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Molecules and Cells

Phosphorylated Extracellular Signal-Regulated Kinase 1/2 Immunoreactivity and Its Protein Levels in the Gerbil Hippocampus during Normal Aging

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Phosphorylated extracellular signal-regulated kinase (pERK) mediates neuronal synaptic plasticity, long-term potentiation, and learning and memory in the hippocampus. In this study, we examined pERK1/2 immunoreactivity and its protein level in the gerbil hippocampus at various ages. In the postnatal month 1 (PM 1) group, very weak pERK1/2 immunoreactivity was detected in the hippocampus. In the CA1 region, pERK1/2 immunoreactivity was considerably increased in the stratum pyramidale in the PM 6 group. Thereafter, pERK1/2 immunoreactivity was decreased. In the CA2/3 region, pERK1/2 immunoreactivity increased in an age-dependent manner until PM 12. Thereafter, numbers of pERK1/2-immunoreactive neurons were decreased. However, in the mossy fiber zone, pERK1/2 immunostaining became stronger with age. In the dentate gyrus, a few pERK1/2-immunoreactive cells were observed until PM 12. In the PM 18 and 24 groups, numbers of pERK1/2-immunoreactive cells were increased, especially in the polymorphic layer. In Western blot analysis, pERK1/2 level in the gerbil hippocampus was increased with age. These results indicate that total pERK1/2 levels are increased in the hippocampus with age. However pERK1/2 immunoreactivity in subregions of the gerbil hippocampus was changed with different pattern during normal aging.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs), a family of related serine/threonine kinases, play important roles in regulating the transduction of extracellular signals to intracellular responses (Kyriakis and Avruch, 1996). MAPKs were recently classified into four major classes: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38 MAPK, and big MAPK (BMK)/ERK5 (Yang et al., 2003).

Among the above-mentioned kinases, extracellular signal-regulated kinases (ERKs) regulate a diverse array of functions, including cell growth, proliferation, differentiation, and apoptosis (Grewal et al., 1999). Moreover, it has been reported that the expression of ERK1/ERK2 mRNA and protein is relatively high in the brain, especially in the hippocampus and nucleus accumbens of the rat brain (Boulton et al., 1991; Ortiz et al., 1995). ERK is thought to play important roles in modulating neuronal function (Suzuki et al., 1995). It has been known that ERK is activated by growth factors, oxidative stress, increases of intracellular calcium levels and glutamate receptor stimulation (Aikawa et al., 1997; Boulton et al., 1991; Kurino et al., 1995; Rosen et al., 1994; Sgambato et al., 1998). In addition, phosphorylation of ERK is related to early gene induction and hyperphosphorylation of cAMP/calcium-responsive element binding protein (Sgambato et al., 1998). There is much evidence that phosphorylated ERK (pERK), which is involved in the development and maintenance of dendritic spines, has been shown to mediate neuronal synaptic plasticity, long-term potentiation (LTP), and learning and memory in the hippocampus (Grewal et al., 1999; Kelleher et al., 2004; Sweatt, 2001). In addition, it was reported that pERK immunoreactivity was changed in the gerbil hippocampus after transient forebrain ischemia (Sugino et al., 2000). However, they did not performed experiment in aged gerbils. Aging is one of risk factors for stroke, and it is a primary factor in the development of greater ischemic neuronal damage in aged rats (Yao et al., 1991). We recently reported that the aged brain was more susceptible to ischemia than in the adult brain, based on the changes in ceruloplasmin level in the hippocampus after ischemia/reperfusion injury (Yoo et al., 2006).

Morphological and functional changes occur in the aged brain, and the accompanying neuropathological and neurochemical alterations are observed in the elderly (He et al., 2008; Kim et al., 2003; Tokalov et al., 2007; Tsunemi et al.,

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2005). Although many studies have focused on determining the role of the MAPK/ERK cascade in the brain, there is no information about chronological changes in pERK1/2 expression in the hippocampus of gerbils during normal aging process. Therefore, in this study, we investigated pERK1/2 expression in subfields of the gerbil hippocampus at various ages using immunohistochemistry and western blot analysis.

