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Neurotrophin Receptor Activation and Expression in Human Postmortem Brain: Effect of Suicide

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Abstract

Background—The physiological functions of neurotrophins occur through binding to two different receptors: pan75 neurotrophin receptor ($p75^{NTR}$) and a family of tropomysin receptor kinases (Trks A, B, and C). Recently, we reported that expression of neurotrophins and TrkB were reduced in brains of suicide subjects. Present study examines whether expression and activation of Trk receptors and expression of $p75^{NTR}$ are altered in brain of these subjects.

Methods—Expression levels of TrkA, B, C, and of $p75^{NTR}$ were measured by quantitative RT-PCR and Western blot in prefrontal cortex (PFC) and hippocampus of suicide and normal control subjects. The activation of Trks was determined by immunoprecipitation followed by Western blotting using phosphotyrosine antibody.

Results—In hippocampus, lower mRNA levels of TrkA and TrkC were observed in suicide subjects. In the PFC, the mRNA level of TrkA was decreased, without any change in TrkC. On the other hand, the mRNA level of $p75^{NTR}$ was increased in both PFC and hippocampus. Immunolabeling studies showed similar results as observed for the mRNAs. In addition, phosphorylation of all Trks was decreased in hippocampus, but in PFC, decreased phosphorylation was noted only for TrkA and B. Increased expression ratios of $p75^{NTR}$ to Trks were also observed in PFC and hippocampus of suicide subjects.

Conclusions—Our results suggest not only reduced functioning of Trks in brains of suicide subjects but that increased ratios of $p75^{NTR}$ to Trks indicate possible activation of pathways that are apoptotic in nature. These findings may be crucial in the pathophysiology of suicide.

Keywords

Trk; $p75^{NTR}$; depression; suicide; postmortem brain; gene expression

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Introduction

Neurotrophins are a family of secreted proteins that include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin (NT)-3, and NT-4/5. These neurotrophins are essential for regulating neuronal differentiation in the developing brain but also are crucial for trophic support, maintenance of differentiated neuronal phenotypes, neurogenesis, synaptic formation, and regulation of synaptic connections in adult neurons as well as in activity-dependent plasticity, which is a defining feature of the brain throughout life (1-6). Neurotrophins are unique in using two different classes of cell surface receptors to exert their biological actions: 1) the tropomysin receptor kinase (Trk) and 2) pan75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor α receptor superfamily (7). The dual receptor system accounts for the diverse effects exerted by neurotrophins.

There are several subtypes of Trks, which are characterized by a specific affinity for the different neurotrophins. For example, NGF binds preferentially to TrkA, whereas BDNF and NT-4/5 show high affinity for TrkB. NT-3, on the other hand, binds to TrkC with high affinity but can also bind to TrkA and TrkB with lower affinity (8,9). Structurally, the extracellular domain of Trk receptors consists of a cysteine-rich cluster, followed by three leucine-rich repeats, another cysteine-rich cluster and two immunoglobulin-like domains, involved in ligand binding. The cytoplasmic domain consists of a tyrosine kinase domain surrounded by several tyrosines. Ligand binding to Trk receptors causes their dimerization and results in receptor autophosphorylation of kinases present in the cytoplasmic domain. Once phosphorylated, Trk receptors become scaffolding structures that recruit adaptor proteins that couple the receptor to downstream signaling pathways, resulting in alterations in gene expression and neuronal functioning (10). Tyrosine kinase activity is thus essential for the vast majority of Trk receptor-mediated responses to neurotrophins (1,11,12). Both Trk receptors and $p75^{NTR}$ are expressed highly in human cortical and hippocampal brain areas (13-16).

$p75^{NTR}$ initially discovered as a low-affinity receptor for NGF, is now known as a class of receptor that can bind to all neurotrophins with equivalent nanomolar affinities (17). The 3.8 kb mRNA for $p75^{NTR}$ encodes a 427 amino acid protein containing a 28 amino acid single peptide, a single transmembrane domain, and a 55 amino acid cytoplasmic domain (18). Although $p75^{NTR}$ receptors do not contain a catalytic motif, they interact with several proteins, including Trk receptors, which causes enhancement of ligand specificity and ligand affinities for Trk receptors (19-21).

Several studies suggest that BDNF may be involved in stress and depressive behavior (22-27), and that the beneficial effects of antidepressants are associated with an upregulation of BDNF expression (28-30). In a previous study, we reported that expression of BDNF is lower in postmortem brains of suicide subjects (31), which was associated with decreased expression of its cognate receptor TrkB (31). In addition, we recently reported altered expression of NGF, NT-3, and NT-4/5 in suicide subjects in a brain region-specific manner (32). The role of neurotrophins in suicide is further substantiated by other investigators who showed altered levels of neurotrophins in suicide brains or in peripheral tissues of suicidal patients (33-35).

Since the physiological functions of neurotrophins require binding to Trk receptors and their successive phosphorylation, examining the expression and functional activation of neurotrophin receptors is an important step in understanding the significance of the role of neurotrophins in a disease state. Therefore, in the present investigation, we examined activation of Trks A, B, and C in the postmortem brains of suicide subjects. In addition, we examined the expression levels of TrkA, TrkC, and $p75^{NTR}$ in these brain areas. Our study provides further

insight into the role of neurotrophins and their receptors in the pathophysiologic mechanisms of suicide.

Methods and Materials

Subjects

Brain tissues were obtained from the Maryland Brain Collection at the Maryland Psychiatric Research Center, Baltimore, MD. We used the same brain samples in which we had studied expression of neurotrophins and TrkB (31,32). The study was performed in the PFC (Brodmann's area 9) and hippocampus obtained from suicide subjects (n = 28) and nonpsychiatric control subjects (n = 21), hereafter referred to as normal controls. The demographic characteristics of suicide subjects and normal controls are provided in supplemental Table 1 and dissection of the brains are described in our earlier publications (31,32). Toxicology and presence of antidepressants were examined by analysis of urine and/or blood samples. pH of the brain was measured in cerebellum (36). All the subjects were diagnosed based on the Diagnostic Evaluation After Death (37) and the Structured Clinical Interview for the DSM-IV (38) as detailed in our earlier publications (31,32,39,40). This study was approved by the Institutional Review Board of the University of Illinois at Chicago.

Determination of mRNA Levels of TrkA, TrkC, and $p75^{NTR}$

Total RNA was isolated by $CsCl_2$ ultracentrifugation as described earlier (39,40). Samples showing an absorbance ratio (260/280) greater than 1.8 and exhibiting strong 28S and 18S rRNA bands were used. In addition, all the samples showed RNA integrity number >7, which is an excellent value for mRNA studies.

