cells in our microglial culture was Mac1 positive and the Ron staining signals were detected at the edge of cell shape in those cells, showing that Ron is expressed on the cytoplasmic membranes of microglia (Fig. 1B). Additionally, the Ron signals were closely located in cell nuclei (Fig. 1B), indicating the same staining pattern as the subcellular localization of HGF-receptor/c-Met tyrosine kinase, the structural relative of Ron (27). These signals in immunocytochemical staining for Ron could be eliminated using anti-Ron antibody preincubated with its blocking peptide (data not shown). This also means that the PCR product from microglial culture in Fig. 1A reflect the expression of Ron in microglia but not in contaminating neural cells. The results indicate that microglia is one of the Ron-expressing cell species.

Functional Ron consists of an α- and a β-chain, which are processed from a single proform polypeptide and the alternatively deleted Ron mRNA codes nonfunctional Ron which lacks extracellular domain (12, 31). Therefore, Western blotting was performed using an antibody specific for the α-chain, present in the extracellular domain, to evaluate the expression of functional Ron. Cell lysates of the primary microglia and, as the positive control, murine macrophage cell line J774A.1 were prepared and subjected to Western blotting. A 35-kDa band, corresponding to the size of the Ron α-chain, was clearly detected in both primary microglia and J774A.1 cells (Fig. 1C).

These data revealed that the functional (processed) form of full-length Ron is expressed in microglia.

Effect of HLP on microglial migration

The accumulation of microglia at the affected and marginal regions in the degenerated and injured brain is observed as the results of stimulated proliferation and/or migration of the cells, which are typical actions of the activated microglia (19). At first, the possible effect of HLP on microglial migration was assessed using a transwell chamber system (Fig. 2A). rhHLP (3, 10 ng/mL) was added in the bottom compartment of the chamber and the cells were seeded in the upper compartment which was separated from the bottom chamber with a micropored membrane. After 24 h incubation, the number of cells on the bottom side of the membrane was counted (Fig. 2B and C). Compared with the results from unstimulated wells, a 2- to 3-fold number of cells was detected when HLP was added (Fig. 2C). These results showed that HLP enhances microglial migration and/or proliferation.

Less effect of HLP on microglial survival and proliferation

Since it is reported that HLP promotes keratinocyte survival (4) and proliferation (32), the possibility of these effects of HLP on microglia were assessed. The primary microglia were cultured in the absence and the presence of 10 ng/mL HLP for up to 48 h, and the population of viable and dead cells was analyzed by double staining with Calcein-AM and PI, respectively. With this method, the viable cells exhibited a green signal and dead cells exhibited a red signal (Fig. 3A). The survival ratio at 24 h after seeding was more than 80% in the absence of HLP, and it decreased to 50% 48 h after seeding. This survival profile was nearly equivalent even in the presence of HLP (Fig. 3A, B). The total number of microglia at 48 h after seeding was unchanged in either the presence or in the absence of HLP, indicating that HLP failed to affect the microglial proliferation. Together, the difference in the cell number detected between HLP-presence and -absence in migration assays (Fig. 2B and C) shows that HLP enhanced the cell migration, but not proliferation, in microglia.

Effect of HLP on regulation of cytokine mRNA in microglia

Another feature of the activated microglia is the production of many kinds of cytokines and neurotrophic factors. These mediate either the degenerative or the regenerative functions of microglia in the CNS. To investigate whether HLP changes the expression level of cytokines and neurotrophic factors in microglia, the level of their mRNA was measured (Table 1). Microglia were cultured in the presence and absence of 10 ng/mL HLP for 24 h, and their total RNAs were isolated and subjected to quantitative real-time RT-PCR analysis. The results showed that HLP-treatment decreased HGF and HLP mRNA levels, whereas the treatment did not alter the levels of other neurotrophic factors such as BDNF and CNTF. Similarly, the *Ron* mRNA level did not change with HLP-treatment. On the other hand, the mRNA of proinflammatory cytokines IL-1α, IL-1β, IL-6, TNFα and GM-CSF were markedly upregulated in the presence of HLP. Compared with their controls (HLP-untreated), IL-6 and GM-CSF increased more than 200-fold, and TNFα achieved more than 2.5-fold under the HLP-treatment.

iNOS was upregulated almost 150-fold by HLP-treatment in microglia (Table 1), It is noteworthy that iNOS was suppressed 0.7-fold by HLP in the mouse macrophage cell line, J774A.1(data not

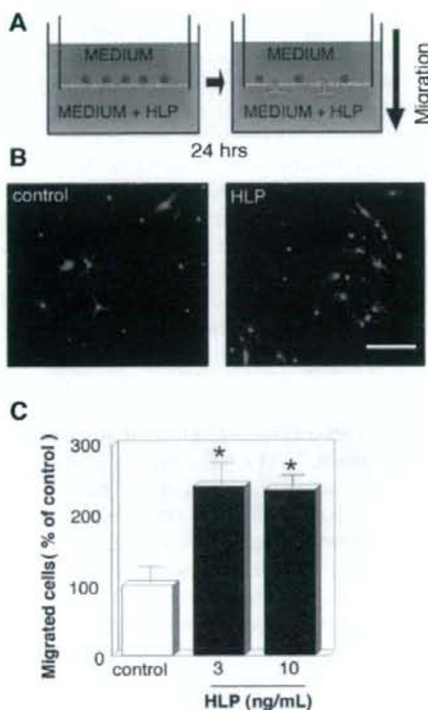

Fig. 2 HLP promotes microglial migration. (A) Schematic representation of the transwell chamber used for migration assays of cultured microglia. Scale bars, 100 µm. (B) Calcein-AM staining of migrated microglia. Microglia translocated to the bottom side of the transwell chamber membrane was stained with Calcein-AM (Green). Left panel shows the effects of the culture for 24 h in the absence of HLP, and right panel shows the effects for 24 h in the presence of 10 ng/mL HLP. (C) Quantification of microglia migrated to the bottom side of the membrane. The number of cells in the absence of HLP was defined as 100%. In HLP-treated wells (3, 10 ng/mL), 2- to 3-fold increase of migrated microglia, compared with controls, were observed ($P < 0.05$). Experiments were done in triplicates and results are shown as mean ± S.E.

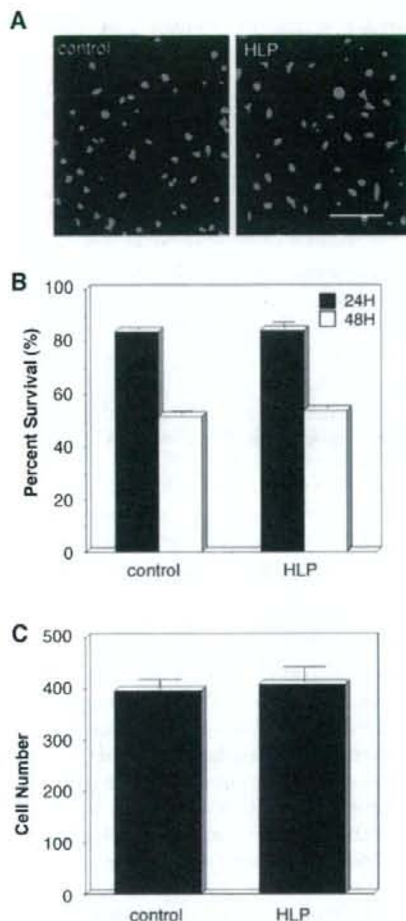

Fig. 3 HLP does not affect microglial survival and proliferation. (A) Double staining of microglia using Calcein-AM and PI. Microglia were seeded at 5×10^4 cells/well and cultured in mN3 medium for 24 and 48 h in both the presence and the absence of 10 ng/mL HLP. Green shows Calcein-AM-stained living cells and red shows PI-stained dead cells. Scale bars, 100 µm. (B) Quantification of percent survival of microglia. Survival was defined as living cells/total cells. (C) Quantification of proliferation of microglia. Proliferation was defined as total cells (both living and dead cells) cultured for 48 h. Experiments were done in triplicate and results are shown as mean ± S.E. The effect of HLP on survival and proliferation under this condition was not significant.

shown). Furthermore, the iNOS level decreased 1/7-fold by HLP-stimulation when the macrophage was under the treatment with LPS (data not shown). These mean that HLP shows diverse effects in iNOS production between macrophage cell line and microglia.

These results predict that HLP enhances inflammatory process in microglia.

DISCUSSION

In the nervous system, neuronal survival and/or neurite extension-promoting activities of HLP for sensory, sympathetic and motor neurons were revealed (5, 6, 24, 26). While the in vivo expression of Ron in microglia of multiple sclerosis patients and mouse model was reported previously (29), any function of

Table 1 *HLP modulates cytokine mRNA in microglia*

Gene	Induction ratio
IL-1α	6.4 ± 0.5
IL-1β	80.1 ± 4.5
IL-6	246.7 ± 14.6
TNFα	2.8 ± 0.6
GM-CSF	221.5 ± 20.5
iNOS	156.5 ± 10.6
BDNF	1.14 ± 0.016
CNTF	1.07 ± 0.04
HGF	0.22 ± 0.003
HLP	0.66 ± 0.2
Ron	1.01 ± 0.014

The amounts of mRNA in control were defined as 1.0, and the levels of indicated mRNA in microglia treated with HLP were calculated in relative value. Experiments were done in triplicates and results are shown as mean ± S.E.

