Increased Levels of Transcription Factors Elk-1, Cyclic Adenosine Monophosphate Response Element-Binding Protein, and Activating Transcription Factor 2 in the Cerebellar Vermis of Schizophrenic Patients

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Background: We investigated the levels of transcription factors associated with activation of the mitogen-activated protein (MAP) kinase pathway in schizophrenics using postmortem brain samples. These studies were done to determine whether our previous findings of abnormal levels of the MAP kinases in the cerebellar vermis were linked to additional downstream targets of this signal transduction pathway.

Method: We measured the protein levels of 3 transcription factors in nuclear fractions of postmortem samples from cerebellar vermis of 10 patients with schizophrenia and 13 control subjects: Elk-1, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), and activating transcription factor 2 (ATF-2). Studies in rats examined the postmortem stability and effect of haloperidol and risperidone on levels of Elk-1, cAMP, and ATF-2 proteins.

Results: We found a significant increase in the protein levels of Elk-1 (mean±SD, 4489±659 vs 2915±583 arbitrary densitometric units [P<.001]), CREB (mean±SD, 2149±1061 vs 904±711 arbitrary densitometric units [P=.003]) and ATF-2 (mean±SD, 1421±854 vs 512±394 arbitrary densitometric units [P=.003]) in the cerebellar vermis of schizophrenic subjects. Complementary studies in rats indicate that these findings can not be attributed to subacute treatment with antipsychotic medications.

Conclusion: Taken together with the alterations of MAP kinases previously reported, and the findings of elevations of downstream transcription targets, we suggest that the MAP kinase signal transduction pathway contributes to the cerebellar abnormalities in schizophrenia.

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The notion that the cerebellum may be an important neuroanatomic region in the pathogenesis of schizophrenia, while not totally new, has been reenergized by several streams of new data. Anatomical and neuroimaging data have provided new insights into the involvement of the cerebellum in both motor and cognitive aspects of behavior. In addition, a variety of neuroimaging and neuropathological studies have identified abnormalities in the cerebellum of subjects with schizophrenia. Volkow and coworkers have reported that medicated patients with chronic schizophrenia had reduced cerebellar metabolism as measured by positron emission computed tomography (PET). A later PET study on a group of schizophrenic patients withdrawn from medications for at least 3 weeks found that they failed to activate a prefrontal-thalamic-cerebellar network during complicated narrative recall, and termed this deficit cognitive dysmetria. Magnetic resonance imaging (MRI) studies examining schizophrenic patients have described a decrease in the size of the cerebellar vermis and, recently, a negative association between cerebellar size, symptom duration, and psychosocial impairment. Reduced Purkinje cell size in the vermis of schizophrenic patients has also been reported.

The cerebellum was selected as the primary focus of the current study based on the above data, as well as data from our earlier studies. For example, we found increased concentrations of nitric oxide synthase (NOS) in the cerebellar vermis. Moreover, we have demonstrated that several intermediates of the mitogen-activated protein (MAP) kinase pathway are altered in the cerebellar vermis, but not in mesopontine tegmentum or frontal pole (Brodmann area 10). Extracellular stimuli, such as nerve growth factor, ion channels associated with the N-methyl-D-aspartate–type glutamate receptors, muscarinic acetylcholine receptors, and nitric
SUBJECTS AND METHODS

SUBJECTS

Brain tissue was collected after consent for autopsy from the Central Veterans Healthcare System (Little Rock, Ark), University of Arkansas for Medical Sciences, and the State of Arkansas Medical Examiner’s Office. All procedures were approved by the Institutional Review Board of the University of Arkansas for Medical Sciences.

A probable lifetime psychiatric diagnosis was established for all subjects by using the Diagnostic Evaluation After Death. This evaluation index uses information from multiple sources, including medical record review and interviews with family. The Diagnostic Evaluation After Death was used whether the patient was being evaluated pre- or postmortem. Many of the elderly psychiatric patients were interviewed premortem, and psychiatric diagnoses were established according to DSM-III-R criteria by one of us (C.N.K.) or another trained psychiatrist. Two subjects had histories of alcohol abuse (Table). Medical records from control subjects were reviewed to determine whether they had an active psychiatric disorder at the time of their death and/or earlier in their lives. Two subjects had a distant history of alcohol abuse, which ceased 8 years before death. A third subject had an uncertain history of alcoholism that may have been ongoing at the time of his death. Finally, 2 patients were prescribed very low doses of amitriptyline and mesoridazine, respectively, for sleep or for pain control. Details of patients and their brains were reported previously. In both groups, the cause of death was generally cancer, cardiac failure, or a terminal respiratory condition.