The mRNA levels of TrkA, TrkC, $p75^{NTR}$, and of housekeeping genes neuron-specific enolase (NSE) and cyclophilin were determined using competitive RT-PCR as described earlier (39, 40). The sequences of external and internal primers are given in Table 1. Decreasing concentrations of TrkA (1.5-0.05 pg for PFC and 6.25-0.19 pg for hippocampus), TrkC (200-12.5 pg), or $p75^{NTR}$ (12-0.75 pg for PFC and 6.25-0.39 pg for hippocampus) internal standard cRNAs and 1.5 μCi [^{32}P]dCTP were added to 1 μg of total RNA. The PCR mixture was amplified for 28 cycles. Following amplification, aliquots were digested with *Xho* I in triplicate and run by 1.5% agarose gel electrophoresis. The results are expressed as attomoles/μg of total RNA.

Preparation of Samples for Immunoprecipitation and Western Blot

Proteins from PFC or hippocampal tissues were extracted using RIPA buffer [20 mM Tris-HCL (pH 8), 150 mM NaCl, 1mM EDTA, 50 mM NaF, 1 mM Na_2MoO_4, 0.5 mM Na_3VO_4, 5 mM $Na_2P_2O_7$, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.01 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mg/ml pepstatin A, and 10 mM benzamidine]. S1 fraction was prepared by centrifugation at 1,000 rpm for 10 min at 4°C. Protein content was determined by the Bradford method (Bio-Rad, CA, USA).

Immunoprecipitation of TrkA, TrkB, and TrkC and Immunolabeling with Phosphotyrosine

Supernatant containing 100 μg protein was incubated with antibodies against TrkA, TrkB, or TrkC (100:1 dilution; Santa Cruz Biotechnology, CA, USA) for 2 h at 0°C. The samples were added to a suspension of protein-A sepharose beads (Amersham, NJ, USA) in Tris-buffered saline and incubated at 4°C for 1h. The pellet was collected by centrifugation at 2,500 rpm for 30 s at 4°C and washed four times with TBS containing 0.5 mM Na_3VO_4 and 0.01 mM PMSF. The pellet was resuspended in 15 ml of 2X sample buffer, boiled for 5 min, and subjected to

10% SDS-polyacrylamide gel electrophoresis as described earlier (39). The blots were incubated overnight at 4°C with anti-mouse phosphotyrosine (1 μg/ml, Chemicon International, Temecula, CA, USA), followed by horseradish-peroxidase-linked secondary anti-mouse IgG (0.3 μg/ml; Bio-Rad) for 5 h at room temperature. The bands on the autoradiograms were quantified using the Loats Image Analysis System (Westminister, MD, USA).

Immunolabeling of TrkA, TrkC, and $p75^{NTR}$

Equal volumes (20 μl) of samples containing 60 μg of protein were electrophoresed on 10% (w/v) polyacrylamide gel. The blots were incubated overnight at 4°C with primary antibodies for TrkA (1:650), TrkC (1:1,000), or $p75^{NTR}$ (1:200, NeoMarkers, Fremont, CA, USA) followed by horseradish-peroxidase-linked secondary anti-goat IgG (TrkA, 1:1000 dilution), anti-rabbit IgG (TrkC, 1:5,000 dilution) or anti-mouse IgG ($p75^{NTR}$, 1:800 dilution) for 5 h at room temperature. The membranes were stripped and re-probed with β-actin monoclonal primary (1:5000 for 1 h, Sigma Chemical Co., St. Louis, MO, USA) and anti-mouse secondary antibody (1:5000 for 1 h). The optical density (O.D.) of each protein was corrected by the O.D of the corresponding β-actin band. The antibody for $p75^{NTR}$ has been well characterized in human brain for Western blotting (41,42,43). We characterized TrkA and TrkC antibodies using positive controls (H4 cell lysate, SK-N-SH cell lysate for TrkA; EOC20 whole cell lysate for TrkC). Also, the specificity for TrkA and TrkC was confirmed by pre-incubating the antibodies with the corresponding antigenic peptides (100-fold excess) (Santa Cruz Biotechnology, CA, USA).

Statistical Analysis

Data analyses were performed using the SPSS version 15 (Chicago, IL, USA). All the dependent variables were first subjected to tests of normality. The assumption of normality was tested using the Shapiro-Wilk test. To adjust for multiplicity of testing based on multiple endpoints (i.e., dependent variables), a multiple analysis of covariance (MANCOVA) was applied to the data for each brain area. Age, gender, pH of the brain, race, and postmortem interval (PMI) were used as covariates. The assumption of homogeneity of variance was tested using Box's test of equality of covariance matrices. In the presence of a significant MANCOVA for a given brain area, ANCOVAs were performed for each dependent variable. For the two-group analysis (normal controls vs. suicide subjects) MANCOVA was followed by ANCOVA. For the three-group analysis (normal controls, depressed suicide subjects, suicide subjects with other psychiatric disorders), if the ANCOVA for that dependent variable was significant, pairwise between-group comparisons were performed for each dependant variable.

The differences in age, gender, pH of the brain, and PMI between suicide subjects and normal controls were analyzed using the independent-sample "*t*" test. The relationships between Trk receptor activation and their respective mRNA and protein levels; mRNA and protein levels of $p75^{NTR}$; and measures of Trk receptors and $p75^{NTR}$ with PMI, age, and pH of the brain were determined by Pearson product-moment correlation analyses. The effects of gender and comparison between depressed subjects who showed antidepressant toxicity at the time of death with depressed subjects who did not were determined by an independent sample "*t*" test.

Results

There were no significant differences in age (t = 0.63, df = 47, P = 0.53), PMI (t = 0.11, df = 47, P = 0.91) or pH of the brain (t = 1.00, df = 47, P = 0.32) between suicide subjects and normal control subjects (supplemental Table 1).

