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HEPATOCYTE GROWTH FACTOR AS AN ENHANCER OF NMDA CURRENTS AND SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS

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Abstract—Hepatocyte growth factor (HGF) promotes the survival and migration of immature neurons, but its role in the mature brain has remained elusive. In the hippocampus of juvenile rats, we found that the HGF receptor c-Met was expressed in neurons. Furthermore, it was highly Tyr-phosphorylated, more so than in the liver under normal conditions, suggesting that the receptor is activated and that HGF may act continuously in the intact brain. Exogenously applied HGF enhanced synaptic long-term potentiation (LTP) in the CA1 region of hippocampus, but did not affect long-term depression. We further found that HGF augmented *N*-methyl-D-aspartate receptor-mediated currents in both slices and dissociated neurons. This augmentation is likely to underlie the enhancement of LTP. Considering that the expression of both HGF and c-Met are known to be induced by ischemic stimuli, this modulation would provide a novel understanding of a neuronal regulatory systems shared with pathogenic ischemic states.

Key words: hepatocyte growth factor, scatter factor, hippocampus, protein kinase C, *N*-methyl-D-aspartate, long-term potentiation.

Hepatocyte growth factor/scatter factor (HGF) is a 103 kDa heterodimeric protein (Michalopoulos et al., 1984; Nakamura et al., 1984) that is currently recognized as a multifunctional signaling molecule stimulating proliferation, motility and morphogenesis in a variety of cell types (for a review, see Maina and Klein, 1999). C-Met is a single known receptor for HGF and it is encoded by the c-*met* proto-oncogene (Bottaro et al., 1991). It is a tyrosine-kinase type receptor, which is composed of a 50 kDa α-subunit and a glycosylated 145 kDa β-subunit (Gonzattihaces et al., 1988).

Previous studies focused on the roles of HGF in the developing brain, and revealed it to be a neural inducer during embryonic development (Streit et al., 1995), an axonal chemoattractant (Powell et al., 2003), a neurotrophic and neuroprotective survival factor (Ebens et al., 1996; Hamanoue et al., 1996; Honda et al., 1995) and a motogen for newborn interneurons (Powell et al., 2001). In the adult brain, HGF and c-*met* are known to be induced by ischemia, injury and other diseases (Hayashi et al., 1998; Zhang et al., 2000; Honda et al., 1995). However, in the normal state, the functions of HGF/c-Met in the organized neuronal network, such as in neuronal transmission, have not been elucidated.

In situ hybridization (Honda et al., 1995; Jung et al., 1994) and immunohistochemical (Korhonen et al., 2000) studies have indicated that the hippocampus is a prominent site for the expressions of HGF and c-Met in the adult CNS. Here, we confirmed that c-Met protein was localized in hippocampal neurons in adult brain, where it showed a relatively high degree of Tyr-phosphorylation. Using ELISA method, we also found a considerable amount of HGF and/or pro-HGF. These results suggested that HGF/c-Met signaling system that is continuously active in intact neurons throughout life. HGF is known to be processed by proteases, one of which, tissue plasminogen activator, is released in a neuronal activity-dependent manner (Thewke and Seeds, 1999). On this basis, we evaluated the effects of HGF on two forms of activity-dependent synaptic plasticity that are observed in the hippocampus, long-term potentiation (LTP) and long-term depression (LTD), both of which are thought to be important for information processing in the brain (Bliss and Collingridge, 1993). Furthermore, enhancement of *N*-methyl-D-aspartate (NMDA) receptor functions was also investigated and is discussed here as a possible mechanism underlying HGF-induced modulation of synaptic plasticity.

EXPERIMENTAL PROCEDURES

Immunochemical staining

Four- to 5-week-old Wistar rats were used. With extreme care to avoid internalization and degradation of c-Met, rats were anesthetized with ether, and then rapidly perfused with ice-cold phosphate buffer, followed by 4% paraformaldehyde. Cryo-sections (20 μm in thickness) were sequentially treated with 0.1% Triton X-100 for 15 min, 1% goat serum for 1 h, a monoclonal antibody to c-Met (1:100 dilution; B-2: sc-8057; Santa Cruz, CA, USA) at 4 °C for 10–16 h, and finally a FITC-conjugated anti-mouse antibody (1:1000 dilution; GE Healthcare Bio-Sciences, Little Chalfont, UK) in 1% goat serum for 3 h. Cultured hippocampal neurons prepared from embryonic day 18 rats (Yamada et al., 2002) were sequentially treated with ice-cold 4% paraformaldehyde for 30 min, 0.1% Triton X-100 for 15 min and 1% goat serum for 1 h. The neurons were then incubated for 10–16 h with antibodies to microtubule-

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Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; AP5, 2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; fEPSPs, field excitatory postsynaptic potentials; HGF, hepatocyte growth factor; LTD, long-term depression; LTP, long-term potentiation; MAP-2, microtubule-associated protein-2; NMDA, *N*-methyl-D-aspartate; PKC, protein kinase C.

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associated protein-2 (MAP-2; 1:4000 dilution; mouse monoclonal; GE Healthcare Bio-Sciences) and c-Met (1:200 dilution; m-Met, rabbit SP260: sc-162; Santa Cruz), and subsequently incubated in Texas Red-conjugated anti-mouse IgG (1:1000 dilution; Invitrogen, CA, USA) and a FITC-conjugated anti-rabbit antibody (1:1000 dilution; GE Healthcare Bio-Sciences) in 1% goat serum at 4 °C for 3 h. Images were acquired with a laser scanning confocal microscope micro-radiance 2000/AG, MRC-1024 system (Bio-Rad, Cambridge, MA, USA) equipped with a TE300 microscope (Nikon, Tokyo, Japan).

