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Activation of ERK by spontaneous seizures in neural progenitors of the dentate gyrus in a mouse model of epilepsy

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Abstract

Cellular changes that are associated with spontaneous seizures in temporal lobe epilepsy are not well understood but could influence ongoing epilepsy-related processes. In order to identify cell signaling events that could occur at the time of spontaneous seizures, the localization of phosphorylated extracellular signal-regulated kinase (pERK) was studied in a pilocarpine mouse model of epilepsy at very short intervals (1.5–2.5 min) after detection of a spontaneous seizure. Within the hippocampal formation, immunolabeling of pERK was evident in a subpopulation of cells in the subgranular zone (SGZ) of the dentate gyrus at these short intervals. Many of these cells had a long vertical process and resembled radial glia, while others had short processes and were oriented horizontally. Labeling with a series of developmental markers demonstrated that virtually all pERK-labeled cells were neural progenitor cells (NPCs). A high percentage (~80%) of the pERK-labeled cells was labeled with either glial fibrillary acidic protein or brain lipid binding protein, indicating that these cells were radial glia-like NPCs. A smaller percentage of labeled cells expressed NeuroD, suggesting that they were later developing NPCs that were assuming a neuronal identity. Early expression of pERK was not detected in immature neurons. Double labeling with proliferation markers demonstrated that approximately 30% of pERK-labeled NPCs expressed Mcm2, indicating that they were actively proliferating. Furthermore, virtually all radial glia-like NPCs that were in the proliferative cycle expressed pERK. These findings suggest that spontaneous seizures and associated ERK activation could contribute to the proliferation of radial glia-like NPCs in this epilepsy model.

Keywords

Extracellular signal-regulated kinase (ERK); hippocampus; dentate gyrus; subgranular zone; neurogenesis; brain lipid binding protein (BLBP); spontaneous seizure; pilocarpine; epilepsy

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Introduction

Temporal lobe epilepsy is characterized by recurrent spontaneous seizures, but the changes that occur at the onset of such seizures *in vivo* are still poorly understood. Likewise, it is unclear whether spontaneous seizures influence ongoing epilepsy-related processes, such as increased neurogenesis, that occur following status epilepticus or other initial insults.

Seizure-induced increases in neurogenesis have been observed in several animal models of epilepsy, not only after status epilepticus (Gray and Sundstrom, 1998; Parent et al., 1998; Parent et al., 1997; Scott et al., 1998), but also after brief induced seizures (Bengzon et al., 1997). The newly-generated neurons originate primarily from neural progenitor cells (NPCs) in the subgranular zone (SGZ) of the dentate gyrus during the first few weeks after the initial insult. Many of the newborn neurons eventually integrate into hippocampal circuitry and may either contribute to the hippocampal network plasticity associated with epilepsy (Jessberger et al., 2005; Overstreet-Wadiche et al., 2006; Parent et al., 1997; van Praag et al., 2002) or, possibly, limit seizure activity (Jakubs et al., 2008).

While the effects of induced seizures on adult neurogenesis are well-documented, the effects of spontaneous seizures are less clear. In particular, the neurochemical changes that occur at the time of a spontaneous seizure and their potential influence on NPCs have received little attention. However, several recent findings have led us to consider possible links between activation of the extracellular signal-regulated kinase 1/2 (ERK) cascade, spontaneous seizures and neurogenesis.

The ERK pathway exhibits dynamic changes following several types of seizure activity. ERK is strongly activated in neurons following severe, chemically-induced seizures (Berkeley et al., 2002; Garrido et al., 1998; Jiang et al., 2005). Our previous studies have also demonstrated that phosphorylated ERK (pERK), the active state of ERK, is increased in many hippocampal neurons following recurrent spontaneous seizures in pilocarpine-treated mice (Houser et al., 2008). These findings suggested that pERK labeling could serve as one of the earliest immunohistochemical indicators of cells that are activated during spontaneous seizures and led to the current studies with even shorter post-seizure intervals.

The ERK cascade also influences proliferation and differentiation of NPCs in the developing central nervous system (Miller and Gauthier, 2007; Yoon and Seger, 2006), and deletion of ERK impairs proliferation of cortical neural progenitors (Samuels et al., 2008). The ERK pathway also appears to be involved in the regulation of seizure-induced neurogenesis during the first few days after status epilepticus, but ERK activation then returns to control levels within one week (Choi et al., 2008). The effects of spontaneous seizures on ERK activation in NPCs have not been determined.

Thus, in the present study, we examined pERK labeling at very short intervals after detection of spontaneous seizures in a mouse model of temporal lobe epilepsy. Interestingly, we found early ERK activation in NPCs in the SGZ at the time of a spontaneous seizure, and this activation preceded the robust increase in pERK expression in dentate granule cells that occurred at slightly later time points. These findings led to additional studies to identify the subtypes and developmental stages of the NPCs that exhibited pERK labeling and to determine if such pERK-labeled cells were in the proliferative cycle. Our findings suggest that spontaneous seizures can trigger activation of the ERK pathway in early developing NPCs and this, in turn, could influence neurogenesis in this epilepsy model.

Materials and Methods

Animals and pilocarpine treatment

Young adult (6–8 weeks of age) C57BL/6 male mice (20–27 g; Harlan, Indianapolis, IN) were used in this study. Sustained seizures were induced in experimental animals by the administration of pilocarpine, a muscarinic cholinergic agonist, and the protocols have been described previously (Peng et al., 2004). Animals were divided randomly into experimental and control groups and were first injected with a low dose of the cholinergic antagonist methyl scopolamine nitrate (1 mg/kg, i.p.) to reduce peripheral cholinergic effects. Thirty min later, animals in the experimental group received an injection of pilocarpine hydrochloride (320–340 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) to induce status epilepticus, and control animals received a similar volume of sterile saline. After the pilocarpine injection, experimental animals were monitored for a minimum of 5 h to assess the severity and duration of the behavioral seizures. Diazepam (7.5 mg/kg, i.p.; Abbott Laboratories, Chicago, IL) was administered at 2 h after the onset of status epilepticus to stop or limit behavioral seizures, and an identical dose of diazepam was given to control animals. Additional diazepam (5 mg/kg) was given if seizures were not reduced sufficiently or recurred in 1 h after the first diazepam injection.

