Research Report

Heparin-binding EGF-like growth factor is required for synaptic plasticity and memory formation

Atsushi Oyagi\textsuperscript{a}, Shigeki Moriguchi\textsuperscript{b}, Atsumi Nitta\textsuperscript{c}, Kenta Murata\textsuperscript{a}, Yasuhisa Oida\textsuperscript{a}, Kazuhiro Tsuruma\textsuperscript{a}, Masamitsu Shimazawa\textsuperscript{a}, Kohji Fukunaga\textsuperscript{b}, Hideaki Hara\textsuperscript{a,}*  

\textsuperscript{a}Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, Gifu 501-1196, Japan  
\textsuperscript{b}Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan  
\textsuperscript{c}Department of Pharmaceutical Therapy & Neuropharmacology, Faculty of Pharmaceutical Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan

\textbf{ABSTRACT}

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of epidermal growth factor (EGF) family, is a potent mitogenic peptide for various types of cells. HB-EGF is widely expressed in central nervous system, including hippocampus and cerebral cortex, and is considered to play pivotal roles in the developing and adult nervous system. In this study, we assessed the role of HB-EGF in learning and memory by testing HB-EGF conditional knock-out mice (KO) in two different learning tasks, and evaluated the long-term potentiation (LTP) in hippocampus slices from these mice. The HB-EGF KO mice were impaired in spatial memory in the Morris water maze and in fear learning in a passive avoidance test. HB-EGF KO mice also showed an impaired LTP, and reduction in activity of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) and phosphorylated GluR1. We also found that the levels of neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), or glial cell line-derived neurotrophic factor (GDNF), were altered in several brain regions in the HB-EGF KO mice. These results confirm the importance of the HB-EGF in synaptic plasticity and memory formation.

© 2011 Elsevier B.V. All rights reserved.

Keywords: CaMKII, HB-EGF, LTP, Memory, Neurotrophic factor

1. Introduction

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the EGF family of growth factors that includes EGF, transforming growth factor (TGF)-\textalpha, amphiregulin, betacelulin, and neuregulin (Barnard et al., 1995; Higashiyama et al., 1991; Watanabe et al., 1994). In the central nervous system, HB-EGF is widely distributed in neurons and neuroglia throughout the brain, and is especially enriched in the hippocampus, cerebral cortex, and cerebellum (Mishima et al., 1996). HB-EGF binds to and activates the EGF receptor (EGF receptor/ErbB1) (Higashiyama et al., 1991), ErbB4 (Elenius et al., 1997), and has been implicated in neuronal survival and glial/stem cell proliferation (Farkas and Krieglstein, 2002; Kornblum et al., 1999; Nakagawa et al., 1998). For instance, HB-EGF has been reported to exert protective effects in rodent models of ischemic brain injury (Jin et al., 2004) and to enhance neurogenesis in the subventricular zone and hippocampal dentate gyrus (Jin et al., 2003; Jin et al., 2004). We have also previously reported that ventral forebrain specific HB-EGF knockout (KO) mice show aggravated...
brain injury induced by a middle cerebral artery occlusion (Oyagi et al., 2011).

We have also previously demonstrated that HB-EGF conditional KO mice exhibit several neurobehavioral abnormalities, including impairments of prepulse inhibition, social interaction, and cognitive function, which were accompanied by aberrant cortical spine morphology (Oyagi et al., 2009). These results indicate that HB-EGF may be one of the important contributors to neuronal development and higher brain function.

HB-EGF is widely expressed in the central nervous system, including the hippocampus and cerebral cortex, where it serves in cognitive functions, however, very few studies have yet examined the involvement of HB-EGF in synaptic plasticity and memory formation. In the present study, we assessed the role of HB-EGF in learning and memory by testing HB-EGF conditional KO in two different learning tasks: the Morris water maze and passive avoidance tests. We also measured long-term potentiation (LTP), activation of various protein kinases in hippocampal slices, and neurotrophic factor levels in various regions of HB-EGF KO mice.