Preparation of membrane fractions and immunoprecipitation

Membrane fractions of the hippocampus and liver were rapidly prepared from 20- to 22-day-old rats. All animal experiments conformed to the Japanese Pharmacological Society guide for the care and use of laboratory animals and guidelines of The University of Tokyo, with care to minimizing the number of animals and their suffering. The tissues were dissected, homogenized in nine volumes of homogenization buffer (0.32 M sucrose, 10 mM Tris–HCl [pH 7.5], 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM Na_3VO_4, 2 mM PMSF) at 1000 r.p.m. for 12 strokes. The homogenates were centrifuged at 1500×*g* for 10 min, and the supernatants were re-centrifuged at 17,500×*g* for 10 min. The resulting pellets were re-suspended in immunoprecipitation buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM PMSF, 2 mM sodium vanadate, 150 mM NaCl, 0.5% Triton X-100) and used as membrane fractions. The vanadate has been reported as an effective inhibitor for protein tyrosine phosphatases in hepatocyte lysate with IC_{50} values of 50 and 20 μM, respectively (Pugazhenthi et al., 1996). The protein concentrations of these fractions were determined by the Bradford method.

For immunoprecipitation, each membrane fraction (8–10 mg protein in 1 ml) was pre-incubated with protein A-Sepharose beads (4 μg; Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 3 h, centrifuged at 800×*g* for 10 min to dissociate protein from the beads and the supernatant was re-centrifuged at 10,000×*g* for 20 min to remove insoluble materials. The supernatant was rotated overnight at 4 °C with a rabbit anti-c-Met antibody (SP260; 3 μg) and protein A-Sepharose beads (4 μg) and was washed three times with immunoprecipitation buffer. The bound proteins were extracted and analyzed by immunoblotting using another antibody for c-Met (B-2). De-glycosylation was performed according to the manufacturer's instructions (Takara Bio, Tokyo, Japan). Briefly, 10 μl (5 U) glycopeptidase F and 2.5 μl 1 M Tris–HCl (pH 8.6) were added directly to the immunoprecipitates and incubated for 21 h at 37 °C.

Immunoblotting

Membrane fractions or immunoprecipitates were boiled for 5 min in sample buffer (10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.005% Bromophenol Blue in Tris–HCl, pH 6.8) and subjected to 5% SDS-PAGE. After electroblotting, the blots were immersed in 5% skimmed milk/PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4) supplemented with 0.1% Tween 20 for 1 h, and then reacted with a monoclonal anti-c-Met antibody (1:250) for 10–16 h at 4 °C. After several washes, the blots were incubated with HRP-conjugated anti-mouse IgG (1:1000 dilution) for 1 h, and then immersed in ECL-plus reagents (GE Healthcare Bio-Sciences) and exposed to films. For labeling with an anti-phosphotyrosine antibody (1:500 dilution; PY20; Transduction Laboratories, Lexington, KY, USA), 5% BSA in TBS-T solution (10 mM Tris–HCl, 100 mM NaCl, 0.1% Tween 20) was used.

For blotting of NR1, cultured hippocampal neurons were solubilized in a solution containing 2% Triton X-100, 25 mM NaF, 100 nM calyculin A, 2 mM Na_3VO_4 and protease inhibitors. The supernatants were collected and probed with an anti-phospho(Ser896/897)-NR1 antibody (1:250 dilution; Upstate Biotechnology Inc., Lake Placid, NY, USA) and an anti-NR1 antibody (1:500 dilution; Chemicon International Inc., Temecula, CA, USA). A solution of 2% BSA was used for blocking. An inhibitor of protein kinase C, calphostin C, was obtained from Sigma-Aldrich. The X-ray films were scanned and the signal intensities were analyzed using the Scion Image software (Scion Corp., http://www.scioncorp.com).

Electrophysiology

Transverse slices of the hippocampus (400 μm) were rapidly prepared from male Wistar rats (3–5 weeks-old), and stored for at least 1 h in a chamber containing artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 26 mM $NaHCO_3$, 10 mM glucose, 5 mM KCl, 1.3 mM $MgSO_4$, 1.24 mM KH_2PO_4 and 2.4 mM $CaCl_2$), which was aerated and circulated with a gas mixture of 95% O_2/5% CO_2 at 35 °C. Bipolar tungsten stimulating electrodes were positioned in the stratum radiatum, and the field excitatory postsynaptic potentials (fEPSPs) from the stratum radiatum were recorded extracellularly. The stimulus intensity was adjusted to obtain a fEPSP slope of about one-half the maximum response. Test stimulations (50 μs duration) were applied at intervals of 30 s. ACSF was continuously perfused, and substituted with ACSF containing drugs for the indicated times. The maximal slopes of the fEPSPs were collected for data analysis.

For recordings of cultured neurons, we used cells prepared from embryonic day 18 embryos after 10–12 days *in vitro*. Glutamate receptors-mediated currents at holding potentials of −55 mV were recorded with a patch pipette filled with the following intracellular solution (140 mM $CsMeSO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES and 4 mM Mg-ATP; adjusted to pH 7.3 with CsOH or HCl). For recording of NMDA-mediated currents, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was added to the perfusion solutions (140 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1.24 mM KH_2PO_4, 10 mM glucose and 10 mM HEPES; adjusted to pH 7.3 with NaOH or HCl with or without 1.3 mM $MgSO_4$; supplemented with 1 μg/ml BSA and 1 μM tetrodotoxin). HGF (recombinant human HGF; a gift from Mitsubishi Pharma Co., Osaka, Japan) was included in the perfusion solution for the indicated duration in Fig. 4B. α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) or NMDA was applied through a thin tube settled near the cells for 3 or 10 s, respectively (Baba et al., 2002). The peaks of the currents were measured; however, the same tendency was observed in the plateau phase of the NMDA currents.

