Research report

Phosphorylation of P42/P44 MAP kinase and DNA fragmentation in the rat perforant pathway stimulation model of limbic epilepsy

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Accepted 20 December 2001

Abstract

The intracellular signaling pathways associated with neuronal injury after perforant pathway stimulation of the rodent hippocampus have not been examined. To determine whether activation of the p42/p44 (Erk1/2) MAP kinase (MAPK) phosphorylation cascade is linked to neuronal injury after perforant pathway stimulation (PPS), we stained for phosphorylated Erk1/2 (P-Erk1/2) and for DNA fragmentation, a marker of cell death after PPS. Eighteen Sprague-Dawley rats underwent PPS for 6 (n=6), 12 (n=6), or 24 (n=6) h and were sacrificed either immediately (n=9) or 48 h (n=9) after stimulation. Sham-operated non-stimulated control animals (n=2) and control animals receiving low frequency stimulation only (n=4) were also examined. Brain sections were stained for DNA fragmentation and P-Erk1/2. DNA fragmentation was evident only in granule cells and CA3 pyramidal cells of the stimulated side 48 h after 24 h of PPS. PPS resulted in robust phosphorylation of Erk1/2 that displayed a stereotyped timecourse, appearing first in hilar neurons on the ipsilateral side and later in hilar neurons, granule cells, hippocampal pyramidal and non-neuronal cell populations on both the stimulated and contralateral sides. Both Erk1/2 phosphorylation and DNA fragmentation show definite and reproducible staining patterns after PPS that vary based on duration of stimulation. Populations displaying Erk1/2 activation appeared to differ from those showing DNA fragmentation and neuronal injury. © 2002 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system

Topic: Epilepsy: human studies and animal models

Keywords: MAP kinase; Perforant pathway stimulation; Epilepsy; Rat; DNA fragmentation

1. Introduction

While selective neuronal injury has been described in a variety of animal models, recent studies have increasingly focused on the mechanisms and pathways leading to cell death. In particular, there has been ongoing debate as to whether seizure-induced cell death is necrotic, apoptotic, or both.

Accumulating evidence suggests that multiple mechanisms lead to cell injury after seizure activity in the rodent. Some studies suggest that granule cells of the dentate gyrus die via the apoptotic route while pyramidal cells of CA1, CA2 and CA3 as well as hilar neurons die via necrosis [2,17,35,36]. Both apoptosis and necrosis have been linked to glutamate excitotoxicity, NMDA receptor activation [30,31], and ischemia [25], but why certain cell populations die in one manner as opposed to another is not known. Granule cells represent a unique population of excitatory neurons within the hippocampus that are ideally positioned and connected to function as seizure-generating cells [26,27]. If these cells die by apoptosis, while other cells in the hippocampus die by necrosis, multiple neuroprotection strategies might be required to block stimulation-induced injury. We have used DNA fragmentation as a readily identifiable marker of cell injury, whose correlation with apoptotic cell death remains controversial [38].
The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that regulates intracellular activity in response to extracellular signals. Two neuronal isoforms of MAPK, p42 (ERK2) and p44 (ERK1) have been immunocytochemically mapped and found to have high concentrations within the cortex, cerebellum and hippocampus of both developing and adult brain [13]. Activation of these proteins requires dual phosphorylation on both serine and tyrosine residues. The presence of activated Erk1/2 in cell bodies and dendrites has implicated this pathway in postsynaptic signal transduction. Activation of Erk1/2 and other MAPKs have been described in response to ischemia [5], growth factors [4,8,16,21], glutamate receptor activity [19,30,31,41], and electroconvulsive shock treatment [1,37] and generalized seizures [15]. Numerous targets for Erk1/2 have been described, including cytoskeletal proteins and synapsin, and activated MAPKs have been shown to translocate from the cytoplasm into the nucleus and act to regulate transcription of early genes [41]. The exact function of activation in vivo, however, is unknown. Moreover, although attempts have been made to correlate the activation of MAPKs with apoptotic cell death [6,20], whether the activation of MAPKs is beneficial or detrimental to a cell undergoing stress is still not known. Interestingly, we have previously shown that inhibition of Erk1/2 activation blocks cell death in a primary neuronal cell culture model of seizure-like activity [23].

