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Epileptiform discharge upregulates p-ERK1/2, growth-associated protein 43 and synaptophysin in cultured rat hippocampal neurons

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ABSTRACT

Extracellular signal-regulated protein kinase, ERK1/2 is activated by phosphorylation (p-ERK1/2) during environmental stress such as epileptiform discharge. We investigated the role of ERK1/2 in abnormal axon growth and synapse reorganization in cultured neurons displaying epileptiform activity.

The cultured neurons displaying epileptiform activity were treated with magnesium-free extracellular fluid for 3 h and monitored epileptiform discharges using whole-cell patch clamp. Two study groups, neurons displaying epileptiform activity and the same neurons treated with ERK1/2 inhibitor U0126, were studied at six time points, 0 min, 30 min, 2 h, 6 h, 12 h, and 24 h following discharge. The expressions of p-ERK1/2, C-fos, growth-associated protein 43 (GAP-43) and synaptophysin (SYP), as markers of axon growth and synapse reorganization, were investigated by double-label immunofluorescence and western blotting.

In the neurons displaying epileptiform activity, p-ERK1/2 was detected immediately following discharge, and expression peaked at 30 min. The expression of C-fos, GAP-43 and SYP followed the same pattern as p-ERK1/2. In the treated group, p-ERK1/2 was inhibited completely, and C-fos, GAP-43 and SYP were reduced.

These findings indicate that epileptiform discharge activates ERK1/2 which regulates C-fos in cultured neurons displaying epileptiform activity, and this cascade may upregulate GAP-43 and SYP to contribute to axon growth and synapse reorganization to potentiate epileptic activities.

1. Introduction

Epilepsy is a clinical syndrome caused by abnormal discharge of a high degree of synchronization of neurons in the brain. The process whereby normal brain tissue undergoes an injury that produces permanent plasticity changes that lead to the occurrence of spontaneous recurrent epileptic seizures, is called epileptogenesis. To study some of the molecular events involved in epileptogenesis, hippocampal neuronal cultures were exposed to 3 h of magnesium-free media that resulted in a permanent alteration in the neuronal culture physiology as evidence by the development of a permanent "epileptiform" phenotype.[1,2] Neurons displaying epileptiform activity provide a useful culture model for investigating the molecular changes associated with epilepsy.

Mossy fiber sprouting, a phenomenon characterized by abnormal axon growth and synapse reorganization, is one of the pathological hallmarks associated with mesial temporal lobe epilepsy and occurs via unknown mechanisms. In general, both GAP-43 and SYP are considered to be markers of mossy fiber sprouting. GAP-43, a neuro-axonal growth protein, is upregulated in neural tissues during development and regeneration[3] and is elevated in injured neurons following ischemic damage.[4] SYP is a calcium binding protein expressed on presynaptic vesicles that plays an important role in synaptogenesis,[5] is directly correlated with the density of synapses, and is thought to be a marker for synapse reorganization.[6,7] Extracellular signal-regulated protein kinase (ERK1/2), a member of the mitogen-activated protein kinase (MAPK) family, is abundantly expressed in the brain,[8] especially in the neuronal cell body and dendrites. ERK1/2 acts as part of a signaling cascade activated in response to extracellular cues such as neurotransmitters or neurotrophic factors and influences synapse reorganization, axonal growth, neuronal excitability and transcription factor activation. ERK1/2 activation occurs when it is phosphorylated by MAP kinase and ERK activator kinase (MEK1/2)[9] and recent reports have found phosphorylated ERK1/2 (p-ERK1/2) expressed in an animal epilepsy model.[10] The transcription factor C-fos, a target for ERK1/2,[11] is upregulated following

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seizures and participates in the formation of epileptogenic foci. In addition to inducing transcription factor activation in the nucleus, p-ERK1/2 activates cytoplasmic substrates, including *MAP2* and *MBP*, thereby contributing to cytoskeletal remodeling and synapse reorganization.[12,13]

To better understand the cellular and molecular mechanisms underlying the process of epileptogenesis, both function and surface expression of GAP-43 and SYP were evaluated, and experimental techniques to modulate p-ERK1/2 and C-fos were employed to study the contribution of this cellular process toward the induction and maintenance of spontaneous recurrent epileptic seizures in this model. The results demonstrate that epileptic activity leads to increase in p-ERK1/2 and C-fos that contributes to the expression of GAP-43 and SYP in the hippocampal culture model.