HLP on microglia had not been defined. This study found the expression of the Ron/HLP receptor in murine primary microglia, which led us to reveal that HLP has the potential to stimulate microglial migration and increase mRNA levels of inflammatory cytokines, including IL-6 and GM-CSF, and inflammatory enzyme, iNOS in the cells. These findings are reminiscent of the involvement of HLP in activation of microglia in the brain.

In response to various neurodegenerative diseases and injuries, microglia proliferate and are recruited to the affected region, where they undergo morphological, immunophenotypical and functional changes (13, 14, 19). During these activation processes in microglia, expression of immunomodulatory cytokines and release of an inflammatory mediator, nitric oxide, are enhanced, and then microglia acquire phagocytotic properties. Similarly, the results from the present study show that HLP stimulates production of inflammatory cytokines and iNOS in primary microglia (Table 1). The in vivo states of microglia just before becoming phagocytotic may be formed with HLP.

The present study shows that HLP was able to promote microglial migration (Fig. 2). It took 18–24 h after HLP-stimulation to observe enhanced migration of microglia, which is much longer than the time required for migration enhanced by adenosine phosphate derivatives, such as ATP or ADP (A study conducted in a similar manner as the present one concluded that ATP and ADP induce microglial migration within 90 min) (11). Similarly, actin reorganization underlying the cell migration could not be observed within 30 min after HLP-stimulation in our experiments (data not shown), whereas the actin reorganization and the subsequent cell membrane ruffling were observed within 5 min following ATP and ADP-stimulation (11). It is possible that HLP-dependent protein synthesis or the secretion of some motogen following HLP-stimulation is required to induce microglial migration by HLP. More importantly, HLP and adenosine derivatives may share the roles in distinct physiological events that enhance microglial migration.

mRNA for IL-1α, IL-1β, IL-6, TNFα, GM-CSF and iNOS were upregulated by HLP-treatment in microglia (Table 1), although mRNA levels of BDNF, CNTF, HGF, and HLP were unchanged or changed little. IL-1α, IL-1β, IL-6, and TNFα are all proinflammatory cytokines induced at an early phase of inflammation. GM-CSF controls granulocyte and macrophage population and has the potential to activate microglia (2, 23). iNOS is an intracellular mediator of inflammation, catalyzing nitric oxide synthesis. These findings suggest that HLP enhances the early phase of inflammation in the brain through the secretion of these factors from microglia. In contrast to the function of HLP on the peripheral macrophages that suppresses inflammatory response of the cells (3, 16, 30), these data suggest that HLP has an ability to enhance inflammatory actions of microglia in the nervous system.

Alternatively, a part of cytokines upregulated by HLP elicit neuroprotective and neurotrophic functions. The microglial conditioned medium could support the survival of mesencephalic neurons isolated from the rat embryo (17). Activated microglia could protect neuronal cell line from hydrogen peroxide toxicity by release of IL-1α and IL-6 (1). Furthermore, among the cytokines upregulated by HLP, GM-CSF exhibits neurotrophic activity against NGF-dependent sympathetic neurons (9), and costimulation by TNFα and IL-1β enhances neurotrophic factor production in astrocytes (28). Therefore HLP may play neuroprotective roles through the upregulation of these cytokines from microglia.

Our present studies predict that HLP is one of the key players during neural degeneration and regeneration through the activation of cytokine production and cell migration in microglia. The mechanisms for in vivo activation of microglia are still elusive, however, the results of the present study propose novel functions of HLP in CNS and demonstrate the possibility that HLP regulates degeneration and regeneration of CNS neurons not only by direct association with neuronal cells but also in an orchestrated manner with the activation of microglia.

Further studies in this regard are necessary to shed light on the distinct biological significance of HLP in protective and degenerative processes in the CNS.

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Hepatocyte growth factor improves synaptic localization of the NMDA receptor and intracellular signaling after excitotoxic injury in cultured hippocampal neurons

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Abstract

To examine the effects of HGF on synaptic densities under excitotoxic conditions, we investigated changes in the number of puncta detected by double immunostaining with NMDA receptor subunits and presynaptic markers in cultured hippocampal neurons. Exposure of hippocampal neurons to excitotoxic NMDA (100 μM) decreased the synaptic localization of NMDA receptor subunit NR2B, whereas synaptic NR1 and NR2A clusters were not altered. Colocalization of PSD-95, a scaffolding protein of the receptor, with the presynaptic protein synapsin I was also decreased after excitotoxicity. Treatment with HGF attenuated these decreases in number. The decrease in the levels of surface NR2B subunits following the addition of the excitotoxic NMDA was also attenuated by the HGF treatment. The decrease in CREB phosphorylation in response to depolarization-evoked NMDA receptor activation was prevented by the HGF treatment. These results suggest that HGF not only prevented neuronal cell death but also attenuated the decrease in synaptic localization of NMDA receptor subunits and prevented intracellular signaling through the NMDA receptor.
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Keywords: Hepatocyte growth factor; Neuronal injury; *N*-methyl-D-aspartate receptor; PSD-95

Introduction

The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of ionotropic glutamate receptors, is highly permeable to Ca^{2+} and Na^{+} (Dale and Roberts, 1985) and plays a pivotal role in the regulation of neuronal development, learning and memory, and neurodegenerative diseases (Dingledine et al., 1999). NMDA receptors are heteromeric complexes of NR1 and 4 types of NR2 (NR2A-2D), or NR3 subunits (Das et al., 1998; Ishii et al., 1993; Monyer et al., 1992; Moriyoshi et al., 1991; Nakanishi, 1992; Nishi et al., 2001). Whereas NR1 is the principal subunit for the channel activity of the NMDA receptor, the NR2 subunits serve to modulate the properties of these heteromeric receptors (Hollmann and Heinemann, 1994).

The postsynaptic density (PSD), which underlies the postsynaptic membrane at excitatory synapses, has been implicated in the linkage of receptors to signaling proteins and to the cytoskeleton (Kennedy, 1997; Kim and Sheng, 2004; Klauck and Scott, 1995; Ziff, 1997). PSD-95, a major protein component of the PSD, interacts with NMDA receptor subunits NR2A and NR2B by binding between PDZ (postsynaptic density-95, PSD-95/Discs large, Dlg/zona occludens-1, ZO-1) domains of PSD-95 and the C-terminal PDZ-binding motif of the receptor proteins (Cho et al., 1992; Kim and Sheng, 2004). PSD-95 also binds to signaling proteins such as neuronal nitric oxide synthase (Brenman et al., 1996) and synaptic Ras-GTPase activating protein p135synGAP (Chen et al., 1998; Kim et al., 1998) and organizes these intracellular signaling complexes. Therefore, it has been implied that PSD-95 links the NMDA receptor to intracellular

Abbreviations: CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; CREB, cAMP-response-element-binding protein; HGF, hepatocyte growth factor; NMDA, *N*-methyl-D-aspartate; PI, propidium iodide; PSD, postsynaptic density.

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signaling pathways at the synapse and plays an important role in synaptic plasticity and learning. With regard to this, in mice carrying a targeted mutation in their PSD-95 gene, NMDA receptor-mediated synaptic plasticity was altered and spatial learning in a water maze was impaired (Migaud et al., 1998).

In contrast to the crucial roles of the NMDA receptor in physiological activities such as learning and memory, an excessive activation of the receptor has been associated with diverse neurological and neurodegenerative disorders, including cerebral ischemia, epilepsy, Parkinson's disease, Alzheimer's disease, Huntington's chorea, and amyotrophic lateral sclerosis (Dingledine et al., 1999). Therefore, it has become an important objective to investigate strategies to protect cells from NMDA receptor-mediated excitotoxicity. The hepatocyte growth factor (HGF), which was found to be a potent mitogen for hepatocytes (Nakamura et al., 1984, 1989), acts as an organotrophic factor for regeneration and has a protective effect in various organs (Balkovetz and Lipschutz, 1999; Matsumoto and Nakamura, 1996; Matsumoto and Nakamura, 2001; Zarnegar and Michalopoulos, 1995). In addition, HGF is known to evoke diverse cellular responses, including motogenic, morphogenic, angiogenic, and anti-apoptotic activities in various types of cells (Matsumoto and Nakamura, 1996; Nakamura et al., 1989; Thompson et al., 2004; Zarnegar and Michalopoulos, 1995). In the central nervous system, HGF and its c-Met receptor were found to function in a variety of ways (Achim et al., 1997; Honda et al., 1995; Sun et al., 2002a,b), including protection of tyrosine hydroxylase-positive midbrain neurons, as well as hippocampal and cortical neurons, against aging-related cell death in culture (Hamanoue et al., 1996; Honda et al., 1995; Machide et al., 1998). We recently demonstrated that HGF prevented *in vivo* ischemic brain injuries (Date et al., 2004; Niimura et al., 2006). Furthermore, HGF improved learning and memory dysfunction of ischemic rats in our previous study (Date et al., 2004). Although HGF exerts protective effects on cultured hippocampal neurons under pathophysiological conditions (Ishihara et al., 2005), it is still not clear whether HGF affects synaptic function of neurons under such conditions. In the present study, to achieve further insight into the reason for the potency of HGF treatment, we examined the effect of HGF on synaptic clustering of NMDA receptor subunits and PSD-95 in hippocampal neurons after excitotoxic injury. To assess the biochemical response to excitatory input, we furthermore evaluated the phosphorylation of cAMP-response-element-binding protein (CREB) mediated by the NMDA receptor. The results obtained show that HGF not only prevented the decrease in the number of synaptic NMDA receptor subunits and PSD-95 but also attenuated the decrease in the surface expression of NDMA receptor subunits. Furthermore, HGF improved phosphorylation of CREB in response to depolarization-evoked activation of the NMDA receptor.

