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Aberrant Extracellular Signal-Regulated Kinase (ERK) 5 Signaling in Hippocampus of Suicide Subjects

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Extracellular signal-regulated kinase 5 (ERK5), the newest member of the mitogen-activated protein (MAP) kinase family, is regulated differently than the other MAP kinases. Emerging evidence suggest the role of ERK5 signaling in promoting cell proliferation, differentiation, neuronal survival, and neuroprotection. The present study investigates whether suicide brain is associated with alterations in components of the ERK5 signaling cascade. In the prefrontal cortex (PFC) and hippocampus of suicide subjects ($n = 28$) and nonpsychiatric control subjects ($n = 21$), we examined the catalytic activities and protein levels of ERK5 and upstream MAP kinase kinase MEK5 in various subcellular fractions; mRNA levels of ERK5 in total RNA; and DNA-binding activity of myocyte enhancer factor (MEF)2C, a substrate of ERK5. In the hippocampus of suicide subjects, we observed that catalytic activity of ERK5 was decreased in cytosolic and nuclear fractions, whereas catalytic activity of MEK5 was decreased in the total fraction. Further, decreased mRNA and protein levels of ERK5, but no change in protein level of MEK5 were noted. A decrease in MEF2C-DNA-binding activity in the nuclear fraction was also observed. No significant alterations were noted in the PFC of suicide subjects. The observed changes were not related to a specific psychiatric diagnosis. Our findings of reduced activation and/or expression of ERK5 and MEK5, and reduced MEF2C-DNA-binding activity demonstrate abnormalities in ERK5 signaling in hippocampus of suicide subjects and suggest possible involvement of this aberrant signaling in pathogenic mechanisms of suicide.
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INTRODUCTION

Mitogen-activated protein kinase (MAPK) signaling cascades constitute a complex network of signaling pathways that play an essential role in the transduction of extracellular signals to cytoplasmic and nuclear effectors, and thereby regulate the genes involved in wide variety of cellular processes, including cell proliferation, differentiation, apoptosis, and synaptic plasticity (Robinson and Cobb, 1997; Johnson and Lapadat, 2002). The mitogen-activated protein (MAP) kinase pathway is composed of a highly conserved three-component cascade containing a MAPK kinase kinase, which upon activation, phosphorylates and activates MAPK kinase. The dual-specificity MAPK kinase then activates threonine and tyrosine residues in the Thr-*X*-Tyr (T*X*Y) motif of MAPK. At least seven members of the MAPK family have been identified, which include extracellular signal-regulated kinase (ERK)-1/2, C-Jun N-terminal kinases, p38 MAPKs, Extracellular signal-regulated kinase 5 (ERK5), ERK6, and ERK7 (Garrington and Johnson, 1999; Chang and Karin, 2001; Pearson *et al*, 2001a).

ERK5 is the newest member of the MAPK family and is almost twice the size of other MAPKs (English *et al*, 1995; Lee *et al*, 1995; Zhou *et al*, 1995). It is widely expressed in many tissues, with the highest levels in the brain (Liu *et al*, 2003; Yan *et al*, 2003). In neurons, ERK5 is activated by neurotrophins, including brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and NT-4 (Cavanaugh *et al*, 2001; Watson *et al*, 2001; Liu *et al*, 2003; Shalizi *et al*, 2003). A number of other extracellular stimuli, such as epidermal growth factor and G-protein-coupled receptors, also activate ERK5 (Kato *et al*, 1998; Marinissen *et al*, 1999). Activation of ERK5 requires phosphorylation of Thr-219 and Tyr-221 residues by upstream MAPK kinase5 (MEK5), which in turn is activated by MEKK2, MEKK3, and Cot (Chao *et al*, 1999; Chiariello *et al*, 2000; Sun *et al*, 2001). ERK5, although activated by a similar Thr-Glu-Tyr dual phosphorylation motif as are ERK1/2 and ERK7, however, contains a unique loop-12 structure and an unusually large C-terminal nonkinase domain, which is absent in other types of MAPKs. This suggests that the regulation and function of this kinase may be different from that of other

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MAPKs (Lee *et al*, 1995; Zhou *et al*, 1995). ERK5 directly or indirectly phosphorylates many transcription factors, such as myocyte enhancer factor (MEF)2, c-Myc, c-Fos, c-Jun, Sap1a, p90 ribosomal S6 kinase (RSK), CREB, and NF-κB (Kato *et al*, 1997; English *et al*, 1998; Kamakura *et al*, 1999; Marinissen *et al*, 1999; Chiariello *et al*, 2000; Pearson *et al*, 2001b). ERK5 also regulates transcription, through a kinase-independent mechanism that involves its unique C-terminal half (Kasler *et al*, 2000; Terasawa *et al*, 2003).

In recent studies, we have observed that expression of BDNF and of the tyrosine receptor kinase B, to which BDNF binds and mediates its function, is reduced in postmortem brain of suicide subjects (Dwivedi *et al*, 2003a). Alterations in expression of other neurotrophic factors, such as NGF, NT-3, and NT-4, in a brain region-specific manner were also noted in these subjects (Dwivedi *et al*, 2005). Interestingly, we found abnormalities in ERK-1/2 signaling in postmortem brain of these subjects, such that expression and activation of ERK-1/2 are decreased and expression of MAPK phosphatase1, involved in dephosphorylation and deactivation of ERK1/2, is increased (Dwivedi *et al*, 2001). Similar attenuation of ERK-1/2 activation has also been reported in postmortem brain of suicide subjects by Hsiung *et al* (2003). In addition, very recently, we reported that in ERK signaling, the upstream kinase B-Raf is less activated in postmortem brain of these subjects (Dwivedi *et al*, 2006). These studies clearly indicate abnormalities in neurotrophic factors and in ERK1/2 signaling in suicide subjects. Although the role of ERK5 in neuronal functions is yet to be fully established in the adult central nervous system, recent studies suggest an important role of ERK5 in neuronal survival (Cavanaugh, 2004). For example, blocking of ERK5 activation diminishes the retrograde survival response initiated by neurotrophin stimulation of axon terminals (Watson *et al*, 2001). In addition, it has been shown that ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing cortical neurons (Liu *et al*, 2003). Yoon *et al* (2005) have reported that electroconvulsive shock can alter the brain region-specific activity of the ERK5-MEF2C pathway in the adult rat brain. Interestingly, in a recent study, Liu *et al* (2006) reported that ERK5 is necessary and sufficient to stimulate generation of neurons from cortical progenitors. Given the emerging role of ERK5 in neuronal survival/neuroprotection, the present study was undertaken to examine whether ERK5 signaling is altered in postmortem brain of suicide subjects. To this end, we examined the expression and activation of ERK5 and the functional status of MEF2C, one of the major substrates of ERK5, in the prefrontal cortex (PFC) and hippocampus of suicide subjects and normal control subjects.

MATERIALS AND METHODS

The study was performed in Brodmann's area 9 (BA9) and hippocampus obtained from suicide subjects ($n = 28$) and nonpsychiatric controls ($n = 21$), hereafter referred to as controls. Brain tissues were obtained from the Maryland Brain Collection at the Maryland Psychiatric Research Center, Baltimore, MD. Brain samples were free of any neuropathological abnormalities or HIV antibodies. Toxicology and presence of antidepressants were examined by analysis of urine and blood samples from these subjects. In each case, screening for the presence of HIV was performed in blood samples, and all HIV-positive cases were excluded. pH of the brain was measured in the cerebellum in all cases as described by Harrison *et al* (1995).