Fig. 1. Physiologic testing using the PPS model. Prestimulation (A–C) extracellular granule cell layer field recordings. Feedback or recurrent inhibition was tested using the 2 Hz frequency. As the interpulse interval (IPI) between stimuli is altered from 100 ms (A) to 60 ms (B) and ultimately 20 ms (C), there is a corresponding diminution of the ratio of the amplitude of the second granule cell spike to the first (downward deflection, long arrow). Afterdischarge spiking (D) was noted for the full duration of PPS. Delivered stimuli using the intermittent stimulation paradigm include a baseline continuous 2 Hz stimuli, or one paired pulse every 500 ms, (H) with superimposed 20 Hz stimulus trains, or 10 single pulses every 500 ms, (I) for 10 s out of every minute. Thick black vertical lines in H and I and grey vertical lines in I represent delivered pulses. After 24 h of stimulation using the intermittent paradigm, recurrent inhibition was lost as evidenced by no change in the ratio of the two granule cell population spikes at 2 Hz frequency when changing the IPI from 100 ms (E) to 60 ms (F) or even 20 ms (G). Multiple granule cell spikes in response to paired pulses (E–G) also indicate lost inhibition.
To study cell death after abnormal neuronal activity, we have utilized a rodent chronic perforant pathway stimulation (PPS) model, in which prolonged repeated stimulation of the angular bundle results in a reproducible pattern of unilateral hippocampal damage. We investigated whether Erk1/2 is phosphorylated in this model and whether phosphorylation occurs in neurons that subsequently manifest injury as demonstrated by DNA fragmentation.

2. Materials and methods

2.1. Perforant pathway stimulation

Male Sprague–Dawley descendant rats (250–350 g, Charles River Labs) were used in all experiments. All experiments were conducted in accordance with the guidelines for animal care set forth by the Subcommittee for Research and Animal Care of Massachusetts General Hospital. Rats were housed on a 12 h light/dark cycle and given ad libitum access to food and water. Rats were anesthetized with urethane (1.25 g/kg s.c.) and placed in a Kopf stereotaxic device. Supplemental doses of urethane were given subcutaneously as needed to maintain surgical anesthesia throughout the experimental period. Rectal temperature was monitored continuously and maintained at 37 °C (± 1 °C) with a heating pad (Harvard Apparatus).

Two holes were drilled in the skull of the animal to accommodate a recording and stimulating electrode. The stimulating electrode (bipolar stainless steel electrode, Rhode Medical Instruments; NE-200; 0.5 mm tip separation) was placed 4.5 mm lateral to the midline suture and 2.2 mm posterior and 2.5 mm lateral to bregma and lowered into the dorsal blade of the entorhinal cortex. The recording electrode (4 M NaCl-filled glass microelectrode, 0.4–1.0 MΩ resistance, 1.5 mm outer diameter) was placed 3.5 mm posterior and 2 mm lateral to bregma and lowered into the dorsal blade of the granule cell layer (approximately 3.5 mm below the brain surface). Exact placement of the recording electrode was determined by optimizing the characteristic shape of the evoked potential. Biphasic current pulses (0.1 ms duration) were generated using a Grass S88 stimulator with a Grass stimulus isolation unit. A supramaximal stimulus voltage was used and was usually 20–30 V. Potentials were amplified by a Grass preamplifier and displayed on a digital oscilloscope (Nicolet).

