Suicide Brain Is Associated with Decreased Expression of Neurotrophins

Yogesh Dwivedi, Amal C. Mondal, Hooriyah S. Rizavi, and Robert R. Conley

Background: Neurotrophins mediate diverse biological responses, including maintenance and growth of neurons and synaptic plasticity in adult brain. This study examined whether suicide brain is associated with changes in the expression of neurotrophins.

Methods: Messenger ribonucleic acid (mRNA) levels of nerve growth factor (NGF), neurotrophin (NT)-3, NT-4/5, and of cyclophilin and neuron-specific enolase (NSE) were measured by quantitative reverse transcriptase polymerase chain reaction, whereas protein levels of neurotrophins were determined by enzyme-linked immunosorbent assay, in prefrontal cortex (PFC) and hippocampus from 28 suicide victims and 21 control subjects.

Results: In hippocampus of suicide subjects compared with control subjects mRNA levels of NGF (p < .001), NT-3 (p < .001), and NT-4/5 (p < .001) were decreased, whether or not they were expressed as a ratio to cyclophilin or NSE. This was accompanied by a decrease in their respective protein levels (NGF [p < .001], NT-3 [p < .001], and NT-4/5 [p < .001]). In PFC, however, mRNA (p = .001) and protein (p < .001) levels of NT-4/5 and only protein level of NGF (p < .001) were decreased; NT-3 levels were unchanged.

Conclusions: Given the role of neurotrophins in synaptic plasticity and maintenance of adult neurons, our findings of altered expression of neurotrophins in postmortem brain of suicide victims suggest that these molecules might play a vital role in the pathophysiology of suicide.

Key Words: Neurotrophins, suicide, depression, prefrontal cortex, hippocampus, mRNA, ELISA

Suicide represents one of the leading causes of death and is a frequent outcome of major psychiatric disorders (Mann 1998). In recent years, several investigators have examined the neurobiology of suicide; however, the precise molecular and pathophysiologic mechanisms are still unclear.

The role of neurotrophins in directing brain growth and neuronal functioning is being increasingly recognized. Neurotrophins not only play an important role in cellular proliferation, migration, and phenotypic differentiation and/or maintenance in the developing central nervous system (Lewin and Barde 1996; McAllister 2001), but their presence is required in the adult central nervous system for maintenance of neuronal functions, structural integrity of neurons, and neurogenesis (Cooper et al 1996; Sofroniew et al 1990), which suggests that neurotrophins are biologically significant over the entire lifespan. In addition, a number of studies have demonstrated that neurotrophic factors regulate structural and synaptic and morphological plasticity to modulate the strength or number of synaptic connections and neurotransmission (reviewed in McAllister et al 1999; Thoenen 2000). It is pertinent to mention that a number of studies demonstrate structural abnormalities in brains of affective disorder patients (reviewed in Duman 2002; Miguel-Hidalgo and Rajkowska 2002; Rajkowska 2000) and of suicide victims (Altshuler et al 1990; Gould et al 2000; Rajkowska 1997), and altered synaptic plasticity in affective disorder patients has been hypothesized (Duman et al 2000).

Neurotrophins are structurally related homodimeric proteins that include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin (NT)-3, and NT-4/5. They exert their effects after binding to receptor tyrosine kinases (Trk), such as TrkA, TrkB, TrkC, and p75NGFR, in a specific manner (Barbacid 1995; Huang and Reichardt 2001). In a recent study, we demonstrated that the expression of BDNF and of its cognate receptor, TrkB, is significantly reduced in prefrontal cortex (PFC) and hippocampus of suicide victims (Dwivedi et al 2003b). These changes were present in all suicide victims, irrespective of psychiatric diagnosis, indicating abnormalities in BDNF expression and in its functioning in the brain of suicide victims. Several studies indicate that in addition to BDNF, other neurotrophins might play an important role in stress, aggressiveness, and anxiety-like behaviors (Bersani et al 2000; Iannitelli et al 1998). To further evaluate the role of neurotrophins in the pathophysiological mechanisms of suicide, we performed the present study to thoroughly examine for the first time the messenger ribonucleic acid (mRNA) and protein expression of NGF, NT-3, and NT-4/5 in PFC and hippocampus obtained from suicide victims and matched nonpsychiatric control subjects. We also investigated whether the changes in expression of these neurotrophins were associated with major depression or were present in all suicide victims. The results of the present study demonstrate alterations in expression of neurotrophins in postmortem brain of suicide victims and suggest the possible involvement of neurotrophins in the pathophysiology of suicide.

Methods and Materials

The study was performed in Brodmann’s area 9 and hippocampus obtained from the right hemisphere of suicide victims (n = 28) and nonpsychiatric control subjects (n = 21), hereafter referred to as normal control subjects. Brain tissues were obtained from the Brain Collection Program of the Maryland Psychiatric Research Center, Baltimore, Maryland. The detailed diagnostic procedures are provided in our earlier publication (Dwivedi et al 2003b). Family members were interviewed after giving informed consent. This study was approved by the institutional review board of the University of Illinois at Chicago.

