Single-prolonged stress induces increased phosphorylation of extracellular signal-regulated kinase in a rat model of post-traumatic stress disorder

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Abstract. The extracellular signal-regulated kinase (ERK) signaling transduction pathway has been implicated in multiple physiological processes. It is not clear whether the ERK1/2 pathway participates in post-traumatic stress disorder (PTSD). The aim of this study was to provide novel insights into the mechanisms of how the amygdala participates in PTSD by investigating changes in the ERK1/2 pathway induced by single prolonged stress (SPS). The level of phosphorylated ERK1/2 (pERK1/2) protein was defined in a single-prolonged stress (SPS) animal model of post-traumatic stress disorder. A total of 100 male Wistar rats were randomly divided into a normal control group and SPS groups of 0, 30, 60 and 120 min. pERK1/2 distribution in the amygdala neurons was observed using immune electron microscopy. The expression of pERK1/2 was examined by immunohistochemistry and Western blotting. The pERK protein was located in some cell organelles, such as the mitochondria and neuraxon. Quantitatively, the expression of pERK protein level was significantly increased in the SPS rats. The results suggest that the ERK signal transduction pathway may play a crucial role in the pathology of PTSD.

Introduction

Post-traumatic stress disorder (PTSD) is an anxiety disorder that develops after exposure to a life-threatening traumatic experience. It is characterized by symptoms that often endure for years, including continuous re-experiencing of the traumatic event, avoidance of stimuli associated with the trauma, numbing of general responsiveness and increased arousal (1). Single-prolonged stress (SPS) (2) was shown to induce enhanced inhibition of the hypothalamic-pituitary-adrenal axis, which is a putative neuroendocrinological hallmark of PTSD (3-5). Subsequently, SPS paradigms were extensively developed and employed in the investigation of PTSD (6,7).

As one of the key regions in the limbic system of the brain, the amygdala has been documented to play a crucial role in fear, rage and emotional memory (8,9). Amygdala hyper-responsivity with apparent exaggerated responses in PTSD has been reported (10-19). Cui *et al* additionally found that behavioral changes in response to SPS may be attributed to the amygdala (20).

Extracellular signal-regulated protein kinases (ERK) are involved in a complex intracellular signaling cascade that controls various neurobiological effects (21). The active form of ERK1/2, namely phosphorylated ERK1/2 protein (pERK1/2), is activated in response to various excitatory stimuli (22) and is crucial for the regulation of the transcription of several transcriptional factors (21,23). The ERK pathway, a highly conserved kinase cascade, mediates the transmission of signals from cell surface receptors to cytoplasmic and nuclear effectors (24). It is well known that ERK1/2 play important roles in numerous types of cells, including neurons. The ERK1/2 pathway is activated by stimuli associated with synaptic activity and plasticity in neurons (25). Several lines of evidence indicate that the ERK1/2 signal pathway is activated by various stimuli (24), including exposure to stress (26). Following neuronal injury, the ERK pathway is activated, suggesting that it plays a role in cell death (27-29). Ding *et al* hypothesized that cellular apoptosis in the amygdala of rats in a PTSD model may be one of the causes of amygdala atrophy, which may decrease the volume of the amygdala (30). However, whether the ERK1/2 pathway is activated in the amygdala during SPS is not as yet clear. In view of the importance of the ERK pathway, we aimed to examine changes in the level of pERK1/2 in the amygdala of SPS rats, to determine whether ERK is involved in SPS. Our findings provide novel insights into the mechanism of the participation of the amygdala in PTSD.

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Materials and methods

Experimental animals. The study utilized 100 healthy male Wistar rats, aged 7 or 8 weeks at the start of the experiment and weighing approximately 180 g, supplied by the Animal Experimental Center of China Medical University. The rats were maintained at 22±1˚C and 55±5% humidity on a 12-h light/dark cycle with free access to food and water, and were allowed to acclimatize for 1 week prior to the experiments. Experiments were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications no. 80-23, revised 1996). All efforts were made to reduce the number of animals used and to minimize animal suffering during the experiment.

