Published in final edited form as:

J Affect Disord. 2010 July ; 124(1-2): 164–169. doi:10.1016/j.jad.2009.10.017.

Altered Levels of Extracellular Signal-Regulated Kinase Signaling Proteins in Postmortem Frontal Cortex of Individuals with Mood Disorders and Schizophrenia

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Abstract

Background—The extracellular-regulated protein kinase (ERK) pathway has been implicated in processes such as neuronal plasticity and resilience in psychiatric disorders including major depressive disorder (MDD), bipolar disorder (BPD), and schizophrenia. The extent of the possible involvement of this pathway in psychiatric disorders remains unknown, as does its potential utility as a pharmacological target for the future development of novel therapeutics.

Methods—Western blot analyses were used to measure levels of different proteins—Rap1, B-Raf, MEK1, MEK2, ERK1/2, RSK1, CREB, NSE, and beta-actin—in the postmortem frontal cortex of individuals with schizophrenia, MDD, and BPD, as well as healthy non-psychiatric controls.

Results—Levels of most studied protein members of the ERK cascade were lower in individuals with psychiatric disorders than controls; differences between psychiatric groups were not statistically significant. In general, protein levels were lower in individuals with schizophrenia than in those with BPD or MDD, but protein levels varied across groups.

Limitations—The small number of individuals in each diagnostic group may limit our interpretation of the results. Factors such as postmortem interval, medication status at time of death, and mood state at time of death may also have influenced the findings.

Discussion—The results are consistent with the hypothesis that the ERK pathway is implicated in reduced neuronal plasticity associated with the course of these psychiatric illnesses. The results

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Conflict of interest

The authors have no conflict of interest to declare, financial or otherwise. Dr. Manji is now with Johnson and Johnson Pharmaceutical Research and Development. This work was completed while he was an employee of the NIMH.

Contributors

Peixiong Yuan: managed the human brain samples; conducted some of the experiments; undertook the statistical analysis; wrote the first draft of the manuscript.

Rulun Zhou, Yun Wang, Xiaoxia Li, and Jianling Li: conducted some of the immunoblot experiments.

Xavier Guitart: managed the literature searches; conducted data analyses; contributed to manuscript preparation.

Guang Chen and Hussein K. Manji: designed the study; wrote the protocol; conducted data analyses; contributed to manuscript preparation.

All authors contributed to and approved the final manuscript.

warrant an expanded investigation into the activity of other members of this pathway as well as other brain areas of interest.

Keywords

Bipolar disorder; major depressive disorder; schizophrenia; brain; cAMP response binding element (CREB); protein regulation

1. Introduction

Brain intracellular signal transduction systems have been found to be altered in patients with different psychiatric illnesses, including mood disorders and schizophrenia. Additional evidence also implicates the existence of such alterations in the brains of genetically-altered animals, as well as in rodent brains after pharmacological treatment. Specifically, human brain imaging and postmortem studies have revealed discrete regional morphological changes in the brains of individuals with bipolar disorder (BPD), major depressive disorder (MDD), and schizophrenia (Drevets, 2000; Miguel-Hidalgo and Rjkowska, 2002; Cahn et al., 2008; van Haren et al., 2008), as well as dysfunctional neurotrophic signaling in the brains of suicidal subjects (Dwivedi et al., 2006). In addition, decreased size, density, or number of neurons and glial cells have been noted in the brains of individuals with these diagnoses, suggesting dysfunctional neurotrophic signaling.

Although the monoaminergic neurotransmitter systems and their alterations have traditionally received much attention in the neurobiological study of mood disorders and schizophrenia, a growing body of experimental findings point to the idea that mood disorders are associated with regional impairments of neuronal plasticity and resilience (Bachmann et al., 2005; Shaltiel et al., 2007). This suggests that different intracellular pathways and their members are possible targets for the development of new pharmacological treatments. The extracellular signal-regulated kinase (ERK) pathway is one of these putative candidates (Chen and Manji, 2006).