2. Results

2.1. Spatial memory acquisition and retention in the Morris water maze test

HB-EGF mRNA and immunoreactivity have been localized in hippocampus (Hayase et al., 1998; Oyagi et al., 2009), which is one of the most well characterized regions for spatial memory formation. We first examined spatial memory acquisition and retention abilities in the Morris water maze test to determine the involvement of HB-EGF in hippocampus-dependent memory formation. Both WT and HB-EGF KO mice exhibited similar escape latencies to the hidden platform with daily training (Fig. 1B), suggesting that the learning kinetics of reference memory were similar between each group [genotype; F(1,19)=1.048, P>0.05, time; F(4,76)=32.604, P<0.01, genotype×time; F(4,76)=1.808, P>0.05]. In a probe trial, WT mice spent significantly more time in the target quadrant than in other quadrants, indicating that they remembered the place where the platform used to be [Figs. 1 A and C] [WT; F(3,48)=44.557, P<0.0001]. In contrast, HB-EGF KO mice spent less time than the WT mice in this area (Fig. 1 C). No significant differences were observed in swim speed between WT and HB-EGF KO mice [WT; 18.4±0.82 cm/s (n=13), KO; 17.6±1.47 cm/s (n=8)].

2.2. Fear memory in passive avoidance test

Next, we conducted a passive avoidance test. Passive avoidance conditioning was used to determine whether HB-EGF KO mice exhibited altered abilities in a learning task; in this paradigm, lasting context-fear association can be imparted with a single training session (Duvarci et al., 2005). During the training session, WT and HB-EGF KO mice showed a similar latency in crossing to the dark side of the chamber (Fig. 2A), whereupon they received a single foot shock. When animals were tested at 24 h after conditioning, HB-EGF KO mice exhibited a significantly decreased latency to cross to the foot shock-paired dark side (Fig. 2A).

2.3. Long-term potentiation (LTP) in hippocampus CA1 neuron

Because impaired memory implies abnormal hippocampal plasticity in HB-EGF KO mice, we next analyzed LTP induced by a high-frequency stimulation (HFS) in hippocampus CA1 neurons using slice preparations. In control slices from WT mice hippocampus, HFS (100 Hz, 2 trains) of the Schaffer collateral/commissural pathways induced LTP in the hippocampal CA1 region, which lasted over 60 min (1 min after HFS; 265.7±23.1% of baseline, 60 min after HFS; 205.0±34.2% of baseline, n=5) (Figs. 3A, B, and C). On the other hand, a marked reduction of LTP was observed in slices from HB-EGF KO mice (1 min after HFS; 143.4±14.6% of baseline, 60 min after HFS; 130.8±16.2% of
baseline, F(3,12) = 18.194, P < 0.0001, n = 5) (Figs. 3A, B, and C). These results indicate that HB-EGF is essential for hippocampal LTP in the CA1 region, and therefore HB-EGF may contribute to cognitive function and memory formation.

2.4. Phosphorylation of various protein kinases in the hippocampus of HB-EGF KO mice

Activation of synaptic proteins, such as CaMKII by high-frequency stimulation (HFS) is essential for hippocampal LTP induction (Fukunaga et al., 1993; Silva et al., 1992). We examined intrinsic CaMKII activity in CA1 slices by western blotting before and after HFS (Figs. 4A, B, C, and D). The summary histogram or relative immunoreactivity demonstrated that basal phospho-CaMKII level was significantly decreased in HB-EGF KO mice, when compared to WT mice (72.5 ± 4.9% of baseline, F(1,8) = 7.228, P < 0.05, n = 5), without changes basal protein levels (Figs. 4A and B). In addition, HFS increased phospho-CaMKII levels in WT and HB-EGF KO mice [WT; 153.2 ± 8.5% of baseline, F(1,8) = 18.589, P < 0.01, KO; 121.6 ± 11.8% of baseline, F(1,8) = 14.717, P < 0.01, n = 5] (Figs. 4A and B). Therefore, autophosphorylated CaMKII level after HFS tended to be lower in HB-EGF mice than in WT mice, but the difference was not statistically significant (Figs. 4A and B). Basal levels of phosphorylated α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor subunit 1 (GluR1) (Ser-831), which is a postsynaptic CaMKII substrate (Derkach et al., 1999), was also significantly lower in HB-EGF KO mice than in WT mice [77.2 ± 4.7% of baseline, F(1,7) = 9.292, P < 0.05, n = 4 or 5] (Figs. 4C and D). On the other hand,
HFS increased the levels of phosphorylated GluR1 (Ser-831) and synapsin I (Ser-603) levels in WT and HB-EGF KO mice [WT pGluR1 (Ser-831); 140.0±10.9% of baseline, F(1,7) = 8.885, P<0.05, KO pGluR1 (Ser-831); 124.3±8.0% of baseline, F(1,8) = 25.725, P<0.01, n=4 or 5, WT pSynapsin I (Ser-603); 128.0±3.6% of baseline, F(1,8) = 37.993, P<0.01, and KO pSynapsin I; 134.8±3.1% of baseline, F(1,8) = 17.229, P<0.01, n=5] (Figs. 4 C and D). No significant changes were observed in the phosphorylation levels of signals such as PKCα, ERK, and β-tubulin. (C) Representative images of immunoblots using antibodies against phosphorylated synapsin (pSyn 1), synapsin 1 (Syn 1), phosphorylated GluR1 (Ser831) (pGluR1), and GluR1. (D) Quantitative analyses of pSyn 1, Syn 1, pGluR1 (Ser831), and GluR1. Values are means ± SEM, WT (n=4 or 5), KO (n=5). * p<0.05, ** p<0.01 vs. WT (Control), ## p<0.01 vs. HB-EGF KO (Control).

