prefrontal cortex of the postmortem BD brain (23). Thus increased cPLA₂ would release more AA, which may interrupt the anti-apoptotic action of 14-3-3 ζ in the brain. Consistent with these findings, a recent study showed that the inhibition of cPLA₂-mediated AA release reduced apoptosis in astrocytes (57). Additionally, mood stabilizers are reported to suppress caspase-3 activity, stimulate Bcl-2 and BDNF expression and enhance neurogenesis in rat hippocampus (14–16). Our findings suggest that deregulation of apoptosis may be involved in BD.

Altered pro- and anti-apoptotic factors may cause changes in neuronal markers. Reports have demonstrated loss of synaptic integrity, associated with decreased expression of the postsynaptic marker drebrin and presynaptic marker synaptophysin, in the Alzheimer disease brain (58,59). We observed a significant decrease in protein levels of synaptophysin and drebrin in BD brain compared with control. These decreases may be responsible for cognitive deficits that have been reported in BD patients.

The limitation of the present study is non-availability of medical diagnosis, and lack of information on whether the patients were in the manic or depressive phase at the time of death. However, since several BD patients died by suicide, they may have been in the depressed phase of their illness. Also, the BD patients had been exposed to various drugs not experienced by the control subjects, which may have confounded the results. Therefore, our findings may be related to differences in drug exposure, rather than the BD trait. However, no statistical differences were found in all genes studied in the present study when the BD subjects were compared with the subgroup of BD subjects that were treated with lithium (data not shown). Also, no statistical significance was found when the BD subjects were compared to the BD subjects that died by suicide. However, future studies should examine apoptotic and synaptic markers in brains of patients with schizophrenia using roughly comparable drug exposure as a control, or with unipolar major depression, or with Alzheimer disease to test for disease specificity.

In summary, postmortem frontal cortex from BD patients compared with control cortex showed significantly decreased anti-apoptotic factor (Bcl-2 and BDNF) protein and mRNA levels, and reduced protein levels of synaptic markers (synaptophysin and drebrin), but increased protein and mRNA expression of pro-apoptotic factors (Bax, BAD and caspase-9/-3). These alterations may enhance apoptosis in the frontal cortex of BD patients. Apoptosis and synaptic loss may occur in the presence of neuroinflammation and excitotoxicity in the BD brain, and may be triggered or interact with these process (24). These multiple pathological processes may be the basis of disease progression, evidenced by reports of progressive mood disturbance, brain atrophy and cognitive decline. Therapeutic strategies aimed at downregulating apoptotic processes and neuronal degeneration might be effective in slowing the progression of BD. Mood stabilizers may help to do this (56,60).

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Abbreviations

AA	arachidonic acid
BD	bipolar disorder
BDNF	brain derived neurotrophic factor
Bcl-2	B-cell lymphoma-2
Bax	Bcl-2-associated X protein
BAD	Bcl-2-associated death promoter

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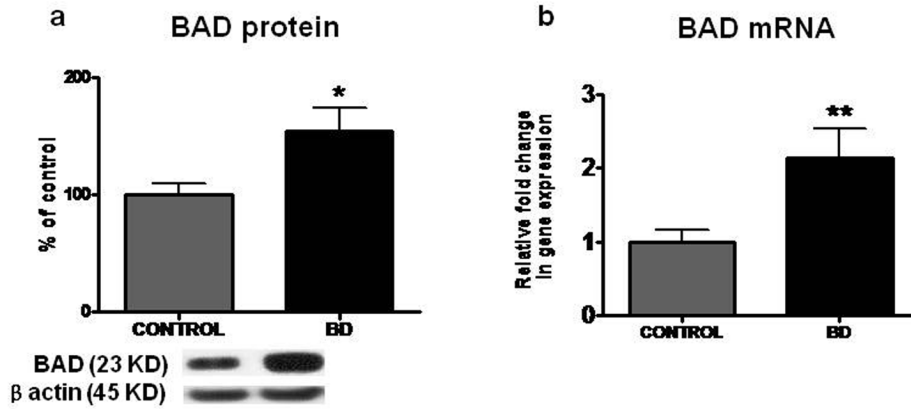


Figure 1.

Mean BAD protein (a) (with representative immunoblot) in control (n = 10) and BD frontal cortex (n = 10). Data are ratios of optical densities of BAD protein to β -actin, expressed as percent of control. mRNA level of bad (b) in postmortem control (n = 10) and BD (n = 10) frontal cortex, measured using real time RT-PCR. Data are mRNA level of bad in the BD patients normalized to the endogenous control (β -globulin) and relative to control level (calibrator), using the $\Delta\Delta C_T$ method. Mean \pm SEM, *p < 0.05, **p < 0.01.