RESULTS

Localization and Tyr-phosphorylation of c-Met in the hippocampus

To confirm the presence of the HGF/c-Met signaling system in the mature hippocampus, the immunohistological localization of c-Met protein in young adult rats was initially studied. Positive signals with an anti-c-Met antibody were evident throughout the hippocampal formation (Fig. 1A). Intense staining was found in the stratum radiatum, stratum pyramidale and stratum oriens, and relatively weak signals were found in the stratum lucidum and alveus hippocampi, which are mainly the axonal pathway (Fig. 1B, C). The staining of the stratum pyramidale was uneven and not all of the somata appeared to be stained. This staining in the somata may reflect an uneven level of c-Met expression among pyramidal cells. These patterns of c-Met ex-

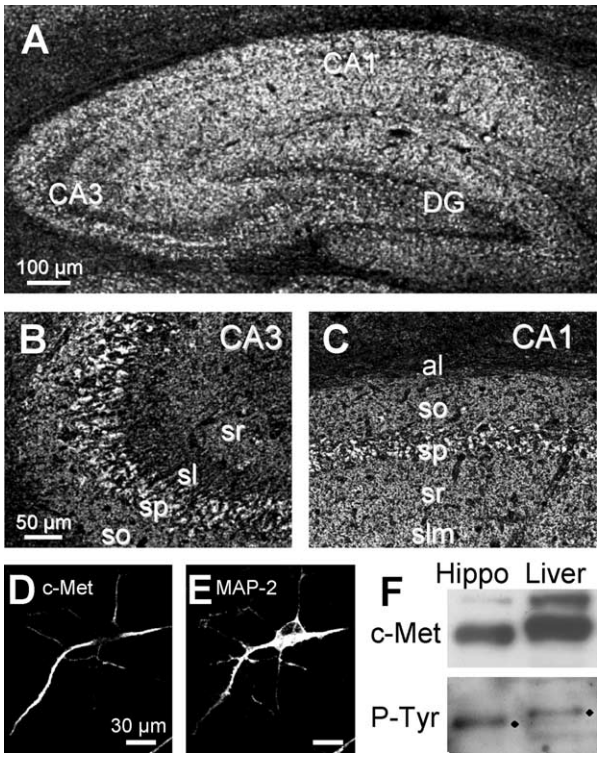

Fig. 1. Localization and phosphorylation of c-Met (the HGF receptor) in the hippocampus. (A–C) Immunoreactivity for c-Met is detected throughout the hippocampus (A), the CA3 area (B) and the CA1 area (C) of a young adult rat (4–5 weeks-old), i.e., the stratum pyramidale (sp), stratum radiatum (sr), stratum lacunosum-moleculare (slm) and stratum oriens (so), except for the stratum lucidum (sl), dentate subgranular zone of dentate gyrus (DG) and alveus hippocampi (al). (D, E) Immunocytochemical localization of c-Met in cultured hippocampal neurons at 10 days *in vitro*. Neurons in the same visual field stained with anti-c-Met (D) or anti-MAP-2 (E), a neuronal dendritic marker, are shown. (F) Equal aliquots of proteins immunoprecipitated by an anti-c-Met antibody from hippocampus (Hippo) and liver (Liver) extracts were detected with anti-c-Met (upper panel) or anti-phosphotyrosine (lower panel) antibodies. The ratio of tyrosine-phosphorylation of c-Met, an index for the activated receptor, is higher in the hippocampus. The same result was obtained in four independent experiments.

pression are mostly consistent with previous reports using a polyclonal antibody (Korhonen et al., 2000) and *in situ* hybridization (Jung et al., 1994). When the localization was further analyzed in dissociated neurons of the hippocampus, positive signals for c-Met were observed in neurites that were also positive for MAP-2, a marker for neuronal dendrites (Fig. 1D, E). These results suggest that c-Met is also expressed in the dendrites of pyramidal cells.

We further characterized c-Met protein expression in the hippocampus by immunoblotting analysis of membrane proteins with an anti-c-Met antibody. A single band was detected, which was 5–10 kDa smaller in molecular weight than the c-Met band in the liver (140–150 kDa). The smaller protein in the hippocampus was found to be c-Met with shorter, or less, N-linked polysaccharides than those found in the liver, since the bands detected by the anti-c-Met antibody in the hippocampus and liver became the same molecular weight after treatment of the immunoprecipitates with endo-*N*-glycosidase (data not shown).

When the immunoprecipitates were detected by immunoblotting with anti-c-Met (Fig. 1F, upper panel) or anti-phosphotyrosine (Fig. 1F, lower panel) antibodies, the ratio of the signal intensity for phosphotyrosine to that for c-Met, an index for the activated receptor, was higher in the hippocampus than in the liver (Fig. 1F) in four independent experiments.

HGF content in the hippocampus

We next measured the HGF content by ELISA using a rat HGF EIA kit (Institute of Immunology, Tokyo, Japan). The HGF, including pro-HGF, in the hippocampus was 5.4 ± 0.6 ng/mg of total protein. The accurate concentration of HGF in the extracellular milieu was hard to estimate from this value, but the total content simply divided by the total volume of the hippocampus was 0.26 ± 0.03 μg/ml, which far exceeds the concentrations that have been used in many experiments. Although the secretion and maturation of HGF should be considered further to elucidate the precise mode of action, our findings confirmed the presence of HGF in the intact hippocampus.

Effects of HGF on neurotransmission and synaptic plasticity

We next investigated the effects of exogenously applied HGF on neurotransmission and synaptic plasticity in Schaffer collateral/commissural fiber-CA1 synapses measured as fEPSP that were evoked in the stratum radiatum. We recorded in CA1 region of the hippocampal slices that were obtained from young adult rats. HGF had no effect on the baseline synaptic transmission (Fig. 2A) or paired-pulse facilitation (Fig. 2B). In contrast, LTP elicited by tetanic stimulation (100 Hz for 1 s) was significantly enhanced in the presence of 10 ng/ml HGF (Fig. 2C). On the other hand, HGF did not affect LTD induced by low-frequency stimulation (1 Hz for 15 min; Fig. 2D). Fig. 2E presents a summary of the effects on the synaptic responses induced after various frequencies of stimulation from 0.33–100 Hz. HGF selectively enhanced the potentiation of synaptic plasticity elicited by the stimulations at 30 Hz and 100 Hz.