2. Materials and methods

2.1. Preparation of neuronal epileptiform discharge model

Neonatal Wistar rats (<24 h old) were obtained from the Experimental Animal Center of Chongqing Medical University, CQ, China. After ether inhalation anesthesia, the brain was exposed and the hippocampus was carefully isolated under a microscope (Nikon, Tokyo, Japan), taking care to remove the meninges and superficial blood vessels. The hippocampus was minced in ice cold D-Hank's medium (Sigma, St. Louis, MO, USA), then incubated in five volumes of 0.125% parenzyme (Sigma) at 37 °C, 5% CO_2 for 30 min. The digestion was stopped by adding an equal volume of growth medium composed of Neurobasal medium (Gibco, Carlsbad, CA, USA), B-27 (2%; Gibco), L-glutamine (0.5 μM; Gibco) and FBS (0.5%; Gibco). Following centrifugation at 1000 rpm for 5 min, the supernatant was discarded and fresh growth medium was added. The tissue was dissociated and the cell suspension was filtered through a 200-mesh cell-sieve. Cells were diluted to a concentration of 5×10^5 cells per ml and plated on coverslips coated with polylysine (0.1%; Gibco) and maintained at 37 °C, 5% CO_2. 24 h after plating, medium was changed to maintenance medium (Neurobasal medium, B-27 (2%)), and L-glutamine (0.5 μM). Half the volume of maintenance medium was changed every 3 days. After 9 days in vitro, the following was performed using previously established procedures.[2] Briefly, cells were placed in magnesium-free media (145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 10 mM glucose, and 0.002 mM glycine, pH 7.3, and adjusted to 325 mOsm with sucrose) for 3 h and epileptiform activity was recorded with whole-cell patch clamp techniques.[2] For patch clamp recording, cultures were placed on the stage of an inverted microscope (Nikon) and continuously perfused with base-recording solution containing 25 mM 2-amino-5-phosphonovaleric acid, 10 mM 6-cyano-7-nitroquinoxaline-2,3-dione, 145 mM NaCl, 2.5 mM KCl, 10 mM Hepes (pH 7.3), 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM glucose. The osmolarity was adjusted to 325 mOsm with sucrose. Patch electrodes (2–4 MΩ resistance) with pipette solution containing 140 mM CsCl, 1 mM $MgCl_2$, 10 mM Hepes (pH 7.2), and 1.1 mM EGTA were used and the solution was adjusted to 310 mOsm with sucrose. In whole-cell current-clamp mode (at zero holding current), the membrane potential was measured immediately. And then neuronal recording was performed with an Axopatch 1D amplifier. Usually, whole-cell recording state could be maintained for a few minutes.

2.2. Double-label immunofluorescence

Wells of cultured neurons were randomly divided into either control, epileptiform, or U0126-treated groups. U0126(Sigma) is used as an inhibitor of ERK1/2 activation. Control neurons were treated with normal cell culture medium throughout the experiment. Neurons displaying epileptiform activity were incubated to magnesium-free medium for 3 h, returned to maintenance medium, and examined at the indicated times. U0126-treated neurons were incubated in magnesium-free medium and U0126 (10 μM) for 3 h, then returned to maintenance medium and examined at the corresponding times. P-ERK1/2 and C-fos were detected absolutely by immunofluorescence. Briefly, cells were washed with PBS for 3–5 min, fixed in 4% paraformaldehyde for 30 min, washed with PBS 3–5 min, treated with 0.5% Triton X-100 (Gibco) for 20 min at room temperature, washed with PBS 3–5 min, and blocked with 10% goat serum for 20 min at room temperature. Cells were incubated with mouse polyclonal anti-p-ERK1/2 and rabbit polyclonal anti-C-fos primary antibodies (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Secondary antibodies (goat anti-mouse FITC and goat anti-rabbit TRITC, 1:50, Santa Cruz Biotechnology) were applied for 2 h at room temperature, and washed with PBS for 3–5 min. For each sample, photos were collected using a laser scanning confocal microscope (Leica, Wetzlar, Germany).

