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ORIGINAL ARTICLE

Altered Balance of Proteolytic Isoforms of Pro–Brain-Derived Neurotrophic Factor in Autism

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Abstract

Defects in synaptic development and plasticity may lead to autism. Brain-derived neurotrophic factor (BDNF) plays a critical role in synaptogenesis and synaptic plasticity. BDNF is synthesized as a precursor, pro-BDNF, which can be processed into either a truncated form or into mature BDNF. Previous studies reported increased BDNF-immunoreactive protein in autism, but the mechanism of this increase has not been investigated. We examined BDNF mRNA by real-time reverse transcription–polymerase chain reaction and BDNF protein by Western blotting and enzyme-linked immunosorbent assay in postmortem fusiform gyrus tissue from 11 patients with autism and 14 controls. BDNF mRNA levels were not different in the autism versus control samples, but total BDNF-like immunoreactive protein, measured by enzyme-linked immunosorbent assay, was greater in autism than in controls. Western blotting revealed greater pro-BDNF and less truncated BDNF in autism compared with controls. These data demonstrate that increased levels of BDNF-immunoreactive protein in autism are not transcriptionally driven. Increased pro-BDNF and reduced truncated BDNF are consistent with defective processing of pro-BDNF to its truncated form. Distortion of the balance among the 3 BDNF isoforms, each of which may exhibit different biological activities, could lead to changes in connectivity and synaptic plasticity and, hence, behavior. Thus, imbalance in proteolytic isoforms is a possible new mechanism for altered synaptic plasticity leading to autism.

Key Words: Autism, Fusiform gyrus, mRNA, pro-BDNF, Protein isoforms, Proteolytic processing, truncated BDNF.

INTRODUCTION

Autistic disorder (AD) is a life-long neurological disorder characterized by abnormal or impaired development in social interactions and communication and repetitive interests or behavior (1). Developmental anomalies of the limbic system and the cortex have been well documented (2–7). Areas of the brain affected in autism are associated with the behavioral characteristics observed in patients with the disorder. For example, patients with autism have abnormalities in face recognition skills and difficulty with face perception (8). Face processing is mediated by the fusiform gyrus, particularly the fusiform face area, an area of occipitotemporal cortex that shows activity during facial recognition tasks (9–11). Hypoactivation of the fusiform gyrus related to face processing in AD has been replicated in a variety of studies (12–14). Therefore, examination of this area using a molecular approach may yield information on neurobiological and neurochemical abnormalities in AD.

The etiology of AD is unknown, but epidemiological and twin studies point to a major role for genetic factors in its pathogenesis (2, 15). Inheritance shows a complex pattern of transmission thought to be the result of multiple interacting genes. Recent advances in the identification of autism-linked susceptibility genes have identified a wide variety of rare genetic events that carry an increased risk for AD. These include chromosomal rearrangements such as maternal duplication of 15q11–q13, copy number variations and mutations in specific synaptic genes (16–25). These genes code for proteins associated with synaptogenesis and excitation/inhibition imbalance. Recent copy number variation (17) and genome-wide association studies implicate additional sets of genes involved in neurogenesis, neurite outgrowth and guidance, and plasticity (20, 26, 27). These findings have focused attention on aberrant neurite outgrowth and synapse formation and connectivity during development as an underlying cause of AD (24, 28).

Brain-derived neurotrophic factor (BDNF) is a synaptic molecule that plays an important role in neuronal survival and differentiation, dendritic spine maturation, and neuritic branching and connectivity during development (29–33). It also plays a critical role in synaptic plasticity and long-term potentiation and is involved in learning, memory, and attention (34–39). Low serum or brain BDNF levels have been reported in schizophrenia (40–42), major depression (43), and Alzheimer disease (44–47).

Brain-derived neurotrophic factor is synthesized as a 32-kDa precursor that can be processed to the 14-kDa mature form by furin, matrix metalloprotease 7, or plasmin (48–50). Both the precursor, pro-BDNF, and the mature form are found in human brain in areas demonstrated to be abnormal in

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patients with autism (4, 51, 52). Pro-BDNF exhibits distinct and opposite functions compared with mature BDNF. It reduces neuronal differentiation and dendritic spines and induces apoptosis and long-term depression in cultured neurons, whereas mature BDNF promotes spine formation, neuronal survival, and long-term potentiation (35, 53–55). A polymorphism in the prodomain of pro-BDNF that interferes with pro-BDNF processing and secretion of mature BDNF has been implicated in memory deficits (56, 57). Moreover, a genetic association has been found between this polymorphism and psychiatric disorders, including schizophrenia (58). This suggests that an imbalance of pro-BDNF and mature BDNF may contribute to neurodegenerative or psychiatric disorders. Pro-BDNF can also be processed at a different site by a calcium-dependent serine proteinase known as membrane-bound transcription factor protease site 1 (MBTPS-1), also known as subtilisin/kexin isozyme (SKI-1). This cleavage produces a 28-kDa truncated BDNF isoform (59). This isoform is not further processed into mature 14-kDa BDNF and, therefore, represents a true final proteolytic product. Although the biological role of this isoform has yet to be elucidated, increased pro-BDNF and mature BDNF and reduced truncated BDNF have been found recently in the serum of patients with schizophrenia (60). Therefore, relative levels of all isoforms of BDNF may be important in psychiatric disorders.