Effects of HGF on NMDA currents

Seeking a key to the mechanism underlying the effects, we examined consecutive fEPSPs evoked by a brief repetitive stimulation (five pulses at 100 Hz). Although 10 ng/ml HGF had no effect on the first two or three fEPSPs in the train, it enhanced the subsequent fEPSPs (Fig. 3A). In contrast to the first phase of fEPSP elicited by the train, which was mainly mediated by AMPA-type glutamate receptors, part of late phase of fEPSPs was 2-amino-5-phosphonopentanoic acid (AP5)-sensitive and therefore mediated by NMDA receptors (data not shown). Then, we induced the NMDA responses as fEPSPs in the Mg^{2+}-free solution containing CNQX, a blocker of the AMPA receptors, since Mg^{2+} in a normal

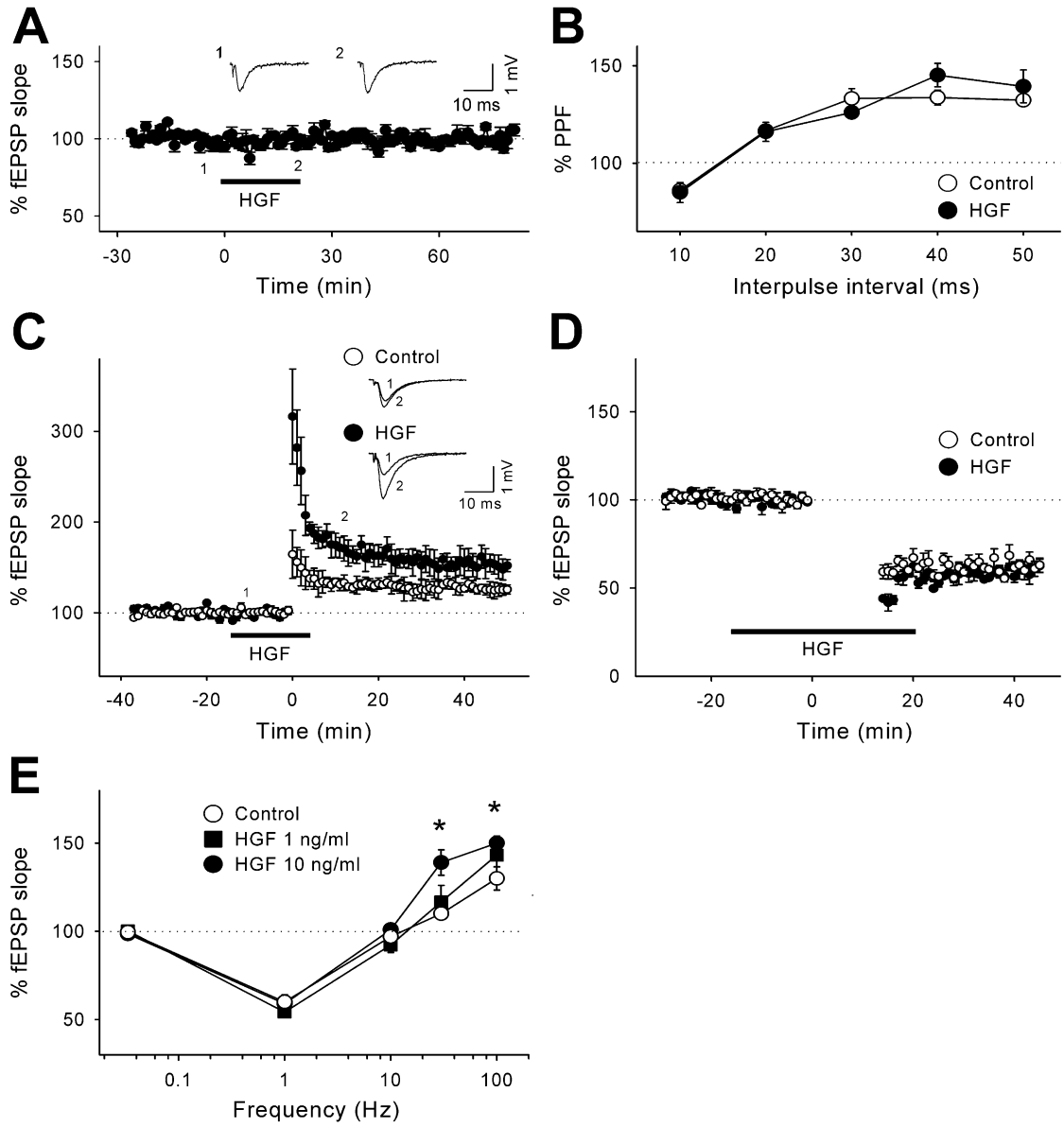

Fig. 2. HGF enhances LTP induction. (A) Bath application of 10 ng/ml of HGF has no effect on basal synaptic transmission. Representative fEPSP recordings at times −5 (1) and 20 (2) are shown in the inset. (B) Paired-pulse facilitation is not significantly affected in the presence of 10 ng/ml of HGF. (C) After perfusing 10 ng/ml of HGF for 15 min, tetanic stimulation at 100 Hz for 1 s was conducted. HGF was also applied for another 5 min after the tetanic stimulation. Representative fEPSP recordings before (1) and 10 min after the tetanus (2) are shown in the inset. (D) Low-frequency stimulation (1 Hz for 15 min) induces LTD and this is not affected by 10 ng/ml of HGF. (E) Summary of the responses in the absence or presence of HGF on the synaptic plasticity after stimulations at different frequencies (0.33 Hz for 60 min, 1 Hz for 15 min, 10 Hz for 90 s, 30 Hz for 30 s and 100 Hz for 1 s). LTP is selectively enhanced in the presence of HGF. * $P<0.05$ vs. Control, Tukey's test. The data represent the mean±S.E.M. of eight to nine slices each.

solution blocks NMDA receptor, as long as neurons are not depolarized. As anticipated, the responses were enhanced by 10 ng/ml HGF (Fig. 3B), indicating the possibility that HGF enhanced LTP by modulating NMDA receptors. When tetanic stimulation (100 Hz for 1 s) was delivered in the presence of 15 μM d-AP5, an antagonist of NMDA receptors, HGF accompanying the stimuli failed to induce LTP (Fig. 3C), suggesting that the enhancement of LTP is not due to the addition of direct potentiation of the AMPA-mediated currents in the long term.