MATERIALS AND METHODS

Experimental animals

We used the progeny of male Mongolian gerbils (*Meriones unguiculatus*) obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea, at postnatal month 1 (PM 1) as the young; PM 3 as the young adult; PM 6 and PM 12 as the adult; PM 18 and PM 24 as the aged. The animals (n = 14 at each age) were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-h light/12-h dark cycle, and provided with free access to food and water. The procedures for handling and caring for animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee at Hallym's Medical Center. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Immunohistochemistry for pERK1/2

The animals (n = 7 per group) were anesthetized with pentobarbital sodium and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were removed and postfixed with the same solution for 6 h. The tissues were cryoprotected by infiltration with 30% sucrose overnight. The brain tissues were then frozen and sectioned with a cryostat at 30 μm, and consecutive sections were collected in six-well plates containing 0.1 M PBS.

To ensure that immunohistochemical data were comparable between groups, the free-floating sections were carefully processed under the same conditions. The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 30 min. They were next incubated with diluted rabbit anti-pERK1/2 (1:200, Chemicon, USA) overnight at 4°C and subsequently exposed to biotinylated goat anti-rabbit IgG (1:200, Vector, USA) and streptavidin peroxidase complex (1:200, Vector, USA). Then, the sections were visualized by staining with 3,3′-diaminobenzidine tetrahydrochloride in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. After dehydration the sections were mounted with Canada balsam (Kanto Chemical, Japan).

A negative control test was carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in all structures.

Western blot analysis

To examine change in pERK1/2 level in the hippocampus at various ages, animals at each ages (n = 7) were used for western blot analysis. After sacrificing animals, hippocampus was removed and then homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N,N,N',N' tetraacetic acid (EGTA) (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylendiamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM PMSF and 1 mM dithiothreitol (DTT). After centrifugation, protein concentration in the supernatants was determined using a Micro BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, USA). Aliquots containing 20 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 15% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, USA). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rabbit anti-pERK1/2 antiserum (1:1,000, Chemicon, USA), peroxidase-conjugated goat anti-rabbit IgG (Sigma) and an ECL kit (Pierce Chemical).

Quantification of data

All measurements were performed in blind conditions in order to ensure objectivity, by two observers for each experiment, carrying out the measures of control and experimental samples under the same conditions.

The studied tissue sections were selected according to anatomical landmarks corresponding to AP -1.4~-1.8 mm of the gerbil brain atlas (Loskota et al., 1974). Twenty sections per animal were selected in order to quantitatively analyze pERK1/2 immunoreactivity. Digital images of the hippocampus were captured with an AxioM1 light microscope (Carl Zeiss, Germany) equipped with a digital camera (Axiocam, Carl Zeiss, Germany) connected to a PC monitor. The number of pERK1/2 immunopositive neurons was counted as a mean number per section of the hippocampal subregion.

The mean intensity of immunostaining in the alveus and mossy fiber zone was measured by a 0-255 gray scale system (white to dark signal corresponded from 255 to 0). Based on this approach, the level of immunoreactivity was scaled as −, ±, + or ++, representing no staining (gray scale value: ≥ 200), weakly positive (gray scale value: 150-199), moderate (gray scale value: 100-149), or strong (gray scale value: ≤ 99), respectively. The density of pERK1/2 immunoreactive alveus and mossy fiber zone was evaluated on the basis of a optical density (OD), which was obtained after the transformation of the mean gray level using the formula: OD = log (256/mean gray level). The OD of background was taken from areas adjacent to the measured area. After the background density was subtracted, a ratio of the optical density of image file was calibrated as % (relative optical density, ROD) using Adobe Photoshop version 8.0 and then analyzed using NIH Image 1.59 software.