Diagnostic Method

At least one family member, after giving verbal informed consent, underwent an interview based on the Diagnostic Evaluation After Death (Salzman *et al*, 1983) and the Structured Clinical Interview for the DSM-IV (Spitzer *et al*, 1995). The interviews were conducted by a trained psychiatric social worker. Two psychiatrists independently reviewed the write-up from this interview, as well as the SCID that was completed from it, as part of their diagnostic assessment of the case. Diagnoses were made from the data obtained in this interview, medical records from the case, and records obtained from the Medical Examiner's office. The two diagnoses were compared and discrepancies were resolved by means of a consensus conference. Controls were verified as free from mental illnesses using these consensus diagnostic procedures. This study was approved by the IRB of the University of Illinois at Chicago.

Preparation of Total and Cytosolic Fractions

Postmortem brain tissues were homogenized in ice-cold homogenizing buffer containing 10 mM HEPES (pH 7.9), 0.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 50 mM sodium fluoride, 0.5 mM dithiothreitol, 10 mM β-phosphoglycerol, 1 mM sodium orthovanadate, 1% NP-40, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 4 μg/ml aprotinin, 10 μg/ml pepstatin A. The homogenate was centrifuged at 800 *g* for 10 min at 4°C. The supernatant was collected and used as total fraction. A portion of supernatant was removed and recentrifuged at 100 000 *g* for 60 min at 4°C. The supernatant was used as cytosol fraction. The protein contents in the total and cytosolic fractions were determined by the procedure of Lowry *et al* (1951) using bovine serum albumin as the standard.

Preparation of Nuclear Extract

Nuclear fraction was prepared according to the procedure described earlier (Dwivedi *et al*, 2001, 2003b). Tissues were homogenized in ice-cold buffer containing 10 mM HEPES (pH 7.4), 1.5 mM HCl, 10 mM KCl, 2 mM sodium pyrophosphate, 4 mM pNPP, 2 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 4 μg/ml aprotinin, and 10 μg/ml pepstatin A. The homogenate was centrifuged at 100 000 g for 30 min. The resulting pellet was suspended in 20 mM HEPES (pH 7.4), 84 mM NaCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 50% glycerol, and protease inhibitors as above. The homogenate was incubated for 15 min on ice with frequent agitation, and the nuclear extracts were separated by centrifugation at 20 000 *g* for 15 min. The protein content of the nuclear extracts was determined by the method of Lowry *et al* (1951).

ERK5 and MEK5 Catalytic Activity Assays

Nuclear and cytosolic (ERK5 activity) or total (MEK5 activity) fraction were subjected to immunoprecipitation by incubating with 10 μl of anti-ERK5 or anti-MEK antibody (Santa Cruz Biotechnology) for 2 h at 4°C, followed by 20 μl of protein A-sepharose (Amersham Pharmacia Biotech) for 2 h. Immunoprecipitates were washed three times with a buffer containing 20 mM Tris (pH 7.5), 500 mM NaCl and 0.05% Tween 20, and 2 mM DTT. To detect the kinase activity, the immune complex was washed once with a reaction buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 2 mM dithiothreitol, and 1 mM PMSF and incubated for 30 min at 30°C with the substrate in a buffer (final volume, 15 μl) containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 μM ATP (2 μCi of [γ-^{32}P]ATP), and 10 μg of myelin basic protein or GST-ERK5 as substrate for ERK5 or MEK5, respectively. Reaction products were separated by SDS-PAGE and visualized with autoradiography. Quantitation of ERK5 and MEK5 activities was achieved by using the Loats Image Analysis system (Westminster, MD, USA).

Western Blot of ERK5 and MEK5

Immunolabeling of ERK5 was determined in nuclear and cytosolic fractions, whereas, immunolabeling of MEK5 was determined in the total fraction by Western blot. Samples were boiled for 5 min, vortexed, and then centrifuged for 2 min. Equal amounts of samples (20 μg protein) were loaded on 7.5% (w/v) polyacrylamide gel using the Mini Protein II gel apparatus (Bio-Rad Laboratories, Hercules, CA). The gels were run using 25 mM Tris base, 192 mM glycine, and 0.1% (w/v) SDS at 150 V. The proteins were subsequently transferred electrophoretically using 25 mM Tris base, 0.2 M glycine, and 20% methanol (pH 8.5) to an enhanced chemiluminescence (ECL) nitrocellulose membrane using the Mini Trans-Blot transfer unit (Bio-Rad Laboratories) at 0.15 A constant current. Membranes were washed with TBST (10 mM Tris base, 0.15 M NaC1, and 0.05% Tween 20) buffer for 5 min. The blots were blocked by incubating with 5% (w/v) powdered nonfat milk in TBST, 0.2% (v/v) Nonidet P-40, and 0.02% (w/v) SDS (pH 8.0) for 1 h and were incubated overnight at 4°C with primary antibody (ERK5, 1 : 800; MEK5, 1 : 500). The membranes were washed 3 times for 10 min each with TBST, and then membranes were incubated with HRP-conjugated anti-goat secondary antibody (1 : 1000 dilution) for 5 h at room temperature. Membranes were washed 3 times, 10 min each with TBST. The membranes were stripped using stripping buffer (Chemicon International, Temecula) and treated with primary β-actin antibody (1.1 mg/ml; 1 : 3000 dilution) for 3 h and secondary HRP-linked anti-mouse antibody (1 : 5000 dilution) for 2 h. β-Actin was used as a housekeeping protein to reduce interblot variability. Membranes were then incubated with a chemiluminescent detection reagent (New England Bio Labs, Inc., Beverly, MA) at room temperature. The membranes were exposed on ECL-autoradiographic films. A pooled brain extract was used in each gel as a control. Before the experiment, the dilution of the antibody and the duration of the exposure of the nitrocellulose membranes on autoradiographic film were standardized. The bands on the autoradiograms were quantified using the Loats Image Analysis System (Westminister, MD), and the optical density (OD) of each sample was corrected by the optical density of the corresponding pooled sample band. The values are presented as a percent of the control.

The specificity of each antiserum was checked by using a 100-fold excess of blocking peptide (relative to the molarity of the antiserum) corresponding to the epitope used to generate ERK5 and MEK5. We also examined the antibodies by including positive cells (Caki-1, HeLa, A-673, and NIH3T3 whole-cell lysates) along with human frontal cortex and hippocampus for Western blot and observed that the bands in the PFC and hippocampus were of the same size as observed in cell lines. In addition, to validate our data, we initially determined the immunolabeling of ERK5 and MEK5 in the PFC and hippocampus of suicide and control subjects using five different concentrations of protein (5–50 μg). It was observed that the OD of the band increased linearly with increased concentration of protein and that the curve shifted toward the right when a decrease in immunolabeling was observed (data not shown).

Determination of mRNA Levels of ERK5

The procedures for RNA isolation and competitive RT-PCR analysis have been described previously (Dwivedi *et al*, 2001). Brain tissues were homogenized in 4 M guanidine isothiocyanate, 50 mM Tris/HCl (pH = 7.4), and 25 mM EDTA, and the total RNA was isolated by $CsCl_2$ ultracentrifugation. The yield of total RNA was determined by measuring the absorbency of an aliquot of the precipitated stock at a wavelength of 260/280 nm. Samples with a ratio below 1.8 were rejected. The degradation of mRNA was assessed using denaturing agarose gel electrophoresis and evaluating the sharpness of 28S and 18S rRNA bands. None of the samples used in this study showed any sign of degradation.