We further investigated the currents induced by direct application of AMPA or NMDA using whole-cell mode patch-clamp recordings on cultured hippocampal neurons, where immunoreactive signals for c-Met were found (Fig. 1D). A brief micro-perfusion of HGF onto neurons enhanced NMDA (50 μM)-induced currents in a concentration-dependent manner (10 ng/ml to 1 μg/ml; Fig. 4B), but did not affect AMPA-induced inward currents recorded in normal Mg^{2+} solution (Fig. 4A). A significant augmenting effect of 100 ng/ml of HGF was observed at various concentrations of NMDA (10 μM to 150 μM; Fig. 4C).

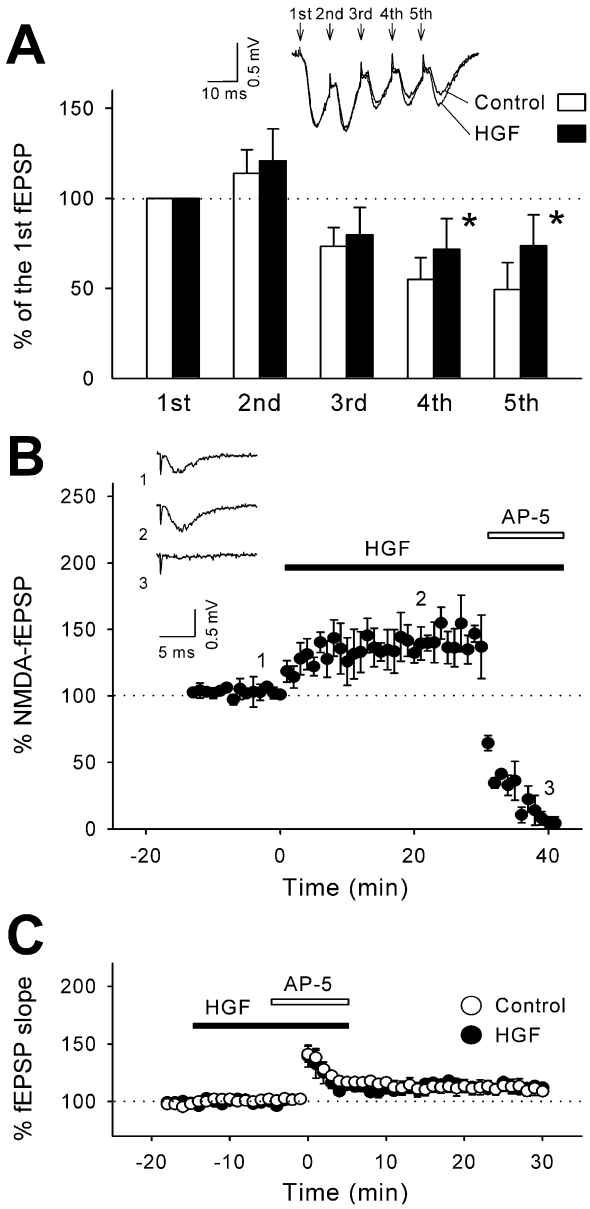

Fig. 3. (A) Effects of HGF on consecutive fEPSPs evoked by brief repetitive stimulations (5 pulses at 100 Hz). Although the first two or three fEPSPs in the train are unchanged 10 min after application of 10 ng/ml HGF, subsequent fEPSPs are significantly augmented. (B) fEPSPs in Schaffer collateral/commissural fiber-CA1 pyramidal cell synapses were recorded in Mg^{2+}-free solution containing 10 μM CNQX to observe NMDA receptor-mediated currents; 10 ng/ml HGF enhances the currents, which are completely abolished by perfusion of 25 μM d-AP5, an antagonist of the NMDA receptor ($n=8$). Representative recordings at −2 (1), 20 (2) and 40 min (3) are shown in the inset. (C) In the presence of 15 μM d-AP5, HGF elicits no difference in the fEPSP after tetanic stimulation (100 Hz for 1 s, at time 0, 109±4%, $n=5$).

Protein kinase C (PKC)-dependent phosphorylation of the NMDA receptor

Since HGF has been shown to activate PKC in neurons (Machide et al., 1998), we tried to detect PKC-dependent phosphorylation of the NMDA receptor. An antibody for the major phosphorylation sites, Ser896/897, of NR1, an essential subtype of the NMDA receptor, was used for immunoblotting, and the level of phosphorylation was found to be increased in neurons treated with HGF (Fig. 5A, B). Pre-treatment with a PKC inhibitor, calphostin C (100 nM), prevented the increase, suggesting that the phosphorylation is PKC-dependent and that is likely to be activated by HGF (Fig. 5B). In contrast, an adenylate cyclase inhibitor, SQ22536 (100 μM), failed to abolish the phosphorylation (123±8.6).

