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Hepatocyte growth factor and c-Met promote dendritic maturation during hippocampal neuron differentiation via the Akt pathway

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Abstract

During central nervous system development, growth factors and their associated receptor protein tyrosine kinases regulate many neuronal functions such as neurite extension and dendrite maturation. Hepatocyte growth factor (HGF) and its receptor, c-Met, can promote formation of neurites and enhance elaboration of dendrites in mature neurons, but their effects on the early stages of dendrite maturation in hippocampal neurons and the signaling pathways by which they promote dendrite formation have not been studied. Exogenous HGF treatment effectively enhanced the phosphorylation and activation of c-Met in cultured hippocampal neurons at 4 days *in vitro*. HGF treatment increased the number of dendrites and promoted dendrite elongation in these neurons. Consistent with these results, HGF activated Akt, which phosphorylates glycogen synthase kinase-3 β (GSK-3 β) to inactivate it, and reduced phosphorylation of microtubule-associated protein 2 (MAP2), which can promote microtubule polymerization and dendrite elongation when dephosphorylated. Conversely, pharmacological inhibition of c-Met with its specific inhibitor, PHA-665752, or genetic knock-down of c-Met with short hairpin RNAs (shRNAs) suppressed HGF-induced phosphorylation of Akt and GSK-3 β , increased phosphorylation of MAP2, and reduced dendrite number and length in cultured hippocampal neurons. Moreover, suppressing c-Met with PHA-665752 or by shRNA decreased MAP2 expression. Inhibiting Akt activity with the phosphoinositide-3-kinase inhibitor LY294002 or Akt inhibitor X suppressed HGF-induced phosphorylation of GSK-3 β , increased MAP2 phosphorylation, and blocked the ability of HGF to enhance dendritic length. These observations indicate that HGF and c-Met can regulate the early stages of dendrite maturation via activation of the Akt/GSK-3 β pathway.

Keywords

HGF; PHA-665752; shRNA; MAP2; GSK-3 β ; neurite

1. Introduction

Growth factors and their associated receptor protein tyrosine kinases can regulate a variety of neuronal functions such as dendrite formation, neurite extension, and migration [1–3]. The importance of hepatocyte growth factor (HGF) and c-Met in development and maintenance of neural circuits is just beginning to be recognized. HGF promotes neurite outgrowth, stimulates

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dendrite growth in mature neurons, and guides axons to targets [4–10]. In addition, c-Met is clustered at excitatory synapses and can stimulate expression of synaptic proteins and promote their clustering at synapses [11].

The downstream signaling pathways by which HGF and c-Met can enhance dendritic development have not been studied. Several downstream signaling proteins activated by growth factor receptors, such as Akt, calcium/calmodulin-dependent protein kinase II (CaMKII), p44/42 mitogen activated protein kinase (MAPK), and glycogen synthase kinase-3 β (GSK-3 β), are crucial regulators of dendritic growth and development [12–16]. Recently Akt has been implicated in signaling downstream of c-Met [17,18]. Among the downstream targets of Akt, GSK-3 β , which is inactivated upon phosphorylation by Akt, can regulate microtubule dynamics by phosphorylating downstream targets, including microtubule-associated protein 2 (MAP2) [19,20]. MAP2 binds to and stabilizes microtubules and thus can regulate microtubule-dependent changes in axons and dendrites [21]. Phosphorylation of MAP2 by GSK-3 β inhibits its association with microtubules and decreases microtubule stability [22,23].

Although the functions of HGF and c-Met in regulating neurite growth and dendritic morphogenesis in neurons suggests crucial roles for these molecules during development, little is known about the intracellular pathways triggered by HGF and c-Met during dendrite maturation in hippocampal neurons. We show that HGF treatment promotes dendrite elongation, induces signaling through the Akt/GSK-3 β pathway, and decreases phosphorylation of MAP2. These effects were reversed by pretreatment with the selective c-Met inhibitor, PHA-665752 [24,25] or by genetic suppression of c-Met expression with use of short hairpin RNAs (shRNAs). Furthermore, inhibitors of Akt blocked the ability of HGF to promote dendrite elongation, but inhibitors of GSK-3 β enhanced this effect. Together, these data indicate that signaling by HGF and c-Met via Akt is important in modulating dendrite maturation during the early stages of hippocampal neuron differentiation.

2. Materials and methods

2.1. Animals

All studies were conducted with a protocol approved by the University of Connecticut Animal Care and Use Committee in compliance with National Institutes of Health guidelines for the care and use of experimental animals.