The quantitation of ERK5 was determined using internal standards. We also determined mRNA levels of cyclophilin, used as a housekeeping gene. Cloning and synthesis of internal standards have been described in our earlier publication.[41] The primer pairs were designed to allow amplification of 88–442 base pairs (bp): forward, 5′ CTCTGTAGCGCCAAGAACCTG (88–108 bp) and reverse, 5′ GGATGATCTGGTGCAGGTCGC (422–442 bp) for ERK5 (GenBank accession u25278), and 118–421 bp: forward, 5′ AGC ACT GGA GAG AAA GGA TTT G (118–139 bp) and reverse, 5′ CCT CCA CAA TAT TCA TGC CTT C (400–421 bp) for cyclophilin (GenBank accession # XM_371409). The internal primer sequence for ERK5 was 5′ CCTAATGCT<u>***C***TCGA***G***</u>GTGGTGACC (256–279 bp). The underlined bases indicate the *Xho*I restriction site, whereas bold and italicized bases indicate the mutation sites. The single-strand internal primers were designed and synthesized so that the restriction site was introduced with only a minimal number of base substitutions, and also such that there was a 24-bp overlap of the primary PCR products. Each of the internal standards was synthesized in two PCR steps, starting with a cDNA template reverse transcribed from the total RNA. The internal standard templates were first cloned into a pGEM4Z vector and then amplified using M13 primers. For cyclophilin, the internal standard was

prepared by deleting 65 bp (220–237/303–320 bp): 5′GGT GGC AAG TCC ATC TAT/AAA TGC TGG ACC CAA CAC.

Quantitative analyses of ERK5 and cyclophilin were performed by competitive RT-PCR as described earlier (Dwivedi *et al*, 2001, 2006). Decreasing concentrations of ERK5 or cyclophilin internal standard cRNAs were added to 1 μg of total RNA. The PCR mixture was amplified for 26 cycles. Following amplification, aliquots were digested with *Xho*I (ERK5) in triplicate and run on 1.5% agarose gel. For cyclophilin, the PCR product was run directly on gel without digestion. To make sure that amplified sequences of ERK5 and cyclophilin match with the corresponding sequences reported in GenBank, the PCR products were sequenced using M13 primer.

To quantitate the amount of product corresponding to the reverse-transcribed and amplified mRNA, 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 *vs* a known amount of internal standard cRNA added to the test sample. The results are expressed as attomoles mRNA/μg total RNA.

Determination of MEF2C-DNA-Binding Activity

The gel mobility shift assay was performed with an oligonucleotide probe containing the MEF2C-binding sequence (5′-GATGCGTCTAAAAATAACCCTGTCG-3′) (Santa Cruz Biotechnology, CA). The probe was end labeled with [γ-^{32}P]ATP (Amersham Biosciences, IL) using T4 polynucleotide kinase and purified by chromatography on a column. For the DNA–protein binding reaction, the samples of nuclear extracts (10 μg of protein) were incubated with 10 fmol of ^{32}P-labeled oligonucleotide containing the consensus MEF2C-binding site at room temperature for 30 min, in 20 μl of binding buffer consisting of 20 mM HEPES, pH 7.9, 0.2 mM PMSF, 6% glycerol, and 2 μg of poly(dI-dC) (Roche Molecular Biochemicals). The DNA–protein complexes were separated from the free DNA probe using electrophoresis on 7% nondenaturing polyacrylamide gels in 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, 0.1 mM EDTA, and 2.5% glycerol. Gels were run at 160 V at 4°C for 3 h, dried, then subjected to autoradiography, and analyzed with Loats Image Analysis system. To show the specificity of MEF2C-DNA-binding activity, nuclear extract from the PFC of controls was incubated in the presence of excess MEF2C antibody for 18 h at 4°C, and then used for the gel mobility shift assay, which resulted in the formation of a supershift band on the gel mobility assay (data not shown). The specificity of the MEF2C-DNA-binding activity was also determined in the presence of unlabeled MEF2C consensus oligonucleotide (Santa Cruz Biotechnology, CA). The MEF2C band was completely blocked by addition of unlabeled MEF2C oligo (data not shown).

Statistical Analysis

Data analyses were performed using the SPSS 8.0 (Chicago, IL) statistical software package. All values reported are the mean $\pm$ SD. The differences in various measures, age, gender, pH of the brain and postmortem interval (PMI), between suicide subjects and normal controls were analyzed using the independent-sample '*t*'-test. An α-level less than 0.05 was considered statistically significant. The relationships between the mRNA and protein levels, as well as catalytic activity of ERK5, and PMI, age, and pH of the brain were determined by Pearson product–moment correlation analysis. The effects of gender on various measures were determined by an independent sample '*t*'-test comparing male and female subjects. Similarly, an independent sample '*t*'-test was used to compare depressed subjects who showed presence of antidepressant(s) during blood/urine analysis at the time of death with depressed subjects who did not. One way ANOVA was performed to examine the effect of major depression on ERK5 measures.

RESULTS

The detailed demographic characteristics of suicide subjects and controls are provided in Table 1. There were 17 male and four female subjects in the control group, and 19 male and nine female subjects in the suicide group. The age range was 21–87 years; the PMI was in the range of 5–32 h. There were no significant differences in age ($t = 0.63$, $df = 47$, $P = 0.53$) or PMI ($t = 0.29$, $df = 45$, $P = 0.77$) between suicide subjects and controls. The mean brain pH values of controls and suicide subjects were 6.06 ± 0.3 and 6.18 ± 0.4, respectively, which were not different between these groups ($t = 1.00$, $df = 47$, $P = 0.32$).

Catalytic Activities of MEK5 and ERK5

The catalytic activity of MEK5 was determined in the total fraction of the PFC and hippocampus and was very similar in these two brain areas. Representative autoradiograms showing MEK5 activity in the PFC and hippocampus are given in Figure 1a, and the mean activity levels are represented in Figure 1b. We observed that the catalytic activity of MEK5 was significantly lower in the hippocampus ($t = 5.7$, $df = 40$, $P < 0.001$) but not in the PFC ($t = 1.3$, $df = 47$, $P = 0.20$) of suicide subjects compared with normal controls.

ERK5 has been shown to be present in the cytosol and is translocated to the nucleus upon activation (Kato *et al*, 1997). A recent study also suggests that ERK5 is localized in the nucleus in the resting state (Raviv *et al*, 2003). Therefore, ERK5 activity was determined in both cytosolic and nuclear fractions. Representative autoradiograms showing ERK5 activities in nuclear and cytosol fractions are given in Figure 2a, and mean activity levels are represented in Figure 2b. We observed that catalytic activity of ERK5 was higher in the hippocampus than the PFC. Also the activity of ERK5 was higher in cytosol fraction than the nuclear fraction. Comparison analysis showed that in the hippocampus of suicide subjects there was significantly lower ERK5 catalytic activity in both nuclear ($t = 5.1$, $df = 40$, $P < 0.001$) and cytosolic ($t = 5.2$, $df = 40$, $P < 0.001$) fractions compared with the activity in controls. On the other hand, no significant change was noted in ERK activity in the PFC of suicide subjects either in the nuclear ($t = 1.4$, $df = 47$, $P = 0.17$) or the cytosolic ($t = 0.56$, $df = 47$, $P = 0.57$) fraction.