Because of its important role in neuronal development, higher than normal levels of BDNF could explain the abnormal synaptic connectivity and neurotransmitter imbalances and the abnormal trajectory of accelerated brain growth seen in subjects with AD. Several studies have reported an increase in BDNF-immunoreactive protein in autism. Neonatal cord blood of children subsequently diagnosed with autism showed a 36% increase in BDNF protein versus controls (61). However, a subsequent report from the same group using a Luminex assay reported no difference in BDNF levels between groups (62). Elevated levels of BDNF immunoreactivity have also been reported in serum (63, 64), in platelet-rich plasma (65), and in basal forebrain tissue of patients with autism (66). However, it is not clear whether this increased BDNF protein derives from transcriptional upregulation or from translational or posttranslational mechanisms because neither BDNF mRNA levels nor protein isoforms have been examined in brain tissue from individuals with autism. Only one study has examined BDNF mRNA levels in AD; elevated BDNF expression in blood lymphocytes of drug-naive male adults with AD compared with controls was reported (67). In this study, we examined postmortem tissue from the fusiform gyrus region to identify differences in BDNF mRNA and protein isoform levels between subjects with autism and controls.

MATERIALS AND METHODS

Human Brain Tissue Samples

Postmortem brain samples from 11 subjects with autism and 14 controls were provided by the Autism Speaks' Autism Tissue Program (Princeton, NJ) via the Harvard Brain Tissue Resource Center (Belmont, MA) and the University of Maryland Brain and Tissue Bank (Baltimore, MD). High quality RNA could not be obtained from 2 autism samples (1 and 8). The 9 remaining AD and all control samples were used for mRNA analysis. All 11 autism and 14 control samples were used for ELISA; 9 autism and 9 control samples were used for Western blot analysis because 2 autism and 5 control samples were obtained at a later date and could not be analyzed simultaneously. All of the tissues were from the fusiform gyrus area of the brain. Clinical data on each tissue sample were obtained through the Autism Tissue Program online portal (http://www.atpportal.org) (Tables 1 and 2). The diagnosis of AD was confirmed using the Autism Diagnostic Interview–Revised (68) postmortem through interviews with the parent(s) and/or caregivers. Tissue samples were stored at −80°C before use.

Reagents

The affinity-purified rabbit polyclonal BDNF antibody, N-20, and the peptide used to raise it (mapping at the amino terminus of the mature form of human BDNF) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti–β-actin was obtained from Sigma (Oakville, Ontario, Canada). Horseradish peroxidase–conjugated donkey anti-rabbit and sheep anti-mouse antibodies were obtained from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada). Human recombinant BDNF was a generous gift from Amgen (Thousand Oaks, CA). The BDNF ELISA kits were purchased from Promega (Madison, WI), Chemicon (Temecula, CA), and R&D Systems (Minneapolis, MN). Trizol reagent was from Invitrogen (Burlington, Ontario, Canada). Aprotinin was obtained from Sigma, pepstatin was from Boehringer Mannheim (Laval, Québec, Canada), and phenylmethylsulfonyl fluoride was from Life Technologies (Burlington, Ontario, Canada). Hybond-P polyvinylidene diflouride membranes were purchased from Amersham Pharmacia Biotech. Prestained broad-range protein molecular weight markers and D_C protein assay kit and reagents were from Bio-Rad (Mississauga, Ontario, Canada). DNase treatment kits were purchased from Ambion (Austin, TX). Reagents for reverse transcription (RT) were obtained from Invitrogen (Carlsbad, CA). Primers were synthesized by MOBIX, McMaster University. Platinum SYBR Green qPCR Supermix UDG was obtained from Invitrogen. All other chemicals were reagent grade.