2.2. Primary dissociated hippocampal neuron cultures

Cultures of dissociated hippocampal neurons from embryonic day 18 rats were prepared as previously described [11,26]. Briefly, hippocampal neurons were dissociated with trypsin and plated on 15 mm diameter coverslips coated with poly-DL-lysine (Sigma) and laminin (ATCC) in Neurobasal medium supplemented with B-27, 25 μ M glutamate and 500 μ M glutamine (Invitrogen) at a density of $\sim 150/\text{mm}^2$ and grown at 37°C in a humidified 5% CO₂/95% O₂ incubator. The cells were fed 5 days after plating and weekly thereafter with plating media without added glutamate.

To test the effects of HGF (R&D Systems) and PHA-665752 (kindly provided by Pfizer), a c-Met inhibitor [27], on c-Met phosphorylation, dissociated hippocampal neuron cultures at 4 days *in vitro* (DIV) were pre-incubated for 2 hr with either 0.0125, 0.05, 0.2, or 1 μ M of PHA-665752 dissolved in dimethyl sulfoxide (DMSO) or an equal volume of DMSO as a control. The cells were then treated for 10 min with 50 ng/ml recombinant human HGF or with an equal volume of carrier buffer (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl, supplemented with 0.1% bovine serum albumin). The cells were then lysed and

processed for immunoblotting as described below. To test the effects of HGF and other inhibitors on dendrite number or length, cells at 3 DIV were treated for 24 hr with carrier buffer or with 50 ng/ml HGF in the presence or absence of 1 μ M PHA-665752, 20 μ M LY294002, 2.5 μ M Akt Inhibitor X (both from EMD Chemicals), or 10 or 20 μ M SB415286 (Tocris), and then fixed and processed for immunocytochemistry. Each inhibitor was added from 1 000X stocks dissolved in DMSO. An equal volume of DMSO was added to control cultures.

2.3. Cell viability assay

Cell viability was tested as previously described [28] with the use of a Cell Counting Kit-8 (Dojindo) according to the manufacturer's instructions. Briefly, dissociated hippocampal neurons at 3 DIV were treated with 0.25, 1, or 5 μ M PHA-665752 or with an equal volume of DMSO for 6 hr or 24 hr, and then incubated with Cell Counting solution for 4 hr at 37°C. Optical density was detected with a plate reader (Bio-Rad) at the wavelength 450 nm.

2.4. Immunocytochemistry

Cells were fixed in -20°C methanol for 20 min and immunostained in pre-block buffer (20 mM phosphate buffer, pH 7.4, 5% normal goat serum, 0.05% Triton X-100, and 450 mM NaCl) at 4°C with the following primary antibodies: rabbit anti-HGF α (American Research Products; 1:100 dilution), mouse anti-c-Met (Upstate; clone DO-24; 1:150 dilution), rabbit anti-phospho-c-Met (pYpYpY1230/1234/1235) (Biosource; 1:150 dilution), or chicken anti-MAP2 (Novus, 1:2 000 dilution), and then with Alexa 488- or 568-conjugated secondary antibodies (Invitrogen) at a 1:1 000 dilution in pre-block buffer for 1 hr at room temperature. Some cells were also incubated with Alexa 555-conjugated phalloidin to stain actin. The cells were mounted as previously described [11], and viewed on a Leica TCS SP2 laser-scanning confocal microscope and scanned with 40X or 100X oil immersion objectives.

To identify the effects of genetic knock-down of c-Met on dendrite morphology, control and shRNA-transfected hippocampal neurons were fixed with 2% paraformaldehyde for 10 min and immunostained with rabbit anti-c-Met (Upstate, 1:100 dilution) and chicken anti-MAP2 antibodies as described above. Images were captured from cells with green EGFP signal.