In addition, the result of the western blot analysis was scanned, and the quantification of the western blotting was done using Scion Image software (Scion Corp., USA), which was used to count relative optical density (ROD): A ratio of the ROD was calibrated as %.

Statistical analysis

Data are expressed as the mean ± SEM. The data were evaluated by a one-way ANOVA SPSS program and the means assessed using Duncan's multiple-range test. Statistical significance was considered at $P < 0.05$.

RESULTS

pERK1/2 immunoreactivity in the CA1 region

In the PM 1 group, very weak pERK1/2 immunoreactivity was

Table 1. The number of pERK1/2 immunoreactive neurons in the gerbil hippocampus during normal aging

Region	Postnatal month (PM)					
	PM 1	PM 3	PM 6	PM 12	PM 18	PM 24
CA1	4.9 ± 3.1	12.0 ± 3.8	63.2 ± 4.6[a,b]	26.6 ± 2.8[a,b]	8.3 ± 0.9[b]	5.7 ± 1.2
CA2/3	8.1 ± 2.8	18.6 ± 1.1[a,b]	21.3 ± 2.0[a]	30.6 ± 2.1[a,b]	6.5 ± 1.0[b]	6.3 ± 0.6
DG	2.7 ± 1.7	5.3 ± 1.0	14.4 ± 1.5[a,b]	13.3 ± 1.8[a]	23.6 ± 2.4[a,b]	21.9 ± 1.5[a]

The numbers of pERK1/2 immunoreactive neurons were counted in the hippocampus of each section.
Values indicate mean ± SEM.
[a]$P < 0.05$, significantly different from the PM 1 group
[b]$P < 0.05$, significantly different from pre-adjacent group

Table 2. The time-course levels of pERK1/2 immunoreactivity in alveus and mossy fiber zone in the gerbil hippocampus during normal aging

Region	Postnatal month (PM)					
	PM 1	PM 3	PM 6	PM 12	PM 18	PM 24
Alveus	-	++	-	-	-	-
Mossy fiber zone	-	+	+	+	++	++

The levels of immunoreactivity were defined as four grades, negative (-), weakly positive (±), moderate (+) and strong (++).

detected in all layers of the hippocampal CA1 region (Figs. 1A and 2A). In the PM 3 group, pERK1/2 immunoreactivity in the CA1 region was similar to that in the PM 1 group (Table 1, Figs. 1B and 2B). Only in this group, on the other hand, pERK1/2 immunoreaction was observed in the alveus (Table 2, Figs. 1B and 4). pERK1/2 immunoreactivity was considerably increased in the PM 6 group, especially in the stratum pyramidale of the CA1 region (Table 1, Figs. 1C and 2C): Many pyramidal neurons showed strong pERK1/2 immunoreactivity. Thereafter, pERK1/2 immunoreactivity was decreased with time until PM 24 (Table 1, Figs. 1D-1F and 2D-2F).

pERK1/2 immunoreactivity in the CA2/3 region

pERK1/2 immunoreactivity was weakly detected in the hippocampal CA2/3 region of the gerbil in the PM 1 group (Fig. 3A). In the PM 3 group, pERK1/2 immunoreactivity was increased, mainly in the stratum pyramidale and mossy fiber zone, compared to that in the PM 1 group (Tables 1 and 2, Figs. 1B and 3B). This pattern was sustained until PM 6 (Figs. 1C and 3C). Many pERK1/2-immunoreactive neurons were observed in the PM 12 group (Table 1, Fig. 3D). In the PM 18 and PM 24 groups, numbers of pERK1/2-immunoreactive neurons were decreased compared to the PM 12 group. However, in the mossy fiber zone, pERK1/2 immunostaining became stronger with age (Tables 1 and 2, Figs. 1E-1F, 3E-3F and 4).

