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Reduced brain-derived neurotrophic factor in prefrontal cortex of patients with schizophrenia

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Anatomical and molecular abnormalities of excitatory neurons in the dorsolateral prefrontal cortex (DLPFC) are found in schizophrenia. We hypothesized that brain-derived neurotrophic factor (BDNF), a protein capable of increasing pyramidal neuron spine density and augmenting synaptic efficacy of glutamate, may be abnormally expressed in the DLPFC of patients with schizophrenia. Using an RNase protection assay and Western blotting, we detected a significant reduction in BDNF mRNA (mean = 23%) and protein (mean = 40%) in the DLPFC of patients with schizophrenia compared to normal individuals. At the cellular level, BDNF mRNA was expressed at varying intensities in pyramidal neurons throughout layers II, III, V, and VI of DLPFC. In patients with schizophrenia; neuronal BDNF expression was decreased in layers III, V and VI. Our study demonstrates a reduction in BDNF production and availability in the DLPFC of schizophrenics, and suggests that intrinsic cortical neurons, afferent neurons, and target neurons may receive less trophic support in this disorder.
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Brain-derived neurotrophic factor (BDNF) and neurotophin-3 (NT-3) are members of the nerve growth factor (NGF) family of peptides and are important molecular regulators of neuronal development and plasticity.[1–4] BDNF, a glutamate neuron trophic factor, increases survival of glutamate neurons *in vitro*, whereas NGF itself is without effect.[5–7] Both BDNF and NT-3 stimulate the growth of dendrites and increase the spine density of glutamate pyramidal neurons in neocortex.[8,9] BDNF is synthesized and released in response to afferent activity, and BDNF, in return, can modulate synaptic density and long-term potentiation of glutamate cortical neurons.[5,8–13] *In vivo*, an increase in cortical BDNF mRNA occurs contemporaneously with cortical neuron dendrite growth and synapse formation, and BDNF is critical for the maturation of excitatory synapses.[14–20]

Cortical glutamate neurons may be dysfunctional in schizophrenia.[21] In post-mortem neurochemical and brain imaging studies, glutamate and neurochemical markers of the integrity of glutamate neurons (*N*-acetyl aspartate or NAA) are reduced in the prefrontal cortex of patients with schizophrenia.[22–25] Recent studies suggest that there may be a loss of mRNAs encoding presynaptic proteins, some of which are abundant in glutamate neurons, in the dorsolateral prefrontal cortex (DLPFC) of patients with schizophrenia.[26–28] Additionally, levels of synapse-associated proteins may be reduced in the frontal cortex of patients with schizophrenia,[29–32] and the density of dendritic spines in layer III pyramidal neurons of the DLPFC of patients with schizophrenia appears reduced.[33,34] Increased neuronal density with a decrease in somal size of pyramidal neurons and a putative diminution of cortical neuropil in the DLPFC[35–38] also support the notion of prefrontal subcellular or synaptic pathology in this disorder. Furthermore, cellular and synaptic alterations in the brain of patients with schizophrenia are not limited to the DLPFC but extend to the hippocampus (for review see Harrison and Eastwood,[39] and Heckers and Konradi[40]).

Molecules that are critical in the development and maintenance of cortical neurons and cortical synapses, such as BDNF, may play a role in the neuropathology of schizophrenia.[41] Upregulation of BDNF increases neuronal cell size and synaptic density,[42] two parameters that are putatively reduced in the brain of patients with schizophrenia, as evidenced above. BDNF is synthesized by neurons in the frontal cortex of rodents[14,43–46] and pyramidal neurons in the dorsolateral prefrontal cortex of *Rhesus* monkeys.[47] In human DLPFC, we expected that BDNF would also be synthesized in pyramidal neurons. We hypothesized that the glutamate-related pathology in the brain of schizophrenic subjects would be associated with abnormal expression of BDNF in the DLPFC.

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Materials and methods

Brain collection

In total, 57 post-mortem human brains were used in this study and a detailed account of which brains were used in each assay can be found in Table 1 (cases are marked with an X if they were used in the RNase protection assay (RPA), the Western blotting or protein measurement assay, and/or the *in situ* hybridization study). Post-mortem brains were obtained from the Office of the Chief Medical Examiner in Washington, DC, USA and were collected at the Clinical Brain Disorders Branch (NIMH, St Elizabeths, Washington, DC, USA and NIH, Bethesda, MD, USA) as previously described.[48] Unfixed coronal slabs (1.5 cm thick) through the entire cerebrum of a hemisected human brain were rapidly frozen at the time of autopsy in a prechilled dry-ice isopentane slurry bath (1 : 1) and stored at −80°C. For the RNA and protein isolations from brain homogenates, DLPFC tissue was excised from the middle third (rostal-caudally) of the superior or middle frontal gyrus immediately anterior to the genu of the corpus callosum. For cryostat-sectioning, tissue blocks were also dissected from the middle one-third of the frozen middle frontal gyrus (this usually included the lower bank of the superior frontal gyrus and the upper and lower banks of the inferior frontal gyrus). Sections were taken in the coronal plane at 14 μm, thaw-mounted on gelatin-coated slides, and stored at −80°C. Brodmann's Area 46 (BA 46) of the DLPFC was defined according to the cytoarchitectural criteria of Rajkowska and Goldman Rakic,[49] as follows: (1) the presence of a well-defined granular layer; (2) columnar arrangement of pyramidal neurons in layer III; (3) an increase in size of pyramidal neurons from superficial layer III to deep layer III; (4) a similar size of pyramidal neurons in deep layer III and layer V; and (5) a clear transition from layer VI to the white matter.

Diagnosis and experimental design

For each of the three separate studies, patients with schizophrenia and normal individuals were matched (all $t<0.34$, $P>0.15$) for age, tissue pH (determined for each case as previously described),[28] post-mortem interval (PMI, defined as time between death and brain freezing), and for gender, race, and brain hemisphere (Table 2). Unfortunately, we could not use all cases for all assays because some cases were not suitable for section-based analysis and because of limited tissue availability (see Table 1). Family members were queried by police and/or by telephone interviews to obtain information regarding medical and/or psychiatric history and/or drug use history (including alcohol abuse and illicit drug use) of the deceased. Any positive history of a psychiatric problem, excessive alcohol or drug use led to the exclusion of that case from the normal control group. All cases that the medical examiner believed to be suspect for illicit drugs or alcohol use had toxicological screens of the blood (the majority of cases studied were tested, see Table 1). All brains were screened for signs of macroscopic pathology at the time of autopsy; brain sections were examined microscopically with the use of Bielschowsky's silver stain on multiple cerebral areas to rule out neuritic pathology as seen in Alzheimer's disease (AD) and other conditions. Cases with an unclear psychiatric diagnosis or any pathological features were excluded.

Diagnosis was determined using DSMIV criteria as previously described.[28] In our study, the 24 patients who were diagnosed with schizophrenia could be classified into the following subtypes: 11 with chronic undifferentiated subtype; 10 with chronic disorganized subtype; and three with chronic paranoid subtype. The average age of disease onset was 24 ± 6.8 ($\pm$SD) years of age (Table 4). All patients diagnosed with schizophrenia had documented auditory hallucinations and paranoid delusions. In all, 19 patients showed evidence of thought disorder, and another 19 exhibited negative symptoms. IQ data were available for 10 patients with schizophrenia (full scale IQ $=82.4\pm12$, $\pm$SD). The total dose of neuroleptic medication given to the patients was calculated by adding the various daily medication levels as determined from available medical records and converting these levels to chlorpromazine (CPZ) equivalents as previously formulated.[50] A median value of drug dosage was then derived from the CPZ equivalents to give the estimated average daily dose; this value was multiplied by the duration of illness (estimated from the earliest age of definable symptoms or age at first hospitalization) to give the estimated lifetime CPZ equivalents.

RNA isolation

Frozen tissue (from $N=26$ controls and $N=23$ patients with schizophrenia, see Table 1) was pulverized over dry ice and ~300–500 mg of tissue was weighed while frozen. Total RNA was extracted using TRIZOL® Reagent (Life Technologies Inc., Grand Island, NY, USA) and a modified version of the manufacturer's recommended method. Frozen DLPFC tissue was thawed in 50 ml sterile Falcon tubes with the addition of 10 ml of TRIZOL® Reagent on wet ice and homogenized for 45 s, using a Polytron tissue homogenizer (Model PT10/35, Brinkmann Instruments, Inc., Westbury, NY, USA) set at the moderate speed of 7. The homogenate was incubated on wet ice for 1 min to allow settling of foam, after which the homogenization steps were repeated. Samples were incubated at room temperature (RT) for 40 min to allow dissociation of nucleoprotein complexes. Chloroform (2 ml) was added to each sample and the tubes were shaken vigorously for 15 s. Tubes were centrifuged at $12\,000\times g$ for 15 min at 4C. The upper aqueous phase was transferred to fresh 50 ml Falcon tubes and RNA was precipitated by the addition of 5 ml of isopropyl alcohol and incubated at RT (20 min). RNA was concentrated by centrifugation at $12\,000\times g$ for 10 min at 4C. After removal of the supernatant, the remaining RNA pellet was washed