Inhibition was assessed as previously described by Sloviter [33]. The presence of feedback or recurrent inhibition, a measure of the animal’s ability to maintain electrophysiological homeostasis in response to granule cell firing within the hippocampus, was tested by examining paired-pulse inhibition as a function of interpulse interval (IPI). The IPI was reduced in steps from 100 to 20 ms (Fig. 1A–C). In normal animals this results in enhanced paired pulse inhibition manifested by a decreased ratio of the amplitude of the second evoked potential to the first evoked potential, whereas when feedback inhibition is reduced, the amplitude ratio between the first and second evoked potentials remains close to unity regardless of the IPI. At the beginning of each experiment animals were examined electrophysiologically to document the baseline integrity of inhibitory pathways (Fig. 1A–C). Evoked epileptiform activity after a 10 s 20 Hz train was also documented in each animal (Fig. 1D). For chronic PPS, paired pulses 40 ms apart were delivered at 2 Hz continuously throughout the 6, 12, or 24 h stimulation period, along with a superimposed 20 Hz/10 s stimulus train delivered once per minute (Fig. 1H and I). The testing protocol described above utilizing varied IPIs was repeated at the conclusion of chronic PPS to assess changes in recurrent inhibition (Fig. 1E–G). Subsequently, animals were perfused immediately (n=9) if they were to be analyzed for P-Erk1/2 or after 48 h (n=9) if they were to be analyzed for DNA fragmentation. Control animals in which electrodes were implanted for 24 h but stimulation was not given (n=2) or continuous 24 h stimulation was given at 2 Hz without superimposed 20 Hz trains (n=4) were sacrificed at appropriate time points for P-Erk1/2 (n=3) or DNA fragmentation (n=3) staining.

2.2. DNA fragmentation

We adopted the protocol developed by Wijsman et al. [39]. At appropriate time points, animals were anesthetized with urethane and perfusion-fixed with 4% paraformaldehyde. After storage in situ overnight at 4 °C, brains were harvested and cut on a sliding vibratome in 0.1 M phosphate buffer (50 μm thick sections).

Free-floating sections were immersed in 2× saline sodium citrate (1× saline sodium citrate contains 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at 80 °C and incubated for 20 min. Sections were treated for 10 min with pronase (1 μg/ml; Boehringer Mannheim) and the digestion process was stopped by immersing the sections in 2% glycine in phosphate-buffered saline (PBS), pH 7.4, for 30 s. Sections were quickly rinsed with double-distilled water and then incubated in a buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM MgCl₂, 10 mM β-mercaptoethanol. DNA fragmentation was assessed by positive immunostaining with antip53 antibody. There was no staining of non-neuronal cells (NNC) and evidence for granule cell staining [G (non-stimulated), H (stimulated) ×31].
toethanol and 0.005% bovine serum albumin for 5 min. Sections were then incubated in a humidified chamber in the presence of 50 μg/ml DNA polymerase I (Promega), 10 μM each of dCTP, dATP, dGTP (Promega) and biotin-21-dUTP (Clontech) at room temperature for 60 min. To detect the incorporation of biotinylated dUTP, sections were incubated in a humidified chamber in 1:100 horseradish peroxidase-conjugated avidin in PBS containing 1% bovine serum albumin and 0.005% Tween-20. Following two rinses in PBS, sections were mounted on slides, counterstained with cresyl violet, coverslipped and examined under the light microscope.

2.3. Phosphorylation of Erk1/2

Brains were harvested and sections cut as above. Free-floating sections were washed in PBS followed by 0.3% Triton (prepared in normal goat serum buffer) and then incubated with a rabbit polyclonal antibody directed against phosphorylated (Tyr-204) p42/p44 MAPK (1:200; New England Biolabs), overnight at 4°C. Sections were washed in PBS and then incubated in the secondary antibody (goat anti-rabbit; 1:200) overnight at 4°C. Sections were again washed in PBS, followed by a 30 min rinse in 0.3% H₂O₂ to extinguish endogenous peroxidases. Immunoreactivity was then visualized with the three-step ABC technique with diaminobenzidine tetrachloride as substrate (Vectastain Elite, Vector Laboratories). Sections were mounted on slides, counterstained with cresyl violet, coverslipped and examined under the light microscope.

3. Results

3.1. Neurophysiology

All animals had intact recurrent or feedback inhibition prior to stimulation. After 6 h of intermittent stimulation, no animals demonstrated an obvious change from baseline in feedback inhibition. After 12 h of stimulation we detected varying degrees of loss of inhibition in 2/6 animals. After 24 h of stimulation, loss of inhibition was apparent in all animals (Fig. 1E–G). All animals receiving 20 Hz superimposed trains exhibited evoked afterdischarges (Fig. 1D) throughout the stimulation period. All sham-operated and low frequency stimulated control animals exhibited normal responses to tests of inhibition.