pERK1/2 immunoreactivity in the dentate gyrus

Very weak pERK1/2 immunoreactivity was detected in all layers of the dentate gyrus in the PM 1 group (Fig. 5A). In the PM3 group, some pERK1/2 immunoreactive neurons were observed in the granule cell layer (Fig. 5B). In the PM 6 and 12 groups, more pERK1/2-immunoreactive cells were observed in the granule cell layer and polymorphic layer (Table 1, Figs 5C and 5D). In the PM 18 group, numbers of pERK1/2-immunoreactive cells were considerably increased in the dentate gyrus, especially in the polymorphic layer (Table 1, Figs. 1E and 5E). Thereafter, this pattern of pERK1/2 immunoreactivity was sustained until PM 24 (Figs. 1F and 5F).

pERK1/2 level in the hippocampus

In the Western blot analysis, age-dependent change in pERK1/2 expression was found in the gerbil hippocampus (Fig. 6). In the PM 3 group, the level of pERK1/2 in the hippocampus was significantly increased in comparison to that in the PM 1 group. In the PM 3-12 groups, the levels of pERK1/2 were similar and did not show any statistically significant differences. The level of pERK1/2 increased significantly with age in the PM 18 and 24 groups.

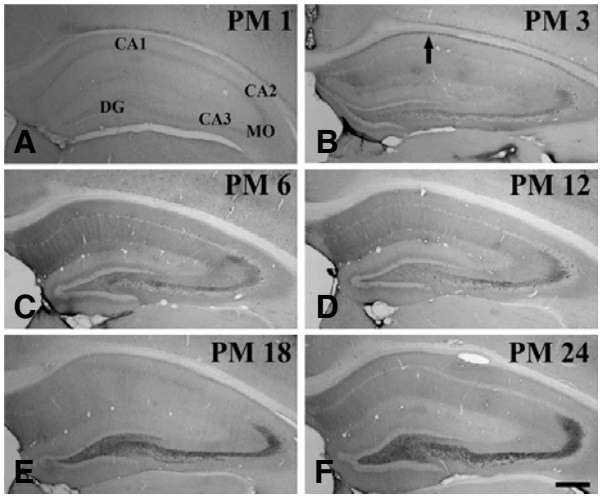

Fig. 1. Low magnification of immunohistochemistry for pERK1/2 in the gerbil hippocampus at postnatal month 1 (PM 1) (A), PM 3 (B), PM 6 (C), PM 12 (D), PM 18 (E) and PM 24 (F). In the mossy fiber zone (MO), pERK1/2 immunostaining became stronger with age. Only in the PM 3 group, pERK1/2 immunoreactivity is considerably increased in the alveus (arrow). CA, conus ammonis; DG, dentate gyrus. Bar = 800 μm.

DISCUSSION

The increasing population growth must be a question of common interests in the aging process (Frye and Walf, 2008; Lukiw et al., 2005). It has well been known that learning and maintenance of spatial memory are controlled by the limbic system of the brain: In particular, the hippocampus plays an important role