After recovery from status epilepticus, the behavior of pilocarpine-treated mice was continuously monitored by direct observation or videotape 5 d/week, using infrared light during the dark phase (6:00 P.M. to 6:00 A.M.). All animal use protocols conformed to National Institutes of Health guidelines and were approved by the University of California, Los Angeles, Chancellor's Animal Research Committee and the Veterans Administration Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their discomfort.

Pilocarpine-treated mice from 2 to 7 weeks following status epilepticus were monitored for spontaneous behavioral seizures, and a total of 17 pilocarpine-treated animals were included in this study. The mice were perfused at different time intervals following a spontaneous seizure, at 1.5–2.5 min (combined and identified as 2 min in subsequent text; $n=6$), slightly longer intervals of 3 min ($n=3$) or 6 min ($n=2$), and 15 min ($n=3$) following seizure detection. Animals that demonstrated additional spontaneous seizures during the previous 12–24 h before perfusion were excluded from the study. For comparison, age-matched epileptic mice (2–6 weeks following status epilepticus) that had not experienced a behavioral seizure in the last 24 h prior to perfusion ($n=3$) were included. Age-matched control animals ($n=14$) were included in the study and were perfused at the same time as the pilocarpine-treated animals.

Behavioral outcomes

Spontaneous behavioral seizures were observed in all pilocarpine-treated animals and were usually detected by 2–3 weeks following induction of status epilepticus. The spontaneous seizures typically consisted of periods of freezing, drooling and mouth movements, clonic movements of the forelimbs, rearing, or rearing and falling, and were often either followed or preceded by a brief (10–20 s) generalized motor seizure. The total duration of the observed behavioral seizure was typically 1 min and no longer than 2 min. For this study, only mice that had clearly observable behavioral seizures, detected directly or on videotape, with rearing or rearing and falling, stage 4 or 5 (Racine, 1972), were included. The mice included in this study were screened to make sure that no behavioral seizures or other overt behaviors, such as jumping and fighting, had occurred during the 12–24 h prior to the last spontaneous seizure. In a single animal, a seizure was observed at 6 h before perfusion, but subsequent analysis showed a similar labeling pattern to others in the same interval group, and thus the animal was retained in the study.

Tissue preparation

Mice were deeply anesthetized by either injection of sodium pentobarbital (90 mg/kg, i.p.) or inhalation of halothane, and then were perfused through the ascending aorta with 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3. Pilocarpine-treated animals were perfused at different time points after detection of a spontaneous behavioral seizure, and control mice were perfused along with the pilocarpine-treated mice at each time point. After perfusion, the brains were maintained *in situ* at 4°C for 1 h and then removed from the skull and postfixed in the same fixative for 1 h at room temperature (RT). After thorough rinsing in phosphate buffer, the brains were cryoprotected in a 30% sucrose solution and sectioned at 30 µm on a cryostat. Forebrain sections containing the rostral half of the hippocampus were sectioned in the coronal plane. Near the middle of the hippocampus, ~2.18 mm posterior to bregma (Franklin and Paxinos, 2008), the brain blocks were reoriented, and the caudal half of the hippocampus was sectioned horizontally. Sections at 300 µm intervals were stained with cresyl violet for general morphological study. Remaining sections were stored in a cryoprotectant solution at -20°C until processing.

Immunohistochemistry

Free-floating sections were processed for immunohistochemistry. Two antisera, raised in either rabbit or mouse, were used for pERK immunohistochemistry (mouse pERK, Cat # 9106S, and rabbit pERK, Cat # 9101S; Cell Signaling, Danvers, MA). Both antibodies detect endogenous levels of phosphorylated p44 and p42 MAP kinase (Erk1 and Erk2) and do not cross-react with the corresponding phosphorylated residues of either SAPK/JNK or p38 MAP kinase (information from manufacturer). These antisera produced nearly identical results, except for slightly less dense labeling of processes with the mouse antiserum. For consistency, rabbit pERK antiserum was used for all single labeling immunohistochemistry.

For single-labeling of pERK, free-floating sections were processed with avidin-biotin peroxidase methods. Sections were first incubated in 1% H₂O₂ for 30 min to eliminate endogenous peroxidase-like activity. After rinsing in 0.1M Tris-buffered saline (TBS), pH 7.3, sections were blocked in 10% normal goat serum in TBS containing 0.3% Triton X-100 and avidin (200 µl / ml) for 3 h to reduce nonspecific binding. Sections were then incubated with a rabbit pERK antiserum in TBS with 2% normal goat serum and biotin (200 µl / ml) overnight at RT. After rinsing in TBS, sections were incubated in biotinylated goat anti-rabbit secondary antiserum (1:1000; Vector Laboratories, Burlingame, CA) at RT for 1 h. To visualize the peroxidase labeling, sections were processed with 0.06% diaminobenzidine tetrahydrochloride (DAB) and 0.006% H₂O₂ diluted in 0.075 M phosphate buffered saline (PBS) for 12 min, and the immunolabeling was enhanced by incubation in 0.003% osmium tetroxide in PBS for 30 s. After rinsing, sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. Images were obtained with a Zeiss (Thornwood, NY) Axioskop 2 microscope equipped with an AxioCam Digital camera system and AxioVision 4.6 software.

Immunofluorescence labeling and confocal microscopy

In order to identify the phenotype of pERK-labeled cells in the SGZ, several neural progenitor cell (NPC) markers were used for double or triple immunofluorescence labeling with pERK. These were: (1) radial glia-like NPC markers that label the early stages of NPCs, including glial fibrillary acidic protein (GFAP), brain lipid binding protein (BLBP), Nestin and Pax6; (2) a marker of intermediate stage NPCs with neuronal lineage potential, NeuroD; (3) immature neuronal markers, including doublecortin (DCX) and neuron-specific class III β-tubulin (Tuj-1); (4) an early granule cell-specific marker, Prox-1; (5) a general neuronal marker, neuron-specific nuclear protein (NeuN); and (6) a marker of mature astrocytes, S100β. Proliferation markers, Ki67 and mini-chromosome maintenance protein 2 (Mcm2), were also

used to determine if pERK-labeled cells were in the proliferative cycle. Further descriptions of the primary antibodies and their concentrations are provided in Table 1.