3.2. Erk1/2 phosphorylation

After 6 h of PPS, Erk1/2 phosphorylation was observed within the ipsilateral dentate hilus. Both neuronal cell bodies and neuritic processes were stained (Fig. 2A, B, Table 1). Additionally there was staining of cells with the morphological features of granule cells located in a single/double cell layer at the inner edge of the granule cell layer adjacent to the hilus. These cells reside in what is known as the subgranular proliferative zone (SGZ), named for the existence of granule cell precursor cells in this location. (Fig. 2B–F, Table 1). Fiber tracts within the hilus consistent with the mossy fiber pathway from granule cells to the hilus and CA3 pyramidal cell layer were also stained on the ipsilateral side. Contralateral staining of neurons and neuritic processes within the hilus, along with presumed mossy fibers, was also visualized but to a much less significant degree (Fig. 2A, Table 1). Contralateral SGZ staining was not seen. Ipsilateral temporal and occipital neo-cortex revealed significant staining within neurons located in layers 2–5 and within small non-neuronal cells throughout all cortical layers (Fig. 3A, Table 1). We observed only rare staining in the contralateral cortex (Fig. 3B, Table 1).

After 12 h of stimulation, strong staining for P-Erk1/2 was observed within the hilus and in the SGZ bilaterally and symmetrically (Fig. 2C–F). Again, both cell bodies and neuritic processes were stained. Marked staining within small round non-neuronal cells was seen within the
Fig. 3. Phosphorylation of p42/p44 MAPK in the CA1 pyramidal cell layer and cortex following PPS. After 6 h of stimulation P-ERK1/2 is well visualized within neurons and non-neuronal cells in several layers of the temporal cortex on the stimulated side (A ×25) with no staining on the non-stimulated side (B ×25). After 12 h of PPS, both the stimulated (C ×25) and non-stimulated cortex (D ×25) stain fairly equally. At this time point, CA1 pyramidal cells and non-neuronal cells stain on the stimulated side (E ×25), whereas only non-neuronal cells stain on the non-stimulated side (F ×25). After 24 h, both the stimulated (G ×25) and non-stimulated (H ×25) CA1 region shows a diffuse and robust staining of non-neuronal cells.
hippocampal fissure, granule cell molecular layer and CA1 molecular layer (stratum lacunosum). These cells, which may represent glia, were seen bilaterally (Fig. 3E–F, Table 1). Additionally there was faint staining of pyramidal cells in the CA1, CA2 and CA3 pyramidal cell layer on the stimulated side only (Fig. 3E–F, Table 1). Mossy fibers, fibers of the CA1 molecular layer (stratum lacunosum) and granule cell molecular layer as well as temporal and occipital neurons was also stained bilaterally (Fig. 3C–F, Table 1).

After 24 h of PPS, P-Erk1/2 was visualized most strongly in the hilus, granule cell layer, SGZ and in non-neuronal cell populations of the CA1 region on the stimulated side (Fig. 2G, H). To a lesser degree, pyramidal cells of CA2 and CA3 region were also stained. Staining was more marked in the ventral blade of the dentate gyrus than in the dorsal blade. Again, both cell bodies and neuronal processes were stained. Additional staining was also seen in non-neuronal cells of the hippocampal fissure, molecular layer (Fig. 3G, H) and occipito–temporal cortex on the stimulated side. Occasional stained neurons in layers 3–5 of the occipito–temporal cortex were also seen (Table 1).

At the 24-h time point, contralateral P-Erk1/2 staining was seen in homologous regions with the exception of the pyramidal cell layer, which was stained only ipsilaterally (Table 1).

Control animals, stimulated for 24 h at 2 Hz without the superimposed 20 Hz trains or sham-operated with electrodes placed and no stimulation given, showed only rare stained cells along the electrode tract.

3.3. DNA fragmentation

After 6 or 12 h of PPS, there was no evidence of DNA fragmentation in any animals. After 24 h, significant DNA fragmentation was apparent within granule cell nuclei. This was more predominant in the ventral than the dorsal blade. Staining was much more marked on the stimulated side (Fig. 4–D, Table 1). Some inter-animal variability in the extent of DNA fragmentation across multiple coronal sections was noted. Staining on the contralateral side also varied considerably from one animal to the next, but was always present to some degree and always clearly less marked than on the stimulated side. Staining was uniformly nuclear. CA3 cells also revealed staining on the stimulated side, mostly in the region of CA3 closest to the hilus. Staining on the contralateral side in the CA3 region was not seen (Fig. 4B, Table 1). Staining was not evident in the cortex at any time point. Controls showed no DNA fragmentation in the hippocampus.