2.3. Western blotting

The levels of SYP and GAP-43 in both control and treated groups were measured by western blotting at the same time points described above. Cells were washed with cold PBS, collected by centrifugation and lysed in cell lysis buffer (100 μl) containing Tris–HCl (50 mM; pH 8.0), NaCl (150 mM), EDTA (1 mM), EGTA (1 mM), Triton X-100 (1%), phenylmethylsulfonyl fluoride (1 mM), and freshly added protease inhibitor cocktail (Calbiochem, San Diego, CA, USA) on ice for 30 min. 50 μg of protein was resolved on a 10% polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) membrane, and blocked for 1 h at room temperature with 5% nonfat dried milk in PBS. Membranes were incubated with primary antibodies (rabbit anti-SYP and mouse anti-GAP-43 at 1:200 or mouse anti-β-actin at 1:2500, Santa Cruz Biotechnology) in blocking buffer. The blots were washed 3–10 min each with PBS plus Tween-20 (0.1%) and incubated with the appropriate diluted HRP-tagged secondary antibody (1:1000, Santa Cruz Biotechnology) for 1 h at room temperature. Blots were developed according to the manufacturer's instructions with Super Signal West Pico Chemiluminescent HRP substrate (Pierce, Rockford, IL, USA), and visualized with exposure to X-ray film. Band intensities were calculated with the Gelwork 4.1 image analysis system.

2.4. Statistics

Significant differences between experimental groups were determined with the Student's *t*-test. Values are presented as mean ± S.E. P values<0.05 were considered significant.

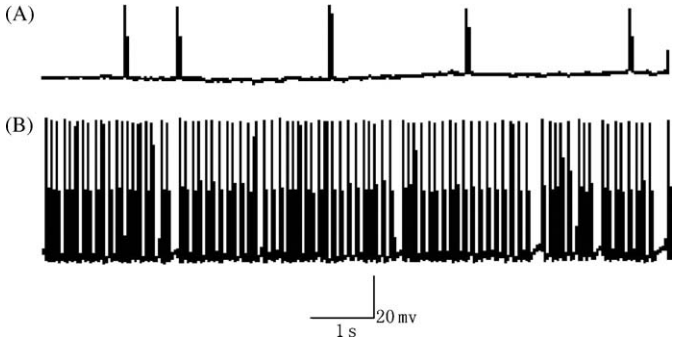

Fig. 1. (A) Before magnesium-free extracellular fluid exposure. (B) Spontaneous epileptiform discharge in cultured neurons induced by magnesium-free extracellular fluid for 3 h.