Table 1 Cohort characteristics for human subjects

Protein	*RPA*	*In situ*	*Case*	*Diagnosis*	*Age (years)*	*Sex*	*Race*	*Side*	*pH*	*PMI (h)*	*COD*	*Manner of death*	*Toxicology*
X			1	CON	53	F	AA	R	5.76	22.0	Burns	Undetermined	NA
X	X		2	CON	34	M	AA	L	6.60	34.5	ASCVD	Natural	Blood EtOH 0.06%, drugs negative
	X		3	CON	68	M	AA	L	6.57	22.5	ASCVD	Natural	NA
X	X		4	CON	47	M	AA	R	6.54	23.5	GSW to chest	Homicide	Blood EtOH 0.10%, drugs negative
X	X		5	CON	58	F	AA	L	6.54	26.5	ASCVD	Natural	Negative
X	X		6	CON	39	F	AA	L	6.34	40.5	ASCVD	Natural	Negative
	X		7	CON	77	F	AA	R	6.00	10.5	Severe thermal burns	Accidental	Negative
	X		8	CON	40	M	C	L	6.44	48.5	ASCVD	Natural	Negative
X	X		9	CON	46	F	AA	L	5.93	19.5	Cardiomyopathy	Natural	Negative
X	X		10	CON	45	M	C	L	6.61	16.0	Crushing injury to chest	Accidental	NA
	X		11	CON	47	M	AA	L	6.03	60.0	Acute bronchial asthma	Natural	Blood morphine, 0.015 mg/dl
X	X		12	CON	46	M	C	L	6.71	27.5	ASCVD	Natural	Negative
X	X		13	CON	77	M	AA	R	6.06	18.5	Occlusive coronary atherosclerosis	Natural	Negative
X	X		14	CON	55	M	AA	R	6.00	9.5	MI (ASCVD)	Natural	Lidocaine 'detected'
X	X		15	CON	48	F	AA	R	6.08	17.0	Pulmonary artery thrombosis	Natural	Phensuximide 'detected'
X	X		16	CON	60	F	C	L	6.40	8.0	ASCVD	Natural	Lidocaine 'detected'
	X		17	CON	61	F	AA	R	6.15	61.0	Multiple blunt force injuries	Accidental	NA
	X		18	CON	26	M	C	L	6.08	13.0	Occlusive coronary atherosclerosis	Natural	NA
	X	X	19	CON	52	F	AA	R	6.87	10.0	Hemopericardium	Natural	Opiates in the occipital lobe
		X	20	CON	35	M	AA	R	5.88	49.5	ASCVD	Natural	Negative
X	X	X	21	CON	41	M	AA	R	6.72	10.0	Stab wounds to chest	Homicide	EtOH
	X	X	22	CON	42	M	AA	R	6.63	40.0	Acute asthma attack	Natural	Negative
		X	23	CON	66	F	C	R	6.37	29.5	Ruptured thoracic anuerysm	Natural	Negative
X	X	X	24	CON	24	M	AA	R	6.59	12.5	Fibrinous pericarditis	Natural	Meperdine in blood
X	X	X	25	CON	38	M	AA	R	6.14	32.5	P E	Accidental	Negative
X		X	26	CON	18	M	AA	R	6.51	14.5	GSW to back	Homicide	Negative
		X	27	CON	83	M	AA	L	6.01	66.5	Pulmonary artery thrombosis	Natural	Negative
	X	X	28	CON	56	M	AA	R	6.09	33.0	P E	Natural	Negative
		X	29	CON	63	M	C	L	6.54	19.0	ASCVD	Natural	Negative
		X	30	CON	52	F	AA	L	6.38	26.0	Acute fibrinous pericarditis	Natural	Lidocaine detected in occipital lobe
X	X	X	31	CON	57	F	AA	R	6.43	19.0	MI (ASCVD)	Natural	Myocardial infarction, ASCVD
X	X	X	32	CON	59	F	AA	R	6.57	37.0	Cirrhosis	Natural	Negative
X	X	X	33	CON	67	F	AA	L	6.69	34.0	Pulmonary edema	Natural	Blood EtOH, 28 mg/dl
X	X		34	CDS	71	F	C	L	6.41	47.5	ASCVD	Natural	Phenothiazine metabolites

													‘detected’
	X		35	CUS	48	M	AA	R	6.42	48.0	ASCVD	Natural	Carbamazepine ‘detected’
X	X		36	CUS	36	M	AA	R	6.56	13.0	Blunt force injuries (fall)	Suicide	Phenothiazine metabolites ‘detected’
	X		37	CUS	46	M	AA	R	6.35	24.5	ASCVD	Natural	Lidocaine & benztropine ‘detected’
X	X		38	CPS	44	F	AA	R	6.51	32.5	Cardiomegaly (hypertension)	Natural	Haloperidol & clonidine ‘detected’
X	X		39	CUS	46	M	AA	R	6.73	25.0	Blunt force injuries (fall)	Suicide	Haloperidol & diphenhydramine ‘detected’
X	X		40	CDS	54	M	AA	R	6.31	26.0	Subarachnnoid hemorrhage	Natural	Etoh 0.32%, benztropine & doxepin ‘detected’
X	X		41	CUS	48	M	C	R	6.29	13.5	Dilutional hyponatremia (hypo-osmolar coma)	Natural	Lidocaine ‘detected’
	X		42	CUS	73	M	C	R	6.00	13.5	ASCVD	Natural	Phenytonin >2.5 μg/ml
	X		43	CUS	34	M	AA	R	6.23	34.5	Acute benztropine intoxication	Undetermined	Benztropine ‘detected’
	X	X	44	CDS	75	M	AA	L	6.29	41.5	Pending	Natural	Negative
	X		45	CUS	64	F	AA	R	6.48	19.5	Asphyxia (aspiration)	Accidental	Negative
X	X	X	46	CDS	67	F	AA	R	6.63	38.5	Bronchial asthma, COPD	Natural	Negative
X	X	X	47	CDS	31	M	C	R	6.46	14.0	Cerebral edema	Natural	Negative
X	X	X	48	CUS	23	M	AA	L	6.48	42.5	Anoxia because of seizure, no Hx seizures	Natural	Negative
X	X	X	49	CPS	60	F	AA	L	6.38	19.0	HCVD	Natural	Amantadine ‘detected’ in brain
	X		50	CUS	30	M	AA	L	6.32	72.5	Pneumonia	Natural	Negative
	X	X	51	CDS	35	M	AA	L	6.70	79.0	Acute pulmonary embolus	Suicide	Negative
		X	52	CPS	80	M	C	L	6.05	13.5	GI bleed	Natural	NA
	X	X	53	CDS	81	F	C	R	6.78	11.0	ASCVD	Natural	Amantadine ‘detected’ in brain, blood negative
X	X	X	54	CUS	61	F	AA	R	6.74	20.0	Asphyxiation’ food bolus	Accidental	Negative
	X	X	55	CDS	38	M	AA	R	6.50	61.0	Ruptured intestine, acute peritonitis	Accidental	Chlorpromazine detected
	X	X	56	CDS	41	F	AA	R	6.08	51.0	ASCVD	Natural	NA
X	X	X	57	CDS	41	M	AA	L	6.63	32.0	ASCVD	Natural	Negative

RPA=RNase protection assay, CON=control, CDS=chronic disorganized schizophrenia, CUS=chronic undifferentiated schizophrenia, CPS=chronic paranoid schizophrenia, F=female, M=male, AA=African American, C=Caucasian, A=Asian, R=right, L=left, ASCVD=arteriosclerotic cardiovascular disease, COPD=chronic obstructive pulmonary disease, MI=myocardial infarction, GSW=gunshot wound, PE=pulmonary embolism, GI=gastrointestinal, NA=not available.

Table 2 Demographic information (±SD) for the three different assays performed

	Number	*Age (years)*	*Gender*	*Race*	*PMI (h)*	*pH*	*Side*	*Freezer time (months)*
RPA cohort								
Normal	26	50 (14)	11F, 15M	5C, 21AA	27.5 (15)	6.4 (0.3)	14R, 12L	78 (45)
Schizophrenic	23	50 (16)	8F, 15M	5C, 18AA	35.0 (18)	6.5 (0.2)	16R, 7L	73 (39)
In situ *cohort*								
Normal	15	50 (17)	6F, 9M	2C, 13AA	30.0 (15)	6.4 (0.3)	11R, 4L	30 (10)
Schizophrenic	12	53 (20)	5F, 7M	3C, 9AA	36.5 (20)	6.5 (0.2)	6R, 6L	42 (14)
Western cohort								
Normal	19	47 (15)	9F, 10M	3C, 16AA	23 (10)	6.4 (0.3)	11R, 8L	132 (49)
Schizophrenic	12	49 (17)	5F, 7M	3C, 9AA	29 (12)	6.5 (0.2)	8R, 4L	133 (42)

with 15 ml of 75% ethanol. This was followed by a final centrifugation at $12\,000 \times g$ for 10 min at 4C. RNA pellets were air dried for 20 min and resuspended in 200 μl 0.1% diethylpyrocarbonate (DEPC)-treated ddH_2O. A spectrophotometer was used to quantitate the micrograms of total RNA in each sample; the quality of RNA was confirmed by agarose gel electrophoresis for every sample, and reisolation was performed as necessary. Total RNA was stored as a stock in DEPC-water or was aliquoted and stored in hybridization buffer (80% deionized formamide, 40 mM PIPES pH 6.7, 400 mM NaCl, 1 mM EDTA) at -80°C in preparation for the RNase protection assay.