All tissues from normal control subjects and suicide victims...
were screened for evidence of neuropathology by experienced neuropathologists at the brain collection program center. The tissues were examined histologically. Fixed sections of PFC were screened with hematoxylin and eosin staining and an antibody to glial fibrillary acid protein. The presence of Alzheimer’s disease, infarcts, demyelinating diseases, or atrophy (or clinical history of these disorders) disqualified use of the subject’s brain from the study. In addition, in each case, screening for the presence of human immunodeficiency virus (HIV) was done in blood samples, and HIV-positive cases were excluded. Toxicology data were obtained by the analysis of urine and blood samples. pH of samples containing reverse-transcribed material were amplified with Hot Tub DNA polymerase, followed by digestion with Xho I and run by 1.5% agarose gel electrophoresis. For cyclophilin, the PCR product was run directly on gel without digestion.

To quantitate, the ethidium bromide-stained bands were excised and counted. The results were calculated as the counts incorporated into the corresponding mRNA amplification product versus the known amount of internal standard (cRNA) added to the test sample. The results are expressed as attomoles/μg of total RNA.

Representative gel electrophoreses showing competitive RT-PCR for NGF, NT-3, and NT4/5 in total RNA isolated from PFC of one normal control subject are given in Figure 1A, B, and C, respectively, whereas competitive PCR analyses of NGF, NT-3, and NT4/5 are given in Figure 1D, 1E, and F, respectively. It was observed that the amplification products for NGF, NT-3, and NT4/5 arise from cRNA at 159 bp, 167 bp, and 167 bp, respectively. A representative competitive RT-PCR gel electrophoresis and the PCR analysis for NSE are presented in Figure 2A and C, respectively, whereas for cyclophilin, the gel electrophoresis and PCR analysis results are given in Figure 2B and D, respectively. The amplification products of NSE and cyclophilin mRNA arise at 304 bp, and 304 bp, respectively, and upon digestion with restriction enzymes generated fragments of 195 + 186 bp for NSE and 239 bp for cyclophilin. To make sure that amplified sequences of NGF, NT-3, NT4/5, NSE, and cyclophilin match the corresponding sequences reported in GenBank, the PCR products were sequenced with M13 primer.

**Table 1. External and Internal Primer Sequences of NGF, NT-3, and NT-4/5 for Amplification**

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Primer</th>
<th>GenBank Accession No.</th>
<th>Nucleotide Position (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>External</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-NGF</td>
<td>F: 5‘ TCA TCC CAT CAC ATC TTC CAC 303–320</td>
<td>E03589</td>
<td>367–387</td>
</tr>
<tr>
<td></td>
<td>R: 5‘ CAG ACA CAG GCC GTA TCT ATC 655–675</td>
<td>M61180</td>
<td>672–692</td>
</tr>
<tr>
<td>NT-3</td>
<td>F: 5‘ GGG GTG ATG TCG TGT GGC ATG CA 220–237</td>
<td>M86528</td>
<td>272–297</td>
</tr>
<tr>
<td></td>
<td>R: 5‘ CCG TGG AGT GTG CAG ACG C 499–518</td>
<td>M22349</td>
<td>596–617</td>
</tr>
<tr>
<td>NT-4/5</td>
<td>F: 5‘ GGG ACT GAG AAA TCC AAG 672–692</td>
<td>XM371409</td>
<td>118–139</td>
</tr>
<tr>
<td></td>
<td>R: 5‘ CTC CAA GGC TTC ACT GTT CTC 400–421</td>
<td></td>
<td>400–421</td>
</tr>
<tr>
<td><strong>Internal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-NGF</td>
<td>5‘ CAG TAC TTT GGG GAC ACC AAG TGC 514–537</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT-3</td>
<td>5‘ CTT GGA GCC CTC GAG CTT GTA TCT C 333–357</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT-4/5</td>
<td>5‘ GCT GAT AAC CTC GAG GAA GAC GCC 427–450</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSE</td>
<td>5‘ GGC AAC AAG CTC GAG ATG CAG GAG TTC 478–504</td>
<td></td>
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</tr>
<tr>
<td>Cyclophilin</td>
<td>5‘ GGT GGC AAG TCC ATC TAT/ 655–675</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bold and italicized letters indicate the mutated bases. Underlined bases indicate the Xho I cleavage site. NGF, nerve growth factor; NT, neurotrophin; F, forward; R, reverse; NSE, neuron-specific enolase.

Determination of mRNA Levels of NGF, NT-3, NT-4/5, Neuron-Specific Enolase, and Cyclophilin by Quantitative Reverse Transcriptase Polymerase Chain Reaction

The detailed procedures for determining mRNA levels by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) are described in our earlier publications (Dwivedi et al 2003a, 2003b). The extent of degradation of mRNA was assessed by evaluating the sharpness of 28S and 18S ribosomal RNA bands. None of the samples used in this study showed detectable signs of degradation.

The quantitation of mRNA levels was performed according to internal standards, as described earlier (Dwivedi et al 2003a, 2003b). The sequences of external and internal primers for NGF, NT-3, NT4/5, neuron-specific enolase (NSE), and cyclophilin are given in Table 1. Each internal standard targeted by the same primers used to amplify the canonic sequence was generated by site-directed mutagenesis to introduce an I restriction site Xho.