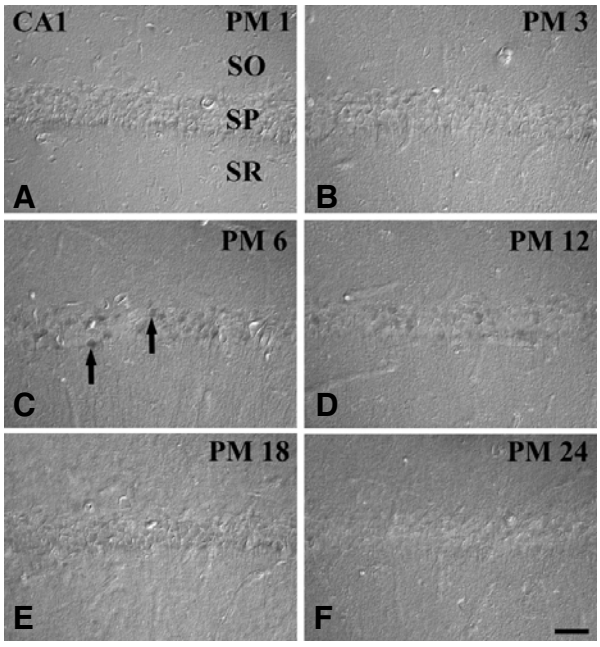

Fig. 2. Immunohistochemistry for pERK1/2 in the hippocampal CA1 region at postnatal month 1 (PM 1) (A), PM 3 (B), PM 6 (C), PM 12 (D), PM 18 (E) and PM 24 (F). pERK1/2 immunoreactivity was considerably increased in the PM 6 group, especially in the stratum pyramidale (SP, arrows). Thereafter, pERK1/2 immunoreactivity is decreased compared to that in the PM 6 group. SO, stratum oriens; SR, stratum radiatum. Bar = 50 μm.

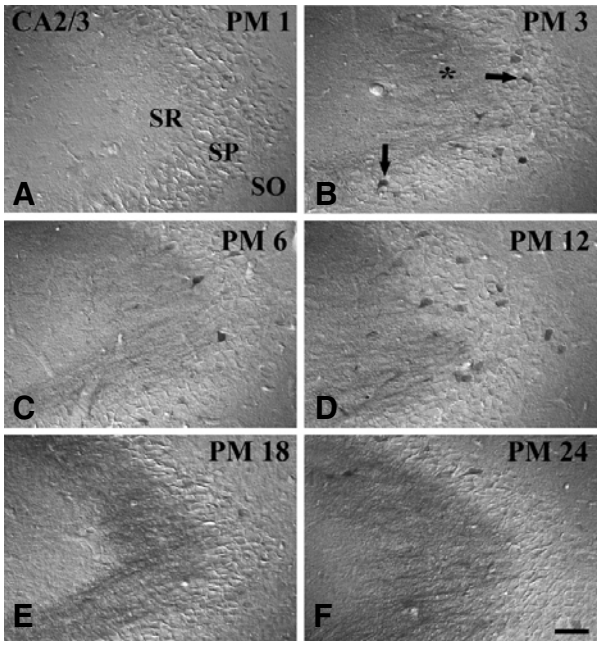

Fig. 3. Immunohistochemistry for pERK1/2 in the CA2/3 region at postnatal month 1 (PM 1) (A), PM 3 (B), PM 6 (C), PM 12 (D), PM 18 (E) and PM 24 (F). In the PM 3-12 groups, strong pERK1/2-immunoreactive cells are observed in the stratum pyramidale (SP, arrows) and mossy fiber zone (asterisk). In the PM 18-24 groups, pERK1/2 immunoreactive neurons are decreased compared to the PM 12. However, the mossy fiber zone is strongly immunostained against pERK1/2. SO, stratum oriens; SR, stratum radiatum. Bar = 50 μm.

in the function of learning and memory (He et al., 2008; Wati et al., 2006).

It has well been known that the ERK1 and ERK2 (ERK1/2) isoforms of MAPKs are activated by some factors, such as neurotrophins and GM1 (Duchemin et al., 2002; Klesse and Parada, 1999). The activated ERK1/2 is thought to be associated with the regulation of differentiative and survival activities in peripheral and central neurons (Mo et al., 2005). In addition, ERK activation has been reported to associate with neuronal protection as well as neuronal degeneration and/or death in many studies using neuronal injury models (Kurino et al., 1995; Kuroki et al., 2001; Namura et al., 2002; Park et al., 2004).

In the present study, pERK1/2 immunoreaction was found in all subregions (CA1, CA2/3 and dentate gyrus) of the hippocampus in all age groups. However, in the PM 1 group, very weak pERK1/2 immunoreactivity was detected in all subregions of the gerbil hippocampus. In the CA1 region, pERK1/2 immunoreactivity was considerably increased in the PM 6 group, thereafter, pERK1/2 immunoreactivity was decreased with age. In the CA2/3 region, pERK1/2 immunoreactive neurons were significantly increased in the PM 12 group, thereafter, numbers of pERK1/2-immunoreactive neurons were decreased. In the mossy fiber zone, pERK1/2 immunostaining became stronger with age. In the dentate gyrus, many pERK1/2-immunoreactive cells were observed in the PM 18 and 24 groups. Western blot analysis revealed that the level of pERK1/2 in the gerbil hippocampus increased significantly with age.