Preparation of riboprobes

A 1.6 kb segment of the human BDNF cDNA (bp 10–1610 X34333) was generously provided in Bluescript vector by Regeneron Pharmaceuticals (Tarrytown, NY, USA). We subcloned the Apa1 fragment (511 bp) of the BDNF cDNA into Bluescript vector (Stratagene, La Jolla, CA, USA) to generate a cDNA template (bp 704–1214, Accession # M61176) for riboprobe synthesis for both the RNase protection assay and the *in situ* hybridization.[3] The specificity of this subclone was confirmed by Northern blotting (see below). To assess any differences in the overall RNA quality in the tissue homogenates and/or in the tissue sections, we determined the mRNA levels for cyclophilin using cDNA templates available from Ambion, Austin, TX, USA. Sense and antisense riboprobes for BDNF were generated from linearized plasmids using a T7 or T3 polymerase (according to insert orientation) and an *in vitro* transcription kit as recommended by the manufacturer (Promega, Madison, WI, USA). The ^{32}P-UTP (Perkin-Elmer, Boston MA, USA) antisense probe (for RNase protection assay and Northern blotting) and ^{35}S-UTP (Amersham, Piscataway, NJ, USA) antisense and sense riboprobes (for *in situ* hybridizations) were labeled to a specific activity of $1–2 \times 10^9$ cpm/μg by addition of radiolabeled UTP and were purified by ethanol precipitation.

Northern blotting

Northern blotting was performed similar to that previously described.[51] A multiple tissue blot (Human Brain Cat# 7755–1, Clontech, Palo Alto, CA, USA) containing poly A RNA from several brain regions of adults (cerebellum, cerebral cortex, medulla, spinal cord, occipital lobe, frontal lobe, temporal lobe, and putamen) was used to verify the specificity of our BDNF cDNA subclone. The blot was prehybridized as previously described.[28] Radioactive BDNF probe at 5 ng probe/ml was added to the hybridization buffer and stringent washes were carried out as previously described.[28]

Ribonuclease protection assay

The RNA samples from the control group and patients with schizophrenia were processed randomly and the experimenter was blind to diagnostic group during the assay and data acquisition. The ribonuclease protection assay (RPA) was performed as detailed previously[52] except with a reduced concentration of RNase A (10 μg/ml). Total RNA (20 μg) was hybridized with the antisense probe for BDNF, whereas 2 µg of total RNA was used for the cyclophilin RPA. Standard curves were generated by adding known amounts of *in vitro* transcribed BDNF sense strand ranging from 0.1 to 100 pg and equating the total amount of RNA to the total RNA from the human brains with the addition of 20 μg of yeast total RNA per microfuge tube. Therefore, the hybridization conditions in the standards and samples are comparable, and both follow the same first-order kinetics of linearity that ensures the quantitative nature of this assay. Hybridization of the riboprobe to standards and samples was allowed to proceed in a 45°C water bath overnight. For RNase digestion of nonprotected fragments of RNA, RNase A (10 μg/ml) was used and inclusion of a no-sense strand RNA (0 pg) in the standard curve was used to verify that the digestion of radiolabeled antisense probe was complete under the RNase conditions used. The protected hybrids were purified by phenol : chloroform extractions and ethanol pre-

cipitations and by 70% EtOH washes, and were size-separated on 5% nondenaturing polyacrylamide gels. The gels were dried with a gel dryer, and BioxMax (Kodak, Rochester, NY, USA) autoradiographic film was used to visualize the position of the radioactive fragments, which were excised and counted in a scintillation counter. The amount of picogram BDNF in the samples was determined by regression analysis from known amounts of BDNF in the standard curve.

Protein extraction

Crude protein extracts, obtained from the same pulverized tissue as the total RNA, were extracted from each case ($N=19$ controls, 17 of which overlapped with the samples used in the RPA and $N=12$ patients with schizophrenia, all of which overlapped with the RPA samples). Those cases with a PMI of over 48 h were not used in the Western blot analysis for BDNF. About 100 mg of pulverized frozen tissue was weighed while frozen, thawed on wet ice, and then homogenized in a hand-held glass and Teflon homogenizer with Tris–glycerol extraction buffer in the presence of protease inhibitors (AEBSF 0.024%, aprotinin 0.005%, leupeptin 0.001%, pepstatin A 0.001%, glycerol 50%, Tris 0.6%) at the following ratio: 10 ml of buffer to 1 g of tissue. Aliquots (50 μl) of the samples were placed into individual Eppendorf tubes and stored at –80°C. For protein quantification, protein concentrations were determined using the Bradford assay. Standard curves (0.0, 12.5, 25.0, 50.0, 75.0, 100.0 μg) were prepared with known quantities of bovine serum albumin (Sigma Chemical Co.; St Louis, MO, USA). Standards and samples (5 μl homogenate mixed with 95 μl ddH_2O) were mixed with 5 ml of Bradford's solution (Bio-Rad; Hercules, CA, USA) and incubated for 5 min at RT. Absorbance at 598 nm was measured in triplicate for each sample using an optical density (OD) spectrophotometer (Beckman, Du-65) and the average value was used to determine the amount of protein extracted from each case.

Western blotting

On the first day of the Western blotting procedure, samples from each case were removed from –80°C and thawed on wet ice. For each sample, identical amounts of total protein content (25 μg for BDNF and 8 μg for β-actin) were removed from the thawed homogenates and prepared for gel electrophoresis by adding ddH_2O to normalize the loading volume. For BDNF, 4 × NuPage LDS sample buffer (10%, glycerol, 141 mM Tris base, 106 mM Tris-HCl, 2% LDS, 0.51 mM EDTA, 0.22 mM SERVA BLUE G250, 0.175 mM Phenol Red—(pH 8.5)) (Invitrogen; Carlsbad, CA, USA) was added to the samples, and for β-actin, 2 × Tris–glycine SDS sample buffer (63 mM Tris-HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol BLUE, dH_2O (pH 6.8)) (Invitrogen) was added to the samples. Samples were then heated to 80°C for 5 min to denature the protein.

Each gel contained about 12 samples, with schizophrenic subjects matched to one or two controls assayed within the same gel; they were matched primarily for PMI and then for age and gender when possible. Subjects with a PMI of over 48 h were excluded from the analysis, because at this time point after death β-actin protein levels start to decline significantly (as determined in controlled studies on the effect of PMI on rodent brain proteins, B Lipska, unpublished observations). The experimenter was blind to the subject status during the assays and quantitation of the immunoreactive bands. The linearity of the BDNF assay and β-actin immunoblotting assays was first determined by assaying increasing amounts of total homogenized protein: for BDNF, 5, 10, 25, and 30 μg ($R=0.99$) and for β-actin, 2.8 , 5.6, 11.3, 22.5, and 45 μg ($R=0.93$). Protein was loaded onto precast 10% Bis-Tris polyacrylamide gels with MES running buffer [MES (pH 7.2), 50 mM Tris base, 0.1% SDS, 1 mM EDTA (pH 7.3), (Invitrogen; Carlsbad, CA, USA) (for BDNF)] or 14% Tris-glycine polyacrylamide gels with Tris/glycine/SDS running buffer [25 mM Tris, 192 mM glycine, 0.1% SDS (pH 8.3), (Bio Rad; Hercules, CA, USA), (for β-actin)]. Protein was separated by electrophoresis at 200 V for 45 min (BDNF) or 125 V for 1.5 h (β-actin) using a single power source, and was transferred onto nitrocellulose membranes for 45 min at 80 V in NuPage transfer buffer [25 mM bicine, 25 mM Bis-Tris (free base), 1.0 mM EDTA, 0.05 mM chlorobutanol (pH 7.2) (Invitrogen) (for BDNF)] or overnight at 4°C at 25 V in Tris/glycine buffer (for β-actin) in a tank transfer system (Amersham, Piscataway, NJ, USA).

Membranes were blocked for 5 h with 6% goat serum (Vector Laboratory, Burlingame, CA, USA) and 5% BSA in Tris-buffered saline (TBS) (pH 7.4) with 0.1% Tween-20 (TBS-T), and then incubated with the primary BDNF rabbit polyclonal antibody (1 : 300 in 6% normal goat serum, 5% BSA, and TBS-T) or with primary β-actin mouse monoclonal antibody (1 : 3000) (Chemicon; Temecula, CA, USA). The BDNF primary antibody[53] was a generous gift from Dr David Kaplan from the Montreal Neurological Institute, Canada. At room temperature, blots were rinsed in TBS-T, incubated in a peroxidase-conjugated secondary antibody of the appropriate species (1 : 10 000; Chemicon; Temecula, CA, USA) in 6% normal goat serum in TBS-T for 2 h, rinsed in TBS-T, developed in ECL-plus (Amersham, Piscataway, NJ, USA), and exposed to Kodak Bio-Max film for several exposure times (range 30 s–20 min). Films from the 5 min (BDNF) and 10 min (β-actin) exposures were digitized using a scanner with standardized parameters for each assay (Hewlett Packard, ScanJet 6300C, Palo Alto. CA, USA). The images were analyzed in NIH image with gel-plotting macros to obtain relative OD values based on band intensity and band width. To control for gel to gel variability in gel running and transfer inherent to the Western blotting method, the OD measurement for BDNF for each case on a gel was divided by the

average OD measurement for normal controls on the same gel and multiplied by 100.