RNA Isolation

RNA isolation, DNase treatment, and real-time RT–polymerase chain reaction were carried out as previously described with modifications (69). Total RNA was isolated using 0.08 to 0.13 g of tissue per sample. Each frozen sample was homogenized in 1 mL/0.1 g wet weight tissue of Trizol reagent on ice using either a Polytron homogenizer (Brinkmann Instruments) or a sonic dismembrator (Fisher Scientific, Ottawa, Ontario, Canada). When using the homogenizer, RNA was purified according to the Trizol reagent instructions. The brain homogenate obtained by the sonication method was purified over RNeasy spin columns (Qiagen, Mississauga, Ontario, Canada) instead of ethanol precipitated. Yield and purity of total cellular RNA were determined by absorbance at 260 and 280 nm.

TABLE 1. Autism Cases

Sample ID No.	Age, yr	Gender	Postmortem Interval, h	Primary Cause of Death	Drug Treatment History	BDNF Protein (Isoform/Total BDNF Ratio)				BDNF Protein (ELISA), pg/mg protein	BDNF mRNA (BDNF/ β-Actin Ratio)
						Pro-BDNF	Truncated BDNF	Mature BDNF	Ratio Total BDNF/β-Actin		
AN01093	56	M	19.48	Anoxic encephalopathy	NA	0.72	0.01	0.27	0.56	76.13	ND
AN00493	27	M	8.3	Drowning	Synthroid	0.34	0.42	0.24	1.83	166.44	1.683
AN00764	20	M	23.7	Auto Trauma	Minocin	0.30	0.45	0.25	2.04	182.46	1.575
AN08792	30	M	20.3	Gastrointestinal bleeding	Phenobarbital, Mysoline, Dilantin, Depakote, Cisapride, Clorazepate, Prolosec, Propulsid, Reglan, Tranxene	0.53	0.17	0.30	4.00	205.82	1.678
AN16115	11	F	12.88	Seizure and drowning in tub	Adderall, Dexadrine, Dilantin, Klonopin, Lamictal, Tegretol, Topomax	0.30	0.44	0.26	1.54	394.25	1.647
AN16641	9	M	27	Seizure disorder	Clonidine, Depakote, Dilantin, Lamictal, Ritalin, Tegretol	0.31	0.36	0.32	2.04	104.48	1.411
UMB1174	7	F	14	Seizure, hypotension	Depakote, Dilantin, Tegretol	0.51	0.10	0.38	0.83	53.11	1.638
UMB1182	9	F	24	Smoke Inhalation	NA	0.84	0.09	0.07	0.31	33.39	ND
UMB797	9	M	13	Drowning	Desipramine	0.25	0.58	0.18	0.86	26.18	1.652
AN08873	5	M	25.5	Asphyxia due to drowning	Prozac	ND	ND	ND	ND	233.65	1.599
AN06420	39	M	13.95	Cardiac tamponade	Synthroid, Depakote, Risperidol, Paxil, Blood pressure medication	ND	ND	ND	ND	133.35	1.740
Mean ± SE	20.1 ± 4.9		18.3 ± 1.8			0.45 ± 0.07	0.29 ± 0.06	0.25 ± 0.03	6.08 ± 1.14	146.30 ± 31.62	1.63 ± 0.03

BDNF, brain-derived neurotrophic factor; F, female; M, male; NA, Information not available; ND, not done.

RNA integrity was examined using agarose gel electrophoresis. RNA samples were stored at −80°C before use.

DNase Treatment and cDNA Synthesis

RNA samples were treated with DNase before RT, according to the manufacturer's protocols (Ambion). cDNA was generated in a reaction mixture containing 2.5 μg of DNase-treated sample, 10 mmol/L each of dATP, dCTP, dGTP, and dTTP (Invitrogen), and 250 ng of random primers (Invitrogen). The samples were incubated at 65°C for 5 minutes followed by addition of 1× First Strand Buffer (3 mmol/L $MgCl_2$, 75 mmol/L KCl, 50 mmol/L Tris-HCl, pH 8.3), 0.01 mol/L dithiothreitol, 40 units of RNaseOUT, and 50 units of Superscript II. The reaction was carried out in a GeneAmp PCR system 2400 thermal cycler (Applied Biosystems, Foster City, CA) at 25°C for 10 minutes, followed by 42°C for 50 minutes, and heat inactivation at 70°C for 15 minutes. A negative control was run in parallel with each RT containing double-distilled water in place of RT (no-RT control).