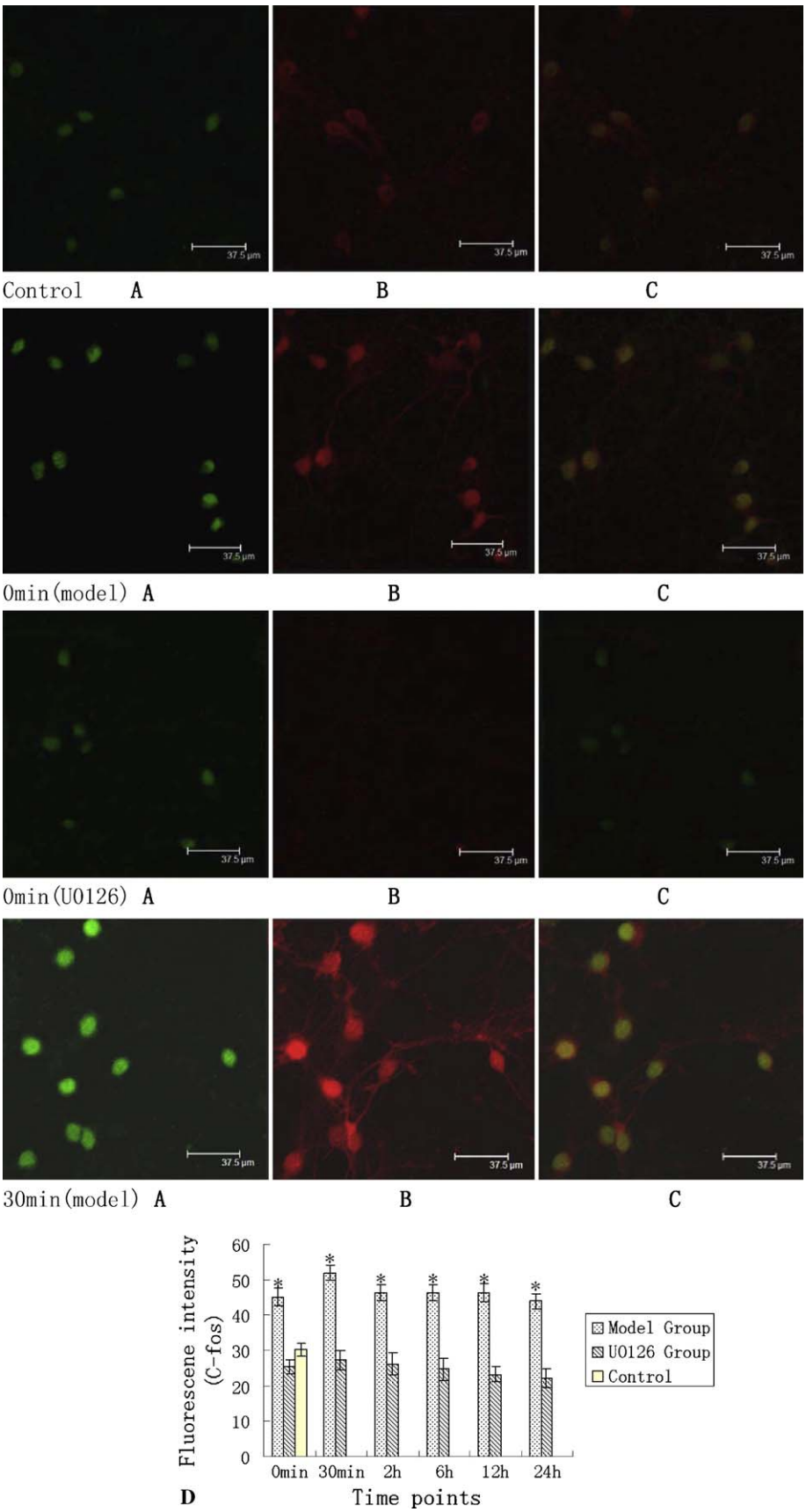

Fig. 2. (Control) C-fos were observed in the nucleus (A), and p-ERK1/2 were detected in the cytoplasm predominantly (B). (Model) Expression of both C-fos and p-ERK1/2 peaked at 30 min (30 min A and B), which were near to at other time points (0 min A and B). (U0126) There were no expression of p-ERK1/2, and similar expression of C-fos were detected at those time points (0 min A and B). (C) was merged images. Scale = 37.5 μm. Fluorescence intensity of C-fos: $^{*}P < 0.01$ (D: model group compared with U0126 group/control group; $n = 20$).

3. Results

3.1. Epileptiform discharge of neuron

Action potentials were recorded by patch clamp in neurons cultured in normal maintenance medium for the duration of the experiment (Fig. 1A). Recordings from neurons that were cultured in magnesium-free medium for 3 h manifested larger and longer duration action potentials. These action potentials progressed into continuous tonic high-frequency burst discharges that resolved into recurrent epileptiform discharges (Fig. 1B).

3.2. Activation of ERK1/2 cascade

Immunofluorescence double labeling found significant differences in the expression of p-ERK1/2 and C-fos between of untreated and U0126-treated neurons displaying epileptiform activity. In control neurons, C-fos was detected in the nucleus (A), and p-ERK1/2 was predominantly detected in the cytoplasm (B). Increased levels of both p-ERK1/2 and C-fos were observed in epiliptiform neurons at 0 min, with expression peaking 30 min after returning the neurons to maintenance medium (Fig. 2, 30 min (model) A and B). In U0126-treated neurons, p-ERK1/2 expression was inhibited completely (Fig. 2, 0 min (U0126) B), and C-fos expression was partially attenuated, with its staining remaining constant at each time point (Fig. 2, 0 min (U0126) A). We selected 20 neurons at random for each time point and compared the absolute fluorescence intensity of C-fos staining between the different experimental groups. Statistical analysis with Student's *t*-tests found significant differences between these groups at all six time points. Values are presented as mean ± S.E. ($\bar{X} \pm s$) and *P* values were <0.01.

3.3. Expression of SYP and GAP-43

We measured the protein levels of SYP and GAP-43 in untreated and U0126-treated neurons displaying epileptiform activity by western blotting. Both SYP (Fig. 3, Con) and GAP-43 (Fig. 4, Con) were detected in control neurons. In untreated neurons displaying epileptiform activity, both SYP and GAP-43 were detected at the first time point analyzed after being returned to maintenance medium (Fig. 3, 0 min and Fig. 4, 0 min) and changes in their expression patterns were similar to p-ERK1/2, with expression peaking at 30 min. In contrast, the expression of SYP and GAP-43 was significantly reduced across all time points in U0126-treated neurons displaying epileptiform activity (Figs. 3 and 4 *0 min–24 h*). The differences in the relative expression levels of SYP and GAP-43 between these groups were found to be significant at all six time points examined. Values are presented as mean ± S.E. ($\bar{X} \pm s$), and *P* values were <0.01.