There are some reports about the alteration of ERK activation in the hippocampus with age. It has also been known that ERK phosphorylation was decreased in older rats (Gooney et al., 2004; Lynch and Lynch, 2001; Zhen et al., 1999). Based on the immunoblot and mean values obtained from densitometric

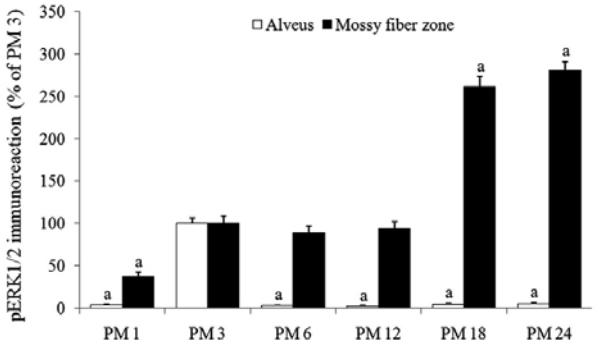

Fig. 4. ROD as % of pERK1/2 immunoreactivity in alveus and mossy fiber zone of the gerbil hippocampus at various ages ($^{a}P < 0.05$, significantly different from the PM 3 group). The bars indicate the means ± SEM.

analysis, it was confirmed that the expression of pERK was decreased in hippocampal preparations obtained from aged rats. It was also reported that ERK2 activity decreased significantly with age in the brains of Balb/c mice (Hu et al., 1998). Moreover, Maher et al. (2005) reported that decrease of ERK activation was observed only in aged rats that failed to sustain long-term potentiation (LTP). They urged that the age-related decrease in ERK was likely to contribute to the deficit in LTP. It was based on the previous studies that LTP expression was ERK-dependent and that LTP was not sustained when ERK was inhibited (English and Sweatt, 1997; Gooney et al., 2004).

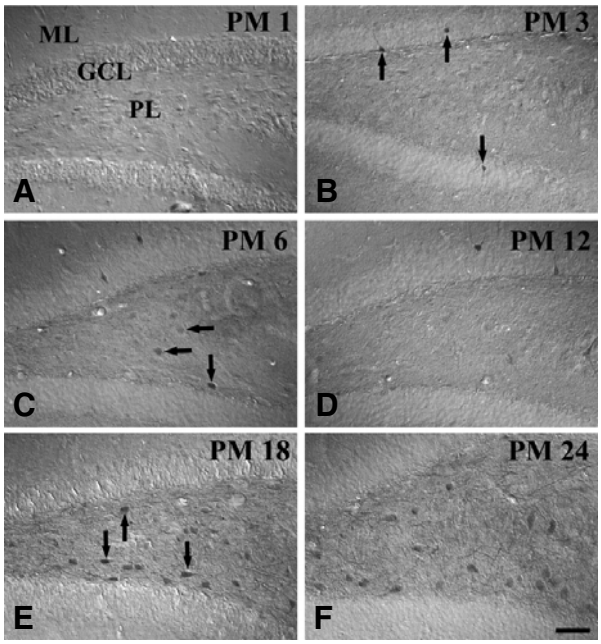

Fig. 5. Immunohistochemistry for pERK1/2 in the dentate gyrus at postnatal month 1 (PM1) (A), PM 3 (B), PM 6 (C), PM 12 (D), PM 18 (E) and PM 24 (F). In the PM3 group, some pERK1/2 immunoreactive cells (arrows) are observed in the granule cell layer (GCL). In the PM 6-12 groups, more pERK1/2 immunoreactive cells are observed in the GCL and polymorphic layer (PL, arrows: polymorphic cells). Thereafter, pERK1/2 immunoreactive cells are considerably increased in the PL. ML, molecular layer. Bar = 50 μm.