PROCEDURES

Neuropathology

All brain specimens were subjected to standard gross and microscopic neuropathological examination by a board-certified neuropathologist (R.E.M.). Silver stains were performed to evaluate neuritic plaques and neurofibrillary tangles. Three schizophrenics and 5 controls showed mild age-related senile changes, and 2 schizophrenics showed small infarctions in regions remote from those evaluated in this study (Table). After collection during autopsy, the brain tissue was dissected and stored at −80°C. Left cerebellar vermis was the tissue between the bisected midline and 1-cm lateral to the midline.

Effects of Postmortem Interval

To determine the effect of postmortem interval (PMI) on the protein levels of the transcription factors, Sprague-Dawley rats (220-250 g) were given subcutaneous injections of either a low (0.15 mg/kg) or high dose (1.5 mg/kg) of haloperidol, a low (0.05 mg/kg) or high dose (0.5 mg/kg) of risperidone, or saline daily for 21 days. High doses for the both drugs were representative of human treatments for severe psychosis. The rats were killed 24 hours after the last treatment and the cerebellum was dissected and stored at −80°C until use.

Effects of Antipsychotic Medications

Sprague-Dawley rats (220-250 g) were given subcutaneous injections of either a low (0.15 mg/kg) or high dose (1.5 mg/kg) of haloperidol, a low (0.05 mg/kg) or high dose (0.5 mg/kg) of risperidone, or saline daily for 21 days. High doses for the both drugs were representative of human treatments for severe psychosis. The rats were killed 24 hours after the last treatment and the cerebellum was dissected and stored at −80°C until use.

Isolation of Nuclear Extracts

Nuclear extracts were isolated as previously described, with slight modifications. Human and animal tissue from cerebellum was homogenized in 10 volumes of homogenization buffer A (20 mmol/L HEPES; pH 7.9; 0.7% Nonidet P-40; 100 mmol/L sodium chloride; 1.5 mmol/L magnesium chloride; 0.5 mmol/L ethylenediaminetetraacetic acid; 0.5 mmol/L dithiothreitol; 10% glycerol, supplemented with the protease inhibitors phenylmethylsulfonyl fluoride [0.5 mmol/L]; leupeptin [5 µg/mL]; and apro tin [5 µg/mL]), and allowed to stand for 10 minutes on ice. The homogenate was centrifuged at 2000g for 10 minutes at 4°C. The pellet was washed in a further 10 volumes of buffer A and centrifugation was repeated. The nuclear pellet was suspended in 2.5 volumes of high-salt buffer B (20 mmol/L HEPES; pH 7.9; 0.5 mol/L potassium chloride; 0.5 mmol/L ethylenediaminetetraacetic acid; 0.5 mmol/L dithiothreitol; 25% glycerol; and the protease inhibitors phenylmethylsulfonyl fluoride [0.5 mmol/L]; aprotinin [5 µg/mL]; and leupeptin [5 µg/mL]). The resulting suspension was incubated for 30 minutes at 4°C on a rocker and nuclear debris was removed by centrifugation at 14000g for 30 minutes at 4°C. The supernatant was designated as the nuclear extract and stored in aliquots at −70°C. The nuclear extracts were used as samples for immunoprecipitation and Western blot analysis. Protein concentrations were determined by the method of Bradford.