2.5. shRNA preparation and transfection

19-mer shRNAs specifically corresponding to the 3'-untranslated region (3'-UTR) of the rat c-Met gene and a control shRNA with the same nucleotides in a scrambled sequence were designed, synthesized (Sigma), and annealed into pmU6pro plasmid vector [29] between the BbsI and XbaI sites. Their sense sequences, beginning at the indicated base pairs after the stop codon, were as follows: 305: 5'-CTATCTCAGTGGAGTTCTA-3'; 612: 5'-GCCACAGACAATGCACTTA-3' Scrambled: 5'-GCACACCAGTACTAGCATA-3'. To form a hairpin, each sense sequence was followed by a loop sequence of 5'-ACA-3' and the corresponding antisense sequence. The c-Met shRNAs or empty pmU6pro plasmid as a control were co-transfected with an EGFP expression plasmid (Clontech) at a 5:1 ratio of shRNA or pmU6pro plasmid:EGFP DNA into dissociated hippocampal neurons (plating density: 2.5×10^5 cells/60 mm dish) at 1 DIV with the use of Lipofectamine LTX following the manufacturer's protocol. After a 2 hr transfection reaction, cells were transferred into normal plating media and incubated for another 3 days.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were isolated from transfected neurons with the use of TRI reagent (Sigma) and reverse transcription was done in 20 μ l reactions containing 5 μ g sample RNA, 2.5 U AMV reverse transcriptase, 2.5 μ M oligo(dT)16 primers, 1 U RNase inhibitor, 1 mM dNTP mixtures, 5 mM MgCl₂, and 2 μ l 10X reaction buffer (Invitrogen). Thirty cycles of PCR amplification

was performed using the following primer pairs against rat c-Met or rat GAPDH as control: c-Met, 5'-CTGGGAGCTCATGACGAGAGG-3' and 5'-GCTAATGTTGTCTTGGGATGGC-3'; GAPDH, 5'-GCATCCTGCACCACCAACTGC-3' and 5'-GTAGGCCATGAGGTCCACCACC-3'. The PCR products were visualized with ethidium bromide on 1% agarose gels.

2.7. Immunoblots

Cells treated with HGF or inhibitors as described above were lysed by boiling in lysis buffer [1% SDS, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulphonyl fluoride, and 1X Halt protease inhibitor cocktail (Pierce)], after which detergent-insoluble materials were removed by centrifugation at 12 000 x g for 10 min. Protein concentrations in the soluble fraction were measured using a BCA protein assay reagent kit (Pierce). Equal amounts of protein were then separated by SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes, and probed with primary antibodies against the following proteins: rabbit anti-HGF α or rabbit anti-phospho-c-Met (pYpYpY1230/1234/1235), each diluted 1:500; rabbit anti-Akt, rabbit anti-phospho-Akt (T308), rabbit anti-phospho-GSK-3 β (S9), rabbit anti-phospho-MAP2 (T1620/16230) (all from Cell Signaling Technology and diluted 1:1 000); mouse anti-MAP2 or rabbit anti-c-Met (both from Upstate and diluted 1:1 000); rabbit anti-GSK-3 β (Chemicon) diluted 1:1 000, or mouse anti- α -tubulin (Sigma) diluted 1:5 000. Bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories) each diluted 1:100 000 and Supersignal West Pico chemiluminescence substrate (Pierce). Staining intensity was quantified from three blots derived from three independent experimental trials. The density of each band or c-Met doublet was quantified with Image J software and normalized to α -tubulin expression.

2.8. Statistical analysis

Confocal microscope images of at least 20 different fields for each treatment from each of three independent experiments were obtained and imported into Image J for quantification and further analysis. The number and length of MAP2-positive primary dendrites protruding from each cell soma were measured and compared between control cultures and cells treated with HGF or the indicated concentrations of inhibitors. Statistical analysis was conducted with the use of Prism software (GraphPad) and performed with one-way ANOVA with Tukey's post-test, unless otherwise indicated. Means $\pm$ SEM are shown, unless otherwise indicated. Equal variances of all data sets were verified by Bartlett's test before conducting the statistical analysis. P values < 0.05 were considered statistically significant.

3. Results

3.1. HGF and c-Met expression is developmentally regulated during neuron differentiation *in vitro*

We investigated the temporal relationship between HGF expression and c-Met expression and activation during the early stages of hippocampal neuron differentiation *in vitro*. HGF expression at 1 DIV was low but detectable by immunoblot. The expression of HGF increased about 5-fold by day 3 and 8-fold by day 7 compared to 1 DIV (Fig. 1A). We examined the expression of the HGF receptor, c-Met, in the same samples. c-Met, which migrates as a 140 and 170 kDa doublet [30], was already apparent at 1 DIV in cultured neurons and increased only slightly through 7 DIV (Fig. 1A). Endogenous phosphorylation of c-Met on its intracellular tyrosine kinase domain was detected at low levels through 3 DIV, but dramatically increased at 4 to 7 DIV (Fig. 1A), an increase in phosphorylation that roughly parallels the increased expression of HGF. We next examined the cellular localization of HGF, c-Met, and phospho-c-Met in hippocampal neurons at 4 DIV. HGF, c-Met, and phospho-c-Met were

present on all neuronal processes (Fig. 1B). In triple-labeled neurons at 4 DIV, c-Met is present on neural processes that also contain actin and MAP2 (Fig. 1C).