In situ hybridization

The fresh frozen prefrontal cortex sections (two sections per case per probe) were removed from the −80°C freezer and thawed at room temperature for 20 min before fixation in 4% formalin in 0.1 M phosphate-buffered saline for 5 min. The normal cases used included 15 controls, nine of which overlapped with those cases used in the RPA, seven of which were also used in the Western blotting (see Table 1). The schizophrenia cases included 12 patients, 11 of which overlapped with the cases used in the RPA and six of which overlapped with those used in the Western blotting (see Table 1). After fixation, the slides were acetylated and dehydrated as described.[54] For BDNF and cyclophilin *in situ* hybridizations, human sections were hybridized with 200 μl of hybridization cocktail containing 5 ng/ml of radiolabeled probe; hybridization was allowed to occur at 55°C overnight in humidified chambers. RNase digestions and stringent posthybridization washes were performed as detailed by Whitfield *et al*.[54] After air-drying, the slides along with ^{14}C standards (American Radiolabeled Chemicals, Inc., St Louis, MO, USA) were apposed to film (BioMax film, Kodak, Rochester, NY, USA) for 14 days for the BDNF and 2 days for cyclophilin. Slides were dipped in NTB2 emulsion (Kodak), dried and developed in D-19 developer (Kodak, Rochester, NY, USA) after 3.5 months (for BDNF mRNA detection).

Image analysis

Autoradiographic images from the films were digitized with a Hewlett-Packard Scanjet Plus flatbed scanner at 300 dpi resolution in 256 grayscale mode into a Macintosh computer and then calibrated using the NIH Image program (W Rasband, NIH). With the aid of a microscope, the boundary of Brodmann's area 46 (BA 46) was delineated on Nissl-stained sections and defined cytoarchitecturally by applying the criteria described by Rajkowska and Goldman-Rakic .[49] Sampling was carried out in BA 46 where the lamina were running parallel to the pial surface. Quantitation of OD from the film was performed blind to diagnosis as previously described.[28]

Silver grain analysis

All slides were assigned a blind number by an individual not analyzing the slides' by taping a new code over the slide label. On a Zeiss Axioplan microscope equipped with a digital camera and personal computer, slides were viewed under bright field at $\times$ 40 magnification and all pyramidal neurons within a field were overlaid with a circle measuring 35 μm in diameter. The illumination was switched to dark field, and bright pixels within the delineated circles were converted to the number of silver grains/cell and counted with the aid of a macroprogram written in Bioquant Image analysis software. In the green measurement window, thresholds for silver grain detection were initially determined from averaging the optimal upper and lower limit for detecting 'bright pixels' in the microscopic field from several slides; this threshold was then held constant for all slides in the analysis. Background levels of silver grains were determined by placing three 35 μm circles over areas of gray matter not containing cell bodies for each field sampled. Care was taken to sample all pyramidal (and spindle-shaped for layer VI) neurons in a field regardless of BDNF mRNA expression levels. For each case, 2–4 fields per layer were analyzed, resulting in 40–75 neurons analyzed per layer per case. Over 1300 neurons in layers II, III, V, and VI from both diagnostic groups were surveyed yielding a total of 5550 neurons analyzed for BDNF mRNA levels. The experimental background measurement was derived for each layer by averaging background measurements from all cases for each layer. A neuron was considered a nonexpresser if its silver grain levels were below two-fold above the average background, a low expresser if it expressed two to three-fold above the average background, a medium expresser if it expressed four to six-fold above the average background, and a high expresser if it expressed six-fold or more above the average background. The number of neurons falling into each category was divided by the total number of neurons sampled in that layer, regardless of expression category, for each case and multiplied by 100 to obtain a percentage by category. In order to compute an average grains per cell measurement, all the neurons in the low, medium, and high expression categories were averaged for each layer for each case.

Statistical analysis

Student's *t*-tests were run to verify that the groups were sufficiently matched on demographic variables. Statistical comparisons between the RNA, protein, and neuron density measurements between the unaffected controls and patients with schizophrenia were accomplished with *t*-tests or ANOVAs. Data for our BDNF measurements were found not to deviate significantly from a normal distribution by the Kolmogorov–Smirnov *D* statistic, so parametric tests were considered appropriate. No cases had BDNF values outside two standard deviations away from the mean, hence no cases were dropped because of outlier measurements. Correlations between RNA yield, protein yield, BDNF measurements and pH, PMI, freezer time and age were tested with a Pearson product moment correlation on the normal control cases only. In the patients with schizophrenia, Spearman correlations were performed between estimated neuroleptic dose and measurements of BDNF. A nonparametric analysis was chosen for correlations with neuroleptic levels and BDNF in the patient group because of the large heterogeneity in drug levels in the patient group. When a variable was found to correlate consistently with a measurement of interest, compar-

isons between the diagnostic groups were also made with an ANCOVA.

Results

DLPFC RNA and protein isolations

The yields of total RNA from frontal cortex did not differ ($t=-0.66$, df$=47$, $P=0.51$) between individuals with schizophrenia (0.61 μg RNA/g tissue) *vs* those without schizophrenia (0.58 μg RNA/g tissue). The quality of the RNA, as assessed by the integrity and intensity of ribosomal RNA bands on agarose gel electrophoresis, was good, and did not differ between controls and schizophrenics. There was no relation between RNA quality and PMI (data not shown), nor was there any correlation between extracted total RNA and age of subject, tissue pH, PMI, or freezer storage time (all R values were between 0.11 and 0.30, all $P>0.15$). We calculated the total amount of protein extracted in the DLFPC from normal individuals (115.9 ± 27.5 mg protein/g of brain tissue) and those suffering from schizophrenia (116.9 ± 26.0 mg protein/g of brain tissue) and found no difference in protein levels ($t=-0.11$, df$=29$ $P=0.92$). Yield of protein in the human DLPFC was not significantly correlated with brain pH, PMI, or age of subject (all $R<0.28$, all $P>0.25$). There was a trend for total protein to correlate with freezer time; however, this was in the opposite direction than what would be predicted if protein deteriorated under the tissue storage conditions, as increased freezer time tended to increase the amount of extractable protein ($r=0.44$, $P=0.057$). Neither RNA yield nor protein yield from DLPFC tissue of patients with schizophrenia was correlated with average daily dose, lifetime dose of neuroleptics or to the most recent dose of neuroleptics (all $R\leq0.10$, all $P>0.29$).

BDNF Northern analysis

Northern analysis revealed that our BDNF riboprobe detected one major transcript at $\sim$4.6 kb in poly A RNA samples extracted from regions of adult human brain (Figure 1). The BDNF hybridizing band at $\sim$4.6 can be delineated in lanes containing cortical (lanes 2, 5, 6, and 7) and subcortical structures (lanes 1, 3, 4, and 8). By visual inspection, BDNF mRNA levels appear to be highest in the spinal cord (lane 4) and the cerebellum (lane 1); also, the BDNF expression is fairly robust and consistent with RNA extracted from multiple cortical regions, including the occipital, frontal, and temporal cortices. The less prominent smaller BDNF transcript can be delineated in RNA from some cortical regions and the medulla, but is not clearly seen in RNA from the spinal cord. These two BDNF transcript sizes are similar to what have been reported previously in cortical regions of the primate brain.[55]

BDNF RNase protection assay

Visual inspection of the autoradiographic films generated in the RNase protection assay revealed

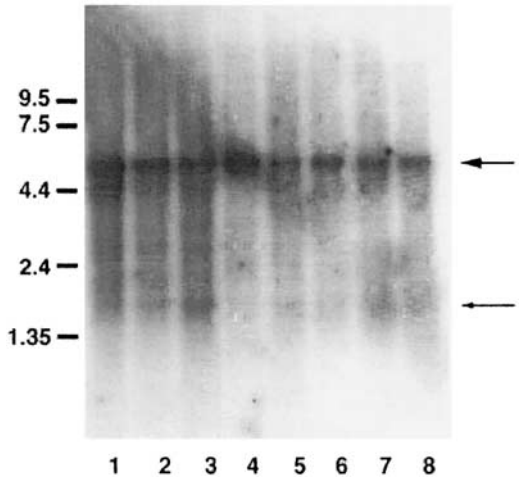

Figure 1 The Northern blot analysis shows one prominent BDNF hybridizing band at 4.6 kb (large arrow) that is detected in all brain regions examined. Another less prominent BDNF mRNA at about 1.6 kb (small arrow) band is also present, but shows greater regional variation in expression. Lanes 1–8 contain poly A RNA extracted from cerebellum (1), cerebral cortex (2), medulla (3), spinal cord (4), occipital lobe (5) frontal lobe (6) temporal lobe (7), and putamen (8).

protected BDNF mRNA in all samples assayed (Figure 2a). The 0 lane of the standard curve contained no apparent signal, verifying that the RNase levels used were sufficient to digest the unprotected single-stranded riboprobe. The RPA was linear over a wide range of input RNA (0.1–50 pg in the standard curve, $R=1.00$). BDNF mRNA was significantly reduced ($t=2.26$, df$=47$, $P=0.03$, mean 23% reduction) in the frontal cortex of patients with schizophrenia (1.71 (±0.17) pg/20 μg total RNA) as compared to unaffected controls (2.23 (±0.16) pg/20 μg total RNA). Levels of BDNF mRNA detected in the RPA showed a weak correlation with age ($r=-0.37$, $P=0.07$). When the data from the BDNF RPA were analyzed by ANCOVA with age as a covariate, the difference between the patients with schizophrenia and controls remained statistically significant (F$=5.73$, df$=1, 46$, $P=0.02$). BDNF mRNA levels did not correlate with PMI ($r=-0.13$, $P=0.52$), tissue pH ($r=0.15$, $P=0.46$), or time in the freezer ($r=0.23$, $P=0.27$). To test the effects of gender or brain hemisphere and diagnosis on BDNF mRNA levels, two-way ANOVAs were performed. While the main effect of diagnosis showed that BDNF mRNA levels were significantly lower in patients in both these analyses (as before), there were no significant main effects of gender or brain hemisphere, nor were there significant interactions between diagnosis and gender or diagnosis and brain hemisphere for BDNF mRNA levels in the DLPFC (all F values <2.0, all P values >0.2). Cyclophilin mRNA levels determined by RNase protection assay did not differ between patients and controls (Figure 2c).