Overall Analysis of Data

All dependent variables in the 2 brain areas were first subjected to tests of normality using the Shapiro-Wilk test. We found non-significant P values (>0.05) for tests of normality for all dependent variables in PFC and hippocampus of both normal control and suicide groups which indicated that we cannot reject the null hypothesis that the data are normally distributed. We used Box's test of equality of covariance matrices to test the assumption of between-group equality. No significant between-group differences were found for covariance matrices in PFC (P = 0.25) or hippocampus (p = 0.38). The overall MANCOVA for all 11 dependent variables adjusted for covariates was significant for PFC (F = 17.84, df = 11, 32, P <0.001) and hippocampus (F = 40.96, df = 11, 30, P <0.001) when the normal control group was compared with the suicide group. In the following sections, we describe the results of the individual ANCOVAs for each dependent variable for PFC and hippocampus. Also, the percent change in various measures of Trks and $p75^{NTR}$ are summarized in Table 2.

mRNA Levels of TrkA, TrkC, and $p75^{NTR}$

Representative gel electrophoreses of the competitive RT-PCR for TrkA, TrkC, and $p75^{NTR}$ mRNA in the PFC from one control subject are given in Figure 1a, b, and c respectively. We found the amplification product arising from the TrkA, TrkC, and $p75^{NTR}$ mRNA template at 267, 464, and 311 bp respectively, and the corresponding digestion products from the cRNA at 135+132, 223+241, and 144+167 bp respectively. Competitive PCR analyses are presented in Figures 1d, e, and f, where the points of equivalence represent the absolute amounts of TrkA, TrkC, and $p75^{NTR}$ mRNA present. The absolute amounts (attomoles/μg total RNA) of Trk receptor and $p75^{NTR}$ mRNAs in PFC and hippocampus of normal controls were as follows: PFC: TrkA, 2.5 ± 0.6; TrkC, 73.17 ± 12.7; $p75^{NTR}$, 12.3 ± 2.4; hippocampus: TrkA, 5.1 ± 0.9; TrkC, 98.3 ± 20.9; $p75^{NTR}$, 4.4 ± 1.4. As can be seen in Figure 2, the expression of TrkC was highest compared with that of TrkA and $p75^{NTR}$ in both PFC and hippocampus. The expression levels of both TrkA and TrkC were greater in hippocampus than PFC. On the other hand, the expression of $p75^{NTR}$ was greater in PFC than hippocampus.

When compared between normal controls and suicide subjects, the mRNA expression of TrkA was significantly decreased in PFC of suicide subjects without any change in mRNA level of TrkC. On the other hand, mRNA levels of TrkA and TrkC were significantly lower in hippocampus of suicide subjects. In contrast, the mRNA level of $p75^{NTR}$ was significantly increased in both PFC and hippocampus of suicide subjects compared with normal controls (Figure 2).

We used NSE and cyclophilin as housekeeping genes. As reported earlier (31,32,39), we did not find a significant difference in mRNA levels (attomoles/μg total RNA) of cyclophilin between normal controls and suicide subjects, either in PFC (controls: 776.6 ± 112.5, suicide: 801.5 ± 117.34; df = 1,42, F = 0.3, P = 0.57) or in hippocampus (controls: 783.5 ± 110.1, suicide: 768.3 ± 102.8; df = 1,40, F = 0.001, P = 0.98). Similarly, no significant differences were observed in mRNA levels of NSE in PFC (controls: 360.2 ± 47.7, suicide: 344.0 ± 43.9; df = 1,42, F = 0.7, P = 0.39) or hippocampus (controls: 349.8 ± 38.2, suicide: 345.9 ± 81.4; df = 1,40, F = 0.2, P = 0.62) between normal controls and suicide subjects. We found similar results when the changes in mRNA levels of TrkA, TrkC, and $p75^{NTR}$ were calculated as ratios to cyclophilin or NSE.

Protein Levels of TrkA, TrkC, and $p75^{NTR}$

Western blot revealed that TrkA and TrkC migrated to 140 kDa, whereas $p75^{NTR}$ migrated to 75 kDa (Figure 3a and 3b). β-Actin was used as a housekeeping protein, and ratios of TrkA, TrkC, and $p75^{NTR}$ vs. β-actin were calculated. Immunolabeling of β-actin was not significantly different in suicide subjects (1.3 ± 0.3 AU) compared with normal controls (1.2 ± 0.3 AU).

Bar diagrams showing ratios of protein levels of neurotrophin receptors and β-actin in PFC and hippocampus are shown in Figure 3c and 3d, respectively. Comparison analysis revealed that immunolabeling of TrkA was significantly decreased in both PFC and hippocampus of suicide subjects. Protein levels of TrkC were significantly decreased in hippocampus but not changed in the PFC of suicide subjects. In contrast, the levels of $p75^{NTR}$ were increased in both PFC and hippocampus of suicide subjects compared with normal controls.

Correlations between mRNA and Protein Levels of TrkA, TrkC, and $p75^{NTR}$

To examine whether the altered protein levels of neurotrophin receptors were associated with their respective mRNAs, we examined the correlations between the mRNA and the protein levels of the neurotrophic receptors in the combined normal control and suicide groups. We observed significant correlations between mRNA and protein levels of TrkA in PFC ($r = 0.39$, $P = 0.006$) and hippocampus ($r = 0.42$, $P = 0.003$). Significant correlations were also observed between mRNA and protein levels of TrkC in hippocampus ($r = 0.52$, $P<0.001$), and of $p75^{NTR}$ in both PFC ($r = 0.42$, $P = 0.002$), and hippocampus ($r = 0.47$, $P = 0.001$).

TrkA, TrkB, and TrkC Phosphorylation

Phosphorylation states of Trks were determined by immunoprecipitation with specific antibodies followed by immunoblotting with phosphotyrosine antibody. Autoradiograms showing the phosphorylation in PFC and hippocampus are given in Figure 4a and 4b respectively and diagrammatically presented in Figures 4c and d respectively. MANCOVA followed by ANCOVA tests showed that there were significant decreases in the phosphorylation of TrkA and TrkB in both PFC and hippocampus of suicide subjects, whereas the phosphorylation of TrkC was decreased only in hippocampus without any change in PFC.

Ratios of $p75^{NTR}$ vs. mRNA and protein levels and phosphorylation of TrkA, TrkB, and TrkC

To examine whether there is an imbalance in the expression and activation of TrkA, B, and C in relation to $p75^{NTR}$ expression, we determined the mRNA and protein expression ratios of $p75^{NTR}$ vs. TrkA, TrkB, and TrkC. In addition, we also determined the ratios of protein expression of $p75^{NTR}$ vs. phosphorylation levels of TrkA, TrkB, and TrkC. As shown in Table 3, we observed significant increase in expression ratios of $p75^{NTR}$ vs. TrkA, B, and C. Similarly, ratios of protein expression of $p75^{NTR}$ vs. phosphorylation of TrkA, TrkB, and TrkC were also increased.

Effects of age, PMI, pH, Means of Death and Antidepressant Toxicology (provided as supplemental material)

We found no significant effects of age, PMI, or pH of the brain on any of the measures in which we found significant differences between normal controls and suicide subjects (supplemental Table 2). We also did not find significant effects of means of suicide (violent vs. nonviolent) or the presence of antidepressant toxicology at the time of death on various measures.