For double and triple immunofluorescence labeling, sections from the same rostral-caudal levels of pilocarpine-treated animals and control animals were processed. Sections were treated with 10% normal goat or donkey serum in 0.1M TBS containing 0.3% Triton for 3 h and then incubated in a mixture of primary antibodies (see Table 2) at RT for 3-4 nights. After thorough rinsing in PBS, sections were incubated for 4 h in a mixture of fluorescent-labeled secondary antisera at RT. The secondary antibodies were labeled with either Alexa Fluor (Invitrogen, Eugene, OR) or Dylight (Jackson ImmunoResearch, West Grove, PA), and all secondary antibodies were at a concentration of 1:500 (for further details see Table 2). After the incubations, all sections were rinsed in TBS for 30 min, mounted on slides, and coverslipped with the antifade medium Prolong Gold (Invitrogen, Carlsbad, CA).

For immunofluorescence labeling with mouse antibodies to pERK, Ki67 and Nestin or rabbit Pax6 antiserum, sections were processed with water bath antigen-retrieval methods to improve labeling before incubation in primary antibodies (Peng et al., 2002). Briefly, sections were incubated in 0.05M sodium citrate solution, pH 8.6, for 30 min at RT and then heated in a water bath in the same solution at 90 °C for 70 min.

Data analysis

To quantitatively evaluate the colocalization of pERK with antigens of interest in the SGZ, double- or triple-labeled sections (from $n=5$ and $n=3$ mice, respectively) at the same dorsal-ventral levels of the hippocampus from animals in the shortest interval groups were analyzed. Sections were scanned with a Zeiss (Thornwood, NY) LSM 510 confocal microscope, and images were obtained with a 40x or 100x objective. The SGZ was operationally defined as a region extending from the interface of the hilus and granule cell layer to a width of approximately two cell bodies into the hilus, as in a previous study (Parent et al., 1997), and single-, double- and triple-labeled cells were counted along the entire SGZ on both sides of each section. Each section was scanned as a 15 μ m thick Z stack spaced by 1 or 0.5 μ m, and included 15-30 Z sections that contained the entire region of interest. Labeled cells were then counted by analyzing each Z section, and the numbers were confirmed by reconstructed Z-sections projected with Zeiss LSM 5 Image Examiner software. For pERK and each antigen of interest, percentages of single-, double-, and triple-labeled cells were calculated.

One section per animal (including the hippocampus on each side) was used for semi-quantitative analysis of double labeling, and 5 animals were used for this analysis. In each hippocampus, all labeled cells within the region of interest were counted, and the numbers varied with the marker. The average numbers of cells counted were: 30 cells/section/animal for pERK, 90 cells/section/animal for GFAP, 39 cells/section/animal for BLBP, 135 cells/section/animal for NeuroD, and 24 cells/section/animal for Mcm2.

To evaluate potential differences in the BLBP-expressing NPCs that were in the proliferative cycle between pilocarpine-treated and control mice, the percentages of single- and double-labeled cells that expressed BLBP and Mcm2 were calculated as described above. Data were analyzed with Student's *t* test, and the level of significance was set at $P<0.05$.

Results

Spontaneous seizures induced early expression of pERK in the SGZ

At short intervals of 1.5 -2.5 min following detection of a behavioral seizure in the pilocarpine-treated mice, distinct pERK labeling was evident in cells of the SGZ, at the interface of the granule cell layer and hilus (Fig. 1C). pERK expression in the SGZ could be observed in both

rostral (coronal) and caudal (horizontal) sections. Strong pERK labeling frequently extended throughout the somata and processes of the cells and was also evident in some nuclei. Such labeling along the base of the granule cell layer was not evident in either control animals (Fig. 1A) or pilocarpine-treated animals that had not exhibited a recent spontaneous seizure (for at least the preceding 24 hrs) (Fig. 1B), as described in our previous study (Houser et al., 2008).

At these short intervals (1.5-2.5 min) following spontaneous seizures, the pERK-labeled cells in the SGZ exhibited two different morphologies. Most of the pERK-labeled cells had an oval-shaped nucleus and often had a long vertical process with very few branches that was directed to the outer part of the granule cell layer (Fig. 1D). Other pERK-labeled cells were oriented horizontally in the SGZ and usually lacked long vertical processes (Fig. 1E).

This pERK labeling in the SGZ was distinct from the robust granule cell labeling that was most prominent at slightly later time points (6 min) after spontaneous seizures (Houser et al., 2008). However, even at the earliest time points, limited granule cell labeling was sometimes found at selected rostral-caudal levels of the hippocampus. Likewise, increased pERK labeling was observed in some other brain regions, including the piriform and entorhinal cortex and amygdaloid nuclei, in several animals. Because shorter post-seizure intervals (e.g. less than 1 min after a spontaneous seizure) were not technically feasible, the brain regions with the earliest increase in pERK labeling could not be determined. However, at any specific level of the dentate gyrus, labeling of cells within the SGZ preceded the labeling of adjacent granule cells. This suggested that the early pERK-labeled cells could have different properties than most granule cells.

pERK-labeled cells in the SGZ expressed markers of neural progenitor cells (NPCs)

To identify the types of cells that expressed pERK at short intervals after spontaneous seizures, we determined the phenotype of pERK-labeled cells in the SGZ at intervals of 1.5-2.5 min after detection of spontaneous seizures. Because the SGZ is the major hippocampal region where adult neurogenesis occurs and many pERK-labeled cells ($80.0 \pm 4.3\%$) showed long vertical processes directed toward the molecular layer, we considered that these cells could be radial glia-like NPCs, sometimes also referred to as type-1 cells. We thus used several NPC markers to confirm the identity of the pERK-labeled cells.

We first determined whether pERK was expressed in GFAP-labeled cells in the SGZ. GFAP labels radial glia-like cells that have been found to be multipotent NPCs in the germinal areas of adult brain, both in the SGZ (Garcia et al., 2004; Seri et al., 2001) and the subventricular zone (Doetsch et al., 1999). GFAP labeling was present in the processes and also often in the somata of numerous pERK-labeled cells (Fig. 2 A-C). By counting the cells which had GFAP labeling in the cell bodies as well as their processes, we found that $77.6 \pm 5.5\%$ of pERK-labeled cells were labeled with GFAP. Most of these double-labeled cells had a long vertical process, suggesting that pERK was expressed in NPCs with radial glia characteristics. However, pERK+GFAP+ cells accounted for a much smaller percentage of GFAP+ cells, as only $22.3 \pm 3.4\%$ of the GFAP-labeled cells in the SGZ expressed pERK.