BDNF Real-time PCR

Primers for BDNF, forward primer 5′-AAA CAT CCG AGG ACA AGG TG-3′ and reverse primer 5′-AGA AGA GGA GGC TCC AAA GG-3′, yielded a 249-bp fragment. Primers for β-actin, forward primer 5′-CTC TTC CAG CCT TCC TTC-3′ and reverse primer 5′-TGT TGG CGT ACA GGT CTT-3′, yielded a 110-bp fragment. Real-time PCR was carried out on a Stratagene MX3000P (La Jolla, CA). Total reaction volume was 20 μL, which included cDNA from 100 ng of RNA and the following components: 300 nmol/L of the BDNF forward and reverse primers, 10 μL of SYBR Green qPCR Supermix UDG (Invitrogen), and 30 nmol/L of Stratagene ROX reference dye. For β-actin, 150 nmol/L of both the forward and reverse primers was used. A negative control containing double-distilled water in place of cDNA was included to ensure that none of the reagents were contaminated. Levels of BDNF and β-actin were determined using absolute quantification with SYBR green. Standards for BDNF were from a BDNF plasmid (pGEM containing the entire BDNF coding region of 755 bp, NCBI Accession No. AY890649), and β-actin standards were from a plasmid obtained from Invitrogen. The thermal profile for BDNF and β-actin: 2 minutes at 50°C, 2 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. PCR product samples analyzed by agarose gel electrophoresis exhibited a single band of the expected size. A dissociation curve after 40 PCR cycles confirmed a single product for both BDNF and β-actin.

Copy numbers and real-time PCR efficiencies were calculated with MXPro MX3000P software (Stratagene). Only experiments in which the real-time PCR efficiency was between 90% and 100% and standard curves yielded $R^2 > 0.990$ were used for analysis. Each sample was run in triplicate. Calculation of differences in BDNF levels between autism and control samples was carried out by 2-tailed Student *t* test

TABLE 2. Control Patients

Sample ID No.	Age, yr	Gender	Postmortem Interval, h	Primary Cause of Death	BDNF Protein (Isoform/Total BDNF Ratio)				BDNF Protein (ELISA), pg/mg protein	BDNF mRNA (BDNF/β-Actin Ratio)
					Pro-BDNF	Truncated BDNF	Mature BDNF	Ratio Total BDNF/β-Actin		
AN12552	56	M	23.61	Traumatic asphyxia and crush injury	0.22	0.63	0.15	0.67	25.51	1.583
AN17344	46	M	25.9	NA	0.41	0.31	0.28	2.05	22.80	1.523
AN14771	30	M	23	Cardiac arrhythmia	0.29	0.49	0.22	1.29	32.99	1.699
UMB818	27	M	10	Multiple injuries	0.29	0.59	0.12	0.45	31.94	1.772
UMB1407	9	F	20	Asthma	0.20	0.67	0.12	0.65	41.23	1.920
UMB1649	20	M	22	Multiple injuries	0.20	0.59	0.21	0.93	61.33	1.595
UMB1706	8	F	20	Rejection of cardiac allograft transplantation	0.17	0.67	0.16	0.42	57.20	1.635
UMB1708	8	F	20	Multiple traumatic injuries	0.26	0.56	0.18	1.05	83.21	1.618
UMB1860	8	M	5	Cardiac Arrhythmia	0.25	0.46	0.30	1.30	42.70	1.625
AN10606	56	M	23	MI	ND	ND	ND	ND	111.62	1.761
AN12240	51	M	4.75	MI	ND	ND	ND	ND	162.14	1.697
AN19760	28	M	23.25	NA	ND	ND	ND	ND	102.66	1.743
AN15240	36	F	18.08	NA	ND	ND	ND	ND	123.01	1.829
AN17425	16	M	26.16	NA	ND	ND	ND	ND	91.65	1.742
Mean ± SEM	28.5 ± 4.8		18.8 ± 1.8		0.25 ± 0.02	0.55 ± 0.04	0.19 ± 0.02	4.89 ± 0.46	71.50 ± 11.15	1.70 ± 0.03

BDNF, brain-derived neurotrophic factor; F, female; M, Male; MI, myocardial infarction; NA, Information not available; ND, not done.

on the means of triplicate C_t values using SPSS (SPSS Inc., Chicago, IL).

BDNF ELISA

Protein isolation and BDNF ELISA were carried out using the BDNF E_{max} ImmunoAssay System (Promega), the human BDNF Quantikine ELISA (R&D Systems), and the ChemiKine BDNF sandwich ELISA kit (Chemicon) according to the manufacturers' protocols. Each sample was initially adjusted to the same protein concentration (0.5 mg/mL for Promega; 1 mg/mL for Chemicon and R&D Systems). In the case of the Promega kit, samples were acidified with 1 mol/L HCl to bring the pH below 2.5 for 15 minutes and then neutralized to pH 7.6 with NaOH. A standard curve using recombinant BDNF protein was run on each ELISA plate. Each sample was tested in triplicate. Two-tailed Student *t* test was carried out on the means of triplicates.