Table 1 Characteristics of Suicide and Control Subjects

Group and subject	Age (years)	Race	Gender	PMI (hr)	Brain pH	Cause of death	Drug toxicity (at the time of death)	Psychiatric diagnosis
Suicide								
1	53	White	Male	23	6.1	Jumping	None	Major depression
2	24	White	Male	7	5.6	GSW	Ethanol	Major depression
3	24	White	Male	22	6.5	Hanging	None	Schizoaffective disorder
4	36	White	Female	10	6.5	GSW	Butalbital, diphenhydramine, acetaminophen	Major depression
5	38	White	Male	24	6.3	Drug overdose	Ethanol, diphenhydramine	Major depression, alcohol abuse
6	46	White	Female	16	6.1	Drug overdose	Nortriptyline	Major depression, agoraphobia
7	21	White	Male	17	6.1	GSW	None	Adjustment disorder
8	44	White	Female	11	5.6	Drug overdose	Nortriptyline	Major depression, alcohol abuse
9	22	Black	Female	16	5.3	Drug overdose	Propranolol	Major depression
10	46	White	Female	21	5.3	Drug overdose	Amitriptyline, desipramine, ethanol	Major depression
11	50	White	Male	7	6.1	GSW	None	No psychiatric illness
12	40	White	Male	26	5.6	GSW	Ethanol	Adjustment disorder
13	68	White	Female	26	6.1	GSW	Amitriptyline	Bipolar disorder
14	37	Black	Male	NA	5.8	CO intoxication	Carbon monoxide	NA
15	51	White	Female	28	6.7	Drug overdose	Amitriptyline, ethanol	Bipolar
16	30	White	Male	32	6.4	Hanging	Cocaine, ethanol	Drug/alcohol abuse
17	24	White	Male	22	6.6	Hanging	None	Schizoaffective disorder
18	75	White	Male	18	6.7	GSW	None	Adjustment disorder
19	43	White	Male	12	6.5	Drug overdose	Acetaminophen, propoxyphen	Major depression
20	21	White	Male	22	6.5	Hanging	None	Adjustment disorder
21	41	White	Female	27	5.9	Drug overdose	Amitriptyline, desipramine, diphenhydramine, nortriptyline, pseudoephedrine, salicylate, ethanol	Major depression, alcohol abuse
22	87	White	Male	16	6.2	GSW	None	Adjustment disorder
23	26	Black	Male	NA	6.5	Hanging	Cocaine	NA
24	41	Black	Male	12	6.3	Multiple injuries	None	No psychiatric illness
25	34	White	Male	16	6.2	GSW	Ethanol	Alcohol abuse
26	27	White	Male	24	6.4	GSW	None	Major depression, alcohol abuse
27	39	White	Male	30	6.5	Asphyxia	Freon, cocaine	Drug abuse
28	36	White	Female	18	6.9	GSW	None	Major depression
Mean	42.9	4 Black	9 Female	18.7	6.1			
SD	13.9	24 White	19 Male	7.6	0.4			
Controls								
29	45	White	Male	22	6.5	ASCVD	None	—
30	22	Black	Male	19	6.2	GSW	None	—
31	83	White	Male	20	5.6	ASCVD	None	—
32	63	White	Female	30	5.7	Ovarian cancer	None	—
33	31	Black	Male	8	5.6	GSW	None	—
34	35	White	Male	24	5.6	Crash injury	None	—

Table 1 Continued

Group and subject	Age (years)	Race	Gender	PMI (hr)	Brain pH	Cause of death	Drug toxicity (at the time of death)	Psychiatric diagnosis
35	33	White	Male	15	6.0	GSW	Acetaminophen	—
36	37	Black	Male	5	6.6	ASCVD	None	—
37	37	White	Male	24	6.3	ASCVD	None	—
38	65	Black	Female	23	5.6	ASCVD	None	—
39	38	Black	Male	16	5.8	Lung sarcoidosis	None	—
40	40	White	Female	7	6.5	ASCVD	None	—
41	23	Black	Male	15	6.7	GSW	None	—
42	42	White	Female	23	6.2	Pneumonia	None	—
43	46	Black	Male	9	6.2	Multiple injuries	None	—
44	48	White	Male	26	6.1	ASCVD	None	—
45	52	White	Male	30	6.3	ASCVD	Ethanol	—
46	37	Black	Male	9.5	6.1	Drug overdose	Ethanol	—
47	43	White	Male	17.5	5.7	ASCVD	None	—
48	41	White	Male	24	6.2	ASCVD	Azacyclonal	—
49	41	Black	Male	27	5.8	Esophageal varices	Ethanol	—
Mean	40.1	12 White	4 Female	19.3	6.2			
SD	16.2	9 Black	17 Male	6.9	0.4			

ASCVD = atherosclerotic cardiovascular disease; GSW = gunshot wound; NA = not available.

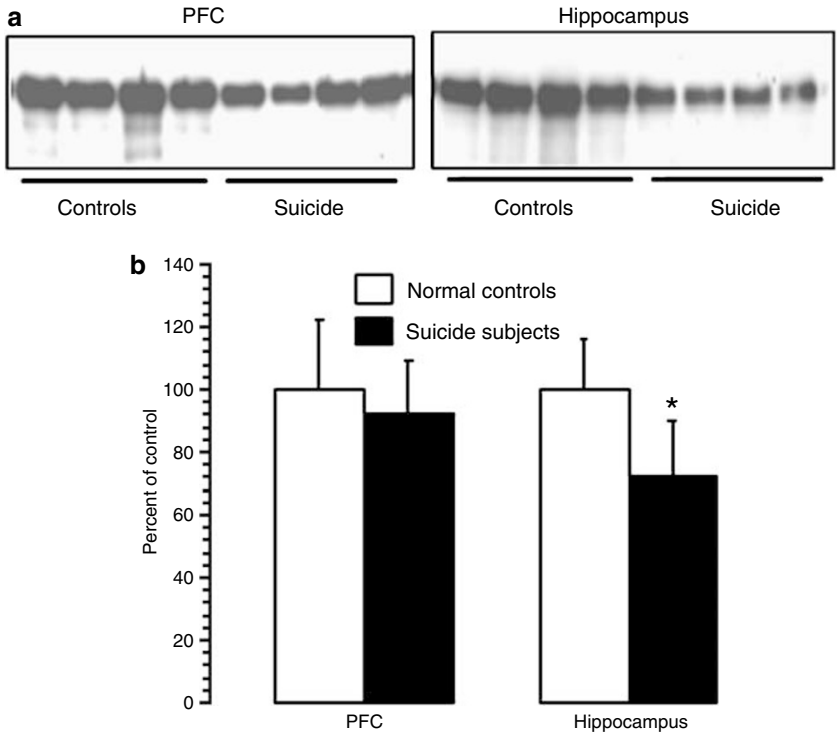

Figure 1 Catalytic activity of MEK5 in total fraction of the PFC and the hippocampus of suicide subjects and normal controls. (a) Representative autoradiograms showing MEK5 activity in the PFC and the hippocampus determined after immunoprecipitation using MEK5 antibody followed by incubation with GST-ERK5 and [γ-^{32}P]ATP. (b) Mean $\pm$ SD of MEK5 catalytic activity in PFC and the hippocampus of suicide subjects and controls. PFC samples were from 21 controls and 28 suicide subjects, and the hippocampus samples were from 21 controls and 21 suicide subjects. *$P<0.001$.

Immunolabeling of MEK5 and ERK5

Immunolabeling of MEK5 (total fraction) and ERK5 (nuclear and cytosolic fractions) was examined in the same nuclear and cytosolic fractions in which their respective catalytic activities were determined. Comparison analysis showed that the level of MEK5 was not significantly altered either in the PFC ($t=67$, df = 47, $P=0.51$) or hippocampus ($t=0.27$, df = 40, $P=0.78$) of suicide subjects compared with controls.