We then determined whether pERK-labeled cells were labeled with S100 β , a marker of mature, non-germinal astrocytes, since the onset of its expression is related to exit from the cell cycle and the maturation of astrocytes (Scotto et al., 1998; Raponi et al., 2007). In the SGZ, the S100 β -labeled cells were usually located horizontally and often had multiple processes. However, S100 β -labeled cells were not limited to the SGZ but were evident throughout the hippocampal formation. Virtually no pERK+ cells in the SGZ were double-labeled for S100 β (Fig. 2D-F). This suggested that the pERK-labeled cells in the SGZ were not mature astrocytes.

To further support the identity of pERK-labeled cells as radial glia-like NPCs, as suggested by the labeling with GFAP, we determined the colocalization of pERK and BLBP (Fig. 3A-F). BLBP is considered to be a specific marker of radial glia-like NPCs, although it may also be found in some intermediate NPCs and astrocytes in the SGZ (Steiner et al., 2006). BLBP labeling had the added advantage of consistently labeling the cell body, in contrast to GFAP that often preferentially labeled the processes. Similar to the data for pERK and GFAP colocalization, $77.6 \pm 4.9\%$ of pERK-labeled cells expressed BLBP. This provided further evidence that early pERK expression following a spontaneous seizure was predominately in the radial glia-like NPCs. Also, pERK+BLBP+ cells accounted for $87.5 \pm 4.8\%$ of BLBP+ cells in the SGZ, a much higher percentage than that of the GFAP-labeled cells that were labeled for pERK ($22.3 \pm 3.4\%$).

We also determined whether pERK was expressed in Nestin- or Pax6-labeled cells in the SGZ. Nestin is an intermediate filament protein that is expressed in many type-1 and type-2 cells (Filippov et al., 2003; Fukuda et al., 2003; Lagace et al., 2007). Pax6 is a transcription factor which is present in NPCs at similar multiple stages of development (Maekawa et al., 2005; Osumi et al., 2008; Sansom et al., 2009). Colocalization of pERK and Nestin was evident in the SGZ, and nestin-labeled processes extended into the granule cell layer (Fig. 4A-C). Coexpression of pERK and Pax6 was also found in the nuclei of cells in the SGZ (Fig. 4D-F). These results further support the identity of pERK-labeled cells as NPCs. Because of the attenuation of pERK labeling by the antigen retrieval treatment required for optimal Nestin and Pax6 labeling, further studies of these double-labeled cells and their percentages were not pursued.

pERK labeling is also expressed in later-stage NPCs

We then determined whether pERK was expressed in later-developing NPCs by studying labeling for NeuroD, a transcription factor that is expressed in more mature NPCs that include late type-2 cells and, primarily, neuronal NPCs that are also referred to as type-3 cells (von Bohlen Und Halbach, 2007). NeuroD is considered to act as a neuronal determination gene (Seri et al., 2004; Tamimi et al., 1996) and is one of the earliest indications of granule cell identity (Liu et al., 2000; Pleasure et al., 2000). NeuroD labeling of nuclei was evident in the SGZ and the inner granule cell layer (Fig. 5D-F). We found that $21.6 \pm 7.1\%$ of pERK cells expressed NeuroD, whereas these double-labeled cells comprised only $3.5 \pm 0.6\%$ of NeuroD-labeled cells in the SGZ (Fig. 5A, D, G). This suggested that pERK was also expressed in a subgroup of NPCs that was directed toward a neuronal lineage. Approximately half ($57.1 \pm 5.4\%$) of the pERK+NeuroD+ cells were located horizontally at the base of the SGZ and exhibited short processes (Fig. 5B, E, H), while the remainder had a long vertical process that extended towards the outer part of the granule cell layer (Fig. 5C, F, I).

To confirm that pERK+BLBP+ cells and pERK+NeuroD+ cells were two different cohorts of cells, we conducted double labeling for BLBP and NeuroD in both control and pilocarpine-treated animals (data not shown) and did not find colocalization of BLBP and NeuroD. This was consistent with the findings of previous studies in which type-2 cells were not found to be both BLBP+ and NeuroD+, and NeuroD labeling was virtually never found in type-1 cells (Steiner et al., 2006). This suggested that the two markers labeled two distinct populations of pERK+ cells.

ERK activation was not evident in immature neurons of the SGZ at the time of a spontaneous seizure

To determine whether pERK was also expressed in immature neurons after the detection of spontaneous seizures, we used DCX and Tuj-1 for double-labeling, since they recognize late mitotic neuronal precursors and newly-generated postmitotic neurons (Brown et al., 2003;

Menezes and Luskin, 1994). Both DCX and Tuj-1 showed labeling in the cytoplasm and long processes of numerous cells along the SGZ. However, virtually no colocalization of pERK and either DCX (Fig. 6A-C) or Tuj-1 (Fig. 6D-F) was observed.

Finally, the potential colocalization of pERK with Prox1, that labels newly-generated as well as mature granule cells, or NeuN, that labels postmitotic neurons, was determined. Labeling for both Prox1 and NeuN was prominent in the nuclei of numerous neurons in the granule cell layer, but no localization was observed in pERK-labeled cells in the SGZ at the short post-seizure intervals (Fig. 6G-L). However, at slightly longer intervals (6 min) after spontaneous seizures, numerous mature granule cells were labeled for pERK, and co-localization of pERK with Prox1 and NeuN could be observed in these granule cells, as reported previously (Houser et al., 2008).