Fig. 4 – Phosphorylation of various protein kinases in the hippocampus of HB-EGF KO mice. (A) Representative images of immunoblots using antibodies against autophosphorylated CaMKII (pCaMKII), CaMKII, phosphorylated PKCα (Ser675) (pPKCα), PKCα, phosphorylated ERK (pERK), ERK, and β-tubulin. (B) Quantitative analyses of pCaMKII, CaMKII, pPKCα, PKCα, pERK, ERK, and β-tubulin. (C) Representative images of immunoblots using antibodies against phosphorylated synapsin (pSyn 1), synapsin 1 (Syn 1), phosphorylated GluR1 (Ser831) (pGluR1), and GluR1. (D) Quantitative analyses of pSyn 1, Syn 1, pGluR1 (Ser831), and GluR1. Values are means ± SEM., WT (n=4 or 5), KO (n=5). * p<0.05, ** p<0.01 vs. WT (Control), ## p<0.01 vs. HB-EGF KO (Control).

2.5. Changes in various neurotrophic factors levels in the brain of HB-EGF KO mice

Next, we investigated the effect of HB-EGF deletion on the production of various neurotrophic factors by measuring the levels of NGF, BDNF, NT-3, and GDNF in the several brain regions in WT and HB-EGF KO mice. In the hippocampus, protein levels of NGF and NT-3 were significantly increased, while GDNF level was decreased in HB-EGF KO mice when compared with WT mice (Fig. 5A). The levels of NGF and BDNF were significantly higher in the cortex of HB-EGF KO mice than in WT mice (Fig. 5B). BDNF level was also upregulated in the striatum of HB-EGF KO mice, compared with WT mice (Fig. 5C). Although only BDNF levels showed a tendency to increase in olfactory bulb of HB-EGF KO mice, no significant changes were observed in the levels of neurotrophic factors in either mouse group (Fig. 5D).

3. Discussion

Neurotrophic and growth factors play distinct roles in development and maturation of the nervous system. HB-EGF is widely distributed in neuron and neuroglia throughout the brain, and is especially enriched in hippocampus, cerebral cortex, and cerebellum (Mishima et al., 1996). This distribution predicts HB-EGF to be an important contributor to neuronal development and higher brain function. In this study, we investigated possible roles for HB-EGF in memory formation and synaptic plasticity.

We first showed that conditional HB-EGF KO mice have cognitive defects, characterized by impairment in both spatial and fear memory. These results correspond to our previous report that HB-EGF KO mice displayed deficits in short term memory in a Y-maze test and in object identification memory in a novel object recognition test (Oyagi et al., 2009). The Morris water maze test is used to investigate hippocampal-dependent learning, including acquisition of spatial and long-term memory (Denayer et al., 2008; Peters et al., 2003). In this test, HB-EGF KO mice were deficient in the probe trial (spatial memory). The HB-EGF KO mice also exhibited impairment of fear memory in
a test session of a passive avoidance test, which was conducted 24 h after a training session. These behavioral dysfunctions suggest that loss of HB-EGF might especially affect memory retention. On the other hand, the latency to platform of HB-EGF KO mice showed a tendency to increase, compared with WT mice, in the acquisition trial (especially at days 3–5) of Morris water maze test, but the difference was not statistically significant. Further studies will be needed to investigate the effect of HB-EGF on memory acquisition.