Effects of Major Depression

We next examined whether the differences in the mRNA and protein levels of neurotrophin receptors and in the phopsphorylation states of Trk receptors were related to depression or were present in all suicide subjects. ANCOVA followed by pairwise between-group comparisons revealed that the mRNA and protein levels of TrkA, TrkC, and $p75^{NTR}$, as well as the activation of TrkA, TrkB, and TrkC, were not different between suicide subjects with major depression ($n = 12$) and suicide subjects with other psychiatric disorders ($n = 16$) in both PFC (Table 4) and hippocampus (Table 5). However, the groups of suicide subjects with major depression and of suicide subjects with other psychiatric disorders both showed significant differences in

these measures in both PFC (Table 4) and hippocampus (Table 5) when compared separately with normal control subjects.

Discussion

In the present study, we found reduced expression of TrkA, C, and increased expression of $p75^{NTR}$ in hippocampus and reduced expression of TrkA and increased expression of $p75^{NTR}$ in PFC of suicide subjects. Decreased phosphorylation of TrkA and B in PFC and TrkA, B, and C in hippocampus was also noted. Our present study provides evidence for the first time that not only Trk receptors are less expressed but also that their activation is compromised in postmortem brains of suicide subjects. In our earlier studies, we found that expression levels of neurotrophins, i.e., BDNF, NGF, NT-3, and NT4/5, as well as TrkB were decreased in the postmortem brains of suicide subjects (31,32). Our previous studies together with the present results clearly demonstrate that there is an overall decrease in expression and functioning of neurotrophins in the brains of suicide subjects.

Our study also indicates that the levels of the receptors for neurotrophins are regulated at the level of transcription, as significant correlations between mRNA and protein levels of these receptors were noted. Promoter sequences for all neurotrophin receptors have been identified, and multiple transcription factors are implicated in the regulation of the expression of these receptors (44,45). For example, many putative transcription factor binding sites within the 5'flanking region of the human TrkA gene have been identified. These include Sp1 and AP-1. Interestingly, the AP-1 site is bound by c-Jun homodimers, which is blocked by methylation. In many cell lines, it has been shown that activation of Trk A expression is caused by direct interference with c-Jun binding to the negative AP-1-like sequence and that the AP-1 binding site plays a crucial epigenetic role in activating TrkA expression. In addition, the 138-bp region located upstream of the transcription initiation site is also crucial for the human TrkA gene. On the other hand, $p75^{NTR}$ transcription is regulated by transcription factor Egr-1. The TrkC gene is regulated by transcription factors AP-1, AP-2, GC, ATF, and Brn2, AML1, and Nkx2.5. Whether these transcription factors and/or epigenetic regulation are involved in the altered expression of neurotrophin receptor genes in the brains of suicide subjects needs to be studied.

Functionally, in contrast to Trk receptors, which contain autophosphorylation sites and are involved in cell survival, $p75^{NTR}$ lacks intrinsic enzymatic activity and can transmit both positive and negative signals (46). It has been shown that $p75^{NTR}$ acts as a positive regulator of TrkA activity in a number of neuronal cell lines (20,47-49). Coexpression of $p75^{NTR}$ and TrkA receptors increases TrkA high affinity binding sites for NGF (19,50) and NGF-mediated TrkA activation (47,49,50). Ligand binding to $p75^{NTR}$ can potentiate TrkA autophosphorylation at a sub-saturating concentration of NGF; this depends upon the relative levels of $p75^{NTR}$ and TrkA (47,50,51). Also, in the presence of $p75^{NTR}$, NT-3 is less effective in activating TrkA, and NT-3 and NT-4 are much less effective in activating TrkB, which thus enhances the affinity for NGF and BDNF to bind to TrkA and TrkB respectively (20,52-54). In contrast to these positive actions, $p75^{NTR}$ can mediate neuronal apoptosis when the cognate Trk receptor is less activated or not activated. For example, in neuronal cell lines, expression of $p75^{NTR}$ in the absence of TrkA receptors induces cell death (55). Similarly, $p75^{NTR}$ can cause developing hippocampal neuronal death induced by any of the neurotrophins in the absence of a Trk receptor (56-58). On the other hand, mice lacking $p75^{NTR}$ show an increased number of cholinergic neurons in the basal forebrain (59). In adult CNS, it has been shown that excitotoxin-induced neuronal apoptosis is accompanied by the induction of $p75^{NTR}$ in the dying neurons (60), which suggests that $p75^{NTR}$ may represent a general stress-induced apoptotic mechanism (44). However, it is pertinent to note that apoptotic mechanisms of $p75^{NTR}$ are active only when Trk receptors are less expressed or less active. Moreover, ectopic expression of the appropriate Trk receptor can convert a proapoptotic neurotrophin to a pro-

survival neurotrophin. Thus it appears that the ratio of expression levels and/or activation states of Trk receptors and $p75^{NTR}$ is quite relevant in neurotrophin-mediated functions. Given that many physiological functions are associated with Trk receptor activation, including cell survival and enhancement of the efficacy of synaptic neurotransmission, and, therefore, neural plasticity, and the strong evidence of a role of $p75^{NTR}$ in the mediation of cell death, our findings of increased expression ratios of $p75^{NTR}$ to Trks appear to be of great relevance to the pathophysiology of mood disorders and suicide. The PFC plays a major role in mood regulation and has been implicated in the pathophysiology of affective disorders and suicide (61). On the other hand, the hippocampus is involved in cognition (62) and is the primary brain area affected by stress (63), one of the major factors in suicidal behavior (64,65). Interestingly, structural abnormalities in cortical and hippocampal brain areas and reduced hippocampal plasticity have been demonstrated in affective disorder patients and during stress (66-72). Some studies even suggest structural abnormalities in the brains of suicide subjects (73,74). Our previously observed reduced expression of neurotrophins (31,32) together with the present findings of reduced expression and activation of Trks and concomitant increased expression $p75^{NTR}$ indicate that the possible consequences is a tipping of the balance away from cell survival, which could be associated with structural abnormalities and reduced neuronal plasticity in suicide brains. In addition to the modulation of hippocampal plasticity (75), recently, Greferath *et al* (76) and Hennigan *et al* (77) showed that $p75^{NTR}$ is involved in negative regulation of plasticity, such that mice lacking $p75^{NTR}$ display intact long-term potentiation but impairment in long-term depression. It is pertinent to mention that recently Saarelainen *et al* (78) demonstrated that normal TrkB signaling is required for antidepressant action and that the phosphorylation of TrkB in response to antidepressants is greater in cortical and hippocampal brain areas after chronic treatment, suggesting that TrkB activation is required to produce the effects of antidepressants. Moreover, stress, a major risk factor of suicide (79-81) causes a decrease in the expression of TrkA, TrkB, and TrkC in rat brain (27). A recent genetic study suggests that the S205L polymorphism, which substitutes serine with leucine residue, of the $p75^{NTR}$ gene is associated with attempted suicide (82), revealing the crucial role of $p75^{NTR}$ in suicide.