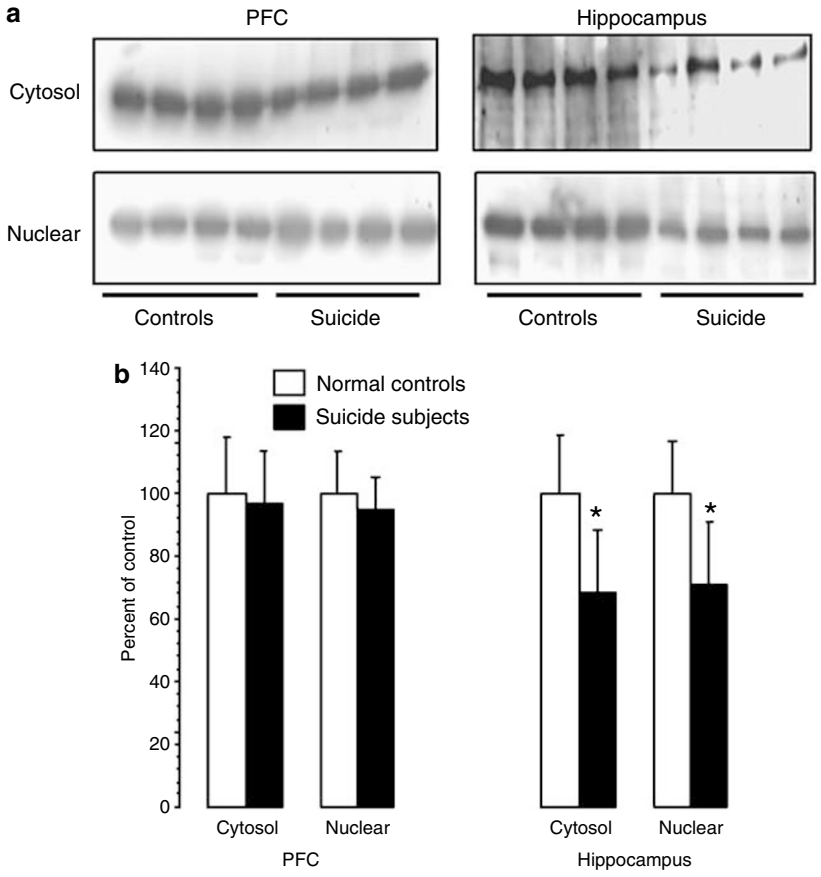

Figure 2 Catalytic activity of ERK5 in cytosolic and nuclear fractions of PFC and the hippocampus of suicide subjects and normal controls. (a) Representative autoradiograms showing ERK5 activity in cytosolic and nuclear fractions of PFC and the hippocampus determined after immunoprecipitation using ERK5 antibody followed by incubation with MBP and [γ-^{32}P]ATP. (b) Mean $\pm$ SD of ERK5 catalytic activities in cytosolic and nuclear fractions of PFC and the hippocampus of suicide subjects and controls. PFC samples were from 21 controls and 28 suicide subjects, and the hippocampus samples were from 21 controls and 21 suicide subjects. *$P<0.001$.

Representative Western blots for ERK5 in cytosolic and nuclear fractions obtained from PFC and hippocampus are depicted in Figure 3a and b, respectively, whereas, bar diagrams showing the comparison of ERK5 between normal controls and suicide subjects in the PFC and hippocampus are depicted in Figure 3c and d, respectively. We observed that protein level of ERK5 was greater in the hippocampus than the PFC and that both hippocampus and PFC showed greater cytosolic ERK5 protein level than the nuclear ERK5 (Figure 3a and b). It was observed that the protein level of ERK5 was significantly lower in both the nuclear ($t=4.6$, df $=40$, $P<0.01$) and the cytosolic ($t=2.5$, df $=40$, $P=0.02$) fraction of the hippocampus of suicide subjects (Figure 3d). On the other hand, no change was observed in protein level of ERK5 either in the nuclear ($t=0.69$, df $=47$, $P=0.49$) or the cytosolic ($t=0.56$, df $=47$, $P=0.58$) fraction of the PFC of suicide subjects compared with controls (Figure 3c).

mRNA Levels of ERK5

The mRNA level of ERK5 was determined in both the PFC and hippocampus of suicide subjects and controls by quantitative RT-PCR using internal standards. Cyclophilin mRNA was determined in these brain areas as a housekeeping gene, and ratios of ERK5 mRNA *vs* cyclophilin mRNA were calculated. A representative gel electrophoresis showing the competitive RT-PCR for ERK5 in total RNA isolated from the PFC of one normal is given in Figure 4a, whereas a competitive PCR analysis of ERK5 is given in Figure 4b. It was observed that the amplification products for ERK5 and cyclophilin arise from the mRNA template at 355 and 304 bp, respectively. The digestion products from ERK5 and cyclophilin arise from cRNA at 180 + 175 and 239 bp, respectively.

Comparison of ERK5 in the PFC and hippocampus showed that the mRNA level of ERK5 was significantly reduced in the hippocampus ($t=6.98$, df $=40$, $P<0.001$) but not in the PFC ($t=0.15$, df $=47$, $P=0.88$) of suicide subjects compared with normals (Figure 4c).

We have used cyclophilin as housekeeping gene in our earlier studies and found no significant differences between normal controls and suicide subjects (Dwivedi *et al*, 2006). In this study, we also did not find a significant difference in cyclophilin mRNA between these two groups either in the PFC (controls: 776.66 $\pm$ 112.5, suicide: 801.53 $\pm$ 117.34 attomoles/μg total RNA; $t=0.74$, df $=47$, $P=0.46$) or in the hippocampus (controls: 783.47 $\pm$ 110.12, suicide: 768.34 $\pm$ 102.76 attomoles/μg total RNA; $t=0.46$, df $=40$, $P=0.65$).

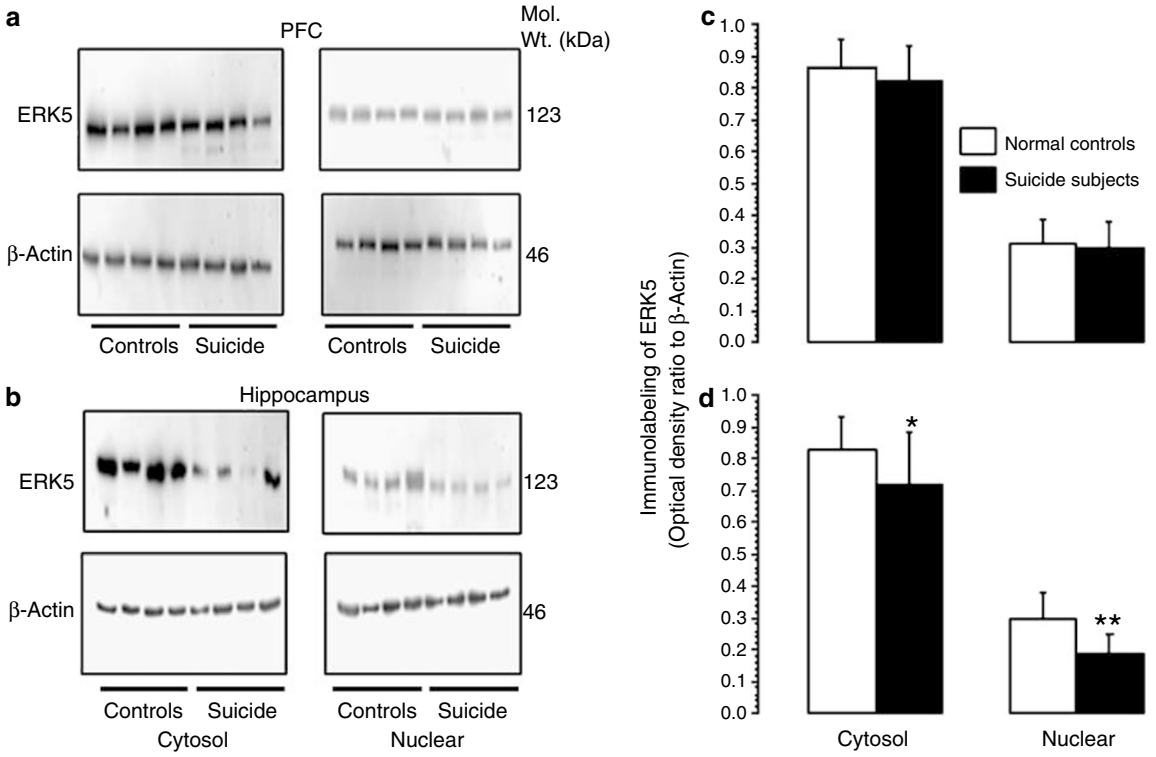

Figure 3 Immunolabeling of ERK5 in the PFC and hippocampus of suicide subjects and controls. Representative Western blots showing the immunolabeling of ERK5 and β-actin in cytosolic and nuclear fractions of PFC (a) and the hippocampus (b) of four controls and four suicide subjects. Protein samples were subjected to 7.5% polyacrylamide gel electrophoresis and transferred to ECL-nitrocellulose membranes, which were then incubated with primary antibody specific for ERK5 or β-actin and corresponding secondary antibody. The bands were quantified as described in Materials and methods. Ratios of the optical density of ERK5 to that of β-actin were calculated. The mean $\pm$ SD of ERK5 immunolabeling in cytosolic and nuclear fractions of PFC (c) and the hippocampus (d) from controls and suicide subjects. PFC samples were from 21 controls and 28 suicide subjects, and the hippocampus samples were from 21 controls and 21 suicide subjects. Suicide group was compared with control group. *$P<0.02$, **$P<0.001$.