Immunoprecipitation, Electrophoresis, and Western Blot Analysis

Samples containing 300 µg of nuclear proteins were subjected to immunoprecipitation with 1-µg anti–Elk-1 (I-20) antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C, followed by incubation with 20-µL protein A/G plus-agarose for 4 hours at 4°C. The immunoprecipitates were centrifuged at 10000g for 10 minutes, and the supernatant was discarded. The immunoprecipitates were washed twice with ice-cold Tris-buffered saline. The final pellet was then solubilized in 100 µL of sodium dodecyl sulfate sample buffer, boiled for 5 minutes, and a 30-µL aliquot was subjected to sodium dodecyl sulfate-polycrylamide gel electrophoresis. Electrophoresis and Western blotting were done essentially as previously described. Briefly, equivalent amounts of protein (25 µg) were loaded on to 10% sodium dodecyl sulfate gels and transferred to the membrane within 1.5 hours of death and kept under refrigeration until autopsied. Cerebellum was dissected and stored at −80°C until use. All procedures were approved by the Institutional Animal Care and Use Committee of University of Arkansas for Medical Sciences.
after electrophoresis, proteins were electrophoretic alliances transferred to a nitrocellulose membrane in transfer buffer (23 mmol/L Tris, 190 mmol/L glycine, and 20% [v/v] methanol). Equal loading and transfer of proteins was confirmed by Ponceau-S staining. The nitrocellulose membranes were blocked with 5% non-fat dried milk in Tris-buffered saline/0.1% Tween 20, pH 7.5, for 1 hour and then incubated overnight at 4°C with Elk-1 (corresponding to human Elk-1 residues 379-392); CREB (generated from human CREB at position 123-137); ATF-2 (generated from human ATF-2 at position 65-77); or signal transducer and activators of transcription (STAT-3) antibodies (New England Biolabs, Beverly, Mass), diluted 1:1000 in Tris-buffered saline/0.1% Tween 20 containing 5% bovine serum albumin. After several washings, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:2000 dilution) in Tris-buffered saline/0.1% Tween 20 containing 5% milk for 1 hour. The enhanced chemiluminescence method was used for detection. The 66-kilodalton (kDa) Elk-1, 43-kDa CREB, and 70-kDa ATF-2 bands were confirmed using control cell extracts (New England BioLabs).

**Densitometry**

The densitometric analysis of autoradiograms was performed on a Computerized Laser Densitometer model 300A (Molecular Dynamics, Sunnyvale, Calif); the images were analyzed by Image Quant software. The optical density of each band is expressed in arbitrary densitometric units.

**STATISTICAL ANALYSIS**

For human postmortem studies, data from experimental groups were compared by the Mann-Whitney U test. Correlations were done using the Spearman rank correlation. For rat experiments, data were analyzed using a Friedman 2-way analysis of variance test and the results are expressed as \( \chi^2 \) statistic. Statistics were done with statistical software Statview for Windows, version 4.5. Values of \( P < .05 \) were considered to be significant.

Oxide\(^{21}\) can trigger activation of extracellular signal-regulated kinases (ERks), members of the MAP kinase family in neurons (Figure 1). The activation of ERK-1 and ERK-2 is mediated via the conserved Ras/Raf/MEK pathway, which plays a critical role in cell growth and differentiation.\(^{22}\) On activation, MAP kinases are translocated to the nucleus and cause subsequent activation of immediate early genes, including c-Fos, through regulation of transcription.\(^{23}\) Recent studies have implicated the MAP kinase pathway to important brain functions, such as long-term memory formation.\(^{24-26}\) The MAP kinase intermediates are expressed at a higher level in areas associated with learning and memory, such as hippocampus, neurocortex, and cerebellum.\(^{27}\) Additional evidence suggested that transcription factors cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and Elk-1 are nuclear targets of MAP kinases during synaptic plasticity and memory consolidation.\(^{24,27,28}\) Given the crucial role of MAP kinases in the integration, amplification, and regulation of signal transduction, it will be not surprising to find that alterations in the expression of various intermediates in this pathway might be involved in the neuropathophysiological events occurring in the brains of patients with schizophrenia. In this study, based on our previous findings of elevated protein levels of ERK-2, we have chosen to determine the protein levels of downstream transcription targets, such as Elk-1, CREB, and activating transcription factor 2 (ATF-2), in cerebellar vermis of schizophrenic and control brains to investigate their potential role in schizophrenia.

**RESULTS**

**ELK-1, CREB, AND ATF-2 LEVELS**

Twenty-three subjects were included in the current study (Table). Brain tissue obtained from 10 patients with schizophrenia (9 men and 1 woman) and 13 controls (11 men and 2 women) were used. Schizophrenics did not significantly differ from controls in age (mean ± SD, 65 ± 13 vs 69 ± 7 years; \( U = 55; P = .54 \)), postmortem interval (PMI) (mean ± SD, 8.9 ± 5.2 vs 6.1 ± 3.4 hours; \( U = 47; P = .26 \)),
or brain weight (mean ± SD, 1279±136 vs 1298±127 g; *U* =43; *P* =.81).