The physiological relevance of Trk receptors is further substantiated by the fact that Trk receptors and $p75^{NTR}$ cross talk to each other at the level of the signal transduction mechanisms that they activate transautophosphorylation of tyrosine leads to the recruitment of proteins containing PTB and SH2 domains. The two major signaling pathways, activated by Trks through these domains, are Ras-Raf-extracellular signal regulated kinase (ERK) and phosphoinositide 3-kinase (PI 3-kinase)-Akt. In addition, phospholipase Cγ binds to activated Trk receptors and initiates an intracellular signaling cascade that results in the activation of protein kinase C. On the other hand, $p75^{NTR}$ stimulates several proapoptotic pathways, which include Jun kinase signaling, sphingolipid turnover, and association with adaptor proteins, such as neurotrophin receptor-interacting MAGE homolog (NRAGE) and $p75^{NTR}$-associated cell death executor (NADE), that directly promote cell cycle arrest and apoptosis (83-86). Trk receptors suppress the major proapoptotic signaling pathway, c-Jun kinase, initiated by $p75^{NTR}$ (87). In sympathetic neurons, Ras-mediated activation of PI3-kinase is required to suppress this signaling pathway (88). Activation of Trk receptors completely suppresses the activation by $p75^{NTR}$ of sphingomyelinase through the association of activated PI3-kinase with acidic sphingomyelinase (89,90). Sphingomyelinase activation results in generation of ceramide which promotes apoptosis by inactivating ERK and PI3-kinase pathways (91-93). Contrary to their proapoptotic action, $p75^{NTR}$ enhances cell survival by activating NF-κB signaling in the presence of Trk receptor activation. Thus, $p75^{NTR}$ acts as a switch between pro- and antiapoptotic actions in neurons. Interestingly, we have reported less-activated ERK1/2 (39), B-Raf (94), and PI-3 kinase (95) in both PFC and the hippocampus of suicide subjects. These findings could be associated with less activation/expression of Trks. These findings also indicate suboptimal activation of prosurvival pathways. Conversely, if $p75^{NTR}$

is more abundantly expressed, this may lead to proapoptotic signaling. Further studies are required to determine whether proapoptotic pathways are activated in brains of suicide subjects and how Trk - and $p75^{NTR}$-mediated signal transduction pathways interplay in the pathophysiology of suicide.

In the present study, we observed that the changes in Trk and $p75^{NTR}$ were present in all suicide subjects regardless of psychiatric diagnosis, suggesting that these changes could be associated with suicide. However, one should be cautious to draw such a conclusion. One of the limitations of the present study is that the study population did not have subjects who had psychiatric disorders and died naturally. In addition, a majority of suicide subjects had some form of mental illness. As mentioned in Introduction, several studies demonstrate alterations in expression of neurotrophic factors and Trk receptors in mood disorders and during stress. For example, decreased expression of neurotrophins or TrkB receptors in depressed patients as well as in animals subjected to several types of stresses have been reported (22-27). Likewise, several genetic studies indicate a linkage of BDNF to bipolar disorder (96-98). Thus, whether the observed changes in Trks and $p75^{NTR}$ are specifically related to suicide or are associated with mental disorders, need to be further clarified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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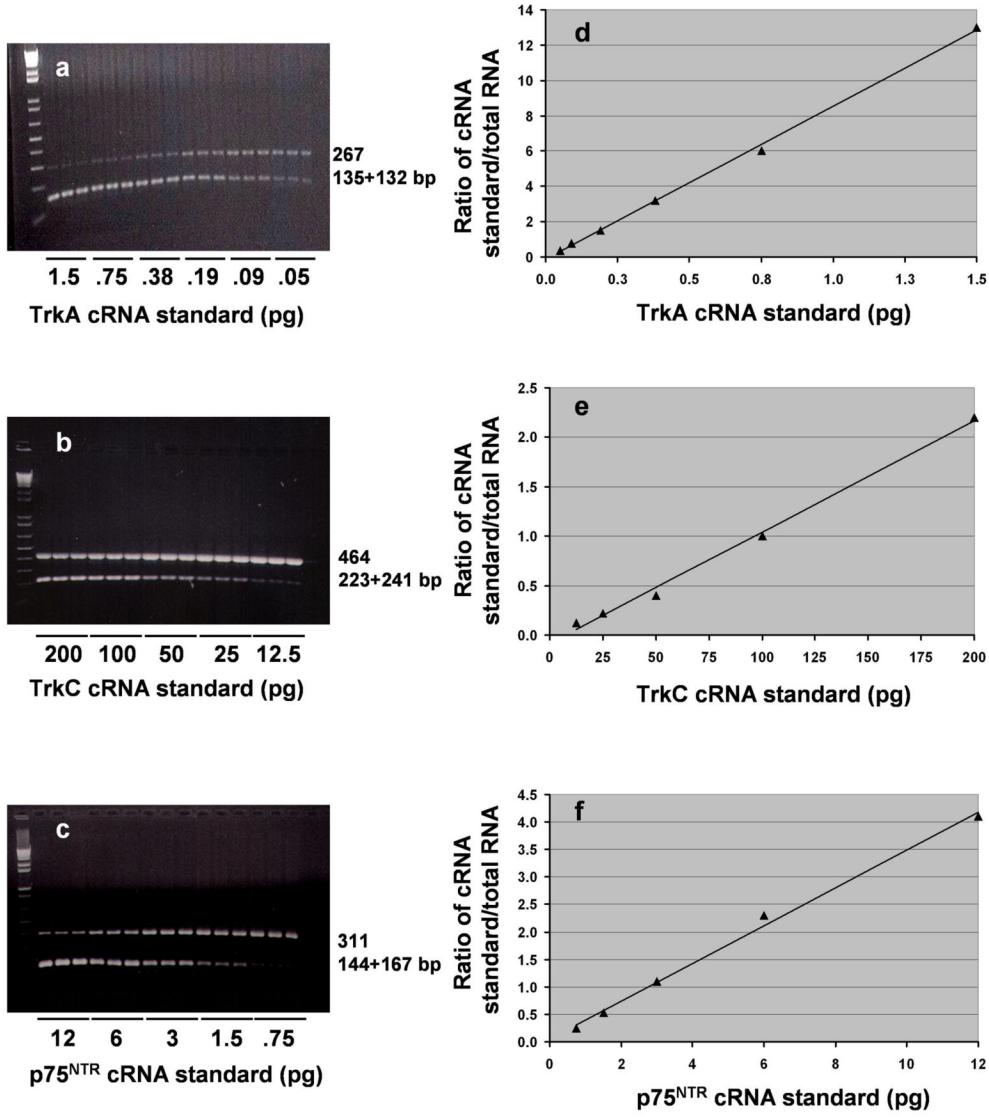

Figure 1.