The ratios of ERK5 *vs* cyclophilin mRNAs in the PFC and hippocampus are given in Figure 4d. It was observed that the mRNA level of ERK5 was still significantly decreased in the hippocampus of suicide subjects when expressed as a ratio to cyclophilin ($t=4.58$, df $=40$, $P<0.001$). There was no significant change in the PFC ($t=0.42$, df $=47$, $P=0.68$).

MEF2C-DNA-Binding Activity

MEF2C-DNA-binding activity was determined in the nuclear fraction by gel-mobility shift assay. A representative autoradiogram showing MEF2C-DNA-binding activity in the hippocampus of three controls and three suicide subjects is depicted in Figure 5a and as a bar diagram in Figure 5b. As can be seen, MEF2C-DNA activity was significantly decreased in the nuclear fraction of the hippocampus of suicide subjects compared with controls ($t=7.49$, df $=40$, $P<0.001$). No significant change was noted in PFC of suicide subjects ($t=0.42$, df $=47$, $P=0.68$) (Figure 5b).

Correlations between: mRNA and Protein Levels of ERK5; Protein Level and Catalytic Activity of ERK5; and Catalytic Activities of ERK5 and MEK5

We examined whether the decreased protein level of ERK5 in the hippocampus was associated with its mRNA level and whether decreased catalytic activity of ERK5 was associated with its protein level. We observed a significant correlation between the mRNA and the protein levels of ERK5 in both cytosolic ($r=0.35$, $P=0.02$) and nuclear fractions ($r=0.36$, $P<0.02$) of the hippocampus. Also significant correlations were noted between the catalytic activity and the protein level of ERK5 in cytosolic ($r=0.31$, $P=0.04$) and nuclear ($r=0.36$, $P=0.019$) fractions of the hippocampus.

We also examined if the decreased catalytic activity of ERK5 was associated with decreased MEK5 catalytic activity in suicide subjects and found a significant correlation between these two measures ($r=0.42$, $P=0.005$).

Effects of Diagnosis and Antidepressants

To examine whether the various measures of ERK5 and MEK5 as well as in MEF2C-DNA-binding activity, in which we found significant differences, between suicide subjects and normal controls were related to depression or were present in all suicide subjects, we examined the effect of major depression on these measures. For this purpose, we divided 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 subjects, 12 had major depression. In the suicide group with other psychiatric disorders ($n=16$), there were five with adjustment, two with schizoaffective disorder, two with bipolar affective disorder, three with drug/alcohol abuse, and three had no diagnosed psychiatric illness; in two suicide subjects the diagnosis was not available. Out of

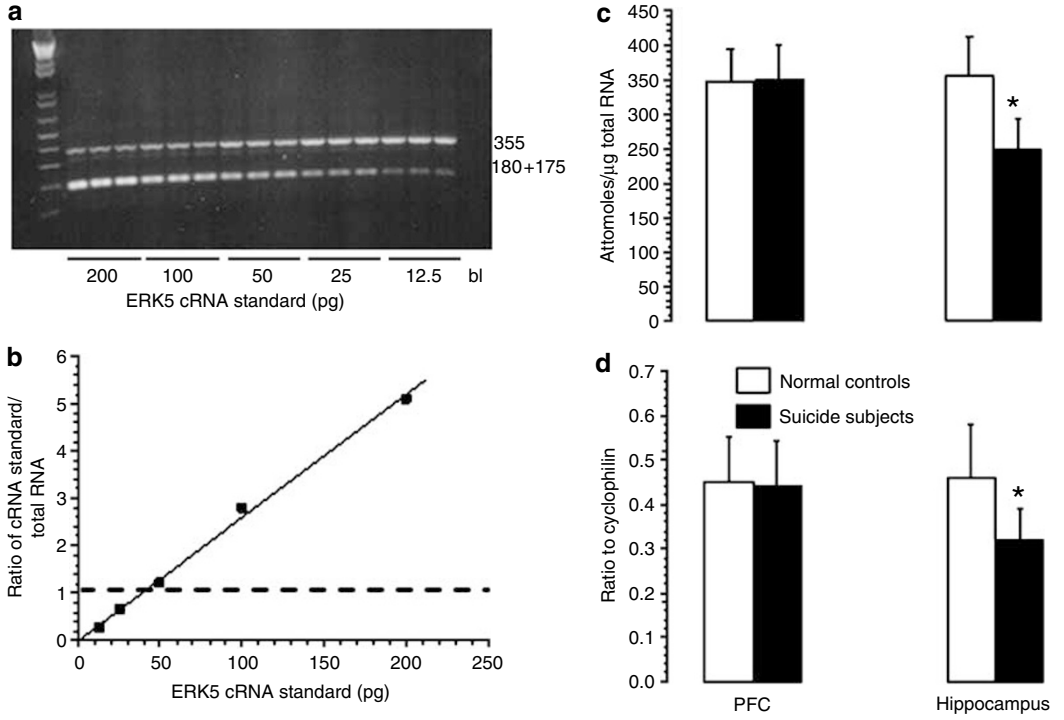

Figure 4 mRNA level of ERK5 in the PFC and hippocampus of suicide subjects and controls. (a) Representative gel electrophoresis showing competitive PCR analysis for ERK5 mRNA contents in the hippocampus obtained from one controls . Decreasing concentrations of standard cRNA (200–12.5 pg) were added to 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 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 bands arise from cRNA generated from the internal standard digested by *Xho*I. bl represent blank. (b) Data derived from the agarose gel are plotted as the counts incorporated into the amplified ERK5 cRNA standard divided by the counts incorporated into the corresponding mRNA amplification product *vs* the known amount of ERK5 internal standard cRNA added to the test sample. The point of equivalence represents the amount of ERK5 mRNA. mRNA levels of ERK5 (c) and as a ratio to cyclophilin (d) in the PFC and hippocampus of suicide subjects and normal controls. The PFC samples were from 21 controls and 28 suicide subjects, and the hippocampus samples were from 21 controls and 21 suicide subjects. Values are the mean ± SD. Suicide group was compared with control group. *$P<0.001$.

28 suicide subjects, the hippocampus was available from 21 suicide subjects. All the normal controls and suicide subjects for hippocampus studies were the same as described. The hippocampi from these suicide subjects were not available: four suicide subjects with major depression, two suicide subjects whose diagnoses of psychiatric illness was not available, and one suicide subject with adjustment disorder. A one-way ANOVA revealed that catalytic activity and mRNA and protein levels of ERK5; catalytic activity of MEK5, as well as MEF2C-DNA-binding activity were not different between suicide subjects with major depression and suicide subjects with other psychiatric disorders in hippocampus (Table 2). However, both suicide subjects with major depression and suicide subjects with other psychiatric disorders showed significant differences in these measures in the hippocampus when compared with controls (Table 2).