Model establishment and grouping. The rats were randomly divided into five groups: the SPS treatment groups (0, 30, 60 and 120 min) and the normal control group. The SPS model consisted of 2 h of whole body restraint in an animal holder, followed by 20 min of immediate forced swimming (temperature, 25˚C; depth, 40 cm). The rats were allowed to recuperate for 15 min, then exposed to ether vapor until loss of consciousness. The rats were then returned to their home cage and left undisturbed until being sacrificed for the experiments. The normal control rats were maintained in their individual home cages with no handling, and were sacrificed after acclimating to their new environment for a week.

Immune electron microscopy. Rats in each group (n=5) were transcardially infused with 200-300 ml of pre-cold saline through the ascending aorta, followed by 300 ml of 0.01 M PBS (pH 7.4) containing 2% PFA and 2.5% glutaraldehyde. The brain was removed and then postfixed overnight. The next day, the amygdala was dissected and cut into small fragments of ~1 mm^3, which were postfixed in 1% osmium tetroxide for 1 h at 4˚C. They were then rinsed in distilled water several times, dehydrated in a gradient series (20-100%) of ethanol and then in acetone, infiltrated with Epon 812, and finally polymerized in pure Epon 812 for 48 h at 65˚C. The basolateral amygdala was localized on semi-thin sections. Ultra-thin sections (70 nm) were cut with a diamond knife and collected on nickel grids. Immune electron microscopy was performed using mouse monoclonal anti-pERK1/2 antibody (Cell Signaling, Beverly, MA, USA) as a primary antibody, and goat anti-mouse IgG colloidal gold particles (5-nm diameter; 1:40 dilution; Boster Biological Technology Ltd., Wuhan, China) as a secondary antibody. In brief, the nickel grid was floated for 5 min on a drop of TBS-BSA (100 mM Tris-HCl, pH 7.6, 150 mM NaCl and 2% bovine serum albumin), then placed on parafilm in a moist chamber. The grid was then floated for 10 min on a drop of 3% H_2O_2, and then washed three times with distilled water and three times with TBS-BSA. The grid was incubated for 20 min on a drop of 10% goat serum at room temperature, followed by incubation for 24 h on a drop of primary antibody solution (diluted 1:100 in TBS-BSA) at 4˚C, and then washed three times. After being incubated for 2 h on a drop of secondary antibody solution (diluted 1:30 in TBS-BSA) at room temperature, the grid was washed three times with TBS-BSA and once with TBS. Finally, the sections were stained with 5% aqueous uranyl acetate, and images were captured using an electron microscope (JEM-1200 EX, Japan). The distribution of pERK1/2 protein in the neurons was assessed by observeing the location of the colloidal gold particles.

Brain tissue preparation and immunohistochemical analysis of pERK1/2. Rats in each group (n=5) were transcardially infused with 200-300 ml of pre-cold saline through the ascending aorta, followed by 300 ml of 4% pre-cold paraformaldehyde. The whole brains were rapidly removed and dissected on ice, followed by 6-10 h of post-fixation in 4% paraformaldehyde at 4˚C. The brain tissue was cut into 15-μm thick slices. After treatment with 1% hydrogen peroxide/methanol, the sections were incubated with 10% normal goat serum for 30 min at 37˚C, the primary antibody (mouse monoclonal anti-pERK1/2 antibody, 1:200 dilution; Cell Signaling) at 4˚C overnight, and the secondary antibody (goat polyclonal anti-mouse IgG, 1:200 dilution; Boster Biological Technology Ltd.) for 1 h at 37˚C. Then, the sections were incubated with avidin-biotin peroxidase complex (1:200 dilution; Boster Biological Technology Ltd.) for 1 h at 37˚C and the immunocomplexes were visualized with 0.05% DAB.

Images from five slices for each rat were obtained by an independent investigator and the results were analyzed by an image analyzer. The average number of positive cells from the amygdala of each rat, located according to the atlas of Paxinos and Watson (31), was calculated as the average of three sequential brain slices throughout the area of interest and measured in both the left and right hemisphere. The counting of positive cells was performed by an individual blinded to the treatment conditions, using the same magnification and identical color scale setting as a correction for background staining.