Long-term potentiation (LTP) at CA1 synapses in the hippocampus, a cellular model for learning and memory, is initiated by the influx of Ca\(^{2+}\) through N-methyl-D-aspartate (NMDA)-type glutamate receptors. The insertion of AMPA-type glutamate receptors into the postsynaptic site and the associated morphology of dendritic spines are believed to be critical for LTP induction (Derkach et al., 2007; Matsuzaki et al., 2004; Shi et al., 1999). In the present study, hippocampal LTP induced by a high-frequency stimulation (HFS) was markedly impaired in HB-EGF KO mice. HB-EGF KO mice also showed the reduction in activity of CaMKII and phosphorylated GluR1.

CaMKII has been implicated as a key molecule in the induction of LTP (Lisman et al., 2002). Phosphorylation of AMPA receptors by CaMKII is reported to be particularly important for LTP induction (Barria et al., 1997; Derkach et al., 1999). Phosphorylation of GluR1 (Ser831) by CaMKII underlies the increase in AMPA receptor-mediated ionic conductance observed in LTP (Ledo et al., 1995; Mammen et al., 1997; Roche et al., 1996). Decreased phosphorylation of CaMKII and GluR1 observed in the hippocampus of HB-EGF KO mice suggest that CaMKII and GluR1 mediate the effects of HB-EGF on synaptic plasticity.

Neurotrophic factors and cytokines display profound neuro-modulatory functions and are involved in the survival and homeostatic maintenance of the central nervous system through regulation of each other’s expression. Disruption of the neurotrophin balance has been associated with pathogenesis of various neurological diseases, such as schizophrenia, amyotrophic lateral sclerosis (ALS), and Alzheimer’s disease (AD) (Narisawa-Saito et al., 1996; Schulte-Herbruggen et al., 2007; Takahashi et al., 2000). Altered neurotrophic factors levels were observed in several brain regions in the HB-EGF KO mice; in particular, NGF, NT-3, or BDNF levels were upregulated in hippocampus and/or cortex of HB-EGF KO mice, compared with WT mice. Since HB-EGF itself has a neurotrophic effect, the absence of HB-EGF may secondarily alter the expression of neurotrophins. Taken together, these findings suggest that the induction of these growth factors compensates for the deficit in HB-EGF and that the imbalance of neurotrophic and growth factors might partly associate with impaired memory function and synaptic plasticity in HB-EGF KO mice.

Typically, HB-EGF is processed from its precursor protein, pro-HB-EGF, which is anchored in the plasma membrane. Pro-HB-EGF is susceptible to proteolytic cleavage, namely ectodomain shedding, and is converted to the mature secreted factor, HB-EGF (Goishi et al., 1995). Accordingly, ectodomain shedding is essential for HB-EGF exerts its biological effects and ADAMs are key enzymes in this pathway (Asakura et al., 2002; Nanba et al., 2003). The administration of an ADAMs inhibitor also impaired memory retention in the passive avoidance test.

In conclusion, the current study demonstrated that HB-EGF KO mice exhibited impairments in spatial and fear memory,
and also showed decreased LTP in hippocampal CA1 neurons. These behavioral and synaptic dysfunctions are associated with impaired activation of CaMKII and GluR1 in the hippocampus. Further studies using in vitro neuronal and non-neuronal cell cultures would provide the proof for a causal link between the behavioral/physiological findings and the molecular profile. These results suggest that HB-EGF plays a significant, but yet to be fully identified, role in synaptic plasticity and memory formation.

4. Experimental procedures

4.1. Animals

Ventral forebrain specific HB-EGF KO mice were generated using the Cre-loxP system, as described previously (Oyagi et al., 2009). All procedures relating to animal care and treatment conformed to the animal care guidelines of the Animal Experiment Committee of Gifu Pharmaceutical University. All efforts were made to minimize both suffering and the number of animals used. The animals (10–15 weeks old) were housed at 24 ± 2 °C under a 12 h light–dark cycle (lights on from 8:00 to 20:00) and had ad libitum access to food and water. In all experiments, we used wild-type (WT) littermates as a control group for the HB-EGF KO mice.