Protein Extraction From Brain Tissue

Protein extraction was performed as previously described with slight modifications (70). Approximately 50 to 100 mg of tissue was homogenized on ice without thawing using a Polytron homogenizer or sonicated on ice using a sonic dismembrator in 10 mL/g wet weight tissue of homogenization buffer (50 mmol/L Tris-HCl pH 7.5, 10 mmol/L ethylenediaminetetraacetic acid, 0.5% Tween-20, 2 μg/mL aprotinin, 2 μg/mL pepstatin, and 100 μg/mL phenylmethylsulfonyl fluoride). The homogenate was incubated for 15 minutes on ice and then centrifuged at 16,000*g* for 15 minutes at 4°C. Supernatants containing solubilized protein were aliquoted and stored at −80°C. Protein concentrations were determined using a D_C protein assay kit.

Western Blotting

Western blotting was carried out as previously described with minor modifications (46, 47). Briefly, 20 μg protein of each sample was resolved in 12% sodium dodecyl sulfate–polyacrylamide gels under reducing conditions. After transfer onto polyvinylidene diflouride membranes, blots were blocked with 10% nonfat milk powder in TBS-T (50 mmol/L Tris-HCl pH 8.0, 133 mmol/L NaCl, 0.1% (v/v) Tween-20), then incubated with primary antibody N-20 (1:1000 dilution in TBS-T) overnight at 4°C and horseradish peroxidase–conjugated donkey anti-rabbit secondary antibody (1:5000 dilution in TBS-T) for 1 hour at room temperature. An enhanced chemiluminescence system (Amersham Pharmacia Biotech) was used for detection. The specificity of the antibody was tested by blocking immunoreactivity with a 5- or 10-fold molar excess of antigenic peptide, according to the manufacturer's protocol (Santa Cruz Biotechnology). All samples were analyzed in 2 to 3 independent Western blots.

Quantification of Western Blots

Each Western blot contained a standard curve consisting of different amounts of protein per lane serving as a control to ensure that the sample loading amount and x-ray film exposure time were in the linear range of detection for BDNF isoforms. The intensities of immunoreactive bands were measured using

ImageJ 1.43 (Wayne Rasband, National Institutes of Health; http://rsb.info.nih.gov/ij) with local background subtracted. For each sample, the relative abundance of each BDNF isoform was expressed as a ratio of that BDNF isoform to the total BDNF signal. Total BDNF was calculated as the sum of pro-BDNF, truncated BDNF, and mature BDNF. For each isoform, the mean of ratios from 2 to 3 blots for each sample was used for statistical analysis. Differences between groups were calculated using 2-tailed Student *t* test.

RESULTS

No Difference in BDNF mRNA Expression Between Autism and Control Samples

There was no significant difference in BDNF mRNA levels in the fusiform gyrus between control (n = 14) and autism (n = 9) samples (2-tailed *t* test, p = 0.35; Fig. 1). Each sample's BDNF C_t value was normalized to its β-actin C_t value. β-actin has been used in the past as a reference gene because its levels do not change in neurological disorders such as Alzheimer disease (45). Investigation of β-actin C_t values showed no significant difference between control and autism samples (p = 0.89, data not shown).

Increased BDNF-Like Immunoreactivity Measured by ELISA in Fusiform Gyrus of Autism Versus Control Subjects

Acid treatment has been found to alter the amount of neurotrophic factor detectable by ELISA in various tissue or sample types (72, 73). Acid treatment disrupts the interaction between BDNF and its receptor, TrkB, or other binding proteins, which may not allow total BDNF to be accurately measured. Therefore, we first tested the effect of acid treatment on our homogenized brain samples. Acidification and subsequent neutralization increased BDNF immunoreactivity detectable by ELISA approximately 4-fold above nonacidified samples (data not shown). Relative differences in BDNF levels between groups did not differ between acidified (Promega) and non-

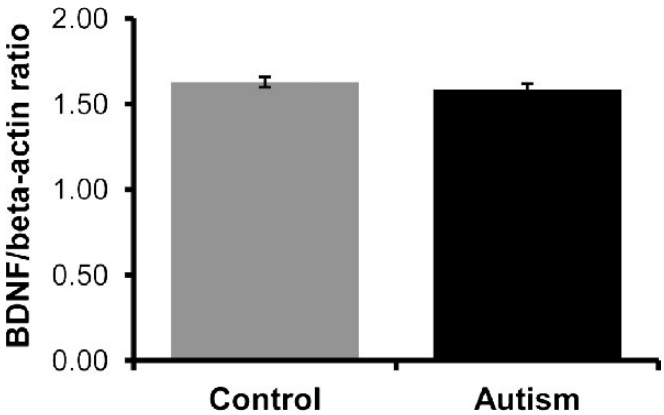

FIGURE 1. Real-time RT-PCR of BDNF mRNA in autism and control samples. BDNF mRNA expression was normalized to β-actin mRNA for each sample. Each sample was measured in triplicate, and the mean was used for statistical analysis. A second independent experiment gave similar results. Mean ± SE is shown for each group. Autism, n = 9; control, n = 14. p > 0.05, 2-tailed *t* test.