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Decreasing concentrations of internal standard complementary RNA (cRNA) were added to 1 μg of total RNA isolated and reverse transcribed. Complementary deoxynucleobase acid aliquots containing reverse-transcribed material were amplified with Hot Tub DNA polymerase, followed by digestion with Xho I and run by 1.5% agarose gel electrophoresis. For cyclophilin, the PCR product was run directly on gel without digestion.

To quantitate, the ethidium bromide-stained bands were excised and counted. The results were calculated as the counts incorporated into the amplified cRNA standard divided by the counts incorporated into the corresponding mRNA amplification product versus the known amount of internal standard (cRNA) added to the test sample. The results are expressed as attomoles/μg of total RNA.

Representative gel electrophoreses showing competitive RT-PCR for NGF, NT-3, and NT4/5 in total RNA isolated from PFC of one normal control subject are given in Figure 1A, B, and C, respectively, whereas competitive PCR analyses of NGF, NT-3, and NT4/5 are given in Figure 1D, 1E, and F, respectively. It was observed that the amplification products for NGF, NT-3, and NT4/5 arise from the mRNA template at 326 bp, 341 bp, and 346 bp, respectively. The digestion products from NGF, NT-3, and NT4/5 arise from cRNA at 167 + 159 bp, 167 + 174 bp, and 167 + 178 bp, respectively. A representative competitive RT-PCR gel electrophoresis and the PCR analysis for NSE are presented in Figure 2A and C, respectively, whereas for cyclophilin, the gel electrophoresis and PCR analysis results are given in Figure 2B and D, respectively. The amplification products of NSE and cyclophilin mRNA arise at 381 bp and 304 bp, respectively, and upon digestion with restriction enzymes generated fragments of 195 + 186 bp for NSE and 239 bp for cyclophilin. To make sure that amplified sequences of NGF, NT-3, NT4/5, NSE, and cyclophilin match the corresponding sequences reported in GenBank, the PCR products were sequenced with M13 primer.

Enzyme-Linked Immunosorbent Assay for NGF, NT-3, NT4/5

Prefrontal cortex or hippocampus was homogenized (1:6, wt/vol) in a buffer containing 100 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deconate, .1% sodium dodecylsulfate, 5 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, leupeptin, pepstatin, and 100 mmol/L sodium orthovanadate. The homogenate was centrifuged at 15,000 g for 10 min at...
4°C, and supernatant was used for all the assays. Protein concentration was determined per Lowry et al (1951).

Levels of \( /H9252\)-NGF, NT-3, and NT-4/5 were determined with the DuoSet ELISA Development System (R & D Systems, Minneapolis, Minnesota). Disposable 96-well plates were coated with 100\( /H9262\) L of capture antibody (mouse antihuman \( /H9252\)-NGF, NT-3, or NT-4/5) and incubated overnight at room temperature. The plates were washed three times with wash buffer (0.05% Tween-20 in phosphate-buffered saline, pH 7.2). Plates were blocked by the addition of 300\( /H9262\) L of blocking buffer (1% bovine serum albumin, 5% sucrose in phosphate-buffered saline with 0.05% NaN\(_3\)) and incubated for 1 hour. After washing with wash buffer, 100\( /H9262\) L of samples were added in each well and incubated for 2 hours at room temperature. The plates were washed, and 100\( /H9262\) L of detection antibody (biotinylated goat antihuman \( /H9252\)-NGF, NT-3, or NT-4/5) were added and incubated for 2 hours at room temperature. Plates were washed, and 1.0 mL of streptavidin–horseradish peroxidase was added and incubated for 20 min at room temperature. After washing the plates with wash buffer, 100\( /H9262\) L of substrate solution (1:1 mixture of color reagent A \( \text{H}_2\text{O}_2\) and B [tetramethylbenzidine]) were added and incubated for 20 min, followed by the addition of 50\( /H9262\) L of stop solution (2 N \( \text{H}_2\text{SO}_4\)). The optical density of each well was read with a microplate reader (Bio-Rad, Hercules, California) at

Figure 1. Representative gel electrophoreses and competitive reverse transcriptase polymerase chain reaction (PCR) analyses of nerve growth factor (NGF), neurotrophin (NT)-3, and NT-4/5. Representative experiments showing competitive PCR analyses for (A) NGF, (B) NT-3, and (C) NT-4/5 mRNA contents in prefrontal cortex of one control subject. Decreasing concentrations of complementary ribonucleic acid (cRNA) for NGF (25–78 pg), NT-3 (12.5–78 pg) or NT-4/5 (12.5–78 pg) were added to a constant amount (1 \( /H9263\)g) of total RNA. The mixtures were reverse transcribed and PCR-amplified in the presence of trace amounts of \( ^{32}\text{P} \) deoxyctydine triphosphate; aliquots were digested by Xho I and electrophoresed on 1.5% agarose gel. The higher molecular size band corresponds to the amplification product arising from the mRNA, whereas the lower band arises from cRNA generated from the internal standard digested by Xho I. Data derived from the agarose gel are plotted as the counts incorporated into the amplified cRNA standard divided by the counts incorporated into the corresponding (D) NGF, (E) NT-3, or (F) NT-4/5 mRNA amplification product versus the known amount of internal standard cRNA added to the test sample. The point of equivalence represents the amount of the respective messenger RNA.