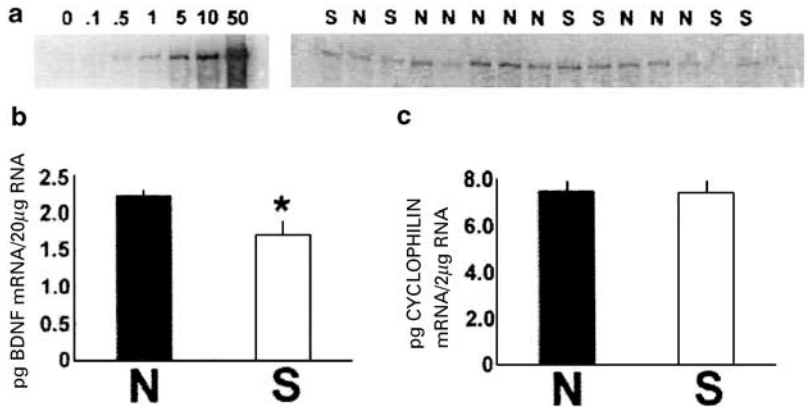

Figure 2 Representative gels containing the standard curve generated from *in vitro* transcribed plus strand and human brain samples after the RPA (a). Increasing amounts of BDNF sense RNA (0, 0.1, 0.5, 1, 5, 10, and 50 pg) were used to determine the amount of BDNF mRNA in the samples. A BDNF protected band is found in all the human brain samples, but varies in intensity (N = normal, S = schizophrenic). The mean picogram (± SE) of BDNF per 20 μg of total RNA (b) and cyclophilin per 2 μg of total RNA (c) is plotted for both groups (* = $P < 0.05$).

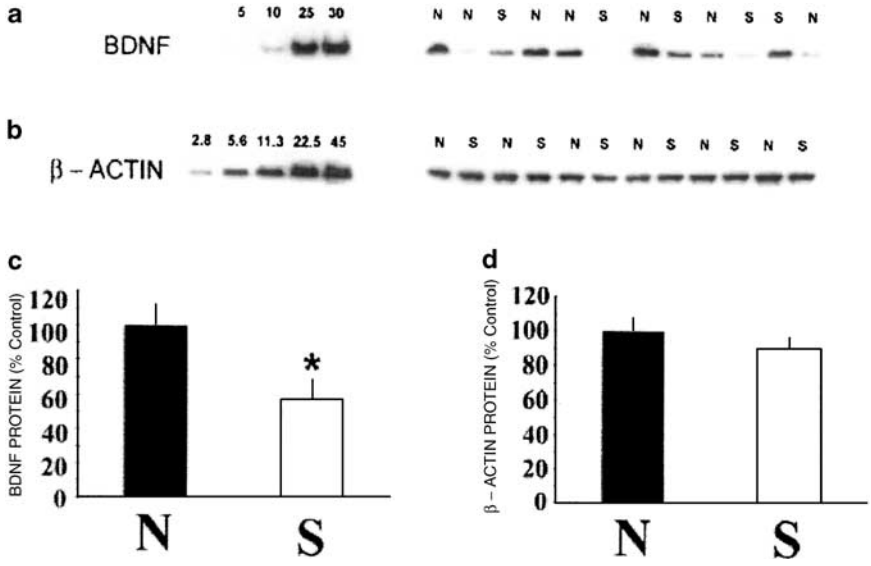

Figure 3 Representative gels from Western blotting using an anti-BDNF antibody. Panels a and b demonstrate the blots containing increasing amounts of total protein (left) and samples from different human brains (right) (N = normal, S = schizophrenic). For BDNF, the immunoreactive bands corresponding to mature BDNF are found just below 14 kDa. A range of 5–30 μg of total protein was used to determine the linear range for quantitation (a). For β-actin, the immunoreactive band is present at 46 kDa. A protein range of 2.8–45 μg was used to determine the linear range for quantitation (b). The mean optical density, expressed as a percent control (± SE) of BDNF per 25 μg of protein (c) and β-actin per 8 μg of total protein, is plotted for both groups (* = $P < 0.05$).

BDNF Western analysis

In human brain homogenates, the rabbit polyclonal antibody raised against BDNF detected a prominent band running at about 13 kDa, consistent with the molecular weight of BDNF protein (Figure 3a). In addition, there were many high molecular weight bands of unknown identity recognized by this antibody, suggesting that in the human frontal cortex this antibody may cross-react with other proteins (data not shown). We determined that BDNF protein (13 kDa band) quantitation was linear over a fairly wide range of protein concentrations ($R = 0.99$) and found a detectable signal in all samples when 25 μg of protein was loaded and size-separated on acrylamide gels. Although the amount of BDNF protein traveling at 13 kDa varied considerably between individuals, we detected a significantly reduced level (by over 40%, $t = 2.02$, df = 29, $P = 0.05$) in the DLPFC of patients with schizophrenia compared to normal controls (Figure 3c). Levels of β-actin, a control protein, assayed from the same homogenates did not differ between the groups (Figure 3b and d, $t = 0.954$, df = 29, $P = 0.35$). Neither BDNF nor β-actin proteins

levels correlated with PMI, tissue pH, age, or freezer time (all $r<0.02$, all $P>0.05$). Similar to the BDNF mRNA analysis, there were no significant main effects of gender or brain hemisphere, nor were there significant interactions between diagnosis and gender or diagnosis and brain hemisphere for BDNF protein levels in the DLPFC (all F values <1.0, all P values >0.4).

BDNF in situ hybridization

We found a distinct laminar pattern of BDNF hybridization signal in the gray matter of the middle frontal gyrus in both schizophrenics and controls. BDNF did not appear homogeneously distributed. There were two bands of increased signal intensity, one at the midcortical (deep III), and deeper cortical level (V/VI) (Figure 4a and b). BDNF mRNA was also expressed at the pial surface and within elongated structures in the white matter, perhaps corresponding to blood vessels. This pattern was distinct from cyclophilin mRNA (Figure 4c and d). On the autoradiographic film, there was no detectable image from sections hybridized with the radiolabeled BDNF sense strand control riboprobe (data not shown). In all layers of the cortex, the BDNF mRNA did not appear evenly expressed, but instead showed a punctate pattern of expression. As a first attempt to quantify BDNF mRNA levels from the *in situ* hybridization, we analyzed the levels of BDNF mRNA from densitometry of the autoradiographic film. While we detected a slight, overall reduction in BDNF mRNA in the cortical layers of patients with schizophrenia, this difference failed to reach statistical significance (data not shown). Additionally, there was no difference in cyclophilin mRNA levels between patients and controls when analyzed from the autoradiographic film (data not shown). At the cellular level, we noted that BDNF mRNA was not expressed uniformly in all cortical neurons (Figure 5), consistent with reports in the monkey cortex.[56,57] Therefore, cell-based quantitation was undertaken as a more sensitive way to measure BDNF expression levels between the two subject groups.

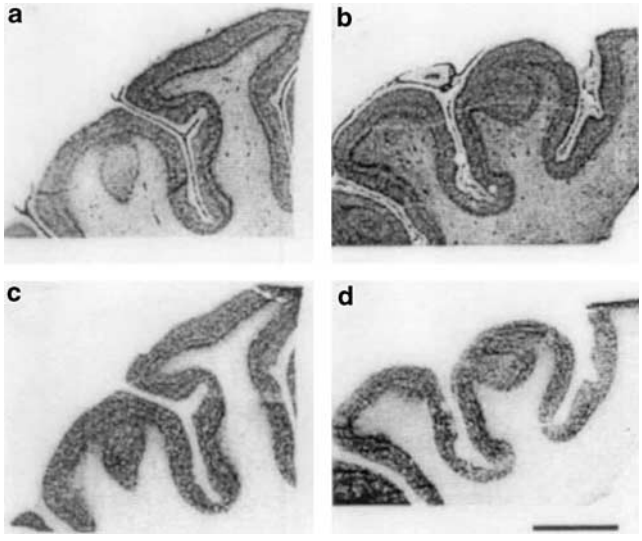

Figure 4 Autoradiographic film images of *in situ* hybridization of BDNF mRNA (a and b) and cyclophilin *in situ* hybridization (c and d). Adjacent sections of human DLPFC from a normal brain (case 26: a and c) and from a schizophrenic (case 57: b and d) demonstrate a similar pattern of hybridization. Robust BDNF hybridization signal is found in the gray matter (with the exception of layer I) relative to the white matter. The BDNF mRNA signal is bilaminar in appearance with one prominent band of expression at the level of the midcortex and another at the deeper cortical levels. BDNF mRNA expression is also detected at the pial surface and in blood vessels of the white matter. Cyclophilin mRNA is more evenly expressed in gray matter DLPFC and is not expressed at the pial surface or in the white matter. Scale bar in (d) equals 1 cm.