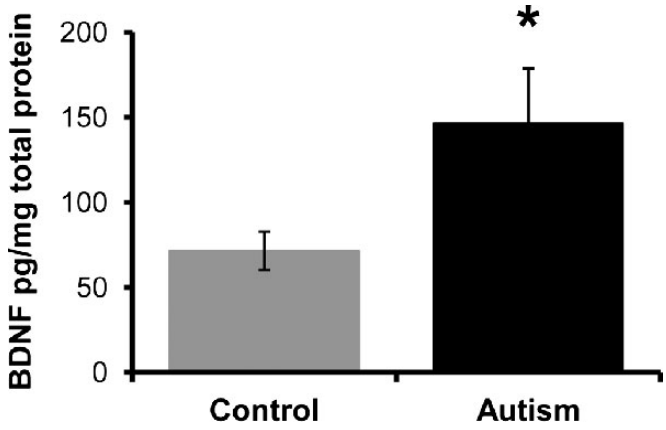

FIGURE 2. BDNF protein levels in fusiform gyrus as measured by ELISA. Each sample was measured in triplicate, and the mean was used for statistical analysis. Data from acidified (Promega) and nonacidified (R&D Systems) assays were combined. Bars indicate mean ± SE. Autism, n = 11; control, n = 14. * p < 0.05 (2-tailed *t* test).

acidified (Chemicon, R&D Systems) methods. Autism subjects exhibited BDNF protein levels in fusiform gyrus 2-fold higher than in controls (p = 0.026; Fig. 2). ELISAs from all 3 kits gave similar results.

BDNF Protein Isoform Balance Measured by Western Blotting

Western blotting using a rabbit polyclonal anti-BDNF immunoglobulin G detected multiple immunoreactive bands in human brain (Fig. 3). Blocking immunoreactivity with an excess of antigenic peptide demonstrated specificity of several bands. The 33-kDa band corresponds to the molecular weight of pro-BDNF. The 28-kDa band in our blots corresponds to a 28-kDa band previously identified as a truncated form of pro-BDNF (59). The 14-kDa–immunoreactive band was identified as mature BDNF because it comigrates with recombinant human BDNF (48, 59). The 24-kDa band has not previously been described and might be an intermediate in the proteolytic processing of pro-BDNF to mature BDNF. This band constituted only a very small fraction (1%–3%) of the total BDNF signal; including it or not in the analysis did not affect the results.

Statistical analysis of the mean ratios of each BDNF isoform to total BDNF pixel values demonstrated a highly significant group × isoform interaction (2-way analysis of

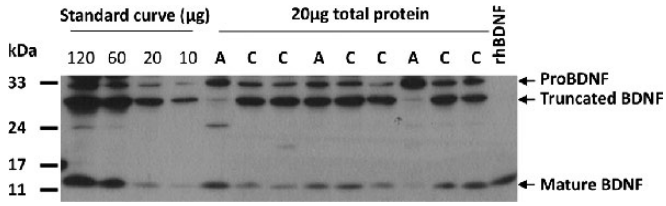

FIGURE 3. Representative Western blot of fusiform gyrus homogenates from autism (A) and control (C) samples. Lanes 1 to 4: different amounts of total protein loaded for standard curve. Lanes 5 to 13: 20 μg of total protein from each autism and control sample. Lane 14: 0.3 ng of recombinant human BDNF.

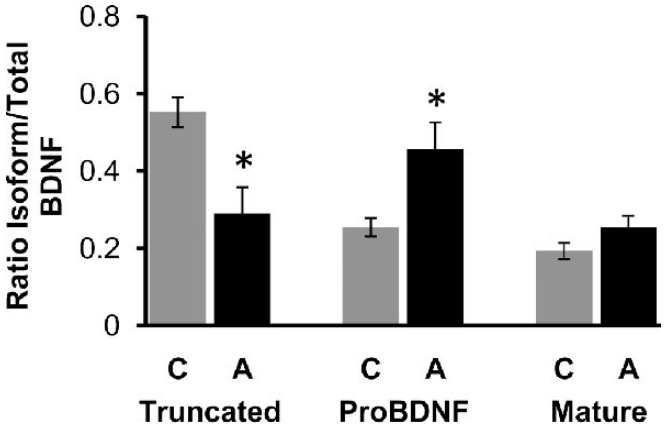

FIGURE 4. Quantification of BDNF in autism and control samples by Western blotting. For each sample, the pixel value of each BDNF isoform was expressed as a ratio to total BDNF pixel values, where total BDNF is the sum of pro-BDNF + 28-kDa truncated BDNF + mature BDNF. ** $p < 0.01$, * $p < 0.05$, 2-tailed *t* test. Bars represent mean ± SE. A = autism, n = 9; C = control, n = 9. Data are averaged from 2 to 3 independent Western blots per sample.