Figure 2. Representative competitive polymerase chain reaction (PCR) analysis of neuron-specific enolase (NSE) and cyclophilin messenger ribonucleic acids (mRNAs). A representative experiment showing a competitive PCR analysis for (A) NSE and (B) cyclophilin mRNA contents in prefrontal cortex (PFC) obtained from one normal control subject. Decreasing concentrations of standard complementary RNA (cRNA) (100–6.25 pg for NSE and 200–12.5 pg for cyclophilin) were added to a constant amount (1 \( /H9263\)g) of total RNA isolated from PFC. The mixtures were reverse transcribed and PCR amplified in the presence of trace amounts of \( ^{32}\text{P} \) deoxyctydine triphosphate; aliquots were digested by Xho I and electrophoresed on 1.5% agarose gel. Cyclophilin cRNA did not require digestion with restriction enzyme. The higher molecular size band corresponds to the amplification product arising from the mRNA, whereas the lower bands arise from cRNA generated from the internal standard digested by Xho I. Data derived from the agarose gels are plotted as the counts incorporated into the amplified cRNA standard divided by the counts incorporated into the corresponding mRNA amplification product versus the known amount of internal standard cRNA added to the test sample. The point of equivalence represents the amount of (C) NSE or (D) cyclophilin mRNA.
the wavelengths of 450 nm and 570 nm. The concentrations of the neurotrophins were determined from the regression line for the standard (generated with recombinant human [rh] β-NGF, NT-3, or NT-4/5, ranging from 15 pg/mL to 480 pg/mL). Neurotrophin concentrations are expressed as nanograms per milligram of protein. All brain samples were assayed in triplicate in determinations. The inter- and intra-assay variations were <4% and <6%, respectively.

The specificity of each antibody used has been tested by the provider. For NGF, the cross reactivities were as follows: ≤0.0625% with rhNT-3, rhBDNF, rhGDNF (glial cell line-derived neurotrophic factor), rhNT-4, rhNeurturin, recombinant rat [rr]GDNF Rα, rrGDNF, rhCNTF (ciliary neurotrophic factor), and rrCNTF; ≤2.61% with recombinant rabbit [rr]β-NGF; ≤35% with recombinant mouse [rm]β-NGF; interference: none with rrGDNF Rα, rhCNTF, rrCNTF, rhBDNF, rhGDNF, rhNT-4, rhGDNF. For NT-3, the cross reactivities were as follows: ≤0.0625% with rhBDNF, rh-BFG, rhNT-4, rhCNTF, and rhGDNF; interference: none with rhBDNF, rhβ-NGF, rhNT-4, rhCNTF, and rhGDNF. For NT-4/5, the cross reactivities were as follows: ≤0.0625% with rhGDNF, rhCNTF, rrCNTF, rrGDNF Rα, rhNeurturin, rhβ-NGF, rhNT-3, rhBDNF, rhGDNF, and rhβ-NGF; interference: none with rhβ-NGF, rhβ-NGF, rhNT-3, rrGDNF Rα, rhCNTF, rrCNTF, rhBDNF, rhGDNF, and rhGDNF.

We also tested the specificity of the antibodies by using human recombinant proteins for NT-3, NT-4/5, and NGF. Protein (100 μg) from hippocampus of two control subjects or 20 ng of recombinant NT-3, NT-4/5, and NGF proteins were electrophoresed on 15% polyacrylamide gels. Blots were probed with NT-3, NT-4/5, and NGF proteins were electrophoresed as described in Methods and Materials. The blots were incubated with (A) anti-NT-3, (B) anti-4/5, (C) or anti-NGF.

Figure 3. Representative Western blots showing the specificities of the neurotrophin antibodies. Recombinant proteins for neurotrophin (NT)-3, NT-4/5 and nerve growth factor (NGF) and two protein samples from hippocampus of normal control subjects (C1 and C2) were electrophoresed as described in detail in Methods and Materials. The blots were incubated with (A) anti-NT-3, (B) anti-4/5, (C) or anti-NGF.

to-noise ratio determination, and with all the neurotrophins the ratio was between 25 and 35.

Statistical Analysis
Data analyses were performed with SPSS 12 software (SPSS, Chicago, Illinois). All values are the mean ± SD. The differences in various measures between suicide victims and normal control subjects were analyzed with the independent-sample t test. All p values were two-tailed. The relationships between the mRNA and the protein levels of NGF, NT-3, NT4/5, and postmortem interval (PMI), age, and pH of the brain were determined by linear regression analyses. The effect of gender within each brain region was analyzed with two-way analysis of variance (ANOVA). The overall group differences in levels of NGF, NT-3, or NT4/5 between normal control subjects, depressed suicide victims, and suicide victims with other psychiatric disorders were evaluated by one-way ANOVA, followed by post hoc (Tukey) comparisons between groups. During analysis of the data, race was included as a potential confounding variable. Statistical significance was assumed at p < .05. The Bonferroni correction for multiple testing was applied.