Experimental procedures

Primary hippocampal cell cultures

Primary hippocampal cell cultures were prepared from fetal rats at gestational day 18 as described previously (Huettner and Baughman, 1986), with slight modifications (Ishihara et al., 2005). Brains were dissected out and the pooled hippocampi were dissociated by incubation at 37 °C for 30 min in Hank's balanced salt solution containing 15 U/ml papain, 210 U/ml deoxyribonuclease I, 1 mM L-cysteine, and 0.5 mM EDTA. The dispersed cells were resuspended in Dulbecco's Modified Eagle's Medium containing 10% horse serum, and plated at a density of 40,000 cells/cm^2 on 12-well plates or in 35-mm dishes coated with poly-L-lysine. At 24 h after plating, the medium was replaced with serum-free Neurobasal medium containing 2% B27 supplement (Gibco-BRL, Rockville, MD, USA) and 0.5 mM glutamine. To inhibit proliferation of non-neuronal cells, we added cytosine arabinoside (1 μM) to each plate or dish. At 3 and 10 days *in vitro* (DIV), one-half of the medium was replaced with fresh Neurobasal medium having the 2% B27 supplement and 0.5 mM glutamine. Cultures were maintained at 37 °C in a 5% CO_2 incubator and used for experiments at 15–18 DIV.

For the experiment of high K^+-induced CREB phosphorylation, 10 μM 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX), 10 μM nifedipine, and 0.3 μM TTX were added 30 min before treatment with 57 mM KCl to inhibit AMPA receptors, L-type calcium channels, and spontaneous synaptic activity, respectively. Hippocampal cells were depolarized for 3 min with 10 mM HEPES buffer, pH 7.4, containing 60 mM KCl, 67 mM NaCl, 2 mM $CaCl_2$–$2H_2O$, 10 mM D-glucose, 10 μM glycine, 10 μM CNQX, 10 μM nifedipine, and 0.3 μM TTX. To inhibit the NMDA receptor, we added 10 μM MK-801 to cultures in some experiments.

Recombinant HGF

Human recombinant HGF was purified from conditioned medium of Chinese hamster ovary cells transfected with an expression vector containing human HGF cDNA as described earlier (Nakamura et al., 1989). The purity of hrHGF was >98%, as determined by SDS-PAGE.

Cell viability assay

Hippocampal cells were washed twice with 10 mM HEPES buffer, pH 7.4, containing 144 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, and 10 mM D-glucose and were then incubated for 15 min at 37 °C in a 5% CO_2 incubator with 100 μM NMDA in 10 mM HEPES buffer, pH 7.4, containing 144 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 10 mM D-glucose, and 10 μM glycine. The hippocampal cells were then washed and maintained in Neurobasal medium containing 2% B27 supplement and 0.5 mM glutamine. After 1, 6 or 24 h of incubation, the cells were incubated with 2 μg/ml propidium iodide (PI) for 20 min. After having been washed with phosphate-buffered saline, cells were fixed in 4% paraformaldehyde to determine the total number of neurons by immunostaining with anti-microtubule-associated protein 2 (MAP-2) antibody. Fluorescent images of cells were captured by a CCD camera (DP50, Olympus, Tokyo, Japan) mounted on an Olympus BX52 microscope equipped with a mercury arc lamp. The number of PI- or MAP-2-positive cells was counted in 10 randomly

chosen areas (245×320 μm) of each well. Results were obtained from 10 frames in 4 wells in 4 independent experiments. HGF was added at the concentration of 30 ng/ml 1 h before the addition of NMDA. The microscopic observations were performed by a person unaware of the study group.

Immunohistochemistry

After having been fixed with ice-cold methanol and blocked, the cells were incubated with the primary antibody, and then with the secondary antibody in blocking solution. They were then incubated with another primary antibody and subsequently with the corresponding secondary antibody. For double immunostaining of mouse anti-NR1 or mouse anti-NR2B antibody with rabbit anti-synapsin antibody, the cells were incubated overnight at 4 °C with mouse anti-NR1 or anti-NR2B antibody (BD Biosciences) and then with biotinylated anti-mouse IgG (Vector Laboratories) for 2 h and streptavidin FITC for 1 h. The cells were incubated with rabbit anti-synapsin I antibody, another primary antibody, for 1 h at 37 °C and subsequently with Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) for 1 h at 37 °C. For double immunostaining of mouse anti-PSD-95 antibody with anti-synapsin antibody, the cells were incubated with mouse anti-PSD-95 antibody for 1 h at 37 °C and then with FITC-conjugated goat anti-mouse IgG (ICN Pharmaceutical, Inc.). The cells were incubated with rabbit anti-synapsin I antibody, another primary antibody, for 1 h at 37 °C and subsequently with Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) for 1 h at 37 °C. Since we could not obtain commercially available mouse anti-NR2A antibody for double immunostaining with rabbit anti-synapsin I antibody, we decided to use rabbit anti-NR2A antibody. In this regard, we used mouse anti-synaptotagmin antibody as a presynaptic marker, since attempts to examine immunostaining of synapsin I using commercially available mouse anti-synapsin I antibody were not successful under conditions in the present study. To provide evidence about the similarity of the two presynaptic markers synapsin and synaptotagmin, we examined double immunostaining of synapsin with synaptotagmin. The cells were incubated with rabbit anti-synapsin I antibody and subsequently with Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch). The cells were incubated with mouse anti-synaptotagmin antibody, another antibody, for 1 h at 37 °C and subsequently with FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch). For double immunostaining of NR2A with synaptotagmin, the cells were incubated overnight at 25 °C with rabbit anti-NR2A antibody and then with biotinylated anti-rabbit IgG (Vector Laboratories) for 2 h and streptavidin FITC for 1 h at 37 °C. The cells were incubated with mouse anti-synaptotagmin antibody, another antibody, for 1 h at 37 °C and subsequently with Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch) for 1 h at 37 °C. Fluorescent images of cells were captured by a CCD camera (DP50) mounted on an Olympus BX52 microscope equipped with a mercury arc lamp. Images were processed by using Adobe Photoshop (Adobe Systems, Mountain View, CA). To count the number of clusters, we evaluated at least 1 dendrite (50 μm length) in each of 5 randomly selected cells in each of 5 separate cultures. The number of immunolabeled puncta with each antibody and double-immunolabeled puncta on basal dendritic segments were manually counted. The microscopic observations were performed by a person unaware of the study group.

Cell-surface biotinylation assay

Surface biotinylation of hippocampal neurons was performed as described previously (Shen et al., 2000) with minor modifications. Hippocampal neurons in culture were washed 3 times with ice-cold PBS, pH 7.4, containing 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, 1.0 mM $CaCl_2$, and 0.5 mM $MgCl_2$. Surface proteins were then biotinylated with 1.0 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL) for 20 min in PBS at 4 °C. For removal of unreacted sulfo-NHS-SS-biotin, cells were washed 3 times with ice-cold 50 mM Tris/PBS, pH 7.4, containing 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, 1.0 mM $CaCl_2$, and 0.5 mM $MgCl_2$. The cells were then lysed