DISCUSSION

Our findings in this study have provided multiple lines of evidence revealing functional presence of HGF/c-Met signaling system in the CA1 region of the hippocampus. We have shown that a high level of HGF is found in the young adult rat hippocampus, and that brain c-Met is more highly Tyr-phosphorylated than liver c-Met. Furthermore, immunohistochemical and immunocytochemical analyses revealed that c-Met was localized predominantly in the dendrites of neurons. In the CA1 region of hippocampal slices, exogenously applied HGF enhanced LTP. In addition, we found that HGF augmented NMDA-induced inward currents in hippocampal slices and neurons. These findings indicate that HGF/c-Met signaling system is functionally important in both the developmental process of the brain and the modulation of synaptic plasticity in the mature CNS.

In the experiment of the endogenous phosphorylation of c-Met, we left the animals on the laboratory bench for at least 1 h, occasionally fed snack foods, before killing. Although further precise experiments on the correlation between the phosphorylation and environmental factors need to be carried out, the level of change in the c-Met phosphorylation might reflect the extent of the experience of animals in the novel environment.

In this paper, we demonstrated that the direct target of the HGF is not the AMPA-type receptors. Since the main mediator of the fEPSP of Shaffer-CA1 synapses in the presence of Mg^{2+} is the AMPA receptors, if HGF directly enhanced the activity of AMPA receptors, the first response shown in inset of Fig. 3A and the slopes of fEPSP shown in Fig. 3C would be affected. In addition to the above, we measured the effect of HGF on AMPA-evoked currents and observed no changes in the response as shown in Fig. 4A.

The AMPA receptors are known to be modulated in the process of LTP formation. It is well-established fact that the enhancement of AMPA-current is the downstream event of the NMDA current induced by high frequency stimulation (Malinow and Malenka, 2002). In fact, we could not entirely explain that the effect on the NMDA receptor is the sole and necessary mechanism for the enhancement of LTP induced by HGF. While multiple mechanisms underlie the modulation of LTP, our work demonstrates that HGF-induced potentiation of NMDA current is one of them.

Our results in Fig. 4C, showing a leftward shift of the dose-response curve of the NMDA-induced currents. We

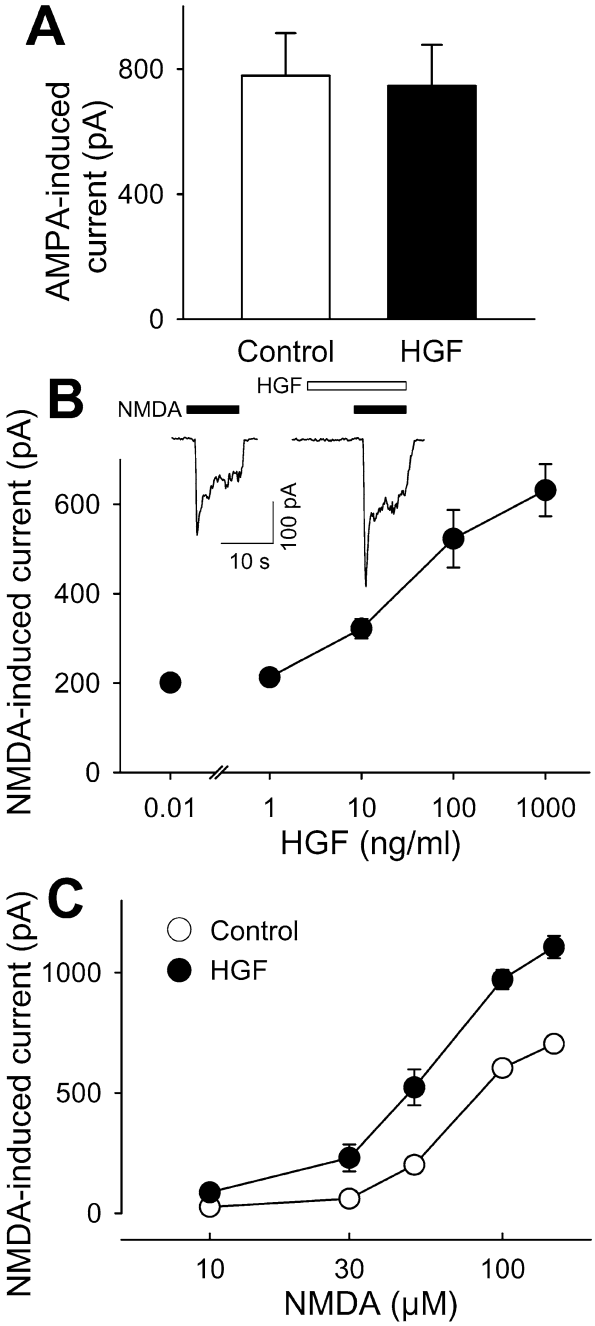

Fig. 4. HGF-induced potentiation of NMDA-induced inward currents in cultured hippocampal neurons. (A) HGF has no effects on AMPA-induced inward currents recorded in normal Mg^{2+} solution. The data represent the means±S.E.M. of four neurons. (B and C) Currents elicited by brief micro-perfusions of NMDA were recorded from cells clamped at −55 mV in Mg^{2+}-free solutions with CNQX and TTX. Representative NMDA (50 μM)-evoked currents in the absence or presence of 100 ng/ml HGF are shown in the inset. The peak amplitudes of the responses were measured as the NMDA-induced currents. (B) HGF augments the currents in a concentration-dependent manner. The data represent the mean±S.E.M. of six to 10 neurons per point. (C) NMDA concentration-response curves in the absence (open circles) or presence (closed circles) of 100 ng/ml HGF. The differences are significant for each concentration of NMDA ($P<0.01$ with HGF vs. without HGF, ANOVA, Tukey's test). The data represent the mean±S.E.M. of 10–12 neurons per point.