Results
The demographic characteristics of suicide victims (n = 28) and normal control subjects (n = 21) have been provided earlier (Dwivedi et al 2003b). The mean (±SD) age of normal control subjects and suicide victims was 42.9 ± 13.9 years and 40.1 ± 16.2 years, respectively. The mean PMI was 18.7 ± 7.6 hours for normal control subjects and 19.3 ± 6.9 hours for suicide victims. The brain pH for normal control subjects was 6.1 ± 4 and for suicide victims was 6.2 ± 4. There were 4 female and 17 male subjects in the control group and 9 female and 19 male subjects in the suicide group. Among control subjects there were 9 blacks and 17 whites, whereas in the suicide group were 5 blacks and 23 whites. There were no significant differences in age [t(47) = .63, p = .53], PMI [t(45) = .29, p = .77], or pH of the brain [t(47) = 1.00, p = .32] between suicide victims and normal control subjects.

Comparison of mRNA Levels of NGF, NT-3, and NT4/5 Between Normal Control Subjects and Suicide Victims
To obtain quantitative measurements it is important that the amount of RNA remain constant between samples. To control for any variation between samples, we used the constitutively expressed cytoskeletal protein cyclophilin as well as NSE (present in both neurons and glia). There were no significant differences in mRNA levels of NSE in PFC [t(47) = 1.4, p = .16] and hippocampus [t(40) = 1.4, p = .17] of suicide victims and

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normal control subjects. Similarly, mRNA levels of cyclophilin were not significantly different between normal control subjects and suicide victims in both PFC \( t(47) = .74, p = .46 \) and hippocampus \( t(40) = .46, p = .65 \).

Scatter plots showing the ratios of NT-4/5 to NSE (NSE: \( t(40) = 4.8, p < .001 \); cyclophilin: \( t(40) = 4.4, p < .001 \)), NT-3 [NSE: \( t(40) = 4.8, p < .001 \); cyclophilin: \( t(40) = 3.5, p = .001 \)], and NT-4/5 [NSE: \( t(40) = 4.7, p < .001 \); cyclophilin: \( t(40) = 3.9, p < .001 \)] were significantly decreased in hippocampus of suicide victims when expressed as a function of the respective NSE (Figure 4B) or cyclophilin mRNA content (Figure 4D).

### Comparison of Protein Levels of NGF, NT-3, and NT4/5 Between Normal Control Subjects and Suicide Victims

Scatter plots showing the results of protein levels of NGF, NT-3, and NT-4/5 are given in Figure 5A and B for PFC and hippocampus, respectively. Comparison between suicide victims and normal control subjects showed that the levels of NGF and NT-4/5 were decreased in both PFC [NGF: \( t(47) = 8.92, p < .001 \); NT-4/5: \( t(40) = 7.72, p < .001 \)] and hippocampus [NGF: \( t(40) = 9.15, p < .001 \); NT-4/5: \( t(40) = 9.2, p < .001 \)] of suicide victims. On the other hand, the level of NT-3 was decreased only in hippocampus \( t(40) = 9.62, p < .001 \) but not in PFC \( t(47) = 1.50, p = .14 \) of suicide victims.

### Correlation Between mRNA and Protein Levels of NGF, NT-3, and NT-4/5

To examine whether the decreases in protein levels of neurotrophins were associated with their respective mRNA levels, we correlated mRNA and protein levels of neurotrophins in the combined control and suicide groups. Interestingly, we observed a significant correlation between mRNA and protein levels of NGF (\( r^2 = .28, p < .001 \)), NT-3 (\( r^2 = .28, p < .001 \)), and NT-4/5 (\( r^2 = .25, p = .001 \)) in hippocampus and between mRNA and protein levels of NT-4/5 (\( r^2 = .24, p < .001 \)) in PFC.

### Effects of Diagnosis

To examine whether the differences in mRNA and protein levels of NGF, NT-3, and NT-4/5 between normal control subjects and suicide victims were related to depression or were present in all suicide victims, we examined the effect of the diagnosis of major depression on these measures. For this purpose, we stratified the suicide victims into those who were diagnosed with major depression and those who were diagnosed with other psychiatric disorders or had no mental illness. Out of 28 suicide victims, 11 had major depression. Of the suicide victims with other psychiatric disorders, there were 4 with adjustment and/or conduct disorders, 2 with schizoaffective disorder, 1 with bipolar affective disorder, 3 with drug/alcohol abuse, 1 with polysubstance abuse, 1 with schizophrenia, and 3 had no diagnosed psychiatric illness. In 2 suicide victims the diagnosis was not available. For the subjects whose hippocampi were available for analysis (\( n = 21 \)), all the suicide victims were
the same as described above, except that not included were 3 suicide victims with major depression, 2 suicide victims whose diagnosis of psychiatric illness was not available, 1 suicide victim with adjustment disorder, and 1 suicide victim with polysubstance abuse. The mRNA and protein levels of NGF, NT-3, and NT-4/5 did not differ between suicide victims with major depression and suicide victims with other psychiatric disorders; however, both these groups showed significant differences when compared with normal control subjects, in both PFC (Table 2) and hippocampus determinations. Suicide group was compared with control group. *p < .001.