We examined the levels of Elk-1 protein by Western blot analysis in nuclear extracts from schizophrenic and age-matched control subjects. The nuclear extracts were first immunoprecipitated and the immune complexes were processed further for immunoblot analysis. The immunoreactive bands were detected by the enhanced chemiluminescence method. Results presented in Figure 2, A, indicated that a protein of approximately 66 kDa molecular weight was detected on the autoradiogram. Difference in the levels of Elk-1 protein was quantified by densitometry of immunoblots from 10 patients with schizophrenia and 13 control subjects (*Figure 3*). We found a significant (about 2-fold) increase of Elk-1 levels in schizophrenic patients (mean±SD, 4489±659 and 2915±583 arbitrary units, for schizophrenics and controls, respectively; *U* =1; *P* ,.001).

For CREB, a protein of about 43 kDa was detected with the CREB antibody on a Western blot from nuclear extracts of patients with schizophrenia and control subjects (Figure 2, B). Quantification by scanning densitometry of immunoblots from 10 schizophrenic and 13 control subjects indicated more than a 2-fold elevation of CREB protein in the brain of patients with schizophrenia (mean±SD, 2149±1061 vs 904±711 arbitrary units; *U* =24; *P* =.003) (*Figure 3*).

For ATF-2, a member of the ATF/CREB protein family, the anti–ATF-2 antibody recognized a protein of approximately 70 kDa molecular weight (Figure 2, C). The data in Figure 3 indicate that the total immunoreactivity detected in the cerebellar vermis from 10 schizophrenic and 13 control subjects, quantified by densitometry, was significantly increased about 3-fold (mean±SD, 1421±854 vs 512±394 arbitrary units; *U* =18; *P* =.003).

Based on our statistical analysis, neither age, PMI, nor brain weight correlated significantly with the levels of Elk-1, CREB, and ATF-2 (3 factors×3 levels=9 total correlations; range, *r* =−0.36 to *r* =0.20). Regarding the correlations between the transcription factors, the correlations between Elk-1 and CREB (*r* =0.45, *P* =.03) and Elk-1 and ATF-2 (*r* =0.52, *P* =.01) reached significance.

To determine whether schizophrenia is associated with specific alterations of MAP kinase nuclear targets or whether there is a global elevation of transcription factors, we compared the levels of STAT-3. No difference was found in the protein levels of STAT-3 between the 2 experimental groups (mean±SD, 519±224 arbitrary units and 560±203 arbitrary units for controls and schizophrenics, respectively; *U* =25; *P* =.44).

### Subject Characteristics*

<table>
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<th>Case No./Sex/Age, y/Time Ill, y</th>
<th>Medication at Death (mg/d)</th>
<th>PMI, h</th>
<th>Weight, g</th>
<th>Neurpathology†</th>
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</table>

*PMI indicates postmortem interval; M, male; and F, female.
†Determined using Consortium to Establish a Registry for Alzheimer Disease (CERAD) criteria.‡
‡Diagnosis not definitive for Alzheimer disease by CERAD criteria.
EFFECTS OF POSTMORTEM INTERVAL AND ANTIPSYCHOTIC MEDICATIONS

We examined the postmortem stability of the protein levels of CREB at time intervals of 0, 3, 6, 9, 12, and 24 hours. Quantification of immunoblots from 6 rats indicated that there was no significant difference in the CREB protein levels after the rat brains were left for 24 hours at 4° to 8°C ($\chi^2=6.28, P=.28$).

Treatment with antipsychotic medications also had no effect on Elk-1, CREB, and ATF-2 in rat cerebellum. Haloperidol (0.15 and 1.5 mg/kg) and risperidone (0.05 and 0.5 mg/kg) were administered to rats (6 per group) by injection for 21 days. Densitometry of the blots presented in Figure 4 shows that long-term treatment with 2 different doses of either haloperidol, risperidone, or saline does not change significantly the levels of transcription factors Elk-1 ($\chi^2=2.27, P=.69$) (Figure 4, A), CREB ($\chi^2=5.47, P=.24$) (Figure 4, B), and ATF-2 ($\chi^2=5.77, P=.21$) (Figure 4, C) in rats.