ERK is activated by many receptors that promote formation of active Ras and Rap1, including several neurotrophic factors. Once activated, Ras and Rap1 may recruit the serine/threonine-kinase Raf to the plasma membrane, where Raf activity is controlled by various protein kinases and the protein phosphatase PP2A (Kolch, 2000). Activated Raf phosphorylates the dual-specificity mitogen activated protein kinase kinase (MEK), which in turn phosphorylates ERK. Phosphorylation of ERK correlates with ERK activity, and activated ERK regulates cytoplasmic and nuclear effectors by phosphorylation. Through stimulation of ribosomal S6 kinase (RSK) and cAMP response element binding (CREB) phosphorylation, the ERK pathway regulates gene expression, including different proteins related to apoptotic and antiapoptotic processes (Einat et al., 2003; Hao et al., 2004; Chen and Manji, 2006; Creson et al., 2009), and could represent a key signaling network underlying some forms of synaptic plasticity (Thomas and Huganir, 2004; Sweatt, 2004). In addition, a number of behavioral studies have investigated the role of the ERK pathway in mediating cognitive function. Notably, rodents treated with a MEK inhibitor had impaired memory consolidation and/or reconsolidation in various paradigms (Thomas and Huganir, 2004), as well as in several behavioral paradigms associated with mood disorders (Einat et al. 2003). Moreover, pharmacological inhibition of MEK in ERK1 knockout mice resulted in impaired reconsolidation of fear memories (Mazzucchelli et al., 2002), despite the fact that these mice showed no cognitive deficits (Cestari et al., 2006). In addition, lithium and valproate, two structurally dissimilar and widely-used mood stabilizers, activate this signaling pathway in rodents (reviewed in Chen and Manji, 2006). These agents are also well-known to enhance cellular functions such as neurogenesis, neurite growth, and neuronal survival (Chuang, 2005; Chen et al., 2005; Chen and Manji, 2006).

Given the important role of the ERK pathway in the central nervous system (CNS), as well as its potential role in modulating mood behavior, we investigated the possibility that some elements of this intracellular cascade might be altered in patients affected with different mood disorders. Moreover, the fact that schizophrenia is thought to be a neurodevelopmental disorder led us to study the possibility that altered levels of these proteins might also be present in this disorder. Thus, we measured levels of different proteins—Rap1, B-raf, MEK1/2, ERK1/2, RSK1, CREB, NSE and beta-actin—in the postmortem frontal cortex of individuals with schizophrenia, MDD, or BPD, as well as in a group of healthy non-psychiatric controls.

2. Methods

2.1 Postmortem brain samples

Frozen postmortem brain samples of frontal cortex from individuals with BPD, MDD, or schizophrenia, as well as from non-psychiatric, healthy controls (“controls”) were obtained from the Stanley Foundation Brain Bank (Rockville, MD, 15 samples per group). Samples were matched for age, gender, and postmortem interval (Table 1). The demographic and clinical characteristics of individual subjects have been extensively detailed (Jarskog et al., 2000; Torrey et al., 2000). All samples were stored at -80° C until used, and all experiments were performed blind to diagnosis.

2.2 Tissue homogenization

Frontal cortex tissue (100 mg approximately) was homogenized in an extraction buffer of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphatase, 1 mM beta-glycerophosphate, and a protease inhibitor mixture (Sigma, St. Louis, MO) and then sonicated for 10 seconds. Homogenates were centrifuged at 14,000 × g for 15 seconds to remove undissolved debris, and supernatants were assayed for total protein concentration by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

2.3 Quantitative western blot

Immunoblotting was performed as previously described (Yuan et al., 2001). Briefly, total protein contents were adjusted to the same level for all samples, and the amount of total protein was loaded on the gels within the linear range of detection. Proteins were denatured in SDS sample buffer and separated on 10% Tris-glycine gels followed by transferring to Opti-Tran nitrocellulose membranes (Midwest Scientific, St. Louis, MO). Each sample was run in duplicate, and molecular weight markers and a pooled sample were run on each gel for quality control and interblot normalization. Primary antibodies to CREB, ERK (Cell Signaling Technology, Beverly, CA), Rap1, B-Raf, MEK1, MEK2 (Santa Cruz Biotechnology, Santa Cruz, CA), beta-actin, and neuronal specific enolase (NSE) (Chemicon, Temecula, CA) were used. The antibodies were diluted according to the manufacturers' recommendations. A secondary antibody from GE Healthcare (Piscataway, NJ) was used and the immune-complex was detected using chemiluminescence (ECL, GE Healthcare, Piscataway, NJ). Quantification of the immunoreactive bands was performed by optical densitometric scanning using an image analysis system (NIH Image 1.62).

2.4 Statistical analysis

Statistical analyses were performed by ANOVA, followed by Fisher's PLSD tests. Unpaired *t*-tests were used to compare two groups. *P* < 0.05 was considered statistically significant. Data are expressed as mean ± SEM.