**Effects of Confounding Variables**

The effects of potential confounding variables, namely, age, gender, PMI, or pH of the brain, were evaluated with respect to the mRNA and protein levels of NGF, NT-3, and NT-4/5 in which we had found differences between normal control subjects and suicide victims. We found no significant effects of age on mRNA levels of NGF, NT-3, or NT-4/5 either in PFC (NGF: $r^2 = .06, p = .09$; NT-3: $r^2 = .07, p = .07$; NT-4/5: $r^2 = .03, p = .23$) or in hippocampus (NGF: $r^2 = .006, p = .62$; NT-3: $r^2 = .001, p = .94$; NT-4/5: $r^2 = .001, p = .6$). In addition, we did not find significant effects of PMI on mRNA or protein levels of NGF, NT-3, or NT-4/5 either in PFC (mRNA: NGF, $r^2 = .004, p = .66$; NT-3, $r^2 = .002, p = .79$; NT-4/5, $r^2 = .002, p = .77$; protein: NGF, $r^2 = .01, p = .49$; NT-3, $r^2 = .01, p = .44$; NT-4/5, $r^2 = .002, p = .77$) or in hippocampus (mRNA: NGF, $r^2 = .02, p = .44$; NT-3, $r^2 = .005, p = .67$; NT-4/5, $r^2 = .002, p = .76$; protein: NGF, $r^2 = .001, p = .88$; NT-3, $r^2 = .01, p = .46$; NT-4/5, $r^2 = .009, p = .56$). Furthermore, no significant effects of pH of the brain were observed on mRNA or protein levels of NGF, NT-3, or NT-4/5 either in PFC (mRNA: NGF, $r^2 = .02, p = .36$; NT-3, $r^2 = .02, p = .31$; NT-4/5, $r^2 = .01, p = .41$; protein: NGF, $r^2 = .01, p = .36$; NT-3, $r^2 = .02, p = .28$; NT-4/5, $r^2 = .02, p = .28$) or in hippocampus (mRNA: NGF, $r^2 = .04, p = .19$; NT-3, $r^2 = .09, p = .06$; NT-4/5, $r^2 = .08, p = .61$; protein: NGF, $r^2 = .03, p = .18$; NT-3, $r^2 = .01, p = .52$; NT-4/5, $r^2 = .01, p = .51$).

There were 17 men and 4 women in the control group and 19 men and 9 women in the suicide group. Comparison studies between normal control subjects and total suicide victims showed no significant differences in any of the measures between men and women in mRNA or protein levels either in PFC [mRNA: NGF, $t(47) = .18, p = .86$; NT-3, $t(47) = .53, p = .59$; NT-4/5, $t(47) = .49, p = .62$; protein: NGF, $t(47) = .79, p = .43$; NT-3, $t(47) = .18, p = .86$; NT-4/5, $t(47) = 1.07, p = .29$] or hippocampus [mRNA: NGF, $t(40) = 1.2, p = .24$; NT-3, $t(40) = .24, p = .81$; NT-4/5, $t(40) = .17, p = .87$; protein: NGF, $t(40) = .76, p = .45$; NT-3, $t(40) = 1.50, p = .14$; NT-4/5, $t(40) = 1.50, p = .14$].

**Discussion**

Using human-specific antibodies and enzyme-linked immunosorbent assay, we compared protein levels of neurotrophins within and across two different brain regions in normal control subjects and suicide subjects. We observed that protein levels of NGF, NT-3, and NT-4/5 were similar within PFC and hippocampus, as well as when compared between PFC and hippocampus. Our findings do not seem to be artifact-associated because we observed similar findings when mRNA levels of these neurotrophins were determined quantitatively with human-specific primers and competitive RT-PCR.

When we compared neurotrophin levels between suicide victims and normal control subjects, we observed significant differences. Both mRNA and protein levels of NGF, NT-3, and NT-4/5 were significantly decreased in hippocampus of suicide victims as compared with normal control subjects. On the other hand, whereas both mRNA and protein levels of NT-4/5 were decreased, only protein level of NGF was decreased in PFC of suicide victims. Interestingly, levels of NT-3 were unchanged in PFC. The changes in neurotrophin levels were not correlated with gender, pH of the brain, PMI, or age. In addition, we also observed that the decrease in protein level of NT-4/5 in PFC of suicide victims was almost of the same magnitude as that of its mRNA. Similarly, the decreases in mRNA and in protein levels of NGF, NT-3, and NT-4/5 were of similar magnitude in hippocampus of suicide subjects, and there were significant correlations between protein and mRNA levels of NT-4/5 in PFC and of NGF, NT-3, and NT-4/5 in hippocampus, which suggests the specificity of the measurements of the various neurotrophins in the two brain areas.
The observed decreases in the levels of neurotrophins in both PFC and hippocampus might be of relevance in suicidal behavior. The PFC plays a major role in mood regulation and has been implicated in the pathophysiology of affective disorders and suicide (George et al. 1994). On the other hand, the hippocampus is involved in cognition (Sweatt 2004) and is the primary brain area affected by stress (Sala et al. 2004), one of the major factors in suicidal behavior (Clayton 1985; Monk 1987). Interestingly, structural abnormalities in cortical and hippocampal brain areas and reduced hippocampal plasticity have been demonstrated in affective disorder patients and during stress (MacQueen et al. 2003; McEwen 1999; Miguel-Hidalgo and Rajkowska 2002; Raj