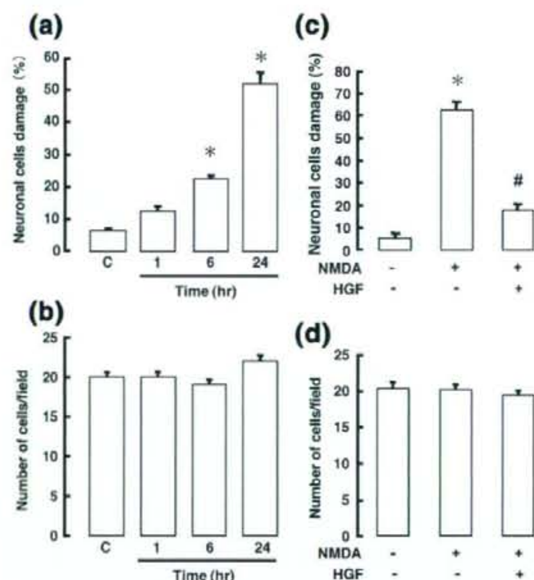


Fig. 1. Time course of changes in NMDA-induced excitotoxic damage to cultured hippocampal cells and the effects of HGF on the damage. (a and b) Hippocampal cells were treated with 100 μM NMDA for 15 min and then incubated in normal conditioned medium without NMDA for 1, 6 or 24 h and thereafter stained with propidium iodide (PI) to detect injured cells and anti-MAP-2 antibody to identify neurons. The number of PI-labeled cells (a) was normalized by total number of MAP-2-positive neurons (b) within the same field (245×320 μm). "C" indicates incubation under normal culture conditions for 24 h without NMDA treatment. (c and d) Cultured hippocampal cells were pretreated or not with 30 ng/ml HGF for 1 h before the addition of 100 μM NMDA. Then, the cells were incubated in normal conditioned medium without NMDA for 24 h. The number of PI-labeled cells (c) was normalized by the total number of MAP-2-positive neurons (d) within the same field (245×320 μm). Results are the means±SEM from 10 fields in 4 wells in 4 independent experiments. *Significant difference from the control group (post hoc Dunnet's *t*-test in a; post hoc Fisher's PLSD in c; $p<0.05$). #Significant difference from the NMDA-treated group (post hoc Fisher's PLSD, $p<0.05$).

with ice-cold lysis buffer (10 mM sodium phosphate, pH 7.4, containing 100 mM NaCl, 0.2% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 μM PMSF, 5 μg/ml antipain, 5 μg/ml aprotinin, 5 μg/ml leupeptin). To isolate biotinylated proteins, we used UltraLink-immobilized neutravidin beads (Pierce). After a 2-h incubation, the beads were washed and bound proteins were eluted with SDS sample buffer.

Western immunoblotting

The hippocampal cells were homogenized in ice-cold 0.32 M sucrose containing 0.2 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of antipain, aprotinin, and leupeptin. Protein concentrations were determined by the method of Lowry et al. (1951). Samples were stored at −80 °C until used and were thawed only once. Proteins (20 μg) were solubilized by heating at 100 °C for 5 min in SDS sample buffer (10% glycerol, 5% β-mercaptoethanol, and 2% SDS, in 62.5 mM Tris–HCl, pH 6.8) and were separated on 8 or 12% polyacrylamide gels. Protein blots were reacted with the appropriate antibodies, and the bound antibody was detected by the enhanced chemiluminescence method (Amersham Biosciences Inc., Piscataway, NJ, USA). Quantification of the immunoreactive bands was performed by using an image analyzer (ATTO Co., Tokyo, Japan). Care was taken to ensure that bands

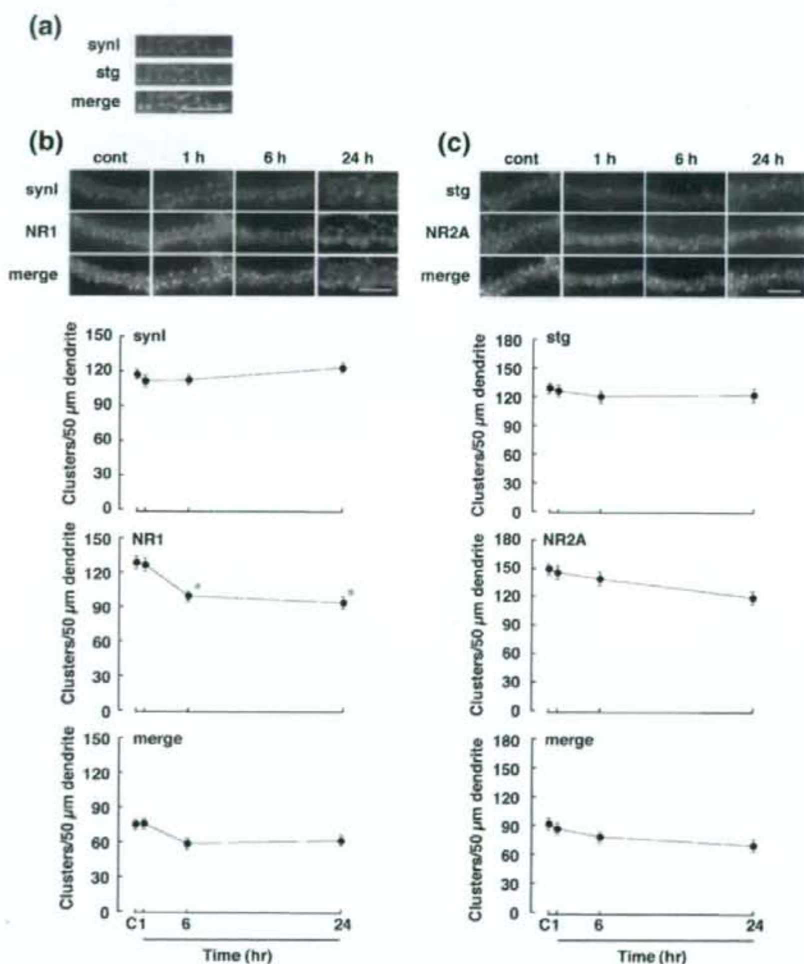


Fig. 2. Time course of changes in the number of clusters including synapsin I and NR1 subunit or synaptotagmin and NR2A subunit after excitotoxicity in cultured hippocampal cells. Hippocampal cells were treated with 100 μM NMDA for 15 min and then incubated in normal conditioned medium without NMDA for 1, 6 or 24 h. (a) Cells were double-immunostained with anti-synapsin I (synI, red) and anti-synaptotagmin (stg, green) antibodies. (b) Hippocampal cells were double-immunostained with anti-synapsin I (synI, red) and anti-NR1 (NR1, green) antibodies. (c) Hippocampal cells were double-immunostained with anti-synaptotagmin (stg, red) and anti-NR2A (NR2A, green) antibodies. Images show magnified regions of dendritic segments. Scale bar, 10 μm. The numbers of clusters positive for synapsin I, NR1, synaptotagmin or NR2A, and those double-positive for synapsin/NR1 or synaptotagmin/NR2A (merged clusters) were counted in a 50-μm dendrite length. Results are expressed as the means ± SEM. *Significant difference from the control group (post hoc Dunnet's t-test, $p<0.05$).

to be semiquantified were in the linear range of response. To remove bound antibodies, we incubated the blots for 30 min at 65 °C in 62.5 mM Tris–HCl buffer, pH 6.8, containing 2% SDS and 0.1 M β-mercaptoethanol. The efficiency of the stripping procedure was confirmed by reacting the stripped blot with secondary antibody alone to ensure that no bound antibodies remained.

Antibodies used for immunoblotting were anti-actin (Sigma-Aldrich), anti-NR1 (BD Biosciences, San Jose, CA), anti-NR2A (Upstate Biotechnology, Inc., Lake Placid, NY), anti-NR2B (BD Biosciences), anti-PSD-95 (Affinity BioReagents), anti-CREB (Cell Signaling Technology), and anti-phospho-CREB (Cell Signaling Technology) antibodies.

Silver staining was performed by using a SilverSNAP Stain Kit II (PIERCE, Rockford, IL). After electrophoresis, the gel slab was fixed in 30% ethanol: 10% acetic acid in water for 30 min. It was washed for 10 min with 10% ethanol in water and then for 10 min with water. The gel was sensitized by incubation for 1 min in the SilverSNAP Sensitizer (SilverSNAP Stain Kit II) and it was washed with water. The gel was incubated in Stain Working Solution (SilverSNAP Stain Kit II) and then developed in Developer Working Solution (SilverSNAP Stain Kit II). When the desired intensity of staining was achieved, the development was terminated with 5% acetic acid.

Statistics

The results were expressed as the means ± SEM. Statistical comparison among multiple groups was evaluated by ANOVA followed by post hoc Dunnet's *t*-test or Fisher's protected least significant difference (PLSD) test. Differences with a probability of 5% or less were considered to be significant ($p < 0.05$).