COMMENT

Previously, we showed alterations of the MAP kinase signal transduction pathway in cerebellar vermis, but not in other brain regions, including mesopontine tegmentum and frontal pole (Brodmann area 10).13 Findings include increased protein levels of ERK-2, and decreased protein levels of the dual-specificity MAP kinase phosphatase (MKP-2). It was not clear from these studies whether the abnormalities of MAP kinases are associated with abnormalities further downstream of the MAP kinase pathway, or if more than 1 intermediate of this signal transduction cascade are altered in the brains of schizophrenics. We now report abnormal levels of several transcription factors within the promoter of the c-Fos gene in the cerebellar vermis from patients with schizophrenia. In cerebellar vermis, we found that the levels of Elk-1, the best-characterized MAP kinase target, were elevated in schizophrenia along with CREB and ATF-2, other targets of signal transduction pathways that are expressed ubiquitously.

Using commercially available phosphospecific antibodies, we were unable to detect the phosphorylated forms of Elk-1, CREB, and ATF-2 in the brain of schizophrenic and control subjects. This could be a result of postmortem events, such as the rapid dephosphorylation caused by endogenous phosphatases, which could affect the expression of the activated phosphorylated forms of the proteins. Therefore, we cannot comment if the observed elevation of transcription factors in cerebellar vermis are caused by increased phosphorylation.

Furthermore, we demonstrated that the protein level of transcription factor STAT-3 that is associated with inflammatory activation does not change in the cerebellar vermis of patients with schizophrenia. These data mediate against an inflammatory process or nonspecific increases in transcription factors in schizophrenia.

Human postmortem studies, especially in elderly patients, can be complicated by the existence of lifetime
exposure to multiple medications. Our sample cohort (Table) consisted largely of elderly male veterans (mean age, >65 years) who died in the hospital with multiple chronic illnesses. Moreover, most of the schizophrenic patients had received antipsychotic drugs over many years during their illness. To provide evidence that our findings are causally related to the schizophrenia and not merely adverse effects of medication exposure, we determined that effect of subacute administration of haloperidol and risperidone in rats did not change Elk-1, CREB, and ATF-2 levels. These results are intriguing, but the very high lifetime exposure of patients with schizophrenia to antipsychotic medications cannot be easily replicated in rat experiments. We are currently designing a study to examine the levels of several transcription factors in nonschizophrenic subjects who have had extensive antipsychotic treatment histories.

In terms of other psychiatric illnesses, Dowlatshahi and coworkers have found a high CREB concentration in the temporal cortex (Brodmann areas 20 and 21) from patients with major depressive disorder treated with antidepressants at the time of death. They have recently reported that bipolar disorder subjects treated with anticonvulsants at the time of death had decreased temporal cortex CREB protein levels relative to those not receiving anticonvulsants. This suggests that antidepressants and anticonvulsants may have opposite effect on CREB levels in patients with mood disorders.

It has been shown that the transcription factors have critical roles in the development and functioning of the normal nervous system, as well as in the adaptation of the nervous system to different stimuli and pathological situations. For example, CREB has been thought to play a role in cortical development and neuronal signaling in the hypothalamus. There is now considerable evidence suggesting a central role of CREB protein in long-term memory formation in both invertebrates and vertebrates. A recent study indicated that a defect of CREB phosphorylation may account for the cognitive dysfunction in patients with Coffin-Lowry syndrome. In addition, ATF-2, c-Jun, and c-Fos play roles in long-term potentiation and learning, during prenatal and postnatal development, and after stimulation by neurotransmitters, hormones, and neurotrophins. Several studies also have shown that ATF-2 is most abundant in the cerebrum, cerebellum, and forebrain. Given the critical role of the MAP kinase pathway in memory consolidation and long-term neuronal plasticity, and also the involvement of the cerebellum in memory, it is not surprising that we found alterations of the downstream transcription factor targets in this brain region in patients with schizophrenia.

**CONCLUSIONS**

This study provides new insights into cerebellar abnormalities of schizophrenia at the level of expression of Elk-1, CREB, and ATF-2 transcription factors targeting the serum response element and cAMP response element DNA regulatory elements, respectively. Future studies using animal models and cell cultures are necessary to elucidate the nature and molecular mechanism underlying these alterations in the brains of patients with schizophrenia.

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