BDNF mRNA—silver grain analysis

In the normal brain, we found that the majority of pyramidal neurons in all four layers examined expressed BDNF mRNA, but to varying degrees (Table 3). BDNF mRNA expression in nonpyramidal neurons was undetectable, consistent with observations in rodent neocortex.[58] Layer II had the lowest percentage of BDNF mRNA-expressing neurons by our criteria (55% of total neurons expressed BDNF mRNA at low, medium, or high levels), while layers with larger pyramidal neurons, layers III, V, and VI had similar percentages of BDNF mRNA-expressing neurons per total neurons sampled (77, 80, and 70%, respectively). Neurons in layers II, III, V, and VI of the DLPFC of patients with schizophrenia mainly had a reduction in the percentage of BDNF mRNA-expressing neurons per total number of neurons sampled (only 42% in layer II, 57% in layer III, 58% in layer V, and 70% in layer VI). Interestingly, neurons expressing the highest level of BDNF mRNA (high expressers) were the most affected in the brain of patients with schizophrenia. We tested whether neurons in any particular cortical layer showed reduced BDNF mRNA by using a one-tailed *t*-test (based on a directional hypothesis that BDNF mRNA levels would be reduced). We found a significant reduction in the percentage of BDNF mRNA-positive neurons expressing high levels of BDNF mRNA in two cortical layers [layer III ($t=-1.88$, df $=25$, $P=0.036$) and layer V ($t=-1.95$, df $=25$, $=0.031$)]. However, the percentage of high BDNF mRNA-expressing neurons in layers II and VI did not differ between patients with schizophrenia and controls ($t=-0.35$, df $=25$, $P=0.37$ and $t=$-0.79, df $=25$, $P=0.22$, respectively).

With a more traditional silver grain quantitative approach, we also tested whether BDNF mRNA levels were reduced by computing the average grains per cell in all the BDNF mRNA-expressing cells regardless of expression category. Once again, we found a reduction in the number of BDNF silver grains/neuron (ranging from 10 to 28%) in all layers in the neocortex of patients with schizophrenia (Figure 6).

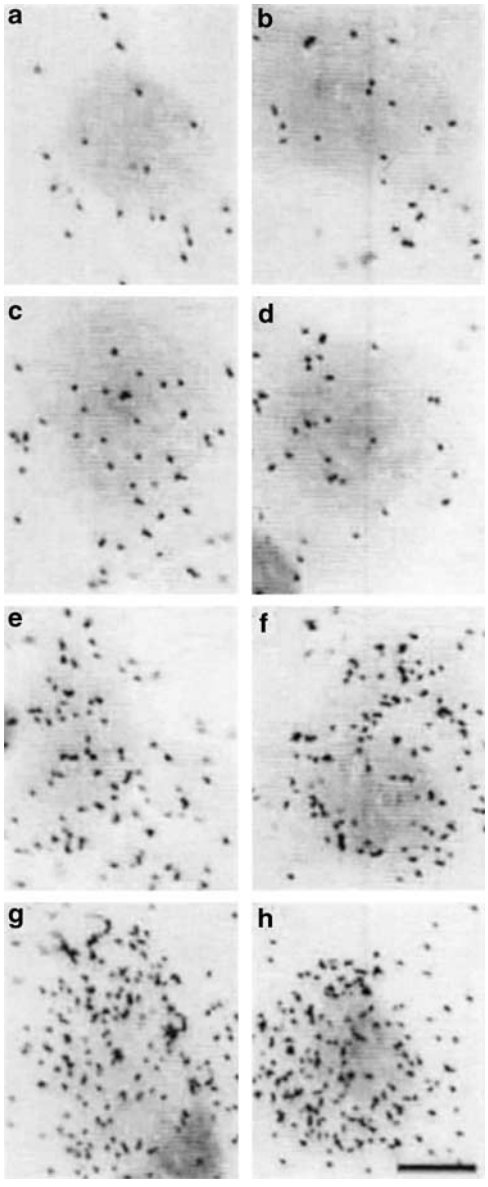

Figure 5 Bright-field photomicrographs demonstrate that BDNF mRNA is not evenly expressed in pyramidal neurons (light gray corresponds to light Nissl staining of cell body and nucleus) of the human DLPFC. The small solid black dots overlying and scattered adjacent to the neurons correspond to the amount of BDNF mRNA for that cell. Neurons in (a) and (b) are not expressing BDNF mRNA significantly above background levels. Neurons expressing BDNF are found to have low (c and d), moderate (e and f) or high levels of BDNF expression (g and h). Layer VI pyramidal neurons are shown from two normal cases (case 26: a, c, e and g; case 24: b, d, f and h). Scale bar in (h) equals 10 μm.

The average number of grains per cell was significantly reduced in layer III ($t=-1.87$, $df=25$, $P=0.037$) in schizophrenics compared to unaffected controls. The reduction in BDNF mRNA in the other layers did not reach statistical significance ($t<-1.2$, $df=25$, $P>0.12$) when the grains per cell were considered.

Table 3 Mean percentage of BDNF-expressing cells falling into three different expression categories (*$P<0.05$)

Diagnosis	*% low*	*% medium*	*% high*
Layer II			
Normal	30	16	9
Schizophrenic	27	8	7
Layer III			
Normal	32	17	28
Schizophrenic	34	14	9*
Layer V			
Normal	35	20	25
Schizophrenic	35	15	8 *
Layer VI			
Normal	31	18	21
Schizophrenic	39	17	14

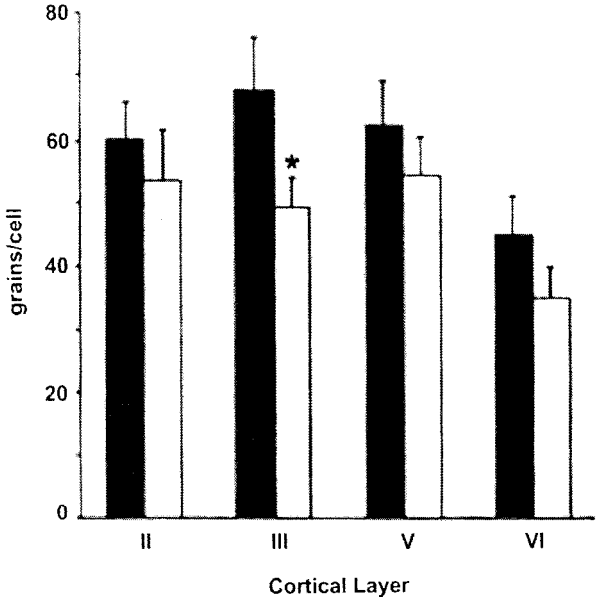

Figure 6 Mean number of grains per BDNF mRNA-expressing cell is graphed for pyramidal neurons in layers II, III, V, and VI. Black bars represent cellular BDNF mRNA levels in unaffected normal individuals and white bars represent the levels in schizophrenics. The error bars represent standard error of the mean (* = $P<0.05$).

BDNF levels in patients—clinical considerations

DLPFC BDNF mRNA levels, detected by RNase protection assay, did not correlate with the average daily dose of neuroleptics ($r=-0.24$, $P=0.30$), with the most recent dose of neuroleptics ($r=-0.12$, $P=0.62$) or with the estimated dose of neuroleptic exposure given throughout the lifetime of the patient ($r=-0.33$, $P=0.15$). BDNF mRNA levels tended to