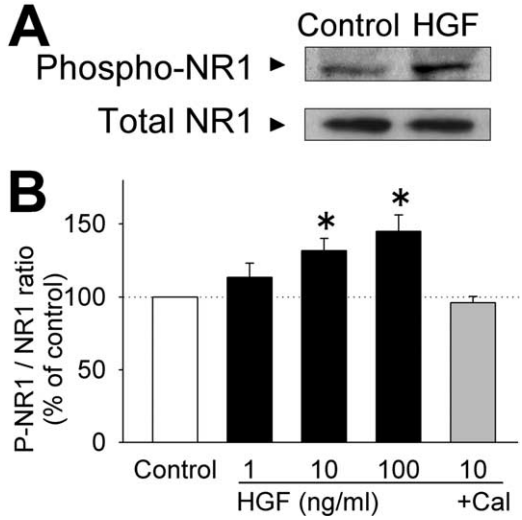

Fig. 5. Effects of HGF on the phosphorylation of the NR1 subunit of the NMDA receptor. Hippocampal neurons were treated with HGF for 10 min. (A) Representative results for immunoblotting of phospho-NR1 (phosphoserine896/897-NR1) and NR1 in the absence (control) or presence of 10 ng/ml HGF. (B) The relative ratios of the phospho-NR1/NR1 immunoreactivities expressed as a percentage of control cultures. HGF increases the phosphorylation of NR1 in a concentration-dependent manner (black columns), and this is blocked by the addition of a PKC-inhibitor, calphostin C (100 nM, gray column). * $P<0.05$ vs. Control, Tukey's test following ANOVA ($n=5$).

do not believe that HGF is capable of direct binding to NMDA receptors. Rather, the quantitative changes in the conductance of NMDA currents, open probability, and/or number of working molecules on the plasma membrane are more probable. Although the sites of NR1 analyzed in Fig. 5 in terms of PKC-phosphorylation do not mediate the activation of the NMDA receptor per se (Zheng et al., 1999; Hisatsune et al., 1997), the PKC-mediated phosphorylation of NR2A or NR2B, another component of the receptor, can enhance the NMDA currents (Mori et al., 1993). This probably occurs via trafficking of the receptor, since PKC-activation has been reported to transfer NR1/NR2A, a type of NMDA receptor complex that might be dominant in synapses, to the cell surface (Lan et al., 2001). Since HGF is known to activate PKC in neurons (Machide et al., 1998), it is possible that HGF mobilizes NMDA receptor molecules and enhances the current via PKC activation.

Here, we propose that HGF-enhanced LTP is mediated, at least in part, by changes in the NMDA receptor system, which is necessary for LTP induction in the CA1 region of the hippocampus. However, it seems inconsistent that HGF failed to affect LTD, which would also require NMDA receptor activation. This may be due to a difference in the subtypes of the NR2 subunit since it has been suggested that NR2A and/or NR2B work for LTP while NR2C and/or NR2D work for LTD (Hrabetova et al., 2000). If the effect of HGF on the NMDA receptor is mediated mainly by PKC, the selectivity for LTP is consistent with the selectivity of the PKC-induced phosphorylation of NR2A and NR2B, and not of NR2C and NR2D (Mori et al., 1993; Liao et al., 2001).

In contrast to HGF, BDNF, which acts on a different but similar tyrosine-kinase type receptor to c-Met, attenuates LTD via PLC (Ikegaya et al., 2002). It may prove interesting to analyze the differences in the downstream molecular profiles of ligands of other tyrosine-kinase receptors that have similar but different effects on plasticity in relation to the stimulus frequency.

Characterizing downstream intracellular effecters of an extracellular signaling molecule is important in order to link the molecule to a higher physiological and pathological phenomenon. In the case of HGF, it has been known to activate PKC in neurons (Machide et al., 1998), and, at the same time, this activation has been demonstrated in ischemia as well as in plasticity. In the ischemic state, there have been many reports showing PKC-mediated phosphorylation and/or activation of the NMDA receptor (Bickler et al., 2004; Cheung et al., 2001; Hammond et al., 1994). It has also been suggested that HGF is expressed and activated under ischemic conditions (Hayashi et al., 1998; Honda et al., 1995; Zhang et al., 2000).

Persistent neural activity that has been observed in some of the working neurons *in vivo* (Egorov et al., 2002) can also cause a local ischemia-like state in firing neurons. Thus, it is possible that the over-activated neurons secrete HGF in the ischemic state, and HGF may then enhance LTP, which would affect subsequent neuronal activity. LTP may therefore be a common mechanism that is shared by normal synaptic plasticity and neuronal responses to ischemia. HGF, which has been shown to be induced in ischemia, might be a key molecule linking normal neuronal function and ischemic responses.

REFERENCES

Baba A, Yamada MK, Nishiyama N, Matsuki N, Ikegaya Y (2002) Different Ca^{2+} dynamics between isolated hippocampal pyramidal cells and dentate granule cells. J. Neurocytol 31:41–48.

Bickler PE, Fahlman CS, Ferriero DM (2004) Hypoxia increases calcium flux through cortical neuron glutamate receptors via protein kinase C. J Neurochem 88:878–884.

Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31–39.

Bottaro DP, Rubin JS, Faletto DL, Chan AML, Kmiecik TE, Vandewoude GF, Aaronson SA (1991) Identification of the hepatocyte growth-factor receptor as the C-Met protooncogene product. Science 251:802–804.

Cheung HH, Teves L, Wallace MC, Gurd JW (2001) Increased phosphorylation of the NR1 subunit of the NMDA receptor following cerebral ischemia. J Neurochem 78:1179–1182.

Ebens A, Brose K, Leonardo ED, Hanson MG, Bladt F, Birchmeier C, Barres BA, Tessier Lavigne M (1996) Hepatocyte growth factor scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. Neuron 17:1157–1172.

Egorov AV, Hamam BN, Fransen E, Hasselmo ME, Alonso AA (2002) Graded persistent activity in entorhinal cortex neurons. Nature 420:173–178.