In summary, pERK was expressed in specific types of NPCs in the SGZ at the onset of a spontaneous seizure (Fig. 7). The majority of these pERK-labeled cells were radial glia-like NPCs, as demonstrated by high percentages of pERK-labeled cells that were labeled with GFAP and BLBP. pERK was also found in early neuronal NPCs as indicated by coexpression with NeuroD. Virtually no coexpression was found in more mature neuronal NPCs which were labeled with DCX, Tuj-1 or Prox1.

pERK-labeled cells in the SGZ showed proliferative properties

NPCs at early stages, especially the radial glia-like progenitors, divide infrequently under normal conditions even though they are abundant (Filippov et al., 2003). We explored the proliferation state of these NPCs in our animal model by using the proliferation marker Mcm2 and the radial glia-like NPC marker BLBP. Mcm2 is expressed from the beginning of the G1 phase of the cell cycle and is present in both slowly-cycling and rapidly-cycling NPCs (Maslov et al., 2004). Mcm2 labeling was found exclusively in nuclei, and these were often clustered in groups of 2-5 in control animals. In pilocarpine-treated animals, the size of the clusters was usually larger than that of control animals, typically with 2-11 nuclei in a cluster. The numbers of Mcm2-labeled nuclei in the SGZ also appeared to be increased in these pilocarpine-treated mice. To determine if the proliferation of radial glia-like NPCs was increased in the pilocarpine-treated animals, the percentages of BLBP-labeled cells that co-expressed Mcm2 were determined in age-matched control and pilocarpine-treated mice (from the 2 min post-spontaneous seizure group). In double-labeled sections, $6.3 \pm 2.5\%$ of the BLBP-labeled cells in the SGZ were Mcm2-positive in control animals, and this percentage increased to $23.7 \pm 6.9\%$ in the pilocarpine-treated animals ($P < 0.01$). This suggested that more radial glia-like NPCs were in the proliferation cycle in the pilocarpine-treated animals than in age-matched controls.

Since proliferation rates could be influenced by the age of the animals and the length of the post-status epilepticus interval, the percentages of double-labeled cells from the above analysis were compared in pilocarpine-treated animals at shorter (3-4 weeks; $n=2$) and longer (5-7 weeks; $n=3$) intervals following status epilepticus and in age-matched controls at each time point ($n=2$ and $n=3$). Although the numbers of animals in each group were too small for statistical analyses, the data suggested a trend toward higher levels of proliferating cells in the animals at shorter intervals following status epilepticus. The percentages of BLBP-labeled cells that were in the proliferation cycle were greater in both groups of pilocarpine animals than in their age-matched controls. In the control groups, 8.1% and 5.7% of the BLBP-labeled cells were labeled with Mcm2 in the younger and older mice, respectively. In contrast, in the pilocarpine-treated groups, 27.5% and 19.1% of the BLBP-labeled cells were labeled for Mcm2 in the mice at shorter and longer intervals following status epilepticus, respectively.

Since pERK was expressed in radial glia-like NPCs at the time of spontaneous seizures and a subset of these radial glia-like NPCs were undergoing proliferation, we used Mcm2, as well as Ki67, a marker that is present during the active phases of G1, S, G2 in the cell cycle (Stoeber et al., 2001), to determine the proliferation state of the pERK-labeled cells in the SGZ in pilocarpine-treated animals at 2 min after detection of a spontaneous seizure. Ki67- and Mcm2-labeled nuclei were scattered along the SGZ, and double labeling of pERK-labeled cells with either Ki67 (Fig. 8E-G) or Mcm2 (Fig. 8A-C) was observed. We then used Mcm2 to determine the percentage of pERK-labeled cells that was in the proliferation cycle and found that $28.7 \pm 5.4\%$ of pERK-labeled cells expressed Mcm2 (Fig. 8D). These double-labeled cells were often in pairs (Fig. 8A-C) or adjacent to cells that were single-labeled for Mcm2. A relatively high percentage of Mcm2+ cells ($44.0 \pm 13.3\%$) showed pERK labeling (Fig. 8D).

To determine whether the radial glia-like NPCs that were in the proliferative cycle expressed pERK, we conducted triple labeling of pERK, BLBP and Mcm2. Approximately two-thirds ($66.5 \pm 4.3\%$) of pERK-expressing cells in the SGZ showed BLBP labeling after a spontaneous seizure, and $29.9 \pm 4.3\%$ of pERK-labeled cells showed Mcm2 expression (Fig. 9E). These results are similar to the percentages obtained in the double-labeling experiments. In addition, $23.0 \pm 2.6\%$ of pERK labeled cells were triple labeled with BLBP and Mcm2 (Fig. 9E). In the analysis of cells that were in the proliferative cycle (Mcm2-labeled), $45.2 \pm 3.4\%$ were labeled for pERK, and $39.2 \pm 2.5\%$ were labeled for BLBP (Fig. 9F). Interestingly, triple labeling revealed that virtually all Mcm2 and BLBP double-labeled cells expressed pERK (Fig. 9A-D, F, G), although some pERK and Mcm2 double-labeled cells were devoid of BLBP expression (Fig. 9F, G). This indicates that essentially all of the radial glia-like NPCs which were in the proliferative cycle showed ERK activation at the time of spontaneous seizures, although the proliferative cells with ERK activation were not exclusive to these NPCs. The proliferative pERK-labeled NPCs that did not express BLBP (approximately 6% of pERK-labeled cells) could be more mature NPCs that were undergoing proliferation.

pERK expression in the SGZ preceded the labeling of granule cells at the time of a spontaneous seizure

At slightly longer intervals (6 min) after detection of a spontaneous behavioral seizure, pERK labeling was evident throughout the granule cell and molecular layers, as described previously (Houser et al., 2008). In order to determine whether the pERK expression in NPCs persisted at longer post-seizure intervals when mature granule cells were labeled, we conducted double labeling with pERK and BLBP at later time points of 6 min (Fig. 10) and 15 min after spontaneous seizures. Limited pERK expression was still evident in BLBP+ cells (Fig. 10A-C), consistent with a gradual decrease in pERK labeling at progressively longer intervals following a spontaneous seizure. However, virtually no colocalization of pERK and DCX was observed at the longer post-seizure intervals (Fig. 10D-F), similar to the findings at the 2 min interval and demonstrating that pERK labeling of DCX+ cells was not simply delayed in time after a spontaneous seizure. In contrast, pERK labeling was evident in numerous granule cells at the 6 minute interval (Fig. 10A,D).

Discussion

A major finding of this study is that ERK is activated in a subpopulation of NPCs in the dentate gyrus at the time of spontaneous seizures in a model of temporal lobe epilepsy. Interestingly, this early ERK activation in NPCs preceded the labeling of mature dentate granule cells at slightly later time points. The NPCs that expressed pERK were predominantly radial glia-like cells, but also included some NPCs that were likely to be progressing to a neuronal lineage. A subpopulation of the pERK-labeled NPCs was in the proliferative cycle, and virtually all of the radial glia-like NPCs that were labeled with a proliferation marker expressed pERK at the

time of spontaneous seizures. These findings suggest that spontaneous seizures and the associated ERK activation could influence neurogenesis in the dentate gyrus.