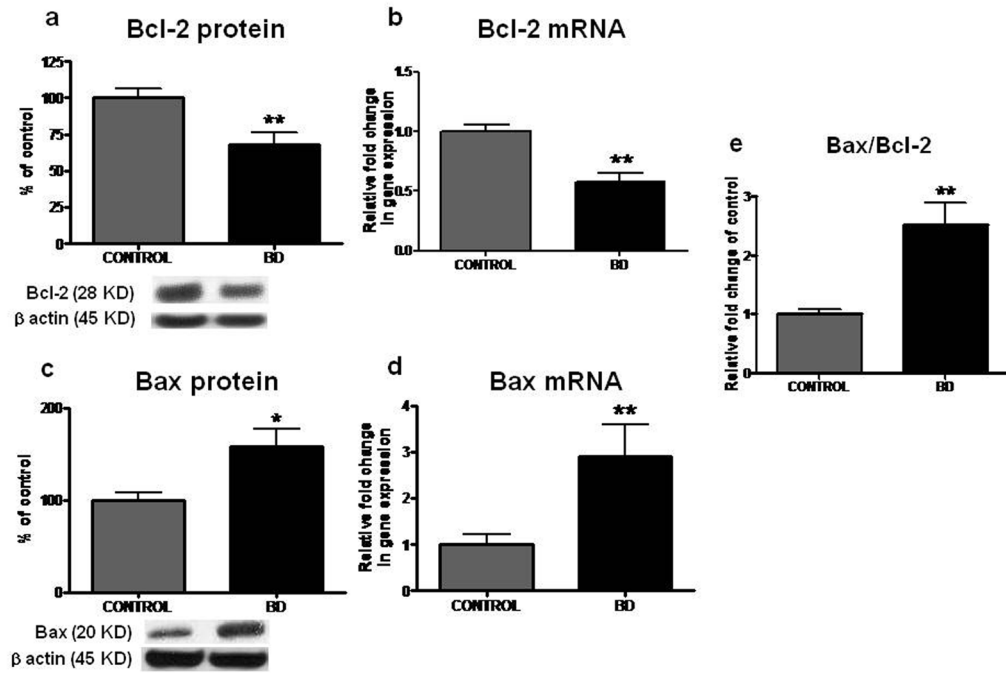


Figure 2. Representative immunoblot of Bcl-2 (a) and Bax protein level (c) in frontal cortex of controls (n = 10) and BD patients (n = 10). Data are ratios of optical density of Bcl-2 and Bax to β -actin, expressed as percent of control. mRNA level of bcl-2 (b) and bax (d) in postmortem control (n = 10) and BD (n = 10) frontal cortex, measured using real time RT-PCR. Bar graph (e) is Bax to Bcl-2 ratio in frontal cortex of controls and BD patients. Mean \pm SEM, * $p < 0.05$, ** $p < 0.01$.