Table 4 Clinical characteristics of patients with schizophrenia

Case #	Age of onset/ duration of illness/ duration of hospitalization	Last CPZ equiv.	Daily CPZ equiv.	Lifetime CPZ equiv.	EtOH/ substance abuse	TD	Auditory/ visual hallucinations	Paranoid/ grandiose delusions	Thought disorder	IQ/CT-MRI	Negative symptoms	Depression/ suicide attempts	Catatonia	Polydipsia
34	15y/56y/44y	100	500	600000	−/−	+	+/+	+/+	+	90/wide sulci	+	+/−	+	+
35	22y/26y/23y	2000	275	2600000	+/−	−	+/−	+/−	+	NA/NA	+	+/+	+	+
36	21y/16y/10y	400	850	5000000	−/−	−	+/−	+/+	+	80/NA	+	+/+	−	+
37	23y/23y/12y	NA	NA	NA	−/−	−	+/−	+/+	+	87/NA	+	−/−	−	−
38	19y/15y/3.5m	200	200	1100000	−/−	+	+/+	+/+	+	NA/NA	+	+/−	−	−
39	36y/10y/5y	300	300	1100000	−/−	−	+/−	+/−	+	NA/NA	+	+/+	+	−
40	23y/31y/27y	2400	800	9100000	+/−	−	+/−	+/+	+	94/NA	+	−/+	−	+
41	33y/15y/1.5y	300	300	1600000	−/−	+	+/+	+/−	+	87/increased VBR, wide sulci	+	+/+	−	+
42	23y/50y/?	450	450	4600000	+/−	−	+/−	+/−	+	NA/NA	+	−/−	+	+
43	26y/8y/2w	NA	NA	NA	+/−	−	+/+	+/+	−	NA/NA	−	−/−	−	−
44	29y/46y/37y	400	400	5300000	+/−	−	+/−	+/−	+	NA/NA	+	−/+	−	−
45	19y/45y/20+y	900	400	5300000	−/−	+	+/+	+/−	+	NA/WNL	+	+/−	+	−
46	30y/37y/5+y	80	100	1300000	−/−	−	+/+	+/−	+	NA/NA	+	−/−	+	−
47	17y/14y/1.5y	200	250	400000	−/−	−	+/+	+/+	+	NA/WNL	−	−/+	−	+
48	21y/2y/2.5m	400	480	33000	−/−	−	+/+	+/−	−	27/30 MMSE/NA	+	+/−	−	−
49	40y/20y/1y	100	100	700000	−/−	−	+/−	+/−	−	NA/NA	+	−/−	−	−
50	18y/12y/2y	1900	500	2200000	−/+	−	+/+	+/−	+	79/NA	−	−/−	−	−
51	22y/13y/9y	NA	NA	NA	−/−	−	+/+	+/−	+	NA/WNL	+	−/+	+	−
52	16y/64y/45y	30	50	700000	−/−	−	+/+	+/+	−	101/wide sulci	+	+/+	+	+
53	27y/54y/26y	100	150	2100000	+/−	−	+/−	+/−	+	NA/NA	+	−/−	+	−
54	34y/27y/14y	NA	200	2000000	−/−	+	+/−	+/+	+	78/increased VBR, wide sulci	+	−/−	−	−
55	16y/27y/18y	800	60	11800000	+/+	+	+/−	+/+	+	27/30 MMSE/ increased VBR	+	−/−	+	−
56	16y/25y/3y	2400	1135	10400000	−/−	−	+/+	+/+	+	58/increased VBR, wide sulci	+	−/−	−	+
57	20y/21y/12y	50	400	2500000	−/−	+	+/−	+/+	+	72/WNL	+	+/+	+	+

CPZ=chlorpromazine, TD=tardive dyskinesia, MMSE=mini mental state exam, CT=computerized tomography, MRI=magnetic resonance imaging, NA=not available, WNL=within-normal limits, VBR=ventricular brain ratio. w=weeks, m=months, y=years.

correlate with illness duration ($r=-0.39$, $P=0.07$), the estimate of which is weighted by age at death. BDNF protein levels within the DLPFC did not correlate with the average daily dose of neuroleptics ($r=-0.01$, $P=0.96$), with the dose of neuroleptics most recently taken ($r=0.18$, $P=0.59$), the lifetime neuroleptic exposure estimate ($r=-0.22$, 0.48), age of onset ($r=0.24$, $P=0.45$), or with the illness duration ($r=-0.33$, $P=0.30$). Additionally, we failed to find any relation between the percentage of BDNF mRNA-positive neurons in the medium or high expression categories and the average daily dose of neuroleptic medication, the cumulative lifetime estimate of neuroleptic exposure or the dose of neuroleptic at the time to death in layers II III, V, or VI (all $P>0.10$). BDNF mRNA levels expressed as grains per cell in layers II, III, V, or VI did not correlate with any of the three measurements of neuroleptic levels used (all $P>0.13$).

Consideration was also given to other characteristics of patients with schizophrenia that may have influenced BDNF mRNA levels (see Table 4). The data on BDNF mRNA levels as detected by RNase protection assay were used for these correlations because we had the largest group of patients in this assay. We were unable to find a correlation between average IQ and BDNF mRNA levels in the DLPFC of patients with schizophrenia (see Table 2, $N=9$). Although subgroups of the patients tended to be relatively small, we did not detect any significant differences in BDNF mRNA levels among schizophrenics with or without the following characteristics: (1) thought disorder, (2) visual hallucinations, (3) depression, (4) suicide attempts, (5) catatonia, (6) polydypsia, or (7) tardive dyskinesia. Also, we did not detect a relation between BDNF mRNA levels and age of onset, tested either as a correlation or with a *t*-test where individuals were grouped into those with an earlier *vs* older age of onset (below and above 23 years of age, $N=23$). There was no significant difference in BDNF mRNA levels among the three diagnostic subtypes of schizophrenia.

Discussion

We report a reduction in a vital neurotrophic factor, BDNF, in the frontal cortex of patients with schizophrenia using a large number of well-characterized brains and three different assay methodologies. The reduction in BDNF mRNA detected by RNase protection assay and *in situ* hybridization is not because of a generalized reduction in mRNA expression, as levels of 'control' or' 'housekeeping' mRNAs are not changed in the same homogenates and in adjacent sections from these cohorts[28] and data not shown. Also, reduced BDNF protein production is not likely to be because of a generalized reduction in protein levels as we did not find a change in overall protein per gram of brain tissue or a difference in β-actin protein levels using similar methodology and the same homogenates. The anatomical distribution of BDNF mRNA in the adult human DLFPC observed in our study is in good agreement with previous reports in the primate neocortex that show prominent but uneven expression of BDNF mRNA in cortical pyramidal neurons of layers III, V, and VI.[47,56,57]

Three nonmutually exclusive models could explain the BDNF reduction in pyramidal neurons of the DLFPC of patients with schizophrenia: (1) BDNF is reduced as an epiphenomenon of having a chronic mental illness and/or because of antipsychotic drug treatment of patients with schizophrenia, (2) BDNF expression is altered in patients with schizophrenia because of the more frequent inheritance of an altered BDNF 'gene', and (3) BDNF fails to be induced during normal brain development because of complex genetic and/or environmental signals, which interact to impair the normal development of the DLFPC in patients with schizophrenia.

Evaluation of the evidence for model 1—epiphenomena

The reduction in BDNF mRNA and protein may be an intrinsic part of the pathology of schizophrenia. It may also be that BDNF reduction is a consequence of this pathology, that is, BDNF could be reduced because of life-long events related to suffering from schizophrenia or any severe chronic psychiatric disorder, such as exposure to neuroleptic drugs and/or to aberrant environmental stimulation of the brain. Indeed, there could be less demand for BDNF because of the pathological circuitry proposed to exist in the DLFPC of patients with schizophrenia.

In our study, BDNF mRNA levels do not appear to be directly related to neuroleptic levels. Not unexpectedly, all the patients with schizophrenia in our study were medicated with typical antipsychotics. In studies of the effect of chronic administration of haloperidol and clozapine on BDNF mRNA levels in the rodent frontal cortex, we and others have failed to find an effect on BDNF mRNA, particularly at the 0.5 mg/kg/day dose of haloperidol that may more closely mimic the therapeutic dose used in humans.[45,59] Other studies in rats have reported that BDNF protein-containing fibers and BDNF concentrations in frontal cortex are downregulated by acute and chronic administration of haloperidol.[60,61] However, one study found that chronic administration of haloperidol to rats resulted in no change in BDNF protein levels in brain.[62] Furthermore, in the human DLPFC, we did not detect any correlation between the most recent dose, average daily dose, or lifetime dose of neuroleptic medication and any measure of BDNF mRNA or BDNF protein. These results suggest that while neuroleptic drugs taken by the patients could influence BDNF levels, they may not be the primary determinant of the BDNF level in the DLFPC of patients with schizophrenia. However, the effects of neuroleptics on brain BDNF levels are not clear at this time.

While we can speculate that BDNF reductions, particularly in BDNF mRNA, are not because of

neuroleptic exposure, we cannot rule out the possibility that other nonspecific illness factors cause a reduction of BDNF in the DLPFC. Telencephalic BDNF mRNA levels are upregulated by increased excitatory neurotransmission, environmental stimulation, and learning processes in both rodents and primates.[12,56,63–66] These are variables that are likely to differ between the patients with schizophrenia and unaffected individuals. Further studies comparing the use of alternative 5′ exons of the BDNF transcript in the brains of patients with schizophrenia would be informative as some promoters are activated by glutamate receptor stimulation whereas others are not.[67–72] Also, studies comparing BDNF in the DLPFC of patients with schizophrenia to other patient groups suffering from a chronic mental illness could test if the change in BDNF is diagnostically specific or could be because of a generalized alteration in cortical stimulation.

BDNF has also been shown in animal studies to have potential antidepressant properties[73] and to be upregulated by antidepressant agents (for review see Vaidya and Duman,[74] and Duman[75]). Furthermore, increased BDNF protein in the hippocampus of depressed patients treated with antidepressants has been reported.[76] In our study, we have tested whether a history of depression may influence BDNF levels in schizophrenia and we have been unable to detect a difference in BDNF mRNA levels between patients with and without a recorded history of depression. Our finding is in accordance with the finding of Chen *et al*,[76] who noted no significant difference in the baseline BDNF levels of depressed patients *vs* normal controls.

Evaluation of the evidence for model 2—genetic etiology

An alteration in BDNF expression may be a primary abnormality in schizophrenia, leading to downstream changes in multiple neurotransmitter systems. Genetic association studies have tested whether endogenous variations in the human BDNF gene, which could lead to changes in BDNF synthesis or BDNF function, are differentially inherited in patients with schizophrenia. Samples of different patient groups and normal controls from around the world have been genotyped at a dinucleotide repeat polymorphism found 1 kb upstream of the BDNF transcription start site. The functional implications of this polymorphism are not known. These studies do not provide any consistent evidence for the differential distribution of BDNF alleles among groups of patients with schizophrenia compared to controls.[77–81] However, these studies, in which the number of patients with schizophrenia ranged from 48 to 262, were somewhat underpowered to find a genetic association between the BDNF genotypes and schizophrenia, particularly as there exist numerous alleles of low frequency with this particular BDNF polymorphism.