3.2. c-Met signaling is required for dendrite elongation in hippocampal neurons

To verify that HGF can activate c-Met at stage 4 of neuronal maturation, we treated cultured neurons at 4 DIV with exogenous recombinant HGF and tested for c-Met phosphorylation with the use of a phospho-c-Met antibody. Phosphorylation of c-Met increased about 4.5 fold by 10 min after application of HGF, but pre-treatment with PHA-665752, a specific c-Met inhibitor [27], suppressed HGF-induced c-Met phosphorylation in a concentration-dependent manner, so that 1 μ M inhibitor nearly completely blocked phosphorylation of c-Met in response to exogenous HGF. Treatment with PHA-665752 alone decreased endogenous phosphorylation of c-Met by about 75%. c-Met expression was not changed significantly by inhibitor treatment (Fig. 2A). The effects of PHA-665752 on cell viability were analyzed. There was no toxicity at 24 hr for neurons treated with 1 μ M or less PHA-665752; only 5 μ M inhibitor treatment was toxic (Fig. 2B).

We further investigated the effect of activating or inhibiting c-Met on dendritic morphology in primary cultured hippocampal neurons. During maturation in culture, hippocampal neurons develop several neurites that develop into a single long axon and multiple dendrites [31]. The dendrites mature by elongating, branching, and acquiring a more tapered appearance starting at stage 4, about 4 DIV [32,33]. To test the effects of c-Met on formation of dendrites, we treated cultured neurons at 3 DIV with 50 ng/ml HGF, 50 ng/ml HGF plus 1 μ M PHA-665752, 1 μ M PHA-665752, or carrier buffer. After a 24 hr incubation, cells were fixed and immunostained against MAP2 to identify dendrites. Cells treated with HGF appeared to have longer dendrites than untreated cells or those co-treated with PHA-665752 (Fig. 2C). HGF treatment increased the number of primary dendrites, an effect completely abolished by pre-treatment with the c-Met inhibitor (Fig. 2C and D). Furthermore, PHA-665752 treatment alone reduced the number of primary dendrites when compared to control neurons (Fig. 2C and D). HGF treatment increased the total primary dendritic length and the average dendritic length (Fig. 2E and F). PHA-665752 treatment blocked the increase in dendritic length in response to HGF and significantly reduced dendritic length compared to neurons treated with control buffer (Fig. 2E and F). The effect of the inhibitor in reducing dendrite number and length compared to control neurons suggests that endogenous signaling by HGF and c-Met contributes to formation and growth of dendrites under basal conditions.

As a second method to test the effect of HGF and c-Met on dendrite maturation in hippocampal neurons, we suppressed c-Met expression with use of shRNAs. To confirm the silencing effect of shRNAs on c-Met mRNA levels, cultured hippocampal neurons were transfected with each shRNA plasmid at 1 DIV and RT-PCR was performed with total RNAs from transfected cells at 4 DIV. Neurons transfected with the 305 shRNA showed about a 60% decrease in the amount of c-Met RT-PCR product compared to cells transfected with an empty control vector, but the PCR product for c-Met was undetectable in cells transfected with the 612 shRNA. c-Met mRNA expression did not change in neurons transfected with a scrambled shRNA sequence. GAPDH mRNA levels as control were not affected by transfection of any c-Met shRNAs (Fig. 3A). To confirm that c-Met protein levels were decreased by the shRNA treatment, whole-cell lysates from hippocampal neurons transfected at 1 DIV with control or c-Met shRNAs were analyzed by immunoblot against c-Met and α -tubulin. Overall c-Met expression was knocked down by 30% and 62% in cultures of neurons transfected with the 305 and 612 shRNAs, respectively, compared to cells transfected with control plasmids. There was no difference in overall c-Met expression in cells transfected with scrambled shRNA when compared to cells transfected with empty control plasmid (Fig. 3B).