To examine whether the observed changes in measures of ERK5, MEK5, and MEF2C in the hippocampus of the suicide group were related to presence of antidepressant(s), we compared the suicide subjects who tested positive for antidepressants during the screen at the time of death ($n=5$) and those who did not ($n=23$). We did not find significant differences in hippocampal mRNA ($t=0.89$, $df=19$, $P=0.38$), protein levels (cytosol: $t=1.0$, $df=19$, $P=0.31$; nuclear: $t=1.0$, $df=19$, $P=0.32$), or catalytic activity (cytosol: $t=0.67$, $df=19$, $P=0.51$; nuclear: $t=0.11$, $df=19$, $P=0.91$) of ERK5 between those who showed presence of antidepressants at the screen at the time of death and those who did not. Antidepressants also did not have a significant effects on MEF2C binding activity ($t=1.8$, $df=19$, $P=0.08$). However, the catalytic activity of MEK5 was significantly higher in the group which showed presence of antidepressant during the screen at the time of death ($t=2.8$, $df=19$, $P=0.01$).

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 various measures in hippocampus in which we had found differences between normal controls and suicide subjects. We found no significant effects of age on mRNA ($r=0.04$, $P=0.82$) or protein levels of ERK5 (cytosol: $r=0.03$, $P=0.86$; nuclear: $r=0.08$, $P=0.59$); however, the catalytic activity of ERK5 only in cytosolic ($r=0.34$, $P=0.02$) but not in the nuclear fraction ($r=0.16$, $P=0.31$) was negatively correlated with age. A similar negative correlation of cytosolic ERK5 catalytic activity with age was noted in the PFC ($r=0.36$, $P=0.01$). On the other hand, age

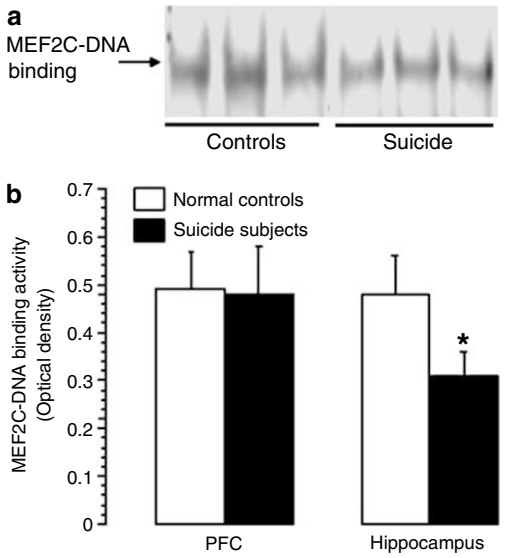

Figure 5 MEF2C-DNA-binding activity in PFC and the hippocampus of suicide subjects and normal controls. (a) A Representative autoradiogram showing MEF2C-DNA-binding activity in nuclear fraction of the hippocampus from three normal controls and three suicide subjects. (b) Mean ± SD of MEF2C-DNA-binding activity in the PFC and hipppocampus of controls and suicide subjects. PFC samples were from 21 controls and 28 suicide subjects, and the hippocampus samples were from 21 controls and 21 suicide subjects. Suicide group was compared with control group. *$P<0.001$.

had no significant effects on the catalytic activity of MEK5 ($r=0.09$, $P=0.51$) or on MEF2C-DNA-binding activity ($r=0.16$, $P=0.30$) in hipppocampus. No significant effects of PMI on mRNA ($r=0.05$, $P=0.72$), protein level (cytosol: $r=0.09$, $P=0.57$; nuclear: $r=0.10$, $P=0.51$), or catalytic activity (cytosol: $r=0.10$, $P=0.50$; nuclear: $r=0.22$, $P=0.15$) of ERK5 were noted in the hippocampus. The catalytic activity of MEK5 ($r=0.03$, $P=0.84$) and MEF2C-DNA-binding activity ($r=0.06$, $P=0.67$) were also not affected by PMI in this brain area. Furthermore, no significant effects of the pH of the brain were observed on mRNA ($r=0.06$, $P=0.70$), protein levels (cytosol: $r=0.03$, $P=0.84$; nuclear: $r=0.07$, $P=0.63$), catalytic activity of ERK5 (cytosol: $r=0.001$, $P=0.99$; nuclear: $r=0.17$, $P=0.27$), or on MEF2-DNA-binding activity ($r=0.24$, $P=0.13$) in the hippocampus. A significant negative correlation was observed between pH and MEK5 activity in the hippocampus ($r=0.33$, $P=0.03$).

We reanalyzed the data for ERK5 and MEK5 catalytic activities in which we observed significant correlation with age and pH respectively, and observed that catalytic activities of ERK5 (cytosolic: df = 2,38, F = 13.8, $P<0.001$; nuclear: df = 2,38, F = 14.4, $P<0.001$) and MEK5 (df = 2,38, F = 18.15, $P<0.001$) were still significantly decreased in the hippocampus of suicide subjects as compared with controls.

There were 17 male and four female subjects in the control group. Comparison studies showed no significant differences in any of the measures between male and female subjects in the hippocampus (ERK5 mRNA: $t=0.19$, df = 19, $P=0.85$; ERK5 protein: cytosol, $t=0.19$, df = 19, $P=0.84$; nuclear, $t=1.6$, df = 19, $P=0.12$; ERK5 catalytic activity: (cytosol, $t=0.44$, df = 19, $P=0.66$; nuclear, $t=2.0$, df = 19, $P=0.06$; MEK5 catalytic activity: $t=0.03$, df = 19, $P=0.97$; MEF2C-DNA-binding activity: $t=1.01$, df = 19, $P=0.99$).

DISCUSSION

In the present study, we noted interesting but contrasting results when ERK5 signaling was examined in the PFC and the hippocampus of suicide subjects. Initially, we determined the catalytic activity of ERK5 in both cytosolic and nuclear fractions, because both these fractions contain ERK5 protein (Kato *et al*, 1997; Raviv *et al*, 2003). We found that the catalytic activity of ERK5 was greater in the cytosolic fraction than the nuclear fraction. When compared between normal controls and suicide subjects, the catalytic activity of ERK5 was decreased in both cytosolic and nuclear fractions of suicide subjects. This decrease was confined only to the hippocampus, without any change in the PFC. Similar results were noted when the protein level of ERK5 was examined, such that the amount of ERK5 protein was greater in the cytosolic fraction than the nuclear fraction and when compared between controls and suicide subjects, protein level of ERK5 was significantly decreased in both these fractions of the hippocampus of suicide subjects. No significant differences were noted in the PFC. We also observed that the mRNA level of ERK5 was decreased in the hippocampus of suicide subjects. Interestingly, Yan *et al* (2001) reported three splice variants for ERK5, that is, ERK5a, ERK5b, and ERK5c, in mouse and showed that mouse ERK5a is the most expressed splice variant and shares 91% homology with human ERK5. They also showed that ERK5b and ERK5c lack a catalytic domain and inhibit ERK5a-mediated MEF2C transactivation, thus acting as dominant negative kinases. Further studies of specific splice variants will be helpful to delineate the role of these variant(s) in suicide.