4. Discussion

Although it is a common contributor to human morbidity, the pathological mechanisms involved in epilepsy are complex and not yet understood. Many researchers have reported that mossy fiber sprouting plays an important role in the pathology of epilepsy, and activation of the ERK1/2 cascade has been observed in animal models of mesial temporal lobe epilepsy,[9,14] although the relationship between these changes remains unresolved. The aim of our study was to clarify the role of ERK1/2 in abnormal axon growth and synapse reorganization following neuronal epileptiform discharges.

Taken together, our results suggest that p-ERK1/2 induces the expression of GAP-43 and SYP, whose gene products are associated with dendritic and synapse growth and reorganization, after epileptiform discharge of cultured rat hippocampal neurons. Inhibition of the p-ERK1/2 pathway with the specific inhibitor U0126, resulted in significantly decreased levels of SYP and GAP-43, suggesting that the ERK1/2 signaling pathway may participate in abnormal axon growth and synapse reorganization by virtue of regulating these genes. Since both abnormal axon growth and synapse reorganization are closely related to mossy fiber sprouting, which is an important pathological component of spontaneous recurrent epileptic seizures, we believe that the ERK1/2 signaling pathway may have an important role in the occurrence and development of spontaneous recurrent epileptic seizures.

Although the activation of ERK1/2 that accompanies the appearance of epileptiform activity suggests a cause–effect relationship between the ERK pathway and epileptiform synchronization,[10,15] there are differing opinions about the role of ERK1/2 in the pathogenesis of mossy fiber sprouting. A previous study concluded that both the ERK1/2 and CREB signaling pathways are highly activated during mossy fiber organization.[16] In contrast, Chen et al.[17] found that inhibiting NR2B-containing NMDARs, whose activation led to ERK1/2 phosphorylation, had no effects on

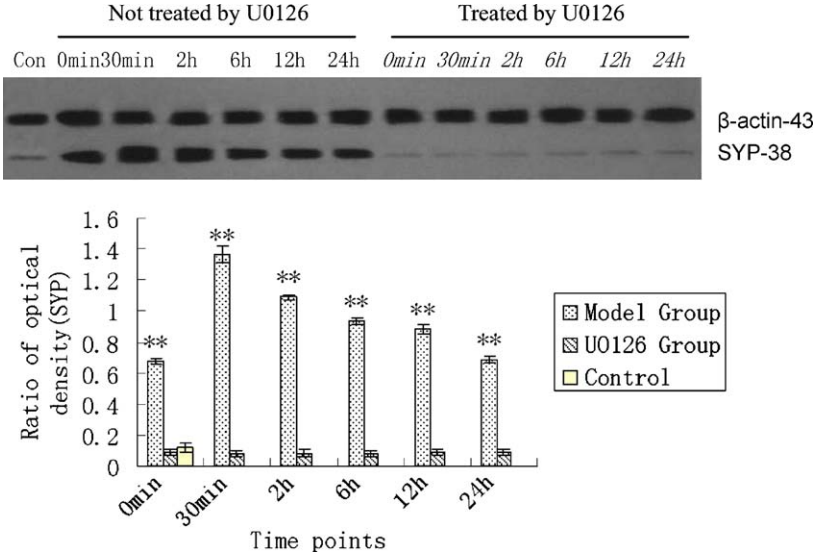

Fig. 3. 0 min–24 h represented model group, and *0 min–24 h* typified U0126 group. The optical density ratio of SYP to β-actin: $^{**}P < 0.01$ (model group compared with U0126 group/control group; $n = 5$).