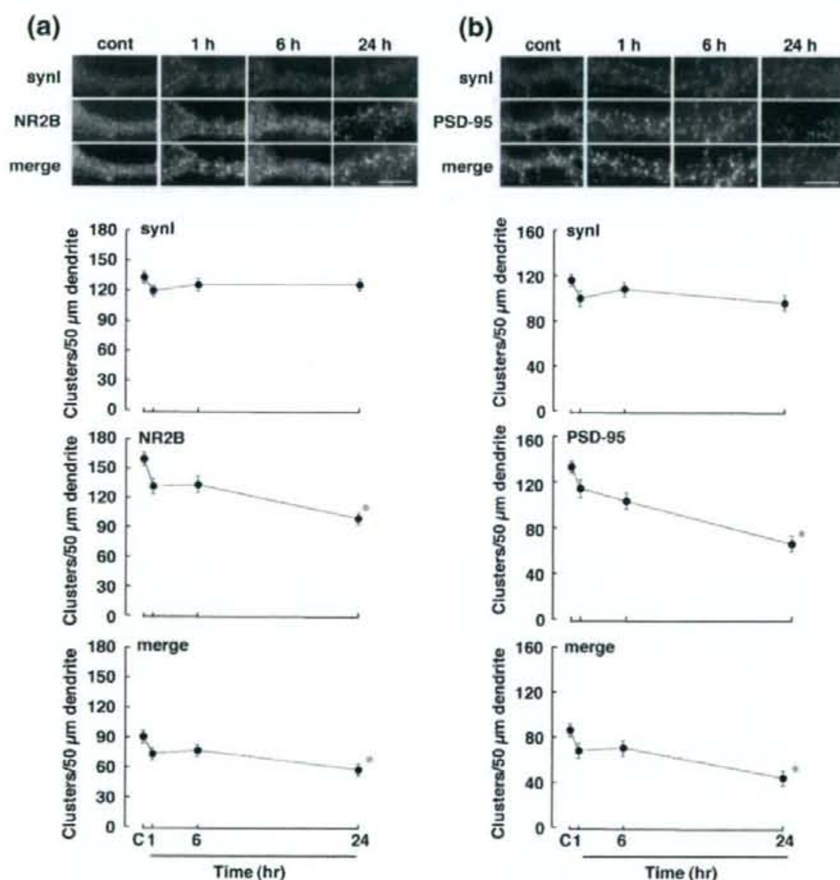
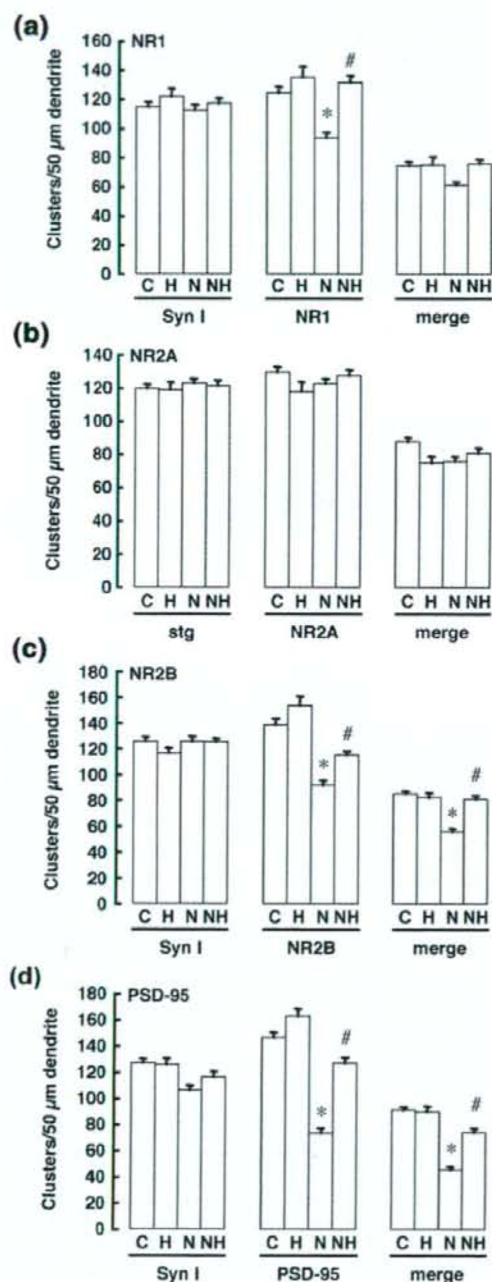


Fig. 3. Time course of changes in the number of clusters including synapsin I and NR2B subunit or synapsin I and PSD-95 after addition of excitotoxic NMDA to cultured hippocampal cells. Hippocampal cells were treated with 100 μM NMDA for 15 min, which treatment was followed by incubation in normal conditioned medium without NMDA for 1, 6 or 24 h. (a) Hippocampal cells were double-immunostained with anti-synapsin I (synI, red) and anti-NR2B (NR2B, green) antibodies. (b) Hippocampal cells were double-immunostained with anti-synapsin I (synI, red) and anti-PSD-95 (PSD-95, green) antibodies. Images show magnified regions of dendritic segments. Scale bar, 10 μm. The numbers of clusters positive for synapsin I, NR2B or PSD-95, and double-positive for synapsin/NR2B or PSD-95 (merged clusters) were counted in a 50-μm dendrite length. Results are expressed as the means ± SEM. *Significant difference from the control group (post hoc Dunnet's *t*-test, $p < 0.05$).

Results

First, we examined the effects of HGF on NMDA-induced neuronal cell damage. The concentrations of NMDA (100 μM) and HGF (30 ng/ml) used in the present study were based on data obtained in our previous study (Ishihara et al., 2005). The number of PI-labeled neurons increased after the application of NMDA without a change in the total number of neurons (Fig. 1a and b). Treatment with HGF attenuated the increase in the number of PI-labeled cells (Fig. 1c) without changing the total number of neurons 24 h after the application of NMDA (Fig. 1d).



Next, the effects of excitotoxicity on clustering and synaptic localization of the NMDA receptor and PSD-95 were examined. Exposure of cultured hippocampal cells to 100 μM NMDA decreased the number of NR1 clusters starting from 6 h after NMDA addition without changing the number of synapsin I, a presynaptic marker protein, -positive puncta (Fig. 2b). Although NR1 clusters were decreased in number, the number of double-positive immunofluorescent puncta containing NR1 subunits and synapsin was not altered (Fig. 2b). We next explored the time course of changes in the number of clusters of NR2 subunits. In the present study, we could not obtain commercially available mouse anti-NR2A antibodies and attempts to examine immunostaining of synapsin I using mouse anti-synapsin I antibody were not successful. Therefore, we decided to examine double immunostaining of NR2A and synaptotagmin, as a presynaptic marker. At first, we identified the localization of different proteins, synapsin I and synaptotagmin. Double immunostaining of synapsin I and synaptotagmin revealed a high degree of cluster colocalization (Fig. 2a). Therefore, the results indicate evidence about the similarity of localization of synapsin I and synaptotagmin. The density of neither NR2A subunits nor synaptotagmin was altered throughout the experiment (Fig. 2c). In contrast, the number of NR2B clusters was decreased at 24 h after the exposure to 100 μM NMDA for 15 min without a change in the number of synapsin clusters (Fig. 3a). The number of NR2B clusters colocalized with synapsin was also decreased at 24 h after the application of NMDA (Fig. 3a). In addition to NMDA receptor subunits, we investigated the number of clusters positive for PSD-95, which is a receptor-anchoring protein. Their number dropped as did the number of double-positive immunofluorescent puncta containing PSD-95 and synapsin at 24 h after the application of NMDA for 15 min (Fig. 3b).

Since the composition of NMDA receptor complex containing PSD-95 at postsynaptic sites would be altered at 24 h after the excitotoxicity, we next examined the effects of HGF on the number of clusters at 24 h after the application of 100 μM NMDA for 15 min. As shown in Fig. 4, treatment with HGF attenuated the decrease in the number of NR1 clusters without changing the number of synapsin clusters or double-positive immunofluorescent puncta (Fig. 4a). Whereas treatment with

Fig. 4. Effects of HGF on the number of clusters after excitotoxicity in cultured hippocampal cells. Cultured hippocampal cells were incubated with (NH) or without (N) 30 ng/ml HGF for 1 h before the addition of 100 μM NMDA. "H" indicates HGF treatment without excitotoxicity. "C" indicates the incubation under normal culture conditions for 24 h without NMDA treatment. Then, the cells were incubated in normal conditioned medium without NMDA for 24 h and double-immunostained with the indicated antibodies. The numbers of clusters containing synapsin I and NR1 (a), synaptotagmin and NR2A (b), synapsin I and NR2B (c) or synapsin I and PSD-95 (d) were counted in a 50-μm dendrite length. Results are expressed as the means ± SEM. *Significant difference from the control group (post hoc Fisher's PLSD, $p<0.05$). #Significant difference from the NMDA-treated group (post hoc Fisher's PLSD, $p<0.05$).

HGF had no effect on the number of NR2A clusters (Fig. 4b), the decrease in the number of NR2B (Fig. 4c) or PSD-95 (Fig. 4d) clusters was attenuated with HGF treatment without a change in the number of synapsin clusters. Treatment with HGF also lessened the decrease in the number of double-positive immunofluorescent puncta containing NR2B and synapsin (Fig. 4c) or PSD-95 and synapsin (Fig. 4d). Treatment with HGF had no effect on the number of clusters under non-excitotoxic conditions (Fig. 4).