### Table 2. mRNA and Protein Expression of NGF, NT-3, and NT-4/5 in Prefrontal Cortex of Suicide Subjects with MDD and Suicide Subjects with Other Psychiatric Disorders

| Variable    | Control Subjects (n = 21) (1) | Suicide Subjects (n = 28) | Overall ANOVA | Multiple Comparison
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Mean SD</td>
<td>Mean SD</td>
<td></td>
<td>df F p 1 vs. 2 1 vs. 3 2 vs. 3</td>
</tr>
<tr>
<td>NGF mRNA</td>
<td>24.95 8.47</td>
<td>24.37 7.28</td>
<td>2.44 1.17 .32</td>
<td>.84 .15 .29</td>
</tr>
<tr>
<td>NT-3 mRNA</td>
<td>36.14 10.88</td>
<td>33.96 10.58</td>
<td>2.44 .15 .86</td>
<td>.59 .91 .69</td>
</tr>
<tr>
<td>NGF Protein</td>
<td>.30 .06</td>
<td>.19 .01</td>
<td>2.44 37.59 &lt;.001</td>
<td>&lt;.001 &lt;.001 .49</td>
</tr>
<tr>
<td>NT-3 Protein</td>
<td>.31 .06</td>
<td>.28 .10</td>
<td>2.44 .85 .43</td>
<td>.43 .21 .75</td>
</tr>
<tr>
<td>NT-4/5 Protein</td>
<td>.33 .03</td>
<td>.23 .06</td>
<td>2.44 28.52 &lt;.001</td>
<td>&lt;.001 &lt;.001 .96</td>
</tr>
</tbody>
</table>

mRNA, messenger ribonucleic acid; NGF, nerve growth factor; NT, neurotrophin; MDD, major depressive disorder; ANOVA, analysis of variance.

| Variable    | Control Subjects (n = 21) (1) | Suicide Subjects (n = 28) | Overall ANOVA | Multiple Comparison
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Mean SD</td>
<td>Mean SD</td>
<td></td>
<td>df F p 1 vs. 2 1 vs. 3 2 vs. 3</td>
</tr>
<tr>
<td>NGF mRNA</td>
<td>32.72 10.02</td>
<td>19.74 7.36</td>
<td>2.39 11.62 &lt;.001</td>
<td>.001 &lt;.001 .99</td>
</tr>
<tr>
<td>NT-3 mRNA</td>
<td>45.91 13.11</td>
<td>30.09 8.07</td>
<td>2.39 9.43 &lt;.001</td>
<td>.002 &lt;.001 .93</td>
</tr>
<tr>
<td>NT-4/5 mRNA</td>
<td>47.85 7.50</td>
<td>34.50 8.48</td>
<td>2.39 12.12 &lt;.001</td>
<td>.002 &lt;.001 .59</td>
</tr>
<tr>
<td>NGF Protein</td>
<td>.46 .02</td>
<td>.35 .05</td>
<td>2.39 50.71 &lt;.001</td>
<td>&lt;.001 &lt;.001 .06</td>
</tr>
<tr>
<td>NT-3 Protein</td>
<td>.35 .04</td>
<td>.22 .02</td>
<td>2.39 45.42 &lt;.001</td>
<td>&lt;.001 &lt;.001 .68</td>
</tr>
<tr>
<td>NT-4/5 Protein</td>
<td>.35 .05</td>
<td>.23 .03</td>
<td>2.39 42.5 &lt;.001</td>
<td>&lt;.001 &lt;.001 .50</td>
</tr>
</tbody>
</table>

mRNA, messenger ribonucleic acid; NGF, nerve growth factor; NT, neurotrophin; MDD, major depressive disorder; ANOVA, analysis of variance.

For multiple comparison, the p values are compared with a Bonferroni-adjusted $\alpha = .05/6 = .008$. Multiple comparison tests with $\alpha = .008$ were considered significant.

mRNA equals attomoles per microgram of total RNA.

Protein values are expressed as nanograms per microgram of protein.
kowska 2000; Sheline et al 2003; Sapolsky 1996, 2000; Woolley et al 1990). Some studies even suggest structural abnormalities in brain of suicide victims (Altshuler et al 1990; Gould et al 2000; Rajkowska 1997). The reduced expression of neurotrophins we observed could possibly be associated with such structural abnormalities and reduced hippocampal plasticity.

Interestingly, we observed a significant correlation between the mRNA and protein levels of NT-3 and of NT-4/5 in PFC and hippocampus of suicide victims. This suggests that the decrease in amount of these neurotrophins could be due to reduced transcription. On the other hand, whereas a significant correlation was observed in mRNA and protein levels of NGF in hippocampus of suicide victims, a dissociation between mRNA and protein levels of NGF was noted in the PFC. Although NGF functions in PFC might be decreased because of decreased protein levels, the mechanism for this decrease is not clear. It is quite possible that regulation of NGF mRNA expression might be different in different brain regions. For example, regulation of NGF mRNA is unique in hippocampus, where NGF is localized in a particular type of γ-aminobutyric acid (GABA)ergic interneurons. It has been shown that a majority of the paravallum-positive neurons express NGF, but only a portion of calretinin-positive neurons are positive for NGF (Pascual et al 1998). Because GABAergic septohippocampal fibers terminate on hippocampal NGF-positive neurons, NGF expression might be modulated by GABA.