3. Results

Protein levels were measured by Western blotting for Rap1, B-raf, MEK1, MEK2, ERK1/2, RSK1, CREB, NSE, and actin. Protein levels for most of the proteins studied were reduced in the patient groups compared with controls, although the patterns of these decreases differed from protein to protein and did not always achieve statistical significance (Figure 1 shows quantitative measurements for some ERK pathway proteins). No differences between psychiatric groups were significant. Protein levels were not significantly correlated ($p>0.05$) with age, postmortem interval (PMI), or pH, except for Rap1 and MEK1 with pH in the schizophrenia group ($r=-0.615$, $p=0.0175$, and $r=0.591$, $p=0.0242$, respectively), and CREB with PMI ($r=-0.675$, $p=0.0065$) in the MDD group.

Overall, more proteins were statistically significantly reduced in individuals with schizophrenia than any other diagnostic group. In individuals with schizophrenia, levels of B-raf, MEK1, MEK2, RSK1, CREB, and Rap1 were all significantly reduced compared with matched controls (B-raf: 48% of control, $t=2.73$, $df=28$, $p=0.01$; MEK1: 53% of control, $t=3.47$, $df=28$, $p=0.002$; MEK2: 52% of control, $t=2.349$, $df=28$, $p=0.0261$; RSK1: 41% of control, $t=2.58$, $df=26$, $p=0.0158$; CREB: 23% of control, $t=4.12$, $df=28$, $p=0.0003$; Rap1: 48% of control, $t=2.136$, $df=28$, $p=0.0416$).

Levels of several proteins—MEK1, MEK2, CREB, and Rap1—were also statistically significantly reduced in individuals with MDD (MEK1: 55% of control, $t=3.36$, $df=28$, $p=0.002$; MEK2: 59% of control, $t=2.255$, $df=28$, $p=0.0322$; CREB: 32% of control, $t=3.61$, $df=28$, $p=0.001$; Rap1: 47% of control, $t=2.162$, $df=28$, $p=0.0394$).

Levels of most proteins were reduced in individuals with BPD; however, these reductions reached statistical significance for only two proteins: B-raf and MEK1 (B-raf: 43% of control, $t=3.02$, $df=28$, $p=0.001$; MEK1: 60% of control, $t=2.48$, $df=28$, $p=0.02$). Notably, 61% of individuals with BPD had reduced CREB levels compared with matched controls, but this difference did not reach statistical significance, mainly due to large individual variations ($t=1.65$, $df=28$, $p=0.11$).

Overall, MEK1 was the only protein whose levels were statistically significantly reduced in all three patient groups (Fisher's ANOVA multiple comparison test for MEK1: $F(3,56)=4.326$, $p=0.0079$). Results varied for all other proteins, although there was a clear trend towards a generalized decrease in protein levels for all the psychiatric conditions, even when levels did not reach statistical significance. Fisher's ANOVA multiple comparison test results: (B-raf: $F(3,56)=3.767$, $p=0.0156$; RSK1: $F(3,52)=2.462$, $p=0.0728$; CREB: $F(3,56)=7.353$, $p=0.0003$, ANOVA; Rap1: $F(3,56)=2.856$, $p=0.045$; MEK2: $F(3,56)=2.546$, $p=0.0651$).

There were no significant differences in protein levels of ERK1/2, NSE, or beta-actin between controls and any psychiatric subgroup (see Figure 2). Furthermore, no statistically significant differences were observed between medicated ($n=36$) and non-medicated subjects ($n=24$; p values ranged from 0.3429 to 0.9393 for the nine proteins). Within each diagnostic subgroup, only three subjects were not medicated. Therefore, comparisons between non-medicated subjects from each disease group ($n=3$) and controls ($n=15$) were not possible.

4. Discussion

In this study, we showed that the ERK pathway is altered in the brains of individuals with BPD, MDD, and schizophrenia compared with controls. To the best of our knowledge, this is the first comparative study to investigate the expression of several proteins in the ERK pathway in the brains of individuals with these disorders.

Overall, several interesting patterns emerged. First, not all proteins involved in the ERK pathway were regulated to the same degree, or similarly across diagnoses. Indeed, only one protein—MEK1—was decreased at statistically significant levels in all three patient groups, although most patient groups showed decreased protein levels compared with controls, even when these did not reach statistical significance. Furthermore, levels of some proteins—ERK1/2, beta-actin, and NSE—were not reduced in any of the patient groups. Second, protein levels were statistically significantly reduced more often in the schizophrenia group than in individuals with BPD or MDD; of the proteins investigated, levels of six were reduced in individuals with schizophrenia (B-raf, MEK1, MEK2, RSK1, CREB, Rap1). Conversely, levels of four proteins were reduced in individuals with MDD (MEK1, MEK2, CREB, Rap1), and levels of only two proteins were reduced in individuals with BPD (B-raf, MEK1).