ERK pathway is activated in cells of the subgranular zone (SGZ) at very short intervals following detection of a spontaneous seizure

The labeling in cells of the SGZ was the earliest observable change in pERK expression in the hippocampus at the time of a spontaneous seizure and occurred prior to a robust increase in pERK expression in the majority of mature granule cells at a slightly later timepoint. Previous studies of seizure-related increases in ERK activation have generally been focused on changes that occur following induced seizures or status epilepticus (e.g. Berkeley et al., 2002; Choi et al., 2008; Choi et al., 2007; Garrido et al., 1998; Gass et al., 1993; Kim et al., 1994; Lugo et al., 2008) and have emphasized the acute activation of dentate gyrus granule cells and other hippocampal neurons, but not cells of the SGZ. However, in a recent study, Choi et al. (2008) observed an increase in pERK labeling in the SGZ at two days following status epilepticus, and this increase was considered to serve as a stimulus for neurogenesis through activation of the IGF-1 receptor. Such labeling in the SGZ was no longer observed by 5 days following status epilepticus (Choi et al., 2008), and this is consistent with our observations of low levels of pERK labeling throughout the hippocampal formation during the chronic period in epileptic mice that had not experienced a recent spontaneous seizure (Houser et al., 2008). The current findings demonstrate a dynamic change of pERK expression in cells of the SGZ following an acute spontaneous seizure.

ERK is activated in neural progenitors at the time of a spontaneous seizure

The early activation of ERK in cells of the SGZ led to questions about the identity of the labeled cells, and, specifically, whether they were neural progenitors or mature glial cells that were reacting to seizure activity. We found that the majority of the pERK-labeled cells showed radial glia-like morphology and expressed GFAP and BLBP, both of which are considered to be markers of radial glia-like (or type-1) NPCs (Anthony et al., 2004; Garcia et al., 2004; Seri et al., 2001). The NPC identity was also supported by the colocalization of other NPC markers such as Nestin and Pax6. These markers are expressed not only in type-1 cells but also in some type-2 cells that are referred to as intermediate NPCs and are at a slightly later developmental stage (Filippov et al., 2003; Fukuda et al., 2003; Osumi et al., 2008; Wang et al., 2005). In addition to pERK-labeled cells with a radial glia-like morphology, some labeled cells were oriented horizontally at the base of the granule cell layer where mature glial cells can be found. However, no coexpression of pERK and the astrocytic marker S100 β was observed, suggesting that the cells with early ERK activation in the SGZ were not mature astrocytes.

Previous fate mapping and clonal analysis have demonstrated that around 90% of newborn neurons are generated from BLBP-labeled radial glia-like neural progenitors during development (Anthony et al., 2004). Likewise, the vast majority of newborn neurons in the normal adult SGZ were found to develop from GFAP-expressing NPCs (Garcia et al., 2004). Since radial glia-like NPCs can give rise to neurons and are the principal source of constitutive neurogenesis in the adult SGZ, we utilized additional markers to determine if ERK is activated in more mature, neuronal NPCs and newly-generated neurons. Interestingly, pERK labeling was found in a subpopulation of NPCs in the SGZ that expresses NeuroD, a marker that is present at later stages than Pax6 in the sequential transcription factor expression in adult hippocampal neurogenesis and is one of the first neuronal lineage markers (Pleasure et al., 2000; Steiner et al., 2006). These findings suggest that ERK activation at the time of spontaneous seizures occurs primarily in two major groups of NPCs (Fig. 11). In separate analyses of pERK-labeled cells with each marker, a high percentage (~80%) of pERK-labeled cells had the morphological characteristics of radial glia-like NPCs and expressed BLBP (Fig.

11A, B). A smaller but sizeable group (~20%) was labeled for NeuroD and thus appeared to be at a more mature stage and, potentially, committed to a neuronal lineage (Fig. 11A, B).

ERK is activated in radial glia-like NPCs that are in the proliferative cycle at the time of a spontaneous seizure

The identification of ERK activation predominantly in radial glia-like NPCs led to further questions about the proliferation state of these labeled cells. Interestingly, we found that a substantial number of pERK-labeled NPCs were in the proliferative cycle as demonstrated by double labeling with Mcm2 or Ki67. Furthermore, many of these proliferative pERK-labeled cells expressed BLBP and were thus radial glia-like cells. This finding was somewhat unexpected since radial glia-like NPCs, although numerous, are considered to be relatively quiescent and few of them are recruited into the proliferation cycle during adulthood (Huttmann et al., 2003; Ledergerber et al., 2006). Instead, more mature, transiently amplifying cells appear to be the major cell type that proliferates in the adult SGZ in response to physiological stimuli such as running and environmental enrichment (Hodge et al., 2008; Kronenberg et al., 2003).

However, increasing evidence suggests that different types of stimuli could exert their effects on cells at different stages of neurogenesis. Whereas radial glial-like NPCs may remain quiescent following several types of physiological stimuli (Kronenberg et al., 2003), severe seizure activity, such as status epilepticus, may induce a significant increase in the proliferation of the radial glia-like NPCs in the SGZ (Huttmann et al., 2003; Zhu et al., 2005; Steiner et al., 2008). Our current findings support this idea for the percentage of BLBP-labeled cells that were in the proliferative cycle was significantly greater in the pilocarpine-treated animals than in age-matched controls. Furthermore, the current findings suggest that this group of cells is highly susceptible to ERK activation at the time of a spontaneous seizure, since essentially all BLBP-labeled cells that expressed the proliferation marker Mcm2 were labeled for pERK.

Potential influence of ERK activation on neurogenesis following spontaneous seizures

The functional effects of ERK activation in NPCs at the time of spontaneous seizures are unknown. However, previous studies have suggested that the ERK pathway plays a vital role in neurogenesis in the developing central nervous system (Miller and Gauthier, 2007; Yoon and Seger, 2006), and deletion of ERK impairs proliferation of cortical neural progenitors during development (Samuels et al., 2008). Recent studies have also suggested a potential influence of ERK activation on neurogenesis immediately following status epilepticus, since disruption of the ERK pathway with a MEK inhibitor reduced seizure-induced NPC proliferation at 2 days after status epilepticus (Choi et al., 2008). The current findings are the first to demonstrate increased ERK activation in NPCs at very short intervals after spontaneous seizures and raise the possibility that ERK activation at the time of spontaneous seizures could contribute to the proliferation of radial glia-like NPCs.