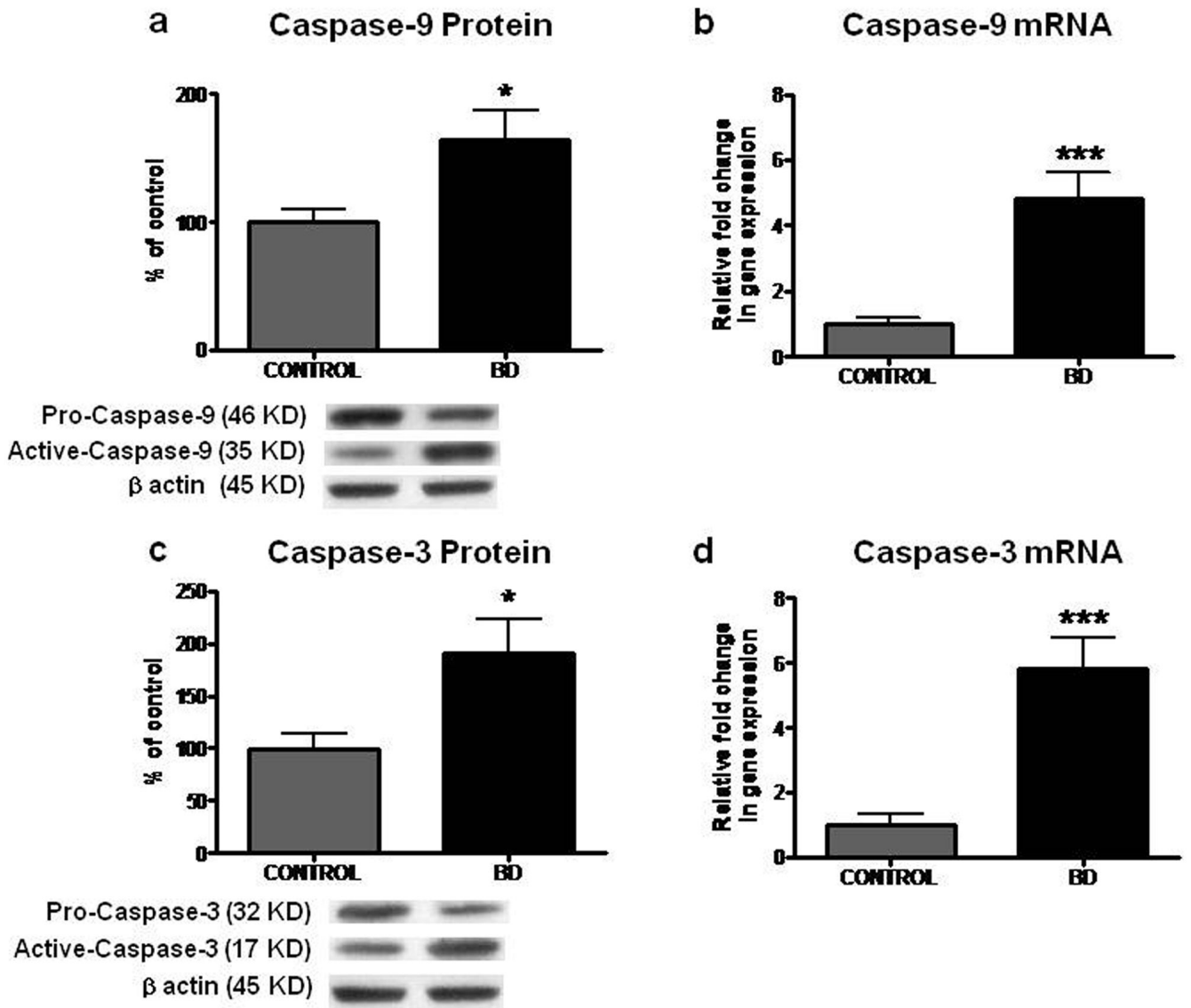


Figure 3.

Representative immunoblot of Caspase-9 (a) and -3 (c) protein levels in frontal cortex of controls (n = 10) and BD patients (n = 10). Data are ratios of optical density of Caspases to β -actin, expressed as percent of control. mRNA levels of Caspase-9 (b) and -3 (d) in postmortem control (n = 10) and BD (n = 10) frontal cortex, measured using real time RT-PCR. Mean \pm SEM, *p < 0.05, ***p < 0.001. (e) Representative histology of control and BD frontal cortex. Active Caspase-3 was detected by kit as described in materials and methods. Scale bar, 100 μ m (lower) and 20 μ m (upper). Red stains indicated by arrows represent the expression of active Caspase-3 in BD frontal cortex. Counterstaining was performed using methyl green.

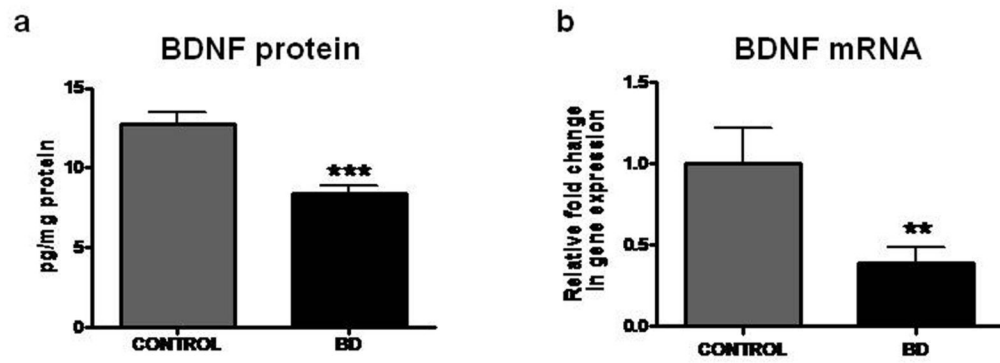


Figure 4. Protein level of BDNF (a) was determined by ELISA. mRNA levels of BDNF (b) in postmortem control (n = 10) and BD (n = 10) frontal cortex, measured using real time RT-PCR. Mean \pm SEM, **p < 0.01, *** < 0.001.