Our findings led us to hypothesize that downregulation of several components of this signal transduction pathway might contribute in part to the pathophysiology of these mental disorders or could represent the downstream/upstream result of other aspects of the illnesses. Despite the differences regarding which proteins were downregulated across diagnoses, the findings have several implications for the involvement of the ERK pathway in mental disorders. For instance, levels of the protein Rap1, a member of the Ras superfamily, were decreased in the postmortem brain tissue of individuals with schizophrenia and MDD. Together, Ras and ERK are necessary for long-term potentiation (Zhu et al., 2002). Rap1 has been reported to regulate long-term potentiation and spatial memory storage by coupling cAMP signaling to a membrane-associated pool of p42/p44 MAPK (Morozov et al. 2003), but its other possible synaptic functions are less understood (Jordan et al., 2004; Kim et al., 2005). Recent data suggest that Rap1 may regulate synaptic strength and induce loss of surface alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors (Fu et al., 2007); Rap1 exerts its effects via MAPK signaling pathways, resulting in removal of AMPA receptors with GluR2/3 subunits. AMPA receptors are the main contributors of excitatory neurotransmission and are involved in early response to glutamate in the synaptic space (Sanacora et al, 2008). Under normal conditions, the glutamatergic system plays a prominent role in synaptic plasticity, learning, and memory, but in pathological conditions it is known to be a potent neuronal excitotoxin, triggering either rapid or delayed neurotoxicity. Thus, reduced Rap1 protein expression might lead to over-activation of the AMPA signaling system and cause atrophic changes relevant to affective psychiatric disorders.

One important consideration concerns the activation of the proteins in the ERK pathway. Because their activation depends on their phosphorylation state, it can be argued that changes in total protein may not be that crucial, or that the changes observed in several of the pathway members could account for a final outcome of reduced phosphorylated CREB and then reduced translation of regulated proteins. Both CREB and brain derived neurotrophic factor (BDNF) are downstream targets of the ERK pathway, although levels of BDNF were not measured in our study. In addition, because BDNF binding to the tyrosine receptor kinase B (TrkB) receptor is not the only way that the ERK cascade is regulated or that CREB is phosphorylated (Nestler et al., 2002; Coyle and Duman, 2003), the relevance of other receptors and their ligands cannot be ruled out.

It should be pointed out that this study has several limitations associated with both its design and its interpretation. First, it is important to note that both PMI and pH are known potential confounding factors on biochemical measures in postmortem tissue (Lipska et al, 2006; Li et al., 2003; Harrison et al, 1995). Here, we found a significant negative correlation between pH and Rap1 levels in the schizophrenia group, a positive correlation between MEK1 levels and pH in the schizophrenia group, and a negative correlation between CREB levels and PMI in the MDD group. These correlations are unlikely to be simply due to PMI- or pH-related protein degradation. Although the precise molecular basis for these correlations is unknown, one

plausible explanation is that the correlations are due to unknown, disease-related, peptide-specific proteases that are sensitive to changes in PMI and pH. We found that CREB levels were significantly lower in the MDD group and that PMI was negatively correlated with CREB levels in this group. However, average PMIs were similar between control and MDD groups. Taken together, these data do not support the notion that lower CREB levels in the MDD group were due to PMI alone. We also found that Rap1 and MEK1 levels were significantly lower in the schizophrenia group, and that pH levels were negatively or positively correlated with Rap1 and MEK1 levels respectively; average pH levels were similar between the control and MDD groups. These data also do not support the notion that lower Rap1 and MEK1 levels in the schizophrenia group were likely due to pH levels alone.

The findings presented here should be considered preliminary and interpreted with caution. When using postmortem brain tissue to uncover either proteins or genes, protein phosphorylation within the intracellular signaling pathways should be strongly considered (Duman, 2002). We previously found that in mice the postmortem interval rapidly decreased levels of protein phosphorylation (Li et al., 2003); CREB phosphorylation, for instance, was almost abolished after four hours at 4°C. Therefore, no antibodies against the phosphorylated forms of all the proteins studied in this pathway were used. As a result, a relevant change in the phosphorylation degree for any of the unchanged proteins cannot be ruled out. Moreover, it is possible that proteins and protein phosphorylation in the human brain do not degrade in the same manner as in the rodent brain. However, although the postmortem interval and degradation of proteins cannot be ruled out, reductions in protein levels would likely be similar in all the brain samples regardless of their origin (from psychiatric subjects or controls), thus eliminating some of this possible bias. A related issue is that factors regulating total protein levels in the brain (or in this case the levels of the studied proteins) are unknown. For instance, levels of phosphorylated CREB cannot be measured in postmortem brain tissue in a reliable manner, making it difficult to determine the functional importance of decreased or unchanged levels of CREB uncovered in this study.