4.2. Morris water maze test

A circular pool (diameter 120 × height 45 cm) was filled to a depth of 30 cm with water (21–23 °C). Four equally spaced points around the edge of the pool were designated as four starting positions. The pool was placed in a dimly lit, sound-proof test room with various visual cues. A hidden platform (diameter 10 cm) was set 0.5 cm below the surface of the water in a fixed position. Mice were placed in the water facing the wall and trained with 4 trials per a day for 5 days. In each trial, the starting position was changed, and the mice swam until they found the platform, or after 60 s were guided to the platform; the mice were then placed on the platform for 15 s before being picked up. Three days after the last training trial, the mice were given a probe test without the platform. In this test, mice were placed in the pool once and allowed to search for 120 s. Mean duration to the platform, and the time spent in the quadrant where the platform had been, was recorded using a video camera-based Ethovision XT system (Noldus, Wageningen, The Netherlands).

4.3. Passive avoidance test

Mice were tested using a two-compartment box with foot shock grid (Neuroscience, Tokyo, Japan). On habituation day, mice were placed in the lighted compartment, facing away from the dark compartment and allowed to explore for 30 s. After 30 s, the guillotine door was lifted. When the mice entered the dark compartment with all four paws, the guillotine door was closed, and the latency to enter was recorded (from the time the door is lifted). Thirty seconds after the foot shock, the mice were removed to the home cage. On test day (at 24 h after training), the mice were returned to the lighted compartment, facing away from the dark compartment. After 30 s, the guillotine door was lifted. When the mice entered the dark compartment with all four paws, the guillotine door was closed, and the latency to enter the dark compartment was recorded (from the time the door was lifted). The mice were removed and returned to the home cage. Animals who failed to enter the dark compartment within 300 s were assigned a maximum test latency score of 300 s.

An ADAM inhibitor, KB-R7785 (30 and 100 mg/kg), was dissolved in carboxymethyl cellulose (CMC) and subcutaneously administered to ICR mice (6 weeks old, SLC, Shizuoka, Japan) once a day for 4 days. The passive avoidance test was conducted on the third day (training trial) to fourth day (test trial), 30 min after KB-R7785 administration. Scopolamine HBr (3 mg/kg) dissolved in saline was intraperitoneally administered to mice.

4.4. Electrophysiology

Preparation of hippocampal slices was performed as described previously (Moriguchi et al., 2008). Briefly, the brain was rapidly removed from each ether-anesthetized male WT or HB-EGF KO mouse and the hippocampus was dissected out. Transverse hippocampal slices (400 μm thickness), prepared using a vibratome (microslicer DTK-1000, Dosaka, Kyoto, Japan), were incubated for 2 h in continuously oxygenized (95% O2, 5% CO2) artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 5 mM KCl, 26 mM NaHCO3, 1.3 mM MgSO4·7H2O, 1.26 mM KH2PO4, 2.4 mM CaCl2·2H2O, and 1.8% glucose at room temperature (28 °C). After a 2 h recovery periods, a slice was transferred to an interface recording chamber and perfused at a flow rate of 2 ml/min with ACSF warmed to 34 °C. Field excitatory postsynaptic potentials (fEPSPs) were evoked by a 0.05 Hz test stimulus through a bipolar stimulating electrode placed on the Schaffer collateral/commissural pathway and recorded from the stratum radiatum of CA1, using a glass electrode filled with 3 M NaCl. A single-electrode amplifier (CEZ-3100, Nihon Kohden, Tokyo, Japan) was used to record the responses, and the maximal value of the initial fEPSP slope was collected and averaged every 1 min (3 traces) using an A/D converter (PowerLab 200, AD Instruments, Castle Hill, Australia) and a personal computer. After a stable baseline was obtained, high frequency stimulation (HFS) of 100 Hz and 1 s duration was applied twice with a 10 s interval and test stimuli were continued for the indicated periods.