A number of studies suggest that neurotrophins are regulated in response to stress (Alfonso et al 2004; Scaccianoce et al 2000; Smith et al 1995; Ueyama et al 1997). Whether stress might have affected the levels of neurotrophins in the brain of the suicide victims in our cohort is not clear; however, such a possibility cannot be ruled out, because there is a strong relationship between stress and suicidal behavior (reviewed by Lopez et al 1997), and a dysregulated stress system has been demonstrated in suicide victims (Arato et al 1989; Hiroi et al 2001; Lopez et al 1992; Nemeroff et al 1988).

The precise mechanisms are still to be elucidated; however, our findings of decreased levels of NT-3, NT-4/5, and NGF in suicide might be of relevance to its pathophysiology. Neurotrophins play a critical role not only in neuronal and glial development and function, but also in cell survival, maintenance, and the functional and structural integrity of the adult brain. More recently, the role of neurotrophins in neuronal plasticity has been reported, showing that neuronal activity regulates the expression, secretion, and signaling of neurotrophins to induce specific changes in synaptic efficacy and synapse morphology (Poo 2001). Interestingly, an emerging hypothesis of the pathophysiology of affective disorders and suicide emphasizes the modulation of synaptic plasticity, neurogenesis, and cellular viability (Duman 2004; Duman et al 2000). Although this is speculative in nature, it is possible that the reported structural abnormalities in brains of affective disorder patients and of suicide victims, which include the loss of neurons and glia and atrophy of the brain, could be associated with insufficient levels of neurotrophins.

Because depression is a major factor in suicidal behavior, and a recent preclinical study demonstrates the involvement of NGF in depression (Angelucci et al 2000), we determined whether the observed effects on the levels of neurotrophins were specific to depression by comparing suicide victims who were diagnosed with major depression with those who had other psychiatric disorders. Our results demonstrate that the levels of NT-3, NT-4/5, and NGF were decreased not only in depressed suicide victims but also in those suicide victims who had other psychiatric disorders. Although changes in levels of neurotrophins were observed in all suicide subjects irrespective of psychiatric diagnosis, whether these changes are specific only to suicide subjects is not clear at present. The possibility that changes in neurotrophin levels might also occur in non-suicide subjects cannot be ruled out. For example, patients with schizophrenia (Sugai et al 2004; reviewed by Durany and Thome 2004) or depression (Karege et al 2002; Shimizu et al 2003) show altered levels of BDNF in postmortem brain or in serum. Further studies are required to examine the specificity of such changes.

Although from this study it is not possible to localize changes anatomically, many studies show that NT-3 affects the function, sprouting, and regrowth of 5-hydroxytryptamine (5HT)-containing neurons (Mamounas et al 1995), as well as survival and biological activity of dopaminergic and noradrenergic neurons (Arenas and Persson 1994; Hyman et al 1994). It has been shown that chronic infusion of NT-3 into the rat midbrain increases the turnover of 5HT and the levels of noradrenaline in many brain areas (Altar et al 1994; Martin-Iverson et al 1994). In this context, it is pertinent to mention that a number of studies suggest both serotonergic and noradrenergic abnormalities in suicide. For example, we and other investigators have shown altered expression of 5HT2A receptors (Arango et al 1990; Mann et al 1986; Pandey et al 2002) and of pre- and postsynaptic 5HT binding sites (Arango et al 1995) in ventrolateral PFC of suicide victims. Also, abnormalities in norepinephrine transporters (Klimek et al 1997), fewer pigmented neurons (Arango et al 1996), and reduced tyrosine hydroxylase immunoreactivity (Biegon and Field�� 1992) in the locus coeruleus, an important target of NT-3, have been reported in depressed and suicide subjects. As far as NT-4/5 is concerned, it supports the expression of phenotypes and the survival of cholinergic (Alderson et al 1993), noradrenergic (Bothwell et al 1993; Friedman et al 1993), and dopaminergic neurons (Hyman et al 1994). In addition, NT-4/5 increases Ca** buffering capacity and survival of calbindin-expressing neurons (Alexi and Hefti 1996; Copray et al 1994). It has been suggested that calbindin is associated with hippocampal vulnerability to neuronal damage (Sloviter 1989). On the other hand, it has been suggested that NGF ameliorates neuronal degeneration in rat cortex and hippocampus after brain insults (Buchan et al 1990; Shigeno et al 1991), which indicates that NGF possesses protective functions in these brain regions. Also, NGF is implicated in cognitive functions and is well known for its dramatic effects on neurite outgrowth (Thoenen 1991) and plays a role in synaptic plasticity (Prakash et al 1996).

In conclusion, given the role of neurotrophins in synaptic plasticity and in supporting various populations of neurons, our findings of reduced expression of neurotrophins in postmortem brain of suicide victims suggest that these neurotrophins might play a role in the etiology and the pathophysiology of suicide.

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Althuлер LL, Casanova MF, Goldberg TE, Kleinman JE (1990): The hippocampus and parahippocampus in schizophrenia, suicide, and control brains. *Arch Gen Psychiatry* 47:1029–1034.


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