4. Discussion

The main finding of this study is that after PPS, Erk1/2 phosphorylation and DNA fragmentation appear in the hippocampus in incompletely overlapping neuronal populations. Within 6 h of beginning repetitive PPS, P-Erk1/2 is noted within mossy fibers, hilar neurons, cortical neurons, and neurons in the SGZ. By 12 h, pyramidal staining is noted as well and cortical staining peaks. After 24 h, both ipsilateral and to a lesser extent, contralateral staining is seen in broad regions of the pyramidal layer as well as in what appear to be glial cells. By contrast, DNA fragmentation, which appears after 24–48 h is seen mainly in ipsilateral granule cells and occasional pyramidal cells, mostly in CA3, and in contralateral granule cells to a lesser extent. Because DNA fragmentation is more restricted than P-Erk1/2 expression, we conclude that activation of Erk1/2 is not sufficient to commit neurons to early cell death associated with DNA fragmentation.

It is possible that additional P-Erk1/2 expression and/or DNA fragmentation might have been detected had different time points of sacrifice been chosen. For example, we may have missed either later expression of P-Erk1/2 or of DNA fragmentation, thereby generating an incomplete picture of the relation between the two. In previous experiments we have examined the time course of both P-Erk1/2 expression and DNA fragmentation in various
seizure models, including PPS. Based on these studies we selected time points for analysis that we had previously found to yield maximal signals and that we thought most likely to reveal a relationship. It is also possible that our assays were not sensitive enough to detect low but biologically important levels of either DNA fragmentation or phosphorylation of Erk1/2.

Anatomic studies have shown that Erk1/2, in its inactive form, is located throughout the adult rat brain, particularly in the superficial layers of the neocortex, striatum, cerebellum, and hippocampus [3,11]. In the hippocampus, the inactive form of Erk1/2 exists in cell bodies and dendrites of dentate granule cells, CA3 pyramidal cells, and to a lesser extent CA1 pyramidal cells, and in the stratum lucidum (or the combination of mossy fibers and CA3 dendrites) [3,11]. Ours is the first report of an increase in the signal of Erk1/2 phosphorylation after direct brain electrical stimulation, i.e., PPS. Moreover, we have documented the spatial and temporal distribution of P-Erk1/2 after synaptic stimulation. Surprisingly, we found strong bilateral staining for P-Erk1/2 in the hilus and SGZ, regions not traditionally thought to have an abundance of endogenous Erk1/2. Interestingly, Parent et al. recently showed that seizure activity secondary to pilocarpine or PPS led to new cell proliferation in the SGZ. These cells mature into granule cells in both the normal location in the dentate gyrus as well as in ectopic locations with aberrant axonal projections [27]. It is possible that Erk1/2 activation is involved in this proliferative response. We also found bilateral staining for P-Erk1/2 in granule cells. It seems likely that contralateral up-regulation of P-Erk1/2 is simply a response to commissural projections from the stimulated side. The relationship of this finding to the clinical phenomenon of secondary epileptogenesis (development of a mirror focus) highlights the potential importance of this observation.

Erk1/2 appears to transduce extracellular events into intracellular signals by triggering a cascade of phosphorylation. MAPKs in general can be activated or up-regulated by neurotrophic factors [8,16,21,28,32], neurotransmitters [19,41] ischemia [5] and seizures [14,15]. Previous studies showed a transient and rapid rise in phosphorylation of Erk1/2 after ECT [1,37], bicuculline-induced generalized seizures [15], pilocarpine [13] and kainic acid [18,22]. Erk1/2 is also rapidly phosphorylated in a cell-culture model of seizure-like activity [23]. At the cellular level, immunostaining has been found in cytoplasm, nuclei and neuritic processes [3,11]. Because Erk 1/2 is activated after numerous excitatory stimuli, is found in dendrites as well as cell bodies, and has been linked to transcription of early genes [41], it appears likely that this kinase cascade is important in mediating long-term effects of brief stimuli, or neuronal plasticity [9]. Moreover, the localization of activated MAPK to dendritic microtubules, which harbor MAP-2, responsible for microtubule assembly in vitro, suggests a role in dendritic remodeling [11,37].