Gonzattihaces M, Seth A, Park M, Copeland T, Oroszlan S, Vandewoude GF (1988) Characterization of the Tpr-Met oncogene-P65 and the Met protooncogene-P140 protein-tyrosine kinases. Proc Natl Acad Sci USA 85:21–25.

Hamanoue M, Takemoto N, Matsumoto K, Nakamura T, Nakajima K, Kohsaka S (1996) Neurotrophic effect of hepatocyte growth factor on central nervous system neurons in vitro. J Neurosci Res 43:554–564.

Hammond C, Crepel V, Gozlan H, Benari Y (1994) Anoxic LTP Sheds light on the multiple facets of NMDA receptors. Trends Neurosci 17:497–503.

Hayashi T, Abe K, Sakurai M, Itoyama Y (1998) Inductions of hepatocyte growth factor and its activator in rat brain with permanent middle cerebral artery occlusion. Brain Res 799:311–316.

Hisatsune C, Umemori H, Inoue T, Michikawa T, Kohda K, Mikoshiba K, Yamamoto T (1997) Phosphorylation-dependent regulation of *N*-methyl-D-aspartate receptors by calmodulin. J Biol Chem 272:20805–20810.

Honda S, Kagoshima M, Wanaka A, Tohyama M, Matsumoto K, Nakamura T (1995) Localization and functional coupling of HGF and c-Met/HGF receptor in rat brain: implication as neurotrophic factor. Brain Res Mol Brain Res 32:197–210.

Hrabetova S, Serrano P, Blace N, Tse HW, Skifter DA, Jane DE, Monaghan DT, Sacktor TC (2000) Distinct NMDA receptor subpopulations contribute to long-term potentiation and long-term depression induction. J Neurosci 20:RC81–RC85.

Ikegaya Y, Ishizaka Y, Matsuki N (2002) BDNF attenuates hippocampal LTD via activation of phospholipase C: implications for a vertical shift in the frequency-response curve of synaptic plasticity. Eur J Neurosci 16:145–148.

Jung W, Castren E, Odenthal M, Vande Woude GF, Ishii T, Dienes HP, Lindholm D, Schirmacher P (1994) Expression and functional interaction of hepatocyte growth factor-scatter factor and its receptor c-met in mammalian brain. J Cell Biol 126:485–494.

Korhonen L, Sjoholm U, Takei N, Kern MA, Schirmacher P, Castren E, Lindholm D (2000) Expression of c-Met in developing rat hippocampus: evidence for HGF as a neurotrophic factor for calbindin D-expressing neurons. Eur J Neurosci 12:3453–3461.

Lan JY, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, Zheng X, Bennett MVL, Zukin RS (2001) Protein kinase C modulates NMDA receptor trafficking and gating. Nat Neurosci 4:382–390.

Liao GY, Wagner DA, Hsu MH, Leonard JP (2001) Evidence for direct protein kinase-C mediated modulation of *N*-methyl-D-aspartate receptor current. Mol Pharmacol 59:960–964.

Machide M, Kamitori K, Nakamura Y, Kohsaka S (1998) Selective activation of phospholipase C gamma1 and distinct protein kinase C subspecies in intracellular signaling by hepatocyte growth factor/scatter factor in primary cultured rat neocortical cells. J Neurochem 71:592–602.

Maina F, Klein R (1999) Hepatocyte growth factor, a versatile signal for developing neurons. Nat Neurosci 2:213–217.

Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci 25:103–126.

Michalopoulos G, Houck KA, Dolan ML, Luetteke NC (1984) Control of hepatocyte replication by 2 serum factors. Cancer Res 44:4414–4419.

Mori H, Yamakura T, Masaki H, Mishina M (1993) Involvement of the carboxyl-terminal region in modulation by TPA of the NMDA receptor channel. Neuroreport 4:519–522.

Nakamura T, Nawa K, Ichihara A (1984) Partial-purification and characterization of hepatocyte growth-factor from serum of hepatectomized rats. Biochem Biophys Res Comm 122:1450–1459.

Powell EM, Mars WM, Levitt P (2001) Hepatocyte growth factor/scatter factor is a motogen for interneurons migrating from the ventral to dorsal telencephalon. Neuron 30:79–89.

Powell EM, Muhlfriedel S, Bolz J, Levitt P (2003) Differential regulation of thalamic and cortical axonal growth by hepatocyte growth factor/scatter factor. Dev Neurosci 25:197–206.

Pugazhenthi S, Tanha F, Dahl B, Khandelwal RL (1996) Inhibition of a Src homology 2 domain containing protein tyrosine phosphatase by vanadate in the primary culture of hepatocytes. Arch Biochem Biophys 335:273–282.

Streit A, Stern CD, Thery C, Ireland GW, Aparicio S, Sharpe MJ, Gherardi E (1995) A role for HGF/SF in neural induction and its

expression in Hensen's node during gastrulation. Development 121:813–824.

Thewke DP, Seeds NW (1999) The expression of mRNAs for hepatocyte growth factor/scatter factor, its receptor c-met, and one of its activators tissue-type plasminogen activator show a systematic relationship in the developing and adult cerebral cortex and hippocampus. Brain Res 821:356–367.

Yamada MK, Nakanishi K, Ohba S, Nakamura T, Ikegaya Y, Nishiyama N, Matsuki N (2002) Brain-derived neurotrophic factor promotes the maturation of GABAergic mechanisms in cultured hippocampal neurons. J Neurosci 22:7580–7585.

Zhang L, Himi T, Murota S (2000) Induction of hepatocyte growth factor (HGF) in rat microglial cells by prostaglandin E_2. J Neurosci Res 62:389–395.

Zheng X, Zhang L, Wang AP, Bennett MV, Zukin RS (1999) Protein kinase C potentiation of *N*-methyl-D-aspartate receptor activity is not mediated by phosphorylation of *N*-methyl-D-aspartate receptor subunits. Proc Natl Acad Sci USA 96:15262–15267.

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