The lack of evidence from family studies for linkage of schizophrenia to the chromosomal region where the BDNF gene is located (11q13) suggests that variation in the BDNF gene may not be a major contributor to risk for the disease. Some of the association studies suggest that inheritance of certain BDNF alleles may relate to age of onset of schizophrenia, treatment responsiveness of schizophrenic symptoms, and parietal lobe brain volume in patients with schizophrenia.[79,80] Further work evaluating other polymorphic regions of the BDNF gene in family-based studies or those that use measures of cognitive function may prove more sensitive, and are needed to fully evaluate the validity of this model. Ongoing studies in our laboratory have investigated one such polymorphism, namely that encoding a nonconservative amino-acid change that affects BDNF subcellular distribution, activity-driven BDNF neuronal release, task-related hippocampal deactivation in humans, and memory function in humans.[82] We did not detect an association between this BDNF polymorphism and the schizophrenic phenotype in a family-based gene association study.[82]

Evaluation of the evidence for model 3—lack of normal developmental induction

Little is known about the role of BDNF in the developing human forebrain. However, studies in other mammals show that BDNF plays a key role in neocortical development. An increase in BDNF has been temporally associated with functional maturation of cortical regions and BDNF is necessary for normal cortical development.[14–19,83] The human dorsolateral prefrontal cortex functionally matures fairly late in postnatal life and individuals may not reach their adult level of performance on tasks that depend on the DLPFC until adolescence or young adulthood.[84] Coincident with this final maturation, we have detected a two-fold induction of BDNF mRNA during the young adult period (22 years) compared to the infant and adolescent period in DLPFC[85]. Additionally, we have found a significant increase in mRNA encoding the BDNF receptor, the high-affinity tyrosine kinase-containing form (full-length trk B or $trkB^{TK+}$), in the young adult human DLPFC[86]. The increase in $trkB^{TK+}$ mRNA is prominent in layers II and III, suggesting that cortico-cortical neurons may be especially responsive to the developmental increase in BDNF[86]. These observations are in line with other evidence suggesting that the supragranular layers of the primate frontal cortex mature quite late in postnatal life.[87–89] Furthermore, these findings support the notion that molecular maturation, which may be defined by an increase in BDNF and $trkB^{TK+}$, occurs during young adulthood in the human DLPFC. Interestingly, it is layer III pyramidal neurons that show the most robust decreases in BDNF mRNA levels in patients with schizophrenia.

This temporal induction of BDNF and $trkB^{TK+}$ mRNAs in the DLPFC of normal humans corresponds

to the average age of onset of schizophrenia in our sample and with the average age of onset of schizophrenia in the general population.[90] The induction of BDNF in late adolescence and young adulthood may be abnormal in the brain of patients with schizophrenia; however, since we cannot currently study the BDNF levels in schizophrenic subjects before and after their first break, we cannot test this hypothesis directly. To test this model experimentally, we have asked if a neonatal insult to the ventral hippocampus in the rat, which is postulated to bring about an altered developmental maturation of the prefrontal cortex, could affect the levels of BDNF mRNA within the prefrontal cortex. We found no significant change in baseline prepubertal or adult levels of BDNF mRNA but a trend for a reduction in BDNF mRNA in the rodent prefrontal cortex after developmental damage to the ventral hippocampus.[45,91] In the rodent prefrontal cortex, BDNF mRNA that is normally induced by stress was deficient in young adult rats, which had experienced an early lesion of the ventral hippocampus.[91] These data support the notion that an early alteration in the developing brain may alter the ability of the frontal cortex to upregulate BDNF in response to environmental or hormonal demands later in life.

Potential consequences of reduced BDNF

Since BDNF is known to be trophic for a variety of neuronal subtypes, one would expect a deficiency in BDNF to have pleotrophic effects. BDNF stimulates survival of both glutamate[5–7,92] and GABAergic neurons.[93–97] Reduced production of BDNF in the brain of patients with schizophrenia may have consequences for both neuronal populations. Indeed, we find that pyramidal neurons and nonpyramidal neurons throughout all layers of the adult human DLPFC contain the high-affinity tyrosine-containing receptor for BDNF, $trkB^{TK+}$[86], and are therefore potentially sensitive to a reduction in BDNF. Reductions in BDNF may relate to alterations in pyramidal neurons found in the prefrontal cortex in schizophrenia, as hypothesized in the Introduction to this paper; however, it is possible that the reduction in BDNF that we find may relate to alterations in GABAergic neuron population as well. BDNF could affect GABAergic neuron density, the level of GAD mRNA expression, the number of GABAergic terminals, or the density of GABA receptors, all of which have been reported to be abnormal in the frontal cortex of patients with schizophrenia.[98–105] GABAergic neurons of the striatum are also dependent on cortical BDNF for their survival; BDNF is transported anterogradely to the striatum from neurons in cortical layers III and V.[106–109] Since we find that neurons within layers III and V show significantly less BDNF mRNA, we would predict that striatal GABAergic function might be compromised because of a reduced supply of cortically derived BDNF.[107,110] Measuring the levels of BDNF and trkB receptor within the caudate/putamen and nucleus accumbens of patients with schizophrenia are needed to further explore this hypothesis.

BDNF has long been recognized as a neurotrophic factor for midbrain dopamine neurons,[111,112] and reduced cortical BDNF may cause the putative reduction in cortical dopamine innervation of the DLPFC[113]. Alternatively, a reduced dopamine innervation to the cortex could lead to a downregulation of BDNF, as injections of levodopa or injections of a D1/D5 receptor agonist can upregulate BDNF mRNA levels in the frontal cortex of rats.[114] Cortically derived and anterogradely supplied BDNF, acting through trkB, can regulate the expression of D3 receptor within the basal ganglia and can alter the responsiveness of striatal neurons to dopamine without altering the levels of D1R and D2R[114]. Additionally, reduced BDNF levels can lead to an increase in striatal dopamine concentration and altered stimulated dopamine output, and a hyper-responsiveness to amphetamine administration in rodents,[115–117] all situations reminiscent of aspects of dopamine dysregulation in schizophrenia.

BDNF also leads to a variety of other molecular changes, some of which may be found in the brain of patients with schizophrenia. For example, BDNF can increase α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors, and cortical neurons from BDNF knockout mice have a reduction in AMPA receptor proteins.[118] A reduction in AMPAR binding has been reported in the temporal cortex in patients with schizophrenia,[119] although AMPAR binding may not be consistently reduced in all brain areas.[120] BDNF administration can also increase the expression of some presynaptic terminal proteins, most notably Rab3a,[121] which is reportedly decreased in the thalamus of patients with schizophrenia.[122] Further, the number of thalamic neurons in the medial dorsal nuclei, which send excitatory projections to the DLPFC, may be reduced in patients with schizophrenia.[123–127] The reduction in thalamic neuronal number in schizophrenia could be caused by a reduction in cortically derived BDNF levels in schizophrenia, as neurons in the thalamus are dependent on BDNF for their survival.[128,129] In fact, we have observed a significant reduction of BDNF mRNA in layer III pyramidal neurons. The basal dendrites of these pyramidal neurons can receive input from the thalamus and may also have reduced synaptic spines in patients with schizophrenia.[130] These observations, coupled with those reported here, would be consistent with either less anterograde or less retrograde supply of BDNF to the thalamus in patients with schizophrenia.

Comparison with other post-mortem studies

Previous studies using post-mortem brain tissue from schizophrenic patients have reported an upregulation[131] or no change[62] of BDNF protein in the frontal cortex of schizophrenics. Both the studies used a different assay method for measurement of BDNF protein levels than that applied in the present report

(a commercially available ELISA kit was used in both previous studies[62,112]). Thus, differences between our studies may relate to the differences in specificity of the antibodies used. Alternatively, they could relate to the differences in the ages of the subjects studied (average age of patients in the Durany study was ~77 years, whereas our patients averaged ~48 years of age). Since BDNF levels may decrease with advancing age [19,131] (and current study), it is possible that patients with schizophrenia show altered age-associated changes in BDNF levels. The Takahashi report (2000) utilized DLPFC tissue from nine patients with schizophrenia compared to tissue from 25 normal controls that were not matched for post-mortem interval (over twice as long for the schizophrenics). In our study, we have controlled for the potential confounds of aging and PMI, and we believe that our findings of reduced BDNF in the brains of patients with schizophrenia are valid.

General conclusions

In summary, patients with schizophrenia have reduced BDNF in their prefrontal cortex. While a reduction in BDNF protein could be caused by many factors, including increased protein degradation or increased protein transport away from the DLPFC, our results show that a reduction in BDNF synthesis, because of lower-than-normal levels of BDNF mRNA within the DLPFC pyramidal neurons, may contribute to lower protein levels. While the cause of reduced BDNF mRNA and protein within the DLPFC of patients is unknown, it does not seem to clearly relate to epiphenomenon or genetic vulnerability. The reduction in BDNF in the DLPFC of patients with schizophrenia is positioned to be a central component of the disease process, as BDNF exerts potent effects on the forebrain systems believed to be involved in schizophrenia.

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