Representative gel electrophoreses showing competitive PCR analysis for TrkA (a), TrkC (b), or $p75^{NTR}$ (c) mRNA contents in PFC obtained from one normal control subject. Decreasing concentrations of internal standard cRNA (TrkA, 1.5-0.05 pg; TrkC, 200-12.5 pg; $p75^{NTR}$, 12-0.75) were added to a constant amount (1 μg) of total RNA. The mixtures were reverse transcribed and PCR-amplified in the presence of trace amounts of [^{32}P]dCTP; aliquots were electrophoresed on 1.5% agarose gel. 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. Data derived from the agarose gel are plotted as the counts incorporated into the amplified TrkA (d), TrkC (e), or $p75^{NTR}$ (f) 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 the respective mRNA.

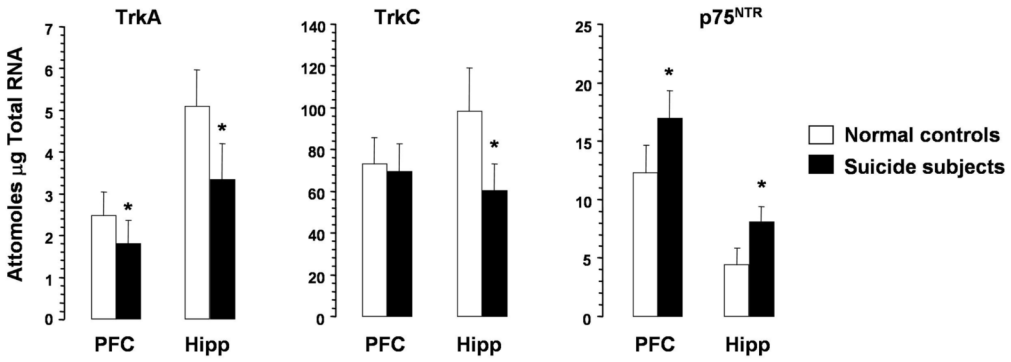

Figure 2.

mRNA levels TrkA, TrkC, and $p75^{NTR}$ in PFC and hippocampus of suicide subjects and normal controls. Data are the mean ± S.D. PFC samples were from 21 normal controls and 28 suicide subjects; hippocampus samples were from 21 normal controls and 26 suicide subjects. Hip, hippocampus. Overall ANCOVA in PFC and hippocampus were as follows: PFC: TrkA, df = 1,40, F = 37, P <0.001; TrkC, df = 1,42, F = 0.5, P = 0.47; $p75^{NTR}$ (df = 1,42, F = 33.6, P <0.001); hippocampus: TrkA, df = 1,40, F = 37, P <0.001; TrkC, df = 1,40, F = 52.1, P <0.001; $p75^{NTR}$, df = 1,40, F = 72.4, P <0.001). * P <0.001.

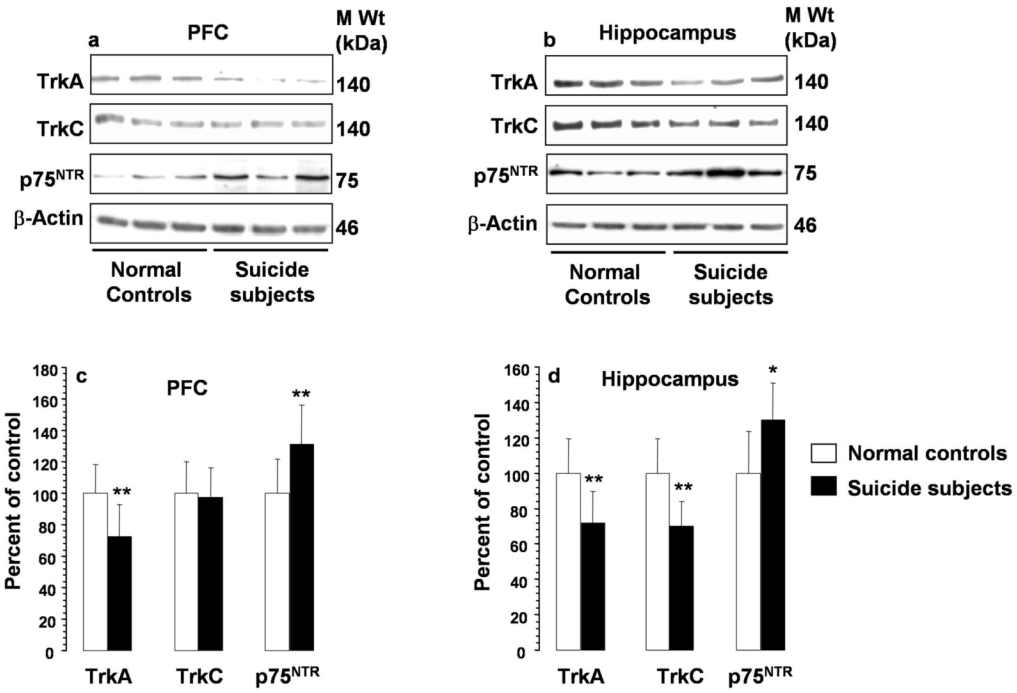

Figure 3.

Western blots showing the immunolabeling of TrkA, TrkC, $p75^{NTR}$ and β-actin in PFC (a) and hippocampus (b) of 3 normal controls and 3 suicide subjects and the mean ± S.D. of immunolabeling of TrkA, TrkC, or $p75^{NTR}$ in PFC (c) and hippocampus (d) from normal controls and suicide subjects. Protein samples were subjected to 10% polyacrylamide gel electrophoresis and transferred to ECL-nitrocellulose membranes, which were then incubated with primary antibody specific for TrkA, TrkC, $p75^{NTR}$, or β-actin and corresponding secondary antibody. The bands were quantified as described in Methods. Ratios of the optical density of TrkA, TrkC, or $p75^{NTR}$ to that of β-actin were calculated. PFC samples were from 21 normal controls and 28 suicide subjects; hippocampus samples were from 21 normal controls and 26 suicide subjects. Suicide group was compared with control group.* Overall ANCOVA: PFC: TrkA, df = 1,42, F = 20.1, P <0.001; TrkC, df = 1,42, F = 0.001, P = 0.98; $p75^{NTR}$, df = 1,42, F = 18.9, P <0.001; hippocampus: TrkA, df = 1,40, F = 21.7, P <0.001; TrkC, df = 1,40, F = 40.3, P <0.001; $p75^{NTR}$, df = 1,40, F = 13.9, P = 0.001. *P = 0.001, ** P <0.001.