In human surgical or autopsy specimens from patients with temporal lobe epilepsy and/or mesial temporal sclerosis, severe damage to CA1 and CA3 pyramidal cells, and dentate hilar interneurons with relative sparing of the granule cell layer and the CA2 pyramidal cells is consistently appreciated. Chronic (24 h) PPS results in a similar pattern of injury initially described by Olney and co-workers in 1983 using cresyl violet staining and electron microscopy [24,33]. Cell loss is found mainly in the hilar and CA3 regions and to a less severe degree in the CA1 region. We used DNA fragmentation as an assay for neuronal injury. Our finding that DNA fragmentation is apparent in only a subset of neurons that have been classically described as suffering injury after PPS supports the notion that in this system DNA fragmentation stains only for certain types of cell death, however, whether DNA fragmentation is specific for apoptotic cell death is controversial [30,31]. Our finding of marked granule cell DNA fragmentation on the stimulated side corresponds well with electron microscopic evidence for apoptosis restricted to the granule cells in the PPS model [36], and supports the notion that DNA fragmentation is a useful marker of apoptosis in this system. Granule cells may be predisposed to apoptotic cell death as demonstrated in previous studies after adrenalectomy or traumatic brain injury in rodents [7,17,35].

DNA fragmentation has been examined in rat brain after seizure activity generated by intra-amygdala or systemic administration of kainic acid, systemic pilocarpine, and maximal electroconvulsive treatment (MECS) [10,12,29,38]. In contrast to our finding after PPS, kainate and MECS fail to induce DNA fragmentation in granule cells [12,29,38]. It seems likely that after PPS, granule cell injury is the result of excessive synaptic input. As such, the PPS model may parallel the clinical phenomenon of neocortical epilepsy in which the hippocampus is only secondarily involved. This situation, where repeated neocortical seizures are associated with distant hippocampal injury, has been described as dual pathology in human epilepsy [34].

The role of Erk1/2 activation in cell death is uncertain. Some studies suggest that the phosphorylation of Erk1/2 actually plays a role in inhibiting apoptosis and neuronal injury [40,42,43]. Other studies, however, suggest that phosphorylation of Erk1/2 is part of a biochemical cascade resulting in neuronal injury [6,22,23]. Because we saw Erk1/2 phosphorylation in many cell groups after PPS that did not reveal DNA fragmentation, we conclude that Erk1/2 phosphorylation is not a sufficient intracellular signal to lead to DNA fragmentation at the times that we surveyed. It remains possible, however, that Erk1/2 phosphorylation is a necessary step in the pathway to injury in a subset of cell death pathways. In the PPS model, 6 h of stimulation results in neither identifiable hippocampal injury nor detectable loss of physiological inhibition. Because the Erk1/2 cascade here identified is turned on after only 6 h
of PPS, prior to irrevocable damage, it is possible that inhibitors of Erk1/2 activation could be neuroprotective in some neuronal populations. In fact we have previously shown that the selective MEK (the enzyme directly upstream of Erk1/2 and responsible for its phosphorylation) inhibitor PD98059 prevents Erk1/2 phosphorylation and cell death in a cell culture model of activity dependent injury [22]. The availability of additional potent MEK inhibitors such as U0126 and SL327 will permit detailed examination of the role of Erk1/2 activation in the development of injury and loss of inhibition.

Acknowledgements

The authors wish to acknowledge the technical expert assistance of Robert S. Sloviter, Ph.D. and Steven F. Ronner, Ph.D. in the perforant pathway stimulation model, Tessa Hedley-Whyte, M.D. for assistance with pathologic analysis, Tom Beer for histological technical assistance, and David Feliciano and Ali Mian for their help with figure preparation. This work was supported by a grant from the Whitehall Foundation (A.J.C.) and the NINDS (NS036224) (A.J.C.)

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