Western blotting of pERK1/2. Rats in each group (n=5) were rapidly decapitated, and the brain was removed and immediately placed in a dish standing on crushed ice. The basolateral amygdala was dissected according to the atlas of Paxinos and Watson (31) using a stereomicroscope, then snap-frozen in liquid nitrogen. Samples from normal control rats and SPS rats were respectively homogenized with a sample buffer containing 200 mM TBS (pH 7.5), 4% SDS, 20% glycerol and 10% 2-mercaptoethanol, and were denatured by boiling for 3 min. The protein fraction (30 μg/lane) prepared from each sample was separated by 12% (w/v) gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a PVDF membrane (Millipore, Bedford, MA, USA) from the gel by a semi-dry blotting apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The membrane was blocked with 5% dried skim milk and 0.05% Tween-20 in TBST at room temperature for 2 h, then incubated with mouse monoclonal anti-pERK1/2 antibody (1:1,000 dilution) overnight at 4˚C.

The blots were washed three times with TBST, incubated with anti-mouse IgG-HRP (1:5,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature, then washed with TBST. After the incubation, the PVDF membrane was washed three times with TBST before visu-

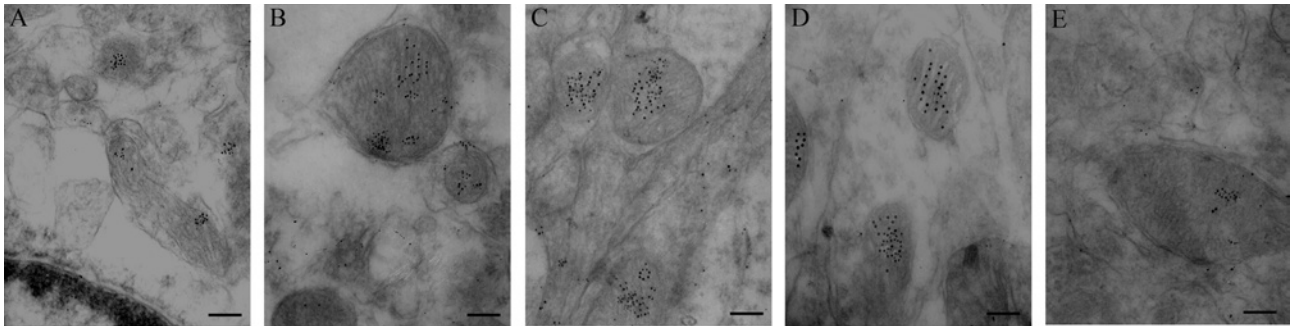

Figure 1. Determination of the distribution of pERK1/2 protein in the amygdala neurons by immune electron microscopy. (A) Control group; (B) SPS 0 min group; (C) SPS 30 min group; (D) SPS 60 min group; (E) SPS 120 min group. Bar, 100 μm.

alization by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). To confirm equal protein loading, the same blots were reincubated with antibodies specific for β-actin (dilution 1:2,500; Abcam, UK). Immunoreactivity for β-actin was detected with ECL. The optical density (OD) was analyzed on the Gel Image Analysis System. Each pERK band was normalized with respect to its corresponding β-actin band, and the values were expressed as the intensity ratio.

Statistical analysis. Values are presented as the means ± standard error of the means (SEM) and were analyzed using SPSS 11.5 software. Statistical significance was determined by one-way analysis of variance (ANOVA) follwed by the Tukey test when appropriate. Differences with a p-value <0.05 were considered statistically significant.

Results

Immune electron microscopy. The distribution of pERK1/2 protein in the neurons was determined by observing the location of the colloidal gold particles. The results of immune electron microscopy showed that the pERK protein was located in some cell organelles, for example, the mitochondria and neuraxon (Fig. 1). However, the location of pERK1/2 was observed in the cell only, and was not quantitatively analyzed.

Immunohistochemical analysis of pERK1/2. The results of immunohistochemistry are shown in Fig. 2. The pERK1/2 protein was located in cytoplasm, as indicated by brown staining (Fig. 2A-E). In the normal control group, there were few numbers of pERK1/2-positive cells, while in the SPS rats the numbers of positive pERK1/2 cells were significantly increased and peaked at 60 min after exposure to SPS ($p<0.01$) (Fig. 2F).

Western blot analysis of pERK1/2. The Western blotting results of pERK1/2 are shown in Fig. 3. The pERK1/2 proteins were detected in two bands at 42 and 44 kDa (Fig. 3A). The density of the pERK1/2 bands indicated that pERK1/2 was significantly increased in the SPS rats (0, 30, 60 and 120 min) ($p<0.01$) and peaked in the SPS 60 min group, exhibiting a 4- to 5-fold increase compared to the control group ($p<0.01$) (Fig. 3B). These results are consistent with those of the immunohistochemical analysis.