We next focused on the surface expression of the NMDA receptor. To assess this, we performed a cell-surface biotinylation assay using cultured hippocampal cells. First, we confirmed that actin was not contained in the biotinylated proteins after the application of NMDA for 15 min (Fig. 5a). The protein composition of biotinylated samples under excitotoxic conditions was comparable to that under normal conditions throughout the experiment (Fig. 5b and c). As shown in Fig. 5d, the surface expression of NR1 subunit was not altered under excitotoxic conditions. Conversely, the amounts of NR2A and NR2B subunits at the cell surface were significantly decreased starting 1 h after the application of NMDA, and the decreased levels were maintained throughout the experiment (Fig. 5e and f).

We next determined the effects of HGF on the surface expression of NMDA receptor subunits. After the application of NMDA for 15 min, the cells were incubated in normal conditioned medium without NMDA for 24 h. Biotinylated proteins

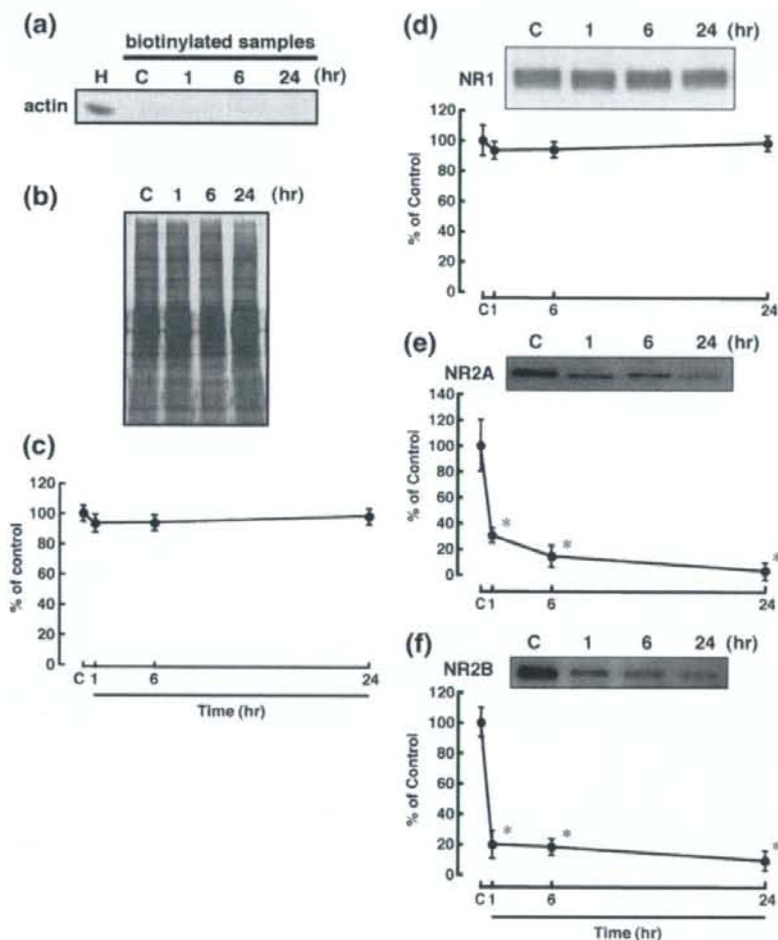


Fig. 5. Time course of changes in the levels of proteins at the cell surface after excitotoxicity in cultured hippocampal cells. Hippocampal cells were treated with NMDA for 15 min and then incubated in normal conditioned medium without NMDA for 1, 6 or 24 h. Thereafter, proteins at the cell surface were labeled with membrane-impermeable sulfo-NHS-SS-biotin, which labeling was followed by precipitation with UltraLink-immobilized neutravidin beads. (a) Biotinylated proteins were probed with anti-actin antibody to confirm that actin was not present in the biotinylated samples. "H" indicates total brain homogenate. (b) Total protein composition of biotinylated samples was determined by silver staining. (c) The gel in silver staining was scanned. Total protein composition was not changed throughout the experiment. Results are expressed as the mean percentages of control ± SEM. (d, e, and f) Biotinylated proteins were immunoblotted with anti-NR1 (d), anti-NR2A (e), and anti-NR2B (f) antibodies. Bands corresponding to NR1, NR2A, and NR2B were scanned. Results are expressed as the mean percentages of control ± SEM. *Significant difference from the control group (post hoc Dunnet's t-test, $p<0.05$).

were not reactive with the antibody against actin regardless of treatment or not with HGF (Fig. 6a). The protein composition of biotinylated samples was unaffected irrespective of HGF treatment (Fig. 6b and c). HGF treatment did not alter the surface expression of NR1 subunit throughout the experiment (Fig. 6d). The decrease in NR2B subunit density at the surface was

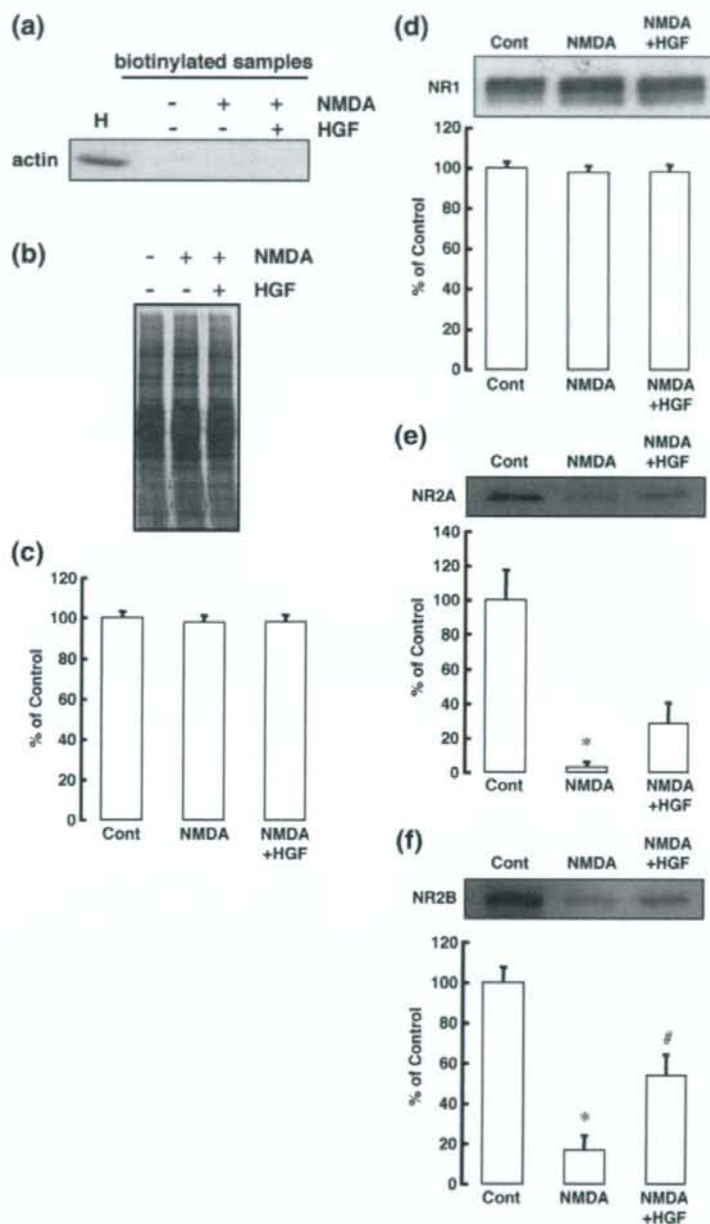


Fig. 6. Effects of HGF on the levels of proteins at the cell surface after excitotoxicity in cultured hippocampal cells. Hippocampal cells were treated with or without HGF for 1 h before the addition of NMDA for 15 min. Then, the cells were incubated in normal conditioned medium without NMDA for 24 h. Proteins at the cell surface were labeled with membrane-impermeable sulfo-NHS-SS-biotin and then precipitated with UltraLink-immobilized neutravidin beads. (a) Biotinylated proteins were probed with anti-actin antibody to confirm that actin was not present in the biotinylated samples. "H" indicates total brain homogenate. (b) Total protein composition of biotinylated samples was determined by silver staining. (c) The gel in silver staining was scanned. Total protein composition was not changed throughout the experiment. Results are expressed as the mean percentages of the control ± SEM. (d, e, and f) Biotinylated proteins were immunoblotted with anti-NR1 (d), anti-NR2A (e), and anti-NR2B (f) antibodies. Bands corresponding to NR1, NR2A, and NR2B were scanned. Results are expressed as the mean percentages of the control ± SEM. *Significant difference from the control group (post hoc Fisher's PLSD, $p<0.05$). #Significant difference from the NMDA-treated group (post hoc Fisher's PLSD, $p<0.05$).