To examine whether the decreased activation of ERK5 is due solely to the decrease in its expression or whether an upstream kinase is also involved in such decreased activation, we examined the catalytic activity of MEK5, which specifically activates ERK5 (English *et al*, 1995; Zhou *et al*, 1995; Kato *et al*, 1997). MEK5 exists in at least two spliced forms, α and β. MEK5β is ubiquitously distributed and is primarily cytosolic, whereas MEK5α is expressed highly in the brain and is present in the particulate fraction (English *et al*, 1995). As the antibody we used does not distinguish between these two splice variants, we determined the catalytic activity and protein level of MEK5 in the total fraction and found that catalytic activity of MEK was decreased in the hippocampus of suicide subjects without any change in its protein level. No change was observed in catalytic activity as well as protein level of MEK5 in the PFC. It has been shown that both α and β splice variants of MEK5 activate ERK5. However, mainly the α isoform is involved in differential subcellular localization of MEK5. This suggests a possible role of MEK5 in generating specificity in ERK5 signaling. Recently, Cameron *et al* (2004) reported that MEK5β inhibited epidermal growth factor-induced ERK5 activation and MEKα-induced MEF2 transcription activity, suggesting dominant negative behavior of MEK5β on ERK5 activation. Further study may clarify which isoform of

Table 2 Effect of Diagnosis on mRNA and Protein Levels of ERK5, Catalytic Activities of ERK5 and MEK5, and MEF2C-DNA Binding Activity in Hippocampus of Suicide Subjects

	Normal controls (n = 21)		Suicide subjects (N = 21)				Overall ANOVA			Multiple Comparison			
	1		With a history of MDD (n = 8) 2		With a history of other psychiatric disorders (n = 13) 3								
Variable	Mean	SD	Mean	SD	Mean	SD	df	F	P	1 VS 2	1 VS 3	2 VS 3
ERK5 mRNA[a]	355.76	56.38	249.75	45.33	245.46	44.43	2, 39	23.8	<0.0001	<0.0001	<0.0001	1.0
ERK5 Immunolabel-ing[b]												
Cytosol	0.83	0.11	0.68	0.16	0.75	0.17	2, 39	3.87	0.02	0.03	0.01	0.72
Nuclear	0.31	0.09	0.18	0.22	0.20	0.72	2, 39	10.9	<0.0001	0.001	0.002	1.0
ERK5 activity[c]												
Cytosol	100.00	18.54	69.50	25.84	67.53	17.37	2, 39	13.6	<0.0001	0.002	<0.001	1.0
Nuclear	100.00	16.37	76.50	27.17	68.08	13.74	2, 39	13.7	<0.0001	0.01	<0.001	0.92
MEK5 activity[c]	100	16.31	81.8	17.29	66.00	10.96	2, 39	20.8	<0.0001	0.02	0.001	0.07
MEF2C-DNA-Binding activity[d]	0.48	0.08	0.34	0.05	0.30	0.05	2, 39	29.4	<0.0001	<0.0001	<0.001	0.62

ANOVA = analysis of variance.
[a]Attomoles/μg total RNA.
[b]Optical density ratio to β-actin.
[c]Percent of control.
[d]Optical density.

MEK5 is involved in suicide and its respective role in the molecular pathophysiology.

Our correlational data analysis demonstrate significant correlations between the catalytic activity and the protein level of ERK5, as well as between the mRNA and the protein levels of ERK5, which suggests that the decrease in protein level of ERK5 is associated with a decrease in its mRNA level and that the decrease in its catalytic activity could be due to a decrease in the amount of ERK5 present. In addition, we observed a significant correlation between decreased catalytic activities of MEK5 and ERK5, suggesting that besides decreased expression level of ERK5, decreased catalytic activity of ERK5 could also be related to decreased MEK5 activity.

We also investigated the functional response of ERK5 by examining the DNA binding activity of MEF2C. MEF2C is a member of the family of transcription factor MEF2 (Kato *et al*, 1997; Marinissen *et al*, 1999) and is highly expressed in developing and adult brain (Leifer *et al*, 1993, 1994; McDermott *et al*, 1993; Lyons *et al*, 1995). Activation of ERK5 leads to phosphorylation and therefore enhanced transactivation capacity of MEF2C (Yang *et al*, 1998). In this study, we observed that MEF2C-DNA-binding activity was significantly decreased in the nuclear fraction of the hippocampus obtained from suicide subjects.

The observed changes in ERK5, MEK5, and MEF2C were not related to confounding variables, such as PMI, gender, or age of subjects. However, we observed that MEK5 catalytic activity was negatively correlated with pH of the brain. Interestingly, MEK5 catalytic activity was greater in the hippocampus of those suicide subjects who showed presence of antidepressants at the time of death, which suggests a possible reversal of decreased MEK5 activity by antidepressants. Further studies are required to confirm this finding in a larger number of antidepressant-treated subjects. Because depression is a major factor in suicidal behavior, we determined whether the observed effects on ERK5 signaling were specific to depression by comparing suicide subjects who were diagnosed with major depression with those who had other psychiatric disorders. It was found that the measures of ERK5, MEK5, and MEF2C were altered in all suicide subjects irrespective of psychiatric diagnosis.

Our finding of altered ERK5 only in the hippocampus but not in the PFC suggests brain region-specific changes and signifies a specific role of ERK5 in the hippocampus of suicide subjects. Although this has not been studied, it is quite possible that the ERK5 pathway may be distinctively regulated in different brain areas. It is pertinent to mention that recently we observed that the level of NGF was decreased in the hippocampus of suicide subjects (Dwivedi *et al*, 2005). Interestingly, it has been reported that internalization and retrogate transport of TrkA, receptors for NGF, activates specifically the ERK5 signaling pathway in neurons (Watson *et al*, 2001). Also, it has been shown that Epac suppresses muscle-specific A-kinase anchoring protein-associated ERK5 activity in a PKA-independent manner (Dodge-Kafka *et al*, 2005), and recently, we reported that Epac level is increased in postmortem brain of suicide subjects (Dwivedi *et al*, 2006). Whether such upstream regulation of ERK5 plays a role in the brain region-specific alteration in ERK5 is not clear and needs to be studied.

Our finding appears to be important and raises a fundamental question: what is the functional implication of an altered ERK5 pathway in the brain of suicide subjects? Emerging evidence suggests a role for ERK5 during development and in adult CNS functions (reviewed in Cavanaugh, 2004; Wang and Tournier, 2006). Recently, Watson *et al* (2001) reported that ERK5 is activated locally by NGF in the distal axons of DRG neurons and that activated ERK5 promotes retrograde neuronal survival through activation of p90 ribosomal S6 kinase, which in turn activates another transcription factor, CREB, involved in regulation of the transcription of survival and pro-apoptotic genes (Watson *et al*, 2001). ERK5, by directly phosphorylating proapoptotic proteins, such as Bad, participates in cell proliferation and in inhibition of apoptosis (Kato *et al*, 1998; Wang and Tournier, 2006). Kamakura *et al* (1999) showed that endogenous c-Fos induction is prevented by inhibition of ERK5 activation and that the MEK5-ERK5 pathway acts on endogenous SRE to express the c-*fos* gene in PC12 cells. Shalizi *et al* (2003) demonstrated that BDNF induces the ERK5-MEF2C pathway and thereby stimulates transcription of NT-3, specifically in newly generated granule neurons. Disruption of this pathway abrogated the ability of BDNF to promote the survival of newly generated granule neurons. It is pertinent to mention that we previously reported that expression of NT-3 is significantly reduced in postmortem brain of suicide subjects (Dwivedi *et al*, 2005). Recently, Wang *et al* (2004) demonstrated not only the role of NMDA and L-type voltage-gated ion channel-mediated activation of ERK5 in cerebral ischemia but also showed that activation of ERK5 plays a neuroprotective role in the hippocampus after cerebral ischemia (Wang *et al*, 2005). More recently, Yoon *et al* (2005) showed that ECS increased the activation of ERK5 and MEF2C phosphorylation in rat brain, which suggests a possible role of ERK5 in the mechanisms of action of ECS. Thus, given the role of ERK5 signaling in various aspects of physiological functions in the brain, decreased signaling by this pathway in the brain of suicide subjects appears to be significant.

In conclusion, to our knowledge, this is the first study examining ERK5 signaling in psychiatric illness, and demonstrates abnormalities in this signaling cascade at multiple levels in the hippocampus of suicide subjects. Given the importance of the hippocampus in cognitive behavior, and its role in stress and suicide, the decrease in ERK5 signaling in this brain area implicates the possible involvement of ERK5 signaling in the pathogenic mechanisms of suicide.

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