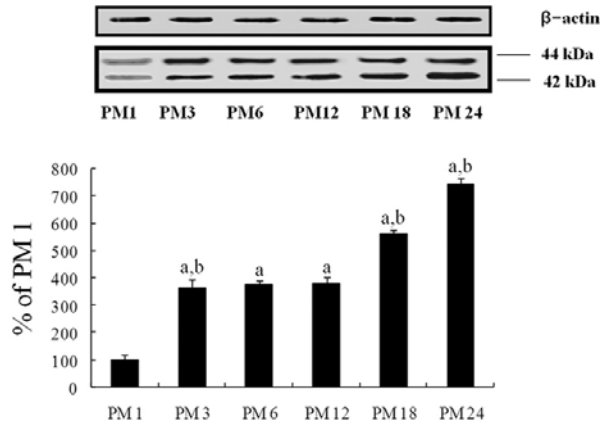

Fig. 6. Western blot analysis of pERK1/2 in the hippocampus derived from various ages. Relative optical density as % values of immunoblot band is represented. Forty four kDa is pERK1, and 42 kDa is pERK2. (n = 7 per group; $^{a}P < 0.05$, significantly different from the PM 1 group, $^{b}P < 0.05$, significantly different from the pre-adjacent group). The bars indicate the means ± SEM.

However, a contradictory report with the above-mentioned studies was recently published. Song et al. (2007) reported that the phosphorylation of ERK1/2 during the aging process was significantly increased in various brain regions, including the hippocampus, of Sprague-Dawley rats. They urged that the increased ERK1/2 activation provided a vulnerable tumorigenic and age-related inflammatory environment in the brain.

With the above-mentioned papers, although there is some discrepancy regarding age-related changes in pERK1/2 phosphorylation in the hippocampus in some animals, our present study shows that the total pERK1/2 level in the gerbil hippocampus increases in an age-dependent manner. This is thought to be due to species specificity. However, pERK1/2 immunoreactivity was decreased in the CA1 and CA2/3 regions of the aged gerbil hippocampus. In contrast, pERK1/2 immunoreactivity in the mossy fiber zone and dentate gyrus increased as the normal aging progresses. Harris et al. (2004) reported that pERK was maximal in the mossy fiber zone of the hippocampus of the apoE4 transgenic mouse, and suggested that neuronal deficits caused zinc release from synaptic termini, which leaded to ERK activation. In addition, the changes in signal transmission and transduction systems were observed in the aged gerbil brain (Hara et al., 1992). These results suggest that regional differences in the age-dependent changes in pERK1/2 immunoreactivity in the gerbil hippocampus may be related to regional heterogeneity as well as age-related changes of the hippocampus.

On the other hand, we observed pERK1/2 immunoreactivity in the alveus of the PM 3 group, not in any other groups. It's too difficult to explain exactly this phenomenon, because there are no studies about it. However, it has been reported that ERK activation could lead to the phosphorylation of myelin basic protein in preparation for myelin synthesis (Stariha et al., 1997). Base on the previous study, we postulated that pERK1/2 might be related to myelin synthesis in the alveus, consisted with CA1 axons, in the PM 3 group. In addition, we observed that pERK1/2 immunoreactivity was found mainly in cytoplasm as well as nucleus of neurons in the gerbil hippocampus. However, Xi et al. (2007) reported that p-ERK in patients with intractable epilepsy were significantly higher than those in the controls: They were mainly expressed in the cytoplasm of neurons and glial cells. This result indicates that the change of pERK1/2 subcellular localization did not occur in the gerbil hippocampus during normal aging.

In conclusion, our present study indicates that total levels of pERK1/2 in the gerbil hippocampus are increased with age. However, pattern of pERK1/2 immunoreactivity is different according to subregion of the gerbil hippocampus during normal aging.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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