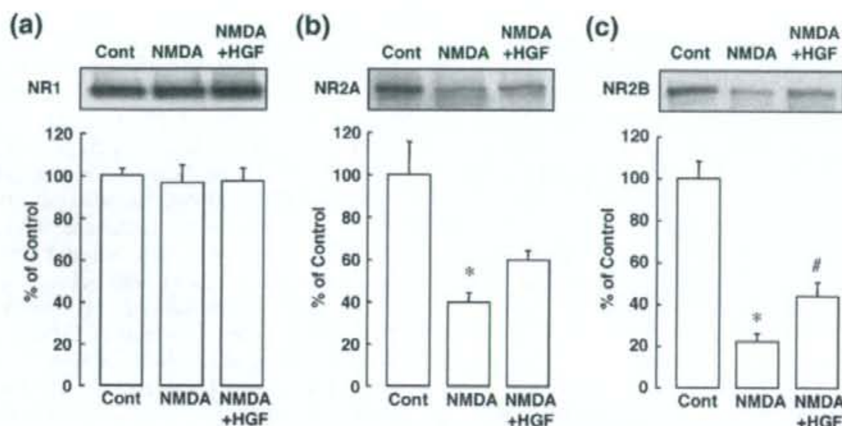


Fig. 7. Effects of HGF on the levels of proteins at the cell surface after excitotoxicity in cultured hippocampal cells. Hippocampal cells were treated with or without HGF for 1 h before the addition of NMDA for 15 min. Then, the cells were incubated in normal conditioned medium without NMDA for 1 h. Proteins at the cell surface were labeled with membrane-impermeable sulfo-NHS-SS-biotin and then precipitated with UltraLink-immobilized neutravidin beads. Biotinylated proteins were immunoblotted with anti-NR1 (a), anti-NR2A (b), and anti-NR2B (c) antibodies. Bands corresponding to NR1, NR2A, and NR2B were scanned. Results are expressed as the mean percentages of the control ± SEM. *Significant difference from the control group (post hoc Fisher's PLSD, $p<0.05$). #Significant difference from the NMDA-treated group (post hoc Fisher's PLSD, $p<0.05$).

inhibited and that in NR2A tended to be attenuated by the HGF treatment (Fig. 6e and f).

We next examined the effects of HGF on the surface expression of NMDA receptor subunits at the early stage. Surface-biotinylated proteins were isolated at 1 h after the application of NMDA. The surface expression of NR1 subunit was not altered regardless of treatment or not with HGF compared with that of control group (Fig. 7a). The decrease in the amounts of NR2B (Fig. 7c) subunits at the cell surface was inhibited and that in NR2A (Fig. 7b) tended to be attenuated by the HGF treatment.

As it is known that stimulation of NMDA receptors of hippocampal neurons induces phosphorylation of CREB, we next examined the effect of HGF on high K^+-stimulated CREB phosphorylation at 24 h after the application of NMDA. Cultured hippocampal cells were pretreated with 10 μM CNQX, 10 μM nifedipine, and 0.3 μM TTX to suppress spontaneous activity. First, we found that basal phosphorylation of CREB was not altered by treatment with 10 μM MK-801 (Fig. 8a). Stimulation with 60 mM KCl induced phosphorylation of CREB, and MK-801 inhibited CREB phosphorylation (Fig. 8b). Under this condition, high K^+-stimulated phosphorylation of CREB was significantly decreased at 24 h after the application of NMDA (Fig. 8c). Treatment with HGF attenuated the decrease in CREB phosphorylation induced by high K^+-stimulation (Fig. 8c).

Discussion

We previously demonstrated that treatment with HGF decreased neuronal death in the *in vivo* ischemic brain (Date et al., 2004; Niimura et al., 2006). Furthermore, in cultured hippocampal neurons, HGF exerted protective effects by acting directly on neurons under the excitotoxic condition (Ishihara et al., 2005). In accordance with this, the protective effects of HGF against neurotoxicity in the present study were comparable to those found in that previous study. Although these effects of HGF may contribute to prevention from learning and memory dysfunction seen after cerebral ischemia (Date et al., 2004), it is still not clear whether synapses under the excitotoxic condition were morphologically and functionally improved by treatment with HGF. So we investigated the effects of HGF on synaptic clustering of the NMDA receptor and PSD-95, and on the biochemical response to excitatory input through the NMDA receptor after the excitotoxic injury.

In control hippocampal cultures, the presynaptic marker proteins synapsin and synaptotagmin were localized at dendritic puncta; and approximately 60% of dendritic clustered NMDA receptor subunits NR1, NR2A, and NR2B were colocalized with these presynaptic marker proteins. Whereas the number of synapsin or synaptotagmin clusters was not altered under the excitotoxic condition, that of NR1 clusters was decreased. Although the limited resolution of fluorescent measurements might preclude identification of smaller individual puncta, the finding that the number of double-positive immunofluorescent puncta containing NR1 subunit and synapsin was not altered suggests that mainly the extrasynaptic NR1 subunits might be decreased in number. The synaptic localization of NR2B was decreased at 24 h after the excitotoxic injury, although the number of synaptic NR2A clusters was not altered throughout the experiment. These results suggest that NR2A subunit would have remained in the NMDA receptor complex at synapses under the excitotoxic condition. Earlier it was shown that cell death mediated by the NMDA receptor was greater in transfected HEK293 cells expressing NR1/NR2A receptors than NR1/NR2B receptors (Anegawa et al., 1995). In addition, the infarct volume after focal ischemia was decreased in mice lacking the NR2A subunit (Morikawa et al., 1998), suggesting that the NR2A subunit plays a key role in neuronal injuries. However,

roles of NR2A subunit in pathological conditions remain unclear and controversial, since the surface expression of NR2A subunits was decreased after excitotoxic injury in the present study. In contrast to NR2A subunit, it is conceivable that NR2B subunits disappeared from synapses. Mutant mice defective in the NR2B subunit showed a failure of neuronal pattern formation and synaptic plasticity (Kutsuwada et al., 1996). Conversely, overexpression of NMDA receptor subunit NR2B in the forebrain of mice resulted in superior ability in learning and memory function in behavioral tasks (Tang et al., 1999). Therefore, the decreased number of NR2B clusters implies the dysfunction of the NMDA receptor. However, a contribution of NR2A subunit to exert physiological function cannot be fully ruled out, since disruption of the NR2A subunit gene reduces the NMDA receptor channel current and results in a deficiency in spatial learning (Sakimura et al., 1995). We also demonstrated that synaptic localization of PSD-95 was decreased at 24 h. In the postsynaptic density, the binding of PSD-95 to NR2 subunits has been implicated in the localization and anchoring of receptors and signaling proteins to the postsynaptic density, and in the regulation of ion-channel function, synaptic activity, and intracellular signaling (Kim and Sheng, 2004; Migaud et al., 1998; Sattler et al., 1999; Yamada et al., 1999). For example, PSD-95-mutant mice showed alteration of synaptic plasticity and failure of spatial learning (Migaud et al., 1998). A marked decrease in the levels of NR2 subunits and PSD-95 would indicate that levels of functional receptors might be decreased. This was consistent with failure of CREB phosphorylation in response to depolarization-evoked NMDA receptor activation in the present study. Therefore, the disturbance of appropriate clustering of NMDA receptors and PSD-95 at synapses may lead to dysfunction of glutamatergic neurotransmission, which ultimately causes impairment of learning and memory function.

In the present study, HGF attenuated the decreased synaptic localization of NR2B and PSD-95. Functional localization of the NMDA receptor at synapses depends on PSD-95 (Barria and Malinow, 2002; Mori et al., 1998; Prybylowski et al., 2002; Steigerwald et al., 2000). Recently, we demonstrated that HGF increased the number of puncta of PSD-95 in young hippocampal neuron (Nakano et al., 2007). These findings suggest that HGF might modulate the synaptic expression of the NMDA receptor by regulating the localization of PSD-95. Interestingly, tyrosine-phosphorylated NMDA receptors induced by tyrosine kinase Src were not truncated by calpain (Rong et al., 2001). PKC, which is one of the upstream components of Src activation (Lu et al., 1999; Macdonald et al., 2005), can be activated by HGF in neurons (Machide et al., 1998). Therefore, the results in the present study suggest the possibility that HGF may prevent the degradation of NMDA receptor subunits through