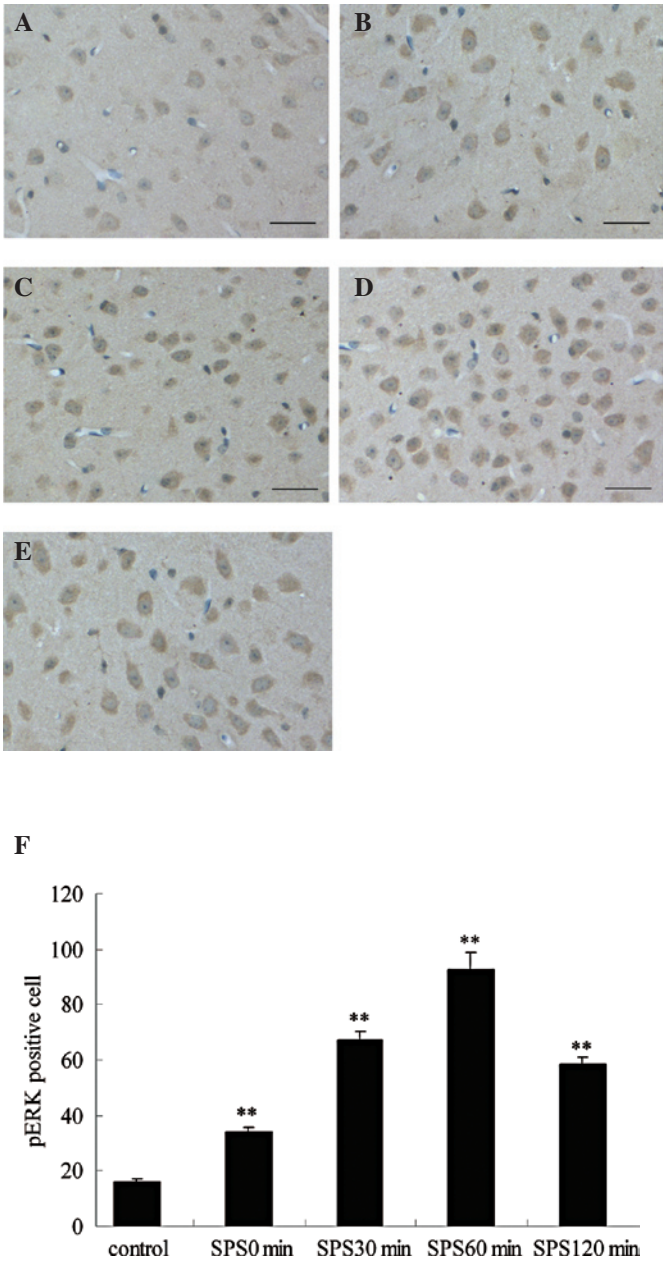

Figure 2. Determination of changes in the pERK1/2 level in the amygdala of SPS rats by immunohistochemical staining. (A-E) Representative photomicrographs of pERK immunostraining. (A) Control group; (B) SPS 0 min group; (C) SPS 30 min group; (D) SPS 60 min group; (E) SPS 120 min group. Bar, 20 μm. (F) Quantification of the changes in the number of pERK-positive cells in the amygdala. $^{**}p<0.01$ compared to the control group.