Activation of the ERK pathway has been implicated in the regulation of NPC proliferation in several previous studies. For example, different extracellular stimuli such as neurotrophic factors, hormones or neuropeptides can influence NPC proliferation via the ERK pathway (Howell et al., 2005; Liu et al., 2009; Ohtsuka et al., 2009). In addition, *in vitro* studies have demonstrated a stimulatory role for ERK on the proliferation of neural stem cells but not their subsequent differentiation (Wang et al., 2009). If similar effects were to occur *in vivo*, they would not only be consistent with the occurrence of ERK activation in the proliferative NPCs, but also could help explain the limited activation of ERK in immature neurons that are undergoing differentiation.

During normal adult neurogenesis, the proliferation of radial glia-like NPCs can be influenced by the microenvironment that includes neurotrophic factors such as fibroblast growth factors

(FGFs), insulin-like growth factor (IGF), brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (Aberg et al., 2000; Bull and Bartlett, 2005; Scharfman et al., 2005; Segi-Nishida et al., 2008). Some of these neurotrophic factors such as FGF2, BDNF and IGF-1, can also be increased by some types of seizure activity (Choi et al., 2008; Hagihara et al., 2005; Jankowsky et al., 2002), and these same factors can serve as upstream signals in the ERK pathway. It is not known if similar changes in the microenvironment occur at the time of spontaneous seizures. However, if such changes occur, upstream molecules in the ERK pathway could be released and act on receptors that are present on radial glia-like cells to stimulate ERK phosphorylation and, potentially, enhance neurogenesis. For example, FGF2 is an upstream factor in the ERK pathway and is also essential for ERK associated promotion of neural stem cell proliferation *in vitro* (Xiao et al., 2007). IGF-1 could have a similar role since previous studies have shown that CREB-dependent IGF-1 can trigger ERK activation, which in turn increases progenitor cell proliferation after status epilepticus (Choi et al., 2008).

Beyond effects on cell proliferation, activation of the ERK cascade in these NPCs could potentially influence cell cycle gene expression (Liu et al., 2009) or regulate the cell fate choice (Kim et al., 2005). The reasons for ERK activation in selected groups of NPCs following spontaneous seizures remain unknown but could be related to selective expression of pertinent receptors on the radial glia-like NPCs during this period.

The current findings suggest that, at certain stages of seizure-induced neurogenesis, spontaneous seizures could further stimulate the neurogenic process, potentially leading to greater numbers of newly generated neurons. A previous behavioral study has suggested that recurrent seizures enhance the production of new neurons (Cha et al., 2004), and the current findings could suggest a mechanism for such enhanced neurogenesis. However, in such studies of animals following status epilepticus, establishing that the presence or absence of spontaneous seizures is the primary variable that accounts for differences in the amount of neurogenesis remains a challenge.

While the precise stimuli and functional effects of the observed ERK activation remain speculative, this study demonstrates that dynamic, cell-type specific increases in ERK activation in the SGZ occur in epileptic animals at the time of spontaneous seizures and suggest that such activation could influence the proliferation of some NPCs in the dentate gyrus.

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Fig. 1.

Phosphorylated ERK labeling in the dentate gyrus of control and pilocarpine-treated mice during the chronic stage or at the onset of a spontaneous seizure. (A) In a control animal, only a few pERK-labeled cells (arrows) are scattered in the dentate granule cell layer (G), and labeled cells are not prominent along the interface of the granule cell layer and hilus (H). (B) In a pilocarpine-treated mouse that had not experienced a recent behavioral seizure, pERK labeling is reduced in the granule cell layer compared to that of a control animal (above). (C) Within 2 min after detection of a spontaneous seizure, numerous pERK-labeled cells are evident in the subgranular zone (SGZ) of the dentate gyrus. At higher magnification of a region in (C), pERK-labeled cells exhibit two different morphologies: (D) one with an oval-shaped nucleus and a long vertical process (arrows) with few branches; and (E) another with horizontally-oriented processes (arrows) at the base of the SGZ. Scale bars: (A-C) 100 μ m, (D, E) 25 μ m.

Fig. 2. Phosphorylated ERK-labeled cells in the SGZ (within 2 min after detection of a spontaneous seizure) expressed radial glia-like neural progenitor cell (NPC) markers but not a mature glial cell marker. (A-C) Colocalization of pERK (green) and GFAP (red) is evident in the process (arrow) of a radial glia-like cell. (D-F) Virtually no colocalization of pERK (green) and S100β (red), a mature astrocyte marker, is evident in cells in the SGZ (arrowheads). Scale bar: 10 μm for all panels.

Fig. 3.

Phosphorylated ERK-labeled cells in the SGZ (within 2 min after detection of a spontaneous seizure) colocalized with BLBP. (A-C) Numerous pERK-labeled cells (green) are double-labeled for BLBP (red) in the SGZ (arrows). (D-F) A representative higher magnification of the region shows that the colocalization of pERK (green) and BLBP (red) is evident throughout the processes and the cell bodies (arrows). Scale bar: 35 μ m for A-C, 10 μ m for D-F.

Fig. 4. Phosphorylated ERK-labeled cells in the SGZ (within 2 min after detection of a spontaneous seizure) exhibited colocalization with type-2 NPC markers. (A-C) Several pERK-labeled cells (green) are double-labeled with Nestin (red), a NPC marker that labels the majority of type-2 and also some radial glia-like NPCs (arrows). (D-F) Colocalization of pERK (green) and Pax6 (red) can be seen in the nuclei of a cell (arrow) in the SGZ. Scale bar: 10 μ m for all panels.

Fig. 5.