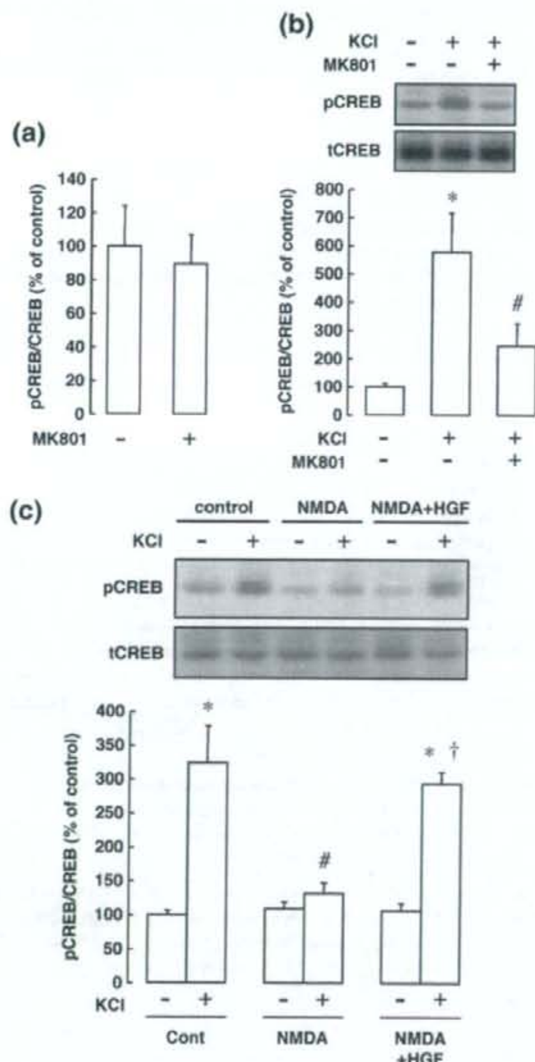


Fig. 8. Effects of HGF on NMDA receptor-mediated CREB phosphorylation after excitotoxicity in cultured hippocampal cells. (a) Cultured hippocampal cells were pretreated with 10 μM CNQX, 10 μM nifedipine, and 0.3 μM TTX for 30 min and then incubated for 3 min with or without 10 μM MK-801. Total cell lysates were probed with anti-phospho CREB (pCREB) and anti-total CREB (tCREB) antibodies. Bands corresponding to pCREB were scanned, and the scanned bands were normalized by tCREB on the same blot. (b) Cultured hippocampal cells were pretreated with 10 μM CNQX, 10 μM nifedipine, and 0.3 μM TTX for 30 min, which pretreatment was followed by stimulation with 60 mM KCl for 3 min with or without 10 μM MK-801. Total cell lysates were probed with anti-phospho CREB (pCREB) and anti-total CREB (tCREB) antibodies. Results are expressed as the mean percentages of control ± SEM. *Significant difference from the untreated group (post hoc Fisher's PLSD, $p<0.05$). #Significant difference from the high-K^+-stimulation group (post hoc Fisher's PLSD, $p<0.05$). (c) Cultured hippocampal cells were pretreated or not with HGF for 1 h before the addition of NMDA for 15 min. Then, the cells were incubated in normal conditioned medium without NMDA for 24 h. Thereafter the cells were incubated with 10 μM CNQX, 10 μM nifedipine, and 0.3 μM TTX for 30 min and subsequently stimulated or not with 60 mM KCl for 3 min. Total cell lysates were probed with anti-phospho CREB (pCREB) and anti-total CREB (tCREB) antibodies. Results are expressed as the mean percentages of the control ± SEM. *Significant difference from the high-K^+-untreated group (post hoc Fisher's PLSD, $p<0.05$). #Significant difference from the high-K^+-stimulated control group (post hoc Fisher's PLSD, $p<0.05$). †Significant difference from the high-K^+-stimulated NMDA-treated group (post hoc Fisher's PLSD, $p<0.05$).

promoting protein phosphorylation. Synaptic structures are not homogenously distributed along a dendrite and are an important determinant of its function. It will be important to determine whether the pathophysiological changes are dependent of the order of dendrites or restricted to apical or basal dendrites.

We further demonstrated that the surface expression of the NMDA receptor subunits NR2A and NR2B, which modulate the properties of heteromeric NR1/NR2 receptors, was decreased by excitotoxicity. In the present study, there was an inconsistency between the number of clusters of NMDA receptor subunits and the surface expression of receptor subunits. A possible reason is suggested to be that the expression of NR2 subunits on cell surfaces is vulnerable to excitotoxic stimulation. On the other hand, the surface expression of NR1 subunit was not altered throughout the experiment, although NR1 clusters were decreased in number. Although immunolabeled puncta on dendritic segments might include intracellular and/or extrasynaptic receptors, our results are consistent with the findings that NR2 subunit is rapidly cleaved after NMDA receptor stimulation with no cleavage of NR1 (Simpkins et al., 2003) and that NR1 immunoreactivity in the cell-surface fraction was not changed after application of glutamate (Wu et al., 2007). We demonstrated that the decreased amounts of NMDA receptor subunits at the cell surface were attenuated by treatment with HGF. This might be due to the protective effect of HGF against neuronal cell death at 24 h after the application of NMDA. In the present study, surface expression of NR2 subunits was significantly decreased at the early period after excitotoxicity. Therefore, we next assessed effects of HGF on the surface expression of NMDA receptor subunits at 1 h after the application of NMDA, when cell death did not occur. We demonstrated that the decrease in surface expression of NR2 subunits tended to be inhibited by HGF treatment. The results suggest that HGF has the ability to attenuate decreases in the NMDA receptor at the cell surface under excitotoxic conditions. The decreases in the surface expression of NR2 subunits may result in a disturbance of intracellular signaling pathways through the NMDA receptor. The prevention of ischemia-induced learning and memory dysfunction by HGF treatment as observed in a previous study (Date et al., 2004) might contribute to the improvement of NMDA receptor localization and intracellular signaling.

The next important issue is whether attenuation of the number of clusters and surface localization of NMDA receptor subunits by HGF treatment is related to an improvement of activity-dependent biochemical response. In this sense, failure of CREB phosphorylation in response to depolarization-evoked NMDA receptor activation was attenuated by HGF treatment. It has been well characterized that CREB is involved in the acquisition of learning and memory (Konradi and Heckers, 2003). Although it is conceivable that CREB phosphorylation in cultured cells is a marker of NMDA receptor signaling and learning and memory function, the results in the present study suggest that HGF improved the ability of intracellular signaling components to phosphorylate CREB via the NMDA receptor.

Whereas the NMDA receptor plays a pivotal role in physiological activities in the central nervous system such as learning and memory function, the excessive activation of the receptor is involved in a variety of neurological and neurodegenerative disorders. Therefore, it is important to explore strategies to protect brain cells from NMDA receptor-mediated injury under pathophysiological conditions. The present findings suggest that treatment with HGF is capable of improving not only synaptic localization of NMDA receptor subunits but also physiological intracellular signaling via the NMDA receptor.

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JPTS FORMAT

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Manuscript type (Original article)

Title: Differing responses of satellite cell activity to exercise training in rat skeletal muscle

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31 **Abstract.** [Purpose] The present study examined satellite cell and hepatocyte growth
32 factor (HGF) responses to exercise intensity in rat soleus muscle. [Methods] HGF levels
33 were assessed during postnatal growth of gastrocneimus muscle. Depression of HGF
34 levels occurred up to postnatal week 4, so 4-week-old rats were used in the exercise
35 training experiment. Rats walk and ran at speeds of 16 or 24 m/min, at -16% grade, 30
36 min/bout. Soleus muscles were removed after 72 h. Animals were injected with the
37 thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) 1 h before sampling. Right soleus
38 muscle was used for immunofluorescence, and left soleus muscle was used to measure
39 HGF protein levels. [Result] HGF levels were unchanged, although numbers of
40 BrdU-positive nuclei increased 2.4-fold in rats running. [Conclusion] The relationship
41 between activation of satellite cells and HGF production after exercise training remains
42 unclear. However, this study indicates the exercise intensity necessary to activate
43 satellite cells. In the future, this result may facilitate the creation of exercise training
44 intensity as an index of satellite cell activity for muscle strength training.

45

46 **Key words:** exercise intensity; satellite cell; HGF

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INTRODUCTION

Muscle strength exercises result in increased muscle fiber size[1], muscle tension and number of myonuclei[2,3]. Skeletal muscle satellite cells play important roles in muscle regeneration and hyperplasia[4]. In adult skeletal muscle, satellite cells are mononuclear myogenic cells[5] that are generally found between the sarcolemma and basement membrane. There, the cells usually remain in a quiescent state[6] and are activated in response to mechanical stimulation[2,7] or muscle injury[8,9], to participate in repair and hypertrophy. Hepatocyte growth factor (HGF) was originally identified as a potent mitogen for hepatocytes[10]. The complete amino-acid sequence of human HGF was described by Nakamura et al.[11] and HGF was later reported as a regenerating factor for the liver, lungs, kidneys, thymus, spleen and skeletal muscle. HGF binds to c-Met, the HGF receptor, on the surface of the satellite cell membrane, causing the cell to enter the G1 phase[12]. HGF is produced from satellite cells by stimulation of mechanical stretch and injury, fulfilling autocrine and paracrine functions[12,13]. However, no studies have examined satellite cell activity and exercise intensity in muscle strength exercises. The present study investigated exercise intensity, satellite cell activity and HGF protein contents to clarify optimum exercise intensity as an index of satellite cell activity.

MATERIALS AND METHODS

Male Sprague-Dawley rats were used in this study. Rats were housed in a temperature-controlled room (20 - 24 °C) with a 12-h light/12-h dark cycle and *ad libitum* access to laboratory chow and water. All procedures for animal care and treatment were performed in accordance with the *Guidelines for the Care and Use of Laboratory Animals* at Kanazawa University, and all protocols had been approved by the Committee on Animal Experimentation at Kanazawa University. To investigate growing changes, 2-, 4-, 8-, 16- and 24-week-old rats were used. Anesthesia was induced using diethyl ether, body weights were measured, and rats were then killed.