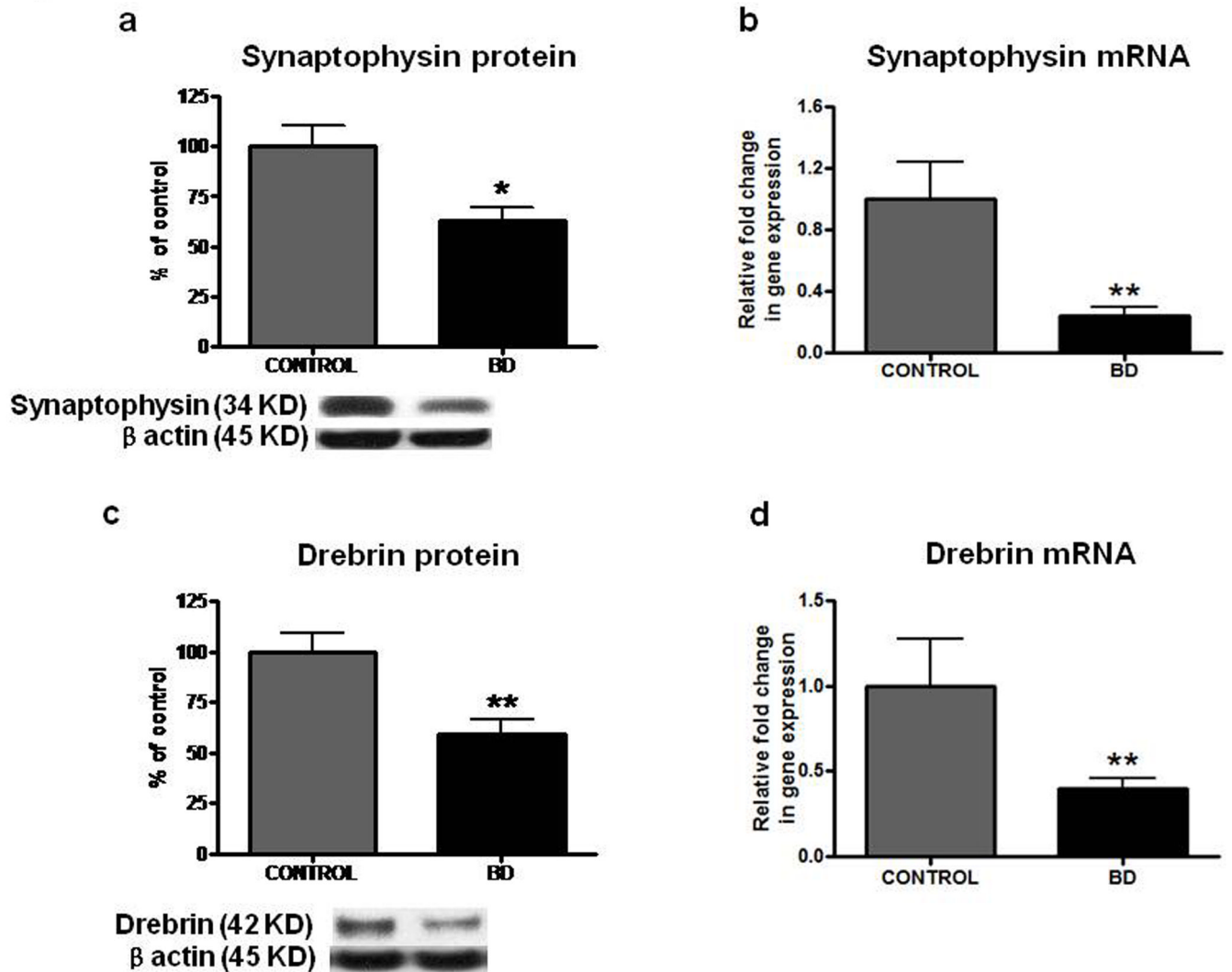


Figure 5.

Representative immunoblot of Synaptophysin (a) and Drebrin (c) protein levels in frontal cortex of controls (n = 10) and BD patients (n = 10). Data are ratios of optical density of synaptophysin and drebrin to β -actin, expressed as percent of control. mRNA levels of Synaptophysin (b) and Drebrin (d) in postmortem control (n = 10) and BD (n = 10) frontal cortex, measured using real time RT-PCR. Mean \pm SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