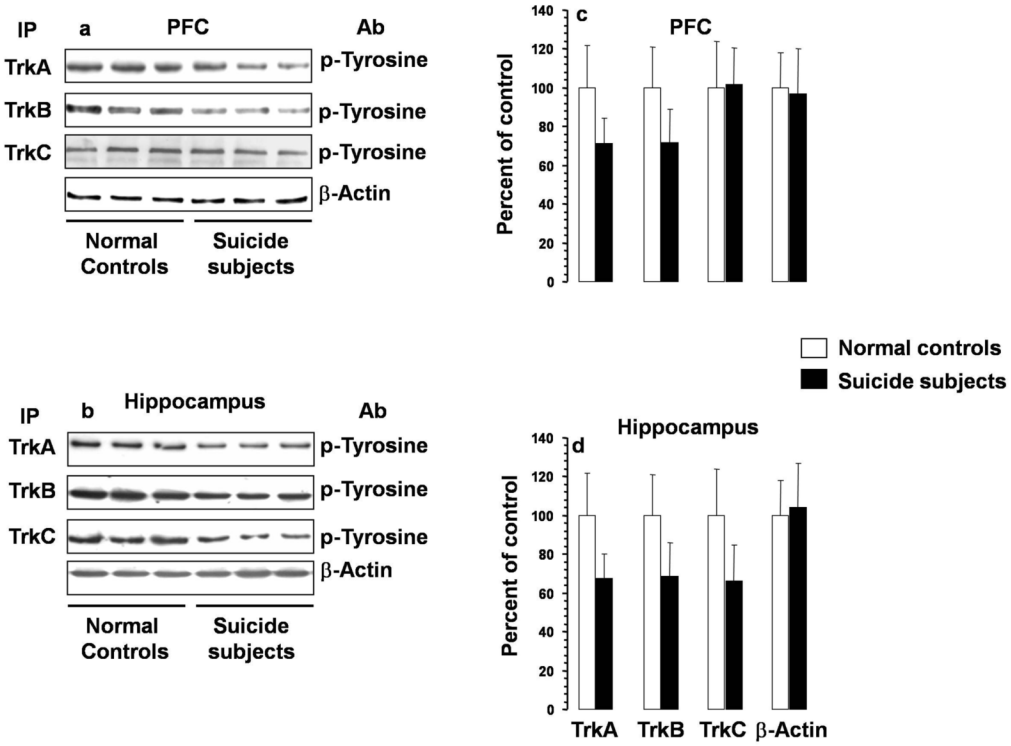

Figure 4.

Activation of TrkA, TrkB, and TrkC in PFC and hippocampus of suicide subjects and normal controls. Autoradiograms showing immunolabeling of phosphotyrosine in PFC (a) and hippocampus (b) determined after immunoprecipitation using TrkA, TrkB, or TrkC antibody. Mean ± S.D. of O.D. of bands of phosphotyrosine depicting activation of TrkA, TrkB, or TrkC in PFC (c) and hippocampus (d) of suicide subjects and normal controls. PFC samples were from 21 normal controls and 28 suicide subjects and hippocampus were from 21 normal controls and 26 suicide subjects. Overall ANCOVA: PFC: TrkA, df = 1,42, F = 26.5, p <0.001; TrkB: df = 1,42, F = 38.5, p <0.001; TrkC, df = 1,42, F = 0.03, p = 0.86; hippocampus: TrkA, df = 1,40, F = 33.7, p <0.001; TrkB, df = 1,40, F = 28.2, p <0.001; TrkC, df = 1,40, F = 27.7, p <0.001. * P <0.001. Ab, antibody; IP, immunoprecipitation.

Table 1

External and internal primer sequences of Trks and $p75^{NTR}$ for Amplification

Primer	Primer sequence	GenBankAccession No.	Nucleotide Position (bp)
External			
TrkA	F: 5'GTGGAGAAGAAGGACGAA ACAC	NM_002529	1222-1243
	R: 5'GTATTGTGGGTTCTCGATGATG		1467-1488
TrkC	F: 5'TACAAGCTTTAACCGGCTCACCACACT CTC	S_76475	408-428
	R: 5'TACGAATTCCCACCACGT TCTCTGCAA TGC		851-871
$p75^{NTR}$	F: 5'CTGCAAGCAGAACAAGCAAGGAGC	NM_002507	831-854
	R: 5'AGGCCTCATGGGTAAAGGAGT		1121-1141
NSE	F: 5'GGGACTGAGAA CAAATCCAAG	NM_001975	295-315
	R: 5'CTCCAAGGCTTCACTGTTCTC		655-675
Cyclophilin	F: 5'AGCACTGGAGAGA AAGGATTTG	XM_371409	118-139
	R: 5'CCTCCACAAT ATTCATGCCTTC		400-421
Internal			
TrkA	F: 5'TGGGATCAACC***T***C***GA***GGCTGTGC TGG		1344-1369
TrkC	F: 5'GTGTGACCTTC***TC***GAGATCAGCGTG		618-642
$p75^{NTR}$	F: 5'ACGCAGACAGCCTC***G***AGCCAGGCCCT		961-990
NSE	5'GGCAACAAGCT***C*** G***AG***ATGCAGGAGTTC		478-504
Cyclophilin	F:5'GGTGGCAAGTCCATCTAT/AAATGCTGGACCCAACAC		220-237/303-320

F, forward; R, reverse; bold and italicized letters indicate the mutated bases. Underline bases indicate the *Xho* I cleavage site.