variance, $p < 0.001$). Western blotting revealed a significant decrease in the truncated BDNF/total BDNF ratio in autism samples versus controls (2-tailed *t* test, $p < 0.01$; Fig. 4) and an increase in pro-BDNF (2-tailed *t* test, $p < 0.05$; Fig. 4). The ratio of mature BDNF/total BDNF exhibited a trend toward increased values in autism samples versus controls (2-tailed *t* test, $p = 0.15$; Fig. 4).

No Influence of Age at Death or Postmortem Interval

There was no statistically significant difference observed between the age at death of the control group and age at death of the autism group ($p = 0.12$ for RNA studies, $p = 0.25$ for ELISA, $p = 0.64$ for Western blotting) (Tables 1 and 2). Similarly, there was no significant difference between mean postmortem interval (PMI) for the control group and mean PMI for the autism group ($p = 0.67$ for RNA studies, $p = 0.85$ for ELISA, $p = 0.81$ for Western). Although the PMIs were longer than the 12-hour period required for preservation of mature BDNF protein as reported by Ferrer et al (71), there was no correlation observed between PMI and BDNF protein levels measured by ELISA ($R^2 = 0.19$, $p = 0.36$) or by Western blotting ($R^2 = 0.0015$, $p = 0.62$). Therefore, PMI had little to no influence on BDNF protein levels in our study. Regression analysis showed that there was no correlation between BDNF mRNA levels and either age ($R^2 = 0.026$, $p = 0.46$) or PMI ($R^2 = 0.065$, $p = 0.24$). We did not find a correlation between BDNF protein levels measured by ELISA and age ($R^2 = 0.007$, $p = 0.97$) or between BDNF protein levels measured by Western blotting and age ($R^2 = 0.001$, $p = 0.88$).

Seizure and Drug History

Because seizures have been found to upregulate BDNF mRNA and protein (74–76), subjects who experienced seizures were excluded from the sample population when the tissue was first acquired. This exclusion criterion was to control for the effect of seizures on BDNF expression. However, after the experiments were conducted, subjects 4, 5, 6 and 7 were discovered to have comorbid seizure disorder. Analysis excluding these subjects did not change the group mean values or significance for either mRNA or protein levels measured by all 3 techniques. Comparison of mRNA and protein levels between subjects with autism who had experienced seizures versus subjects free of seizures (*t* tests) did not show any significant differences for mRNA (seizures, n = 4; no seizures, n = 5; $p = 0.43$), BDNF protein determined by ELISA (seizures, n = 4; no seizures, n = 7; $p = 0.34$), pro-BDNF (seizures, n = 4; no seizures, n = 5; $p = 0.62$), or truncated BDNF (seizures, n = 4; no seizures, n = 5; $p = 0.79$). However, levels of mature BDNF were increased in the group with seizures compared with the group with no seizures (seizures, n = 4; no seizures, n = 5; $p = 0.04$) and to the control group (n = 9; $p = 0.005$).

Anticonvulsant medications have been shown to correct BDNF upregulation caused by seizures (77). There were 5 AD subjects (4–7, 11) who were on anticonvulsant medication. A comparison between the AD subjects who were treated with anticonvulsant medication and those who were not (*t* tests) showed no significant differences in the mean ratio of BDNF mRNA (anticonvulsant, n = 5; none, n = 4; $p = 0.97$), BDNF protein determined by ELISA (anticonvulsant, n = 5; none, n = 6; $p = 0.40$), pro-BDNF (anticonvulsant, n = 4; none, n = 5; $p = 0.62$), or truncated BDNF (anticonvulsant, n = 4; none, n = 5; $p = 0.79$). However, there was a significant difference between groups for mature BDNF. The autism group treated with anticonvulsants exhibited significantly more mature BDNF/total than either the untreated autism group ($p = 0.038$) or the normal control group ($p = 0.008$).