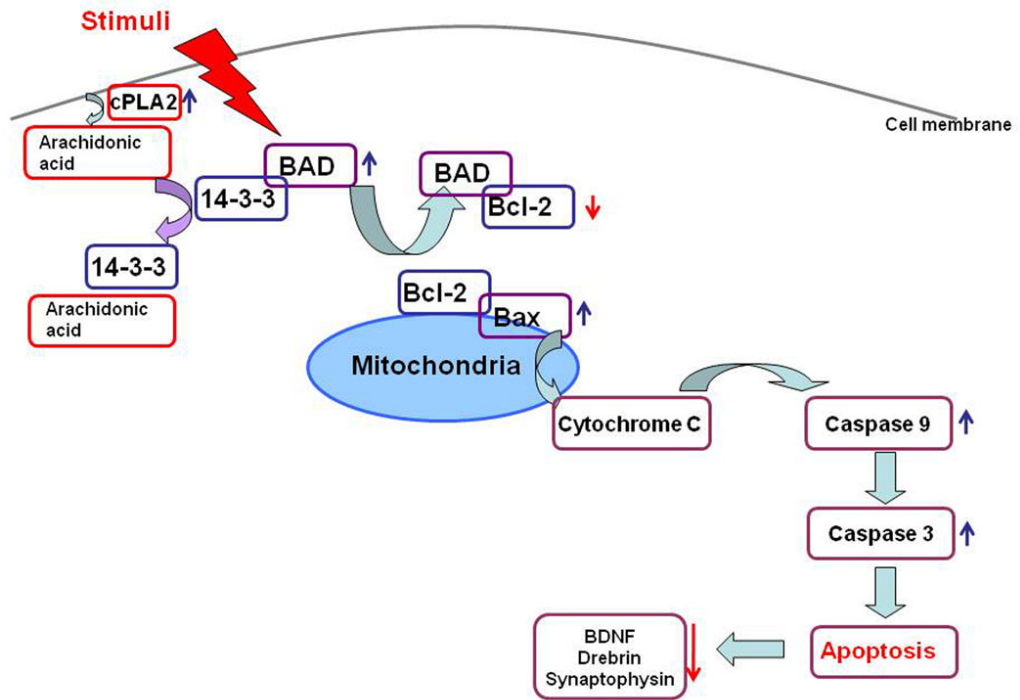


Figure 6. Schematic diagram of apoptotic pathway in brain of bipolar disorder patients. Various stimuli can initiate apoptosis cascade, including diseases. Once triggered, pro-apoptotic factor BAD is dissociated from the complex with 14-3-3 ζ , and replace anti-apoptotic factor Bcl-2, leading to release cytochrome C from mitochondria, which in turn activates Caspases-9 and Caspase-3. Apoptotic change may result in loss of synaptic integrity with decreased expression of synaptic markers, Synaptophysin and Drebrin.

Table 1

Characteristics of control and bipolar disorder subjects

Group	Age, (yr)	Sex	PMI, (h)	Cause of death	Medications
Control	32	F	29	Cardiopulmonary attack	Antibiotics
Control	46	M	30	Cardiopulmonary attack	Insulin
Control	54	M	24	Cardiopulmonary attack	Insulin
Control	36	M	21	Electrocution	Vitamins
Control	41	M	30	Cardiopulmonary attack	None
Control	49	M	27	Cardiopulmonary attack	Vitamins
Control	35	M	20	Cardiac arrest	Not available
Control	35	M	26	unknown	Not available
Control	45	M	24	unknown	Not available
Control	25	M	15	Myocardial Infarction	Not available
BD	29	M	20	Suicide	Paxil
BD	74	M	7	Pneumonia	Neurontin
BD	51	F	35	Ischemic heart disease	Ambien
BD	47	F	16	Major system failure	Lithium carbonate
BD	40	M	30	Suicide	Risperidone
BD	75	M	20	Myocardial infarction	Prozac, Avandia
BD	90	F	19	Ventricular tachycardia	Lithium carbonate,
BD	27	M	20	Suicide	Lithium carbonate
BD	25	F	11	Suicide	Not available
BD	35	M	42	Suicide	Lithium

PMI, postmortem interval; F, Female; M, Male

Table 2

Probabilities and Pearson correlation r squared between brain mRNA levels and subject age, postmortem interval and brain pH.

N=20		BDNF	BCL-2	Caspase-3	Caspase-9	BAD	BAX	Drebrin	Synaptophysin
Age, (yr)	P	0.08	0.07	0.89	0.26	0.58	0.12	0.56	0.911
	r ²	0.15	0.16	0.00	0.06	0.01	0.12	0.01	0.00
PMI, (hr)	P	0.22	0.12	0.83	0.81	0.45	0.12	0.67	0.74
	r ²	0.01	0.00	0.09	0.00	0.03	0.12	0.05	0.00
pH	P	0.70	0.83	0.27	0.27	0.48	0.79	0.55	0.55
	r ²	0.08	0.12	0.00	0.03	0.12	0.00	0.00	0.00

PMI, postmortem interval