Table 2

Percent change in various measures of Trks and $p75^{NTR}$ in PFC and hippocampus of suicide subjects

Brian area	Variables	% Change
PFC		
	mRNA	
	TrkA	↓ 26*
	TrkC	(No change)
	$p75^{NTR}$	↑ 37**
	Immunolabeling	
	TrkA	↓ 28**
	TrkC	(No change)
	$p75^{NTR}$	↑ 31**
	Phosphorylation	
	TrkA	↓ 29**
	TrkB	↓ 30**
	TrkC	(No change)
Hippocampus		
	mRNA	
	TrkA	↓ 34**
	TrkC	↓ 39**
	$p75^{NTR}$	↑ 80**
	Immunolabeling	
	TrkA	↓ 29**
	TrkC	↓ 30**
	$p75^{NTR}$	↑ 30**
	Phosphorylation	
	TrkA	↓ 33**
	TrkB	↓ 32**
	TrkC	↓ 34**

* $p = 0.001$;

** $p < 0.001$.

Table 3

Ratios of $p75^{NTR}$/Trks in PFC and hippocampus of normal controls and suicide subjects

	Group	Mean	SD
PFC			
mRNA			
TrkA	Control	5.18	1.62
	Suicide	9.84	2.74**
TrkB	Control	0.02	0.008
	Suicide	0.05	0.013**
TrkC	Control	0.17	0.04
	Suicide	0.25	0.05**
Immunolabeling			
TrkA	Control	1.03	0.31
	Suicide	1.95	0.59**
TrkB	Control	1.05	0.36
	Suicide	2.12	0.47**
TrkC	Control	1.03	0.25
	Suicide	1.42	0.40**
Phosphorylation			
TrkA	Control	1.04	0.31
	Suicide	2.02	0.69**
TrkB	Control	1.04	0.32
	Suicide	1.97	0.64**
TrkC	Control	1.07	0.42
	Suicide	1.38	0.43*
Hippocampus			
mRNA			
TrkA	Control	0.91	0.35
	Suicide	2.55	0.74**
TrkB	Control	0.002	0.001
	Suicide	0.007	0.002**
TrkC	Control	0.05	0.02
	Suicide	0.14	0.04**
Immunolabeling			
TrkA	Control	1.05	0.37
	Suicide	1.93	0.60**
TrkB	Control	1.02	0.24
	Suicide	2.31	0.61**
TrkC	Control	1.03	0.29
	Suicide	1.91	0.41**
Phosphorylation			
TrkA	Control	1.05	0.34

	Group	Mean	SD
	Suicide	2.00	0.51**
TrkB	Control	1.03	0.30
	Suicide	1.99	0.57**
TrkC	Control	1.04	0.29
	Suicide	2.10	0.60**

* p = 0.013,

** p < 0.001,

SD = standard deviation

Table 4

Effect of major depression on neurotrophin receptors in PFC of suicide subjects

Variable	Normal Controls (n=21) 1		Suicide Subjects (n=28)				Overall ANCOVA		Multiple Comparison		
			With a History of MDD (n=12) 2		With a History of Other Psychiatric Disorders (n=16) 3		F	P	1 VS 2	1 VS 3	2 VS 3
	Mean	SD	Mean	SD	Mean	SD					
Phosphorylation[a]											
TrkA	100	20	69	19	72	14	13	<0.001	0.001	<0.001	0.89
TrkB	100	21	65	15	77	17	21	<0.001	<0.001	<0.001	0.12
TrkC	100	25	107	26	98	19	0.6	0.55	0.38	0.71	0.28
mRNA Levels[b]											
TrkA	2.5	0.5	1.6	0.5	1.9	0.6	8	0.001	<0.001	0.02	0.08
TrkC	73.2	12.7	70.9	11.8	68.2	14	0.8	0.47	0.87	0.27	0.32
$p75^{NTR}$	12.3	2.4	16.9	2.4	16.9	2.45	16.6	<0.001	<0.001	<0.001	0.65
Immunolabeling[a]											
TrkA	100	18	74	19	71	22	10	<0.001	0.006	<0.001	0.65
TrkC	100	20	97	20	98	19	0.3	0.97	0.84	0.92	0.79
$p75^{NTR}$	100	22	126	26	134	25	9.3	<0.001	0.005	<0.001	0.83

[a] = percent of control;

[b] = attomoles/μg total RNA.

Data were analyzed using multivariate analysis of covariance (MANCOVA). Overall MANCOVA (Pillai's Trace test) was found to be statistically significant (F = 2.73, df = 22, 64, P <0.001). The data were then subjected to analysis of covariance (ANCOVA) followed by pairwise between-group comparisons. A total of 11 dependent variables (Activation of TrkA, TrkB, TrkC; mRNA levels of TrkA, TrkC, $p75^{NTR}$, NSE, and cyclophilin; and protein levels of TrkA, TrkC, $p75^{NTR}$) were considered during multivariate analysis. Age, gender, pH of the brain, race, and postmortem interval were used as covariates. df = 2,41

Table 5

Effect of major depression on neurotrophin receptors in hippocampus of suicide subjects

Variable	Normal Controls (n=21) 1		Suicide Subjects (n=26)				Overall ANCOVA		Multiple Comparison		
			With a History of MDD (n=10) 2		With a History of Other Psychiatric Disorders (n=16) 3		F	P	1 VS 2	1 VS 3	2 VS 3
	Mean	SD	Mean	SD	Mean	SD					
Phosphorylation[a]											
TrkA	100	22	60	7	72	14	18.8	<0.001	<0.001	<0.001	0.11
TrkB	100	21	71	19	67	16	13.7	<0.001	0.001	<0.001	0.87
TrkC	100	24	72	22	63	16	13.9	<0.001	0.003	<0.001	0.48
mRNA Levels[b]											
TrkA	5.1	0.9	2.9	0.6	3.6	0.9	19.7	<0.001	<0.001	<0.001	0.19
TrkC	98.2	20.9	62.2	12.6	58.9	13.1	25.7	<0.001	<0.001	<0.001	0.59
p75NTR	4.4	1.4	7.9	1.5	8.2	1.2	35.3	<0.001	<0.001	<0.001	0.81
Immunolabeling[a]											
TrkA	100	19	69	18	73	18	10.6	<0.001	0.004	<0.001	0.86
TrkC	100	19	71	13	69	14	19.8	<0.001	<0.001	<0.001	0.67
$p75^{NTR}$	100	23	137	23	125	18	8.1	0.001	0.001	0.01	0.16

[a] = percent of control;

[b] = attomoles/μg total RNA.

Data were analyzed using multivariate analysis of covariance (MANCOVA). Overall MANCOVA (Pillai's Trace test) was found to be statistically significant (F = 3.65, df = 22, 60, P <0.001). The data were then subjected to analysis of covariance (ANCOVA) followed by pairwise between-group comparisons. A total of 11 dependent variables (Activation of TrkA, TrkB, TrkC; mRNA levels of TrkA, TrkC, $p75^{NTR}$, NSE, and cyclophilin; and protein levels of TrkA, TrkC, $p75^{NTR}$) were considered during multivariate analysis. Age, gender, pH of the brain, race, and postmortem interval were used as covariates. df = 2,39