DISCUSSION

In this study, we found increased BDNF-immunoreactive protein in fusiform gyrus of subjects with autism by ELISA, consistent with earlier reports. Previous studies reported greater BDNF protein in postmortem basal forebrain tissue (66) and in blood and serum (61, 63–65) of autism samples compared with controls. Our study is the first to examine BDNF protein and mRNA in the fusiform gyrus, an area known to be functionally abnormal in autism. Our study is also the first to examine the mechanism of this increase. Using real-time RT-PCR, we found no difference in levels of BDNF mRNA in the fusiform gyrus of autism patients compared with controls, thereby demonstrating that the increase in BDNF-immunoreactive protein in the same samples is not transcriptionally driven. Importantly, Western blotting demonstrated that the increased BDNF immunoreactivity measured by ELISA was caused by significant changes in the relative levels of pro-BDNF and truncated BDNF isoforms between the 2 groups, suggesting that there may be changes in proteolytic processing of pro-BDNF to truncated BDNF in autism. Previous studies have reported increases in other neurotrophins and cytokines in autism brain tissue (78, 79), indicating that many secreted peptides are altered and suggesting that this could be a general mechanism leading to defective synaptic development and plasticity in autism.

It is unlikely that translation or stability could account for these differences in isoforms. Increased translation could

explain the increase in pro-BDNF but cannot explain the decrease in truncated BDNF unless coupled with a corresponding defect in conversion to truncated BDNF. The lack of correlation between BDNF isoform levels and PMI in this study (in addition to our previously published experiments demonstrating no effect of PMI on BDNF levels [46, 47]) argues against postmortem degradation or protein instability as a mechanism. Previous studies indicate that seizures upregulate both BDNF mRNA and mature BDNF protein (74–76), but we found no significant differences in pro-BDNF or truncated BDNF levels between subjects with and without comorbid seizure disorder and subjects on anticonvulsant medication versus those not on such medication. Therefore, it is unlikely that seizures could account for increased pro-BDNF protein in our study without a concurrent increase in BDNF mRNA. Dysregulation of proteolytic processing in autism, unrelated to seizures, is a possible alternative mechanism. The protease SKI-1 converts pro-BDNF to truncated BDNF (59) and is, therefore, a good candidate for the cause of this dysregulation.

The major difference between previous studies using immunoaffinity chromatography and ELISA and our study including Western assays is that the ELISA measures antibody reactivity in solution, whereas in Western blotting, proteins are separated based on size, allowing one to visualize individual protein isoforms. Using Western blotting, we demonstrated a significant increase in pro-BDNF and a significant decrease in truncated BDNF in autism subjects compared with controls. Thus, the sandwich ELISAs that measure increased BDNF in autism likely detect pro-BDNF but are less sensitive toward truncated BDNF. Consistent with this, total BDNF as measured by Western blotting (pro-BDNF + truncated BDNF + mature BDNF, normalized to β-actin) (Tables 1, 2) did not differ between autism and control groups (*t* test, $p = 0.35$) and did not exhibit the 2-fold difference seen using ELISA. On the other hand, the ratio of pro-BDNF/β-actin measured in Western blots exhibited a trend toward an increase in autism compared with that in control subjects (*t* test, $p = 0.07$), suggesting again that the antibodies used for ELISA detect predominantly pro-BDNF.

Increased pro-BDNF and reduced truncated BDNF in AD strongly suggest the importance of proper balance among all 3 BDNF isoforms. The dramatic increase in pro-BDNF likely has undesirable consequences. The biological properties of pro-BDNF suggest that increases in this isoform may be responsible for reduced neuronal differentiation and dendritic spines and altered synaptic connectivity and neurotransmitter levels found in subjects with autism (35, 53–55). Distortion of the balance between BDNF isoforms could lead to changes in connectivity and synaptic plasticity and, hence, behavior. Our results focus attention on defective proteolytic maturation as a possible new mechanism for altered synaptic plasticity leading to autism.

Indeed, several studies support the hypothesis that deficits or imbalances in BDNF isoforms may lead to neuropsychiatric disorders. A polymorphism in the BDNF prodomain, which reduces pro-BDNF processing and BDNF secretion, correlates with deficits in episodic memory (56, 57); reduced proBDNF and BDNF correlate with cognitive decline in mild cognitive impairment and Alzheimer disease (47); and decreased truncated BDNF correlates with cognitive impairment in schizophrenia (60). Animal studies show that decreased BDNF impairs long-term potentiation, learning, and memory (80–82). Therefore, increased pro-BDNF and reduced truncated BDNF might have equally serious effects on learning, memory, and attention in autism. The biological role of the truncated isoform is unknown, and therefore, the full impact of this change in isoform ratios has yet to be determined.

In summary, we have shown that BDNF mRNA levels are unchanged in fusiform gyrus of subjects with autism compared with controls. BDNF-immunoreactive protein measured by ELISA is greater in subjects with autism than in controls, and Western blotting identifies the responsible molecular species as pro-BDNF. Increased pro-BDNF coupled with decreased truncated BDNF suggests that there may be a deficit in the processing of pro-BDNF into the truncated form in subjects with autism. Such a distortion of the balance of BDNF isoforms may have serious neuropsychiatric consequences.

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