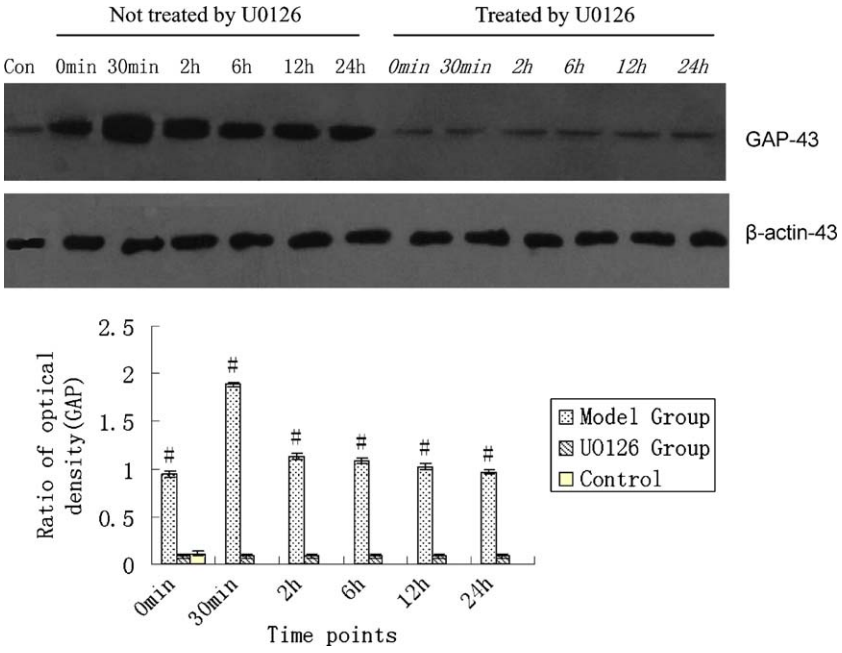

Fig. 4. Western blotting of GAP-43 and β-actin. 0 min–24 h represented model group, and *0 min–24 h* typified U0126 group. The optical density ratio of GAP-43 to β-actin: $^{\#}P < 0.01$ (model group compared with U0126 group/control group; $n = 5$).

epileptogenesis or mossy fiber sprouting. However, in the present study, p-ERK1/2, C-fos, GAP-43 and SYP were all upregulated in a similar time-course in neurons that had been exposed for 3 h to magnesium-free extracellular fluid. Importantly, inhibition of p-ERK1/2 with U0126 resulted in the reduced expression of the other proteins. Upregulation of C-fos expression is suggestive of the ERK1/2 cascade being activated, which was confirmed by a recent report.[12] Moreover, the expression of SYP and GAP-43 also correlates with the activation of the ERK1/2 cascade after neuronal epileptiform discharge. Our results demonstrate that the ERK1/2 pathway does indeed induce the expression of GAP-43 and SYP after epileptiform discharge of neurons; in other words, this suggests that activation of this pathway correlates with axon growth and synapse reorganization. Mossy fiber sprouting, an abnormal axon growth phenomenon, is one of the most important pathological changes after epileptic seizures and is associated with recurrent epileptic seizures.[14] Adachi and colleagues have shown that ERK1/2 is activated in human mesial temporal lobe epilepsy.[9] Therefore, we conclude that ERK1/2 regulates abnormal axon growth and synapse reorganization after epileptic seizures via regulation of GAP-43 and SYP expression, respectively. Our data do not detect mossy fiber sprouting in the epileptic neurons, therefore, a direct relationship between ERK1/2 and mossy fiber sprouting was not found. However, this work provides important clues about the signal transduction underlying recurrent epileptic seizures.

Animal models are used in many studies to investigate epilepsy, but because of the complexity of the nervous system a great number of confounding variables are difficult to control for. Additionally, it has been reported that blocking the connections between the two hippocampi breaks down the formation of a "mirror imaging epileptogenic focus". This indicates that the transmission of the "epileptic wave" between neurons is an important part of the epileptic microenvironment.[18] Accordingly, we followed the procedure described by Sombati[18] and found that after 3 h of exposure to magnesium-free extracellular fluid, 100% of neurons displayed sustained epileptiform discharge. 24 h after restoring normal maintenance media, more than 90% of neurons displayed epileptiform discharges. These data further support the neuronal epileptic discharge model. Our paradigm reveals that p-ERK1/2 is present in both the nucleus and cytoplasm. As a consequence, the expression of its downstream target gene (C-fos) was increased, as reported previously.[6,12]

In summary, these data demonstrate that in cultured neurons displaying epileptiform activity, ERK1/2 is activated and subsequently induces C-fos expression. In turn, this cascade plays a role in upregulating the expression of GAP-43 and SYP, indicating the ERK1/2 signaling pathway participates in the pathogenesis of mossy fiber sprouting. More work is required to determine the role of ERK1/2 in the mechanism of epilepsy pathogenesis. Furthermore, in vivo studies have been performed but not yet published to address the role of the same signal transduction pathway in mossy fiber sprouting, which may lend insight into the mechanism of recurrent spontaneous attacks of epilepsy. These mechanistic findings implicate attractive new targets for the development of drugs that may prevent epilepsy in patients.

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