4.5. Western blotting

Hippocampal CA1 slices were homogenized in a buffer (70 μl) containing 50 mM Tris HCl (pH 7.4), 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 1 mM Na2VO4, 40 mM sodium pyrophosphate, 50 mM NaF, 100 mM calyculin A, 50 μg/ml leupeptin,
25 g/ml pepstatin A, 50 µg/ml trypsin inhibitor, and 1 mM DTT. After the removal of insoluble materials by centrifugation (15,000 rpm for 10 min), the samples were subjected to immuno blotting as previously described (Moriguchi et al., 2009). After determining protein concentration in supernatants using Bradford’s solution, samples were boiled for 3 min in Laemmli sample buffer. Samples containing equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to an immobilon polyvinylidene difluor ide membrane for 2 h at 70 V. After blocking with 50 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5, containing 2.5% bovine serum albumin for 1 h at room temperature, membranes were incubated overnight at 4 °C with anti-phospho CaMKII, [1:5000, (Fukunaga et al., 1988)], anti-CaMKII, [1:5000, (Fukunaga et al., 1988)], anti-phospho-synapsin 1 (site 3) (1:2000, Chemicon, CA, USA), anti-synapsin 1 [1:2000, (Fukunaga et al., 1992)], anti-phospho-GluR1 (Ser831) (1:1000, Upstate) antibodies. Bound antibodies were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK) and analyzed semiquantitatively using the NIH Image software.

4.6. Enzyme immunoassay (EIA)

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial-cell line derived-neurotrophic factor (GDNF) levels were measured with an EIA with a minor modification (Nitta et al., 1999a; Nitta et al., 1999b; Nitta et al., 2004; Niwa et al., 2007). HB-EGF KO and WT mice (12 weeks old) were used in this study. Homogenate buffer [0.1 mol/l Tris–HCl (pH 7.4) containing 1 mol/l sodium chloride (NaCl), 2% bovine serum albumin, 2 mmol/l ethylenediamine-1,3,3′,N′-tetraacetic acid (EDTA), and 0.2% sodium nitride (NaN3)] was added to the brain tissue at a ratio of 1 g wet weight/19 ml of buffer, pulse-sonicated for 100 s, and centrifuged at 100,000 g for 30 min. The supernatant was collected and used for the EIA. Multiwell plates (Falcon 3910; Becton Dickinson and Co., NJ, USA) were incubated with 5 ml of each primary antibody (NGF, MAB256, BDNF, MAB684, NT-3, MAB267, GDNF, and MAB212, R & D Systems, Minneapolis, USA) in 0.1 M Tris–HCl buffer (pH 9.0) (10 mg/ml) per well for 12 h, washed with washing buffer (0.1 M Tris–HCl buffer, pH 7.4, containing 0.4 M NaCl, 0.02% NaN3, 0.1% BSA, and 1 mM MgCl2), and then blocked with washing buffer containing 1% (w/v) skim milk. Tissue extract or each protein standard (30 µl, R & D Systems) in washing buffer was then added to each antibody-coated well; and incubation was carried out for 5 h at 25 °C. After three washes with washing buffer, 30 µl of biotinylated anti-NGF, BDNF, NT-3, and GDNF-antibodies (BAF 256, BAF648, BAF267, and BAF212, respectively; 10 ng/ml, R & D Systems) in washing buffer was added to each well; and the plate was incubated for 12 to 18 h at 4 °C. The biotinylated secondary antibodies were reacted with avidin-conjugated ⋅galactosidase (Boehringer Mannheim GmbH, Mannheim, Germany) for 1 h. After a thorough washing with washing buffer, enzyme activity retained in each well was measured by incubation with the fluorogenic substrate; 4-methylumbelliferyl-β-D-galactoside (100 µM) in washing buffer. The intensity of fluorescence was monitored with 360 nm excitation and 448 nm emission. The detection limit of the EIA was as low as 5 pg/ml. The recovery of each protein (61.8 pg/ml) exogenously added into the homogenizing buffer following disruption of the rat hippocampus was about 80%. The value of protein content thus obtained was used without correction.

4.7. Statistical analysis

All data were expressed as the mean ± SEM. Statistical significance was evaluated by Student’s t-test, Steel test, Mann–Whitney U-test, and one-way or two-way ANOVA test followed by a post hoc Tukey or Scheffe’s tests. A p-value of <0.05 was considered to be statistically significant.

Acknowledgments

The authors thank Dr. Kohichiro Yoshino (Carnabioscience, Kobe, Japan) for the kind gift of KB-R7785.

REFERENCES


Fukunaga, K., Stopinni, L., Miyamoto, E., Muller, D., 1993. Long-term potentiation is associated with an increased activity of Ca2+/calmodulin-dependent protein kinase II. J. Biol. Chem. 268, 7863–7867.