Another important limitation in human brain studies is the issue of medication. Although the confounding effect of medication on gene expression could not be ruled out completely, we observed no statistically significant differences when medicated subjects were compared to non-medicated subjects. One study of B-cell lymphoma 2 (Bcl-2) protein expression (Jarskog et al., 2000) found that individuals with schizophrenia or BPD who had previously taken antipsychotic medications had higher Bcl-2 levels than neuroleptic-naïve subjects; that study also found that lithium-treated individuals with BPD had higher protein levels, though these were not statistically significant. Thus it is possible that although the protein levels described here were lower in the psychiatric subjects than in the controls, without medication these levels would be even lower. In addition, this issue may have affected the different diagnostic subgroups differently. Again, further studies are needed to investigate this issue.

Another key limitation—particularly with regards to the mood disorder subgroups—is that in postmortem studies it is difficult to infer mood state at the time of death. One example of this in the present study concerns our findings regarding CREB. We found that CREB levels were not affected in individuals with BPD, but were decreased in individuals with MDD, an effect opposite to what has been previously described (Dowlatsahi et al., 1998). However, the mood stabilizers lithium and valproate, which are used to treat BPD, have opposite effects on CREB levels than the antidepressants used to treat MDD (Dowlatsahi et al., 1999), a difference with a large potential effect on the results obtained here.

Taken together, our data revealed similarly reduced protein levels (or a tendency towards reduced levels) in the three psychiatric disorders, suggesting a common downstream effect of the ERK pathway in the pathophysiology of psychotic and affective disorders. Given the

relationship of this cascade to neurogenesis, neurite growth, and neuronal survival, it is interesting to note that our results suggest a certain degree of neurodegeneration that may or may not be distinct from classic neurodegeneration observed in disorders such as Alzheimer's and Parkinson's diseases. Despite the preliminary nature of the results, our findings point to a common conceptualization of mood disorders and schizophrenia as “synaptic disorders”, and suggest that the ERK pathway may represent a new target for the development of novel pharmaceuticals for the treatment of these devastating disorders.

Acknowledgments

The authors gratefully acknowledge the Stanley Foundation Brain Bank (Rockville, MD) for providing the postmortem brain samples, and thank Ms. Ioline Henter for her invaluable editorial assistance.

Funding sources

This study was supported by the Intramural Research Program of the National Institute of Mental Health (NIMH; Bethesda, Maryland) and the Stanley Research Foundation (HKM). Neither the NIMH nor the Stanley Foundation had a further role in study design; in the collection, analysis, or interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

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Figure 1.

Altered levels of different proteins in the frontal cortex of individuals with bipolar disorder (BPD), major depressive disorder (MDD), or schizophrenia (SCZ) compared to non-psychiatric controls (Con). Data were acquired from gels and autoradiographs from frontal cortex ($n=15$ for each diagnosis). Unpaired t -tests were performed for two-group comparisons. The upper part of each graph shows a representative Western blot for a given protein.

Figure 2.

Immunoblots and histograms showing levels of ERK1/2 and beta-actin in the frontal cortex of individuals with bipolar disorder (BPD), major depressive disorder (MDD), or schizophrenia (SCZ) compared to non-psychiatric controls (Con). Bars represent the mean $\pm$ SD for each condition (n= 15).

Demographic characteristics of human postmortem brain samples from patients with bipolar disorder, major depressive disorder, or schizophrenia, and from non-psychiatric control subjects. (AA, African American; BPD, bipolar disorder; MDD, major depressive disorder; PMI, post-mortem interval; W, white).

Table 1

Subject	N	Gender	Ethnicity	Age (y) (Mean ± SD)	PMI (h) (Mean ± SD)	pH (Mean ± SD)
Control	15	9M/6F	14W/1AA	48.1±10.7	23.7±9.9	6.3±0.2
BPD	15	9M/6F	14W/1AA	42.3±11.7	32.4±15.9	6.2±0.2
MDD	15	9M/6F	15W	46.5±9.3	23.7±10.7	6.2±0.2
Schizophrenia	15	9M/6F	13W/2AA	44.5±13.1	33.7±14.6	6.2±0.3