Phosphorylated ERK-labeled cells in the SGZ (within 2 min after detection of a spontaneous seizure) expressed neuronal progenitor cell markers. (A-I) pERK (green) is expressed in a subgroup of NeuroD-labeled cells (red) in the SGZ. In double labeled cells, pERK is localized in the cytoplasm, and NeuroD is concentrated in the nucleus. Some of these double-labeled cells have short horizontal processes (B, E, H), while others have a long vertical process that extends into the granule cell layer (C, F, I) when observed at higher magnification. Scale bar: 60 μ m for A,D,G, 10 μ m for all other panels

Fig. 6. Phosphorylated ERK-labeled cells in the SGZ (within 2 min after detection of a spontaneous seizure) exhibited virtually no labeling with immature neuron markers. (A-C) pERK (green) is not colocalized with DCX (red), a marker which labels the somata and processes of immature neurons. (D-F) Colocalization of pERK (green) and Tuj-1 (red), another immature neuron marker, is not evident. (G-I) pERK (green) is not expressed in Prox1-labeled (red) cells, that include both newly-generated and mature granule cells. (J-L) No coexpression of pERK (green) and NeuN (red), a general neuronal marker that labels the nuclei and cytoplasm of postmitotic neurons, is evident. Scale bar: 10 μ m for all panels.

Fig. 7.

Quantification of phenotypes of the pERK-labeled cells in the SGZ within 2 min after detection of a spontaneous seizure. Black bars represent the percentages of pERK-labeled cells that are double-labeled with each NPC marker. Gray bars represent the percentages of each NPC marker that are double-labeled for pERK. NPC markers are indicated on the X-axis, and the percentage values are indicated on the Y-axis. Error bars represent the SEM for each phenotype.

Fig. 8.

Numerous pERK-labeled cells in the SGZ in pilocarpine-treated mice (within 2 min after detection of a spontaneous seizure) were actively proliferating. (A-D) Mcm2 (red), that labels the nuclei of proliferating cells, is expressed in a cluster (arrows) of pERK-labeled cells (green). Quantification of this double labeling data showed that $28.7 \pm 5.4\%$ of pERK-labeled cells expressed Mcm2, while $44.0 \pm 13.3\%$ of Mcm2-labeled cells showed pERK expression (D). (E-G) Ki67 (red), another proliferation marker, labels the nuclei of a pERK-labeled cell (green). Scale bar: 10 μ m for all panels.

Fig. 9.

Phosphorylated ERK was expressed in proliferative radial glia-like NPCs in the SGZ within 2 min after detection of a spontaneous seizure. (A-D) Triple labeling of pERK (green), BLBP (red), and Mcm2 (blue) demonstrated that pERK is expressed in proliferative radial glia-like NPCs in the SGZ. (E) Quantification of triple-labeling shows the percentages of pERK-labeled cells that are double and triple-labeled for BLBP (red) and Mcm2 (blue hatched). (F) Similar quantification demonstrated the percentages of Mcm2-labeled cells that are double- and triple-labeled for pERK (green) and BLBP (red hatched). (G) A Venn diagram shows that all the BLBP+ cells that were in the proliferative cycle (black hatched) expressed pERK. Scale bar: (A-D) 10 μ m.

Fig. 10.

Labeling of pERK and NPC markers at 6 min after detection of a spontaneous seizure. (A-C) Moderate labeling of pERK is still evident in the processes of some radial glia-like NPCs, as shown by double labeling for BLBP (red) and pERK (green). At this time point, there is an increase of pERK labeling throughout the granule cell layer. Arrows indicate examples of cells that are labeled for BLBP and pERK in the SGZ. (D-F) Virtually no colocalization of DCX (red) and pERK (green) is evident. Scale bar: 20 μ m for all panels.

Early ERK activation in NPCs at the time of a spontaneous seizure

Fig. 11.

Summary diagram and suggested lineage of pERK-labeled NPCs in the SGZ at the time of a spontaneous seizure. (A) Summary of the phenotypes of NPCs with ERK activation in the SGZ at 2 min after detection of a spontaneous seizure. (B) Schematic representation of pERK-labeled NPCs in the SGZ at the time of a spontaneous seizure. A high percentage of pERK-labeled cells were identified as radial glia-like progenitors, lower percentages of the pERK-labeled cells were more mature type-2 NPCs, and immature neurons remained unlabeled.

Table 1**Primary Antibodies**

Antibody	Species	Concentration	Source	Catalog No.
phospho-p44/42 MAPK	rabbit	1/5000	Cell Signaling Tech.	9101
phospho-p44/42 MAPK	mouse	1/2000	Cell Signaling Tech.	9106
GFAP	mouse	1/7000	Millipore	MAB3402
Nestin	mouse	1/20	Chemicon/Millipore	MAB353
BLBP	guinea pig	1/8000	Frontier Science	GP-Af291-1
S100 β	mouse	1/5000	Sigma-Aldrich	S2532
Pax6	mouse	1/3000	Chemicon/Millipore	MAB5552
Neuro D (N-19)	goat	1/3000	Santa Cruz	sc-1084
TUJ1	mouse	1/2500	Babco	MMS-435P
Doublecortin	goat	1/12000	Santa Cruz	sc-8066
Prox1	mouse	1/2000	Chemicon/Millipore	MAB5652
NeuN	mouse	1/5000	Chemicon/Millipore	MAB377
Ki67	mouse	1/200	Novocastra Laboratories	NCL-Ki67-MM1
Mcm2	goat	1/1000	Santa Cruz	sc-9839

Table 2

Antibodies used in Double Labeling and Triple Labeling

Primary Antibodies	Secondary Antibodies
Double labeling	
Phospho-p44/42 MAPK (rabbit) with:	
GFAP (mouse)	Goat anti-rabbit IgG (Alexa Fluor 488) + Goat anti-mouse IgG (Alexa Fluor 555)
Nestin (mouse)	“
S100 β (mouse)	“
TUJ1 (mouse)	“
Prox1 (mouse)	“
NeuN (mouse)	“
Ki67 (mouse)	“
NeuroD (goat)	Donkey anti-rabbit IgG (Alexa Fluor 488) + Donkey anti-goat IgG (Alexa Fluor 555)
Doublecortin (goat)	“
Mcm2 (goat)	“
BLBP (guinea pig)	Donkey anti-rabbit IgG (Dylight 488) + Donkey anti-guinea pig IgG (Dylight 549)
Phospho-p44/42 MAPK (mouse) with:	
Pax6 (rabbit)	Goat anti-mouse IgG (Alexa Fluor 488) + Goat anti-rabbit IgG (Alexa Fluor 555)
Triple labeling	
Phospho-p44/42 MAPK (rabbit) with:	Donkey anti-rabbit IgG (Dylight 488) +
BLBP (guinea pig) +	Donkey anti-guinea pig IgG (Dylight 549) +
Mcm2 (goat)	Donkey anti-goat IgG (Alexa Fluor 633)