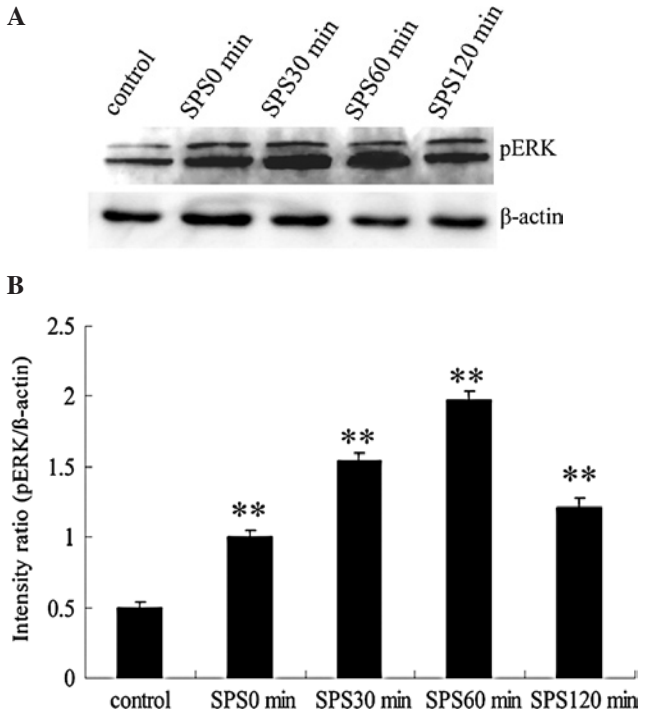

Figure 3. Determination of changes in the pERK1/2 level in the amygdala of normal (control) and SPS rats by Western blotting. (A) Typical results by Western blotting. (B) Relative levels of pERK1/2. Statistical analysis was conducted by one-way ANOVA [$F_{(4,20)}$=93.869, p<0.001] followed by the Tukey test. **p<0.01 compared to the control group.

Discussion

PTSD is defined by symptoms of continuous re-experiencing of the traumatic event, and avoidance of stimuli associated with the trauma and hyperarousal, suggesting a heightened fear response (1). Pathological fear and anxiety may be the manifestation of abnormal modulations in the amygdala and interactions with other regions of the brain, such as the medial prefrontal cortex (mPFC). The amygdala modulates spatial information processing in the hippocampus and is required for the expression of fear (32); indeed, it is the main output center for response to fearful stimuli (33). Neuroimaging studies of PTSD have to date revealed abnormal reductions in mPFC activity (11,34,35), as well as enhanced and distinctive amygdala engagement (10,14), particularly for combat veterans (11). Consequently, this study focused on observing changes in the basolateral nucleus of the amygdala.

The amygdala is divided into three distinct subgroups: the central nucleus, the corticomedial nucleus and the basolateral nucleus (36). The basolateral nucleus is the largest of the three (37), and is the key region for fear initiation. As a result, the basolateral nucleus has been the subject of significant attention. Vyas *et al* reported that chronic unpredictable stress induces atrophy in the bipolar neurons of the basolateral amygdala (38). Therefore, this study focused on changes in the basolateral nucleus. The ERK pathway, which relays signals from the plasma membrane to the nucleus, is one of the pathways in the mitogen-activated protein kinase signaling system (39). Two ERK proteins (ERK1/2) are components of this signaling pathway.

In this study, we found that the pERK1/2-positive cells in the amygdala were significantly increased in SPS rats, suggesting the involvement of the ERK signal transduction pathway in the response to stress. Studies have shown that the brain serotonergic receptors and norepinephrine levels are increased in the amygdala in response to stress (40,41). ERK hyperphosphorylation induced by stress in the amygdala is likely related to excessive monoaminergic neurotransmitters. ERK activation is a common cellular response to monoamine neurotransmitters, such as 5-hydroxytraptamine, norepinephrine and dopamine (42-44). The molecular mechanism of stress-induced ERK hyperphosphorylation may therefore involve the activation of ERK by neurotransmitters.

In the nervous system, the ERKs are activated in neurons in response to synaptic activity (45). ERK phosphorylation is a key step in mediating cellular responses to various extracellular changes, such as gene expression. In the amygdala, activation of the ERK pathway results in long-term changes in synaptic activity, which are thought to underlie the consolidation of fear memory (46,47). Ailing *et al* demonstrated that the levels of pERK and c-Fos were significantly increased in the mPFC during anxiety, and that the inhibition of ERK phosphorylation blocked anxiety-induced c-Fos expression. This suggests that the ERK signal transduction pathway plays a crucial role in anxiety, and that the inhibition of the ERK pathway in the mPFC may produce an anxiolysis effect (48). It is therefore of interest to examine which genes are activated by c-Fos induced by SPS in the central nervous system.

In conclusion, we demonstrated that the levels of pERK were significantly increased in the amygdala during SPS. The results suggest that the ERK signal transduction pathway may play a key role in PTSD. At present, the pathogenesis of PTSD is not entirely clear. PTSD may cause a series of biochemical abnormalities and dysfunction in the amygdala, which leads to brain dysfuction. Thus, the pathogenesis of PTSD requires further study.

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