To test whether suppression of c-Met expression affected dendrite morphology during neuronal development, neurons were transfected at 1 DIV with the shRNA constructs and an EGFP plasmid to mark transfected cells and immunostained against c-Met and MAP2 at 4 DIV. Most hippocampal neurons transfected with empty control plasmid had extended neurites with c-Met and MAP2 distributed throughout the dendrites (Fig. 3C). The distribution of c-Met and MAP2 was similar in cells transfected with the scrambled shRNA plasmid. In contrast, cells transfected with the 305 shRNA plasmid showed strongly reduced staining against c-Met and MAP2 staining was noticeably reduced. In the cells transfected with the 612 shRNA plasmid, the expression of c-Met was nearly undetectable and MAP2 was barely detectable in dendrites of cells transfected with this plasmid (Fig. 3C). Quantification of the transfected cells revealed that cells treated with 305 shRNA had a 20% decrease in the number of primary dendrites, a 35% decrease in total dendritic length, and a 26% reduction in average dendritic length (Fig. 3D–F). Neurons transfected with the 612 shRNA had even fewer primary dendrites (about a 41% decrease from control neurons) and a greater decrease in total (about a 60% reduction) and average (about a 40% reduction) length of primary dendrites compared to both control cells and those transfected with scrambled shRNA (Fig. 3D–F). Thus, inhibiting signaling by c-Met with either the use of an inhibitor or by shRNA caused decreased elaboration of dendrites.

3.3. Inhibition of c-Met reduces MAP2 expression and increases its phosphorylation

MAP2 stabilizes microtubules and is required for neurite extension. Phosphorylation of MAP2 by GSK-3 β modulates its association with microtubules and regulates microtubule stability [19,20]. MAP2 staining was also decreased in neurons transfected with c-Met shRNAs. We thus sought to determine whether c-Met activation or suppression might control expression and phosphorylation of MAP2. Exogenous HGF treatment for 24 hr induced about a 1.5 fold increase in MAP2 expression compared to control neurons. This effect was suppressed by PHA-665752 (Fig. 4A), which also caused an apparent increase in phosphorylation of MAP2. We also examined the phosphorylation of MAP2 in immunoblots in which the total amount of MAP2 was equalized (Fig. 4B). HGF treatment reduced phosphorylation of MAP2, but PHA-665752 treatment increased it (Fig. 4A and B). We also investigated the effect of c-Met shRNAs on the change of MAP2 expression and phosphorylation. MAP2 expression decreased in response to transfection with c-Met shRNAs (Fig. 4C), consistent with the decrease of MAP2 expression in neurons treated with the c-Met inhibitor and the reduced staining against MAP2 in shRNA-transfected neurons. Phosphorylation of MAP2 increased in cultured neurons transfected with either the c-Met 305 or 612 shRNAs. The 612 shRNA, which was most effective at reducing c-Met expression, induced the greatest increase in phosphorylation of MAP2.

3.4. c-Met initiates signaling via the Akt/GSK-3 β pathway to enhance dendrite elongation in hippocampal neurons

Signaling via the Akt/GSK-3 β pathway can regulate dendrite morphology by regulating phosphorylation of MAP2 [19]. Akt is phosphorylated and activated by phosphoinositide 3-kinases (PI3-K), and Akt phosphorylates GSK-3 β to inactivate it. Inactivation of GSK-3 β leads to decreased phosphorylation of MAP2 and its increased binding and stabilization of microtubules. To test whether signaling by c-Met can promote dendrite growth by regulating the Akt/GSK-3 β pathway in cultured hippocampal neurons, we tested for phosphorylation of Akt and GSK-3 β in cells treated with HGF and/or PHA-665752. Phosphorylation of both Akt and GSK-3 β increased in response to treatment with HGF (Fig. 5A), but pre-treatment with PHA-665752 before adding HGF blocked phosphorylation of these proteins. Treatment with the inhibitor alone reduced phosphorylation of Akt and GSK-3 β to about 50–60% of control levels (Fig. 5A). These treatments did not affect Akt and GSK-3 β protein levels. Phosphorylation of Akt and GSK-3 β was also tested in cells transfected with c-Met shRNAs.

Transfection with either 305 or 612 c-Met shRNA plasmids reduced phosphorylation of Akt and GSK-3 β (Fig. 5B).

To confirm that Akt and GSK-3 β are downstream molecules that mediate the effects of HGF on dendrite elongation, we treated cultured hippocampal neurons with LY294002, an inhibitor of PI3-K, which can phosphorylate Akt to activate it, or Akt Inhibitor X, a selective inhibitor of Akt. Both LY294002 and Akt Inhibitor X treatment reduced HGF-induced phosphorylation of GSK-3 β at the site phosphorylated by Akt (Fig. 6A). Since the kinase activity of GSK-3 β is increased when it is unphosphorylated [13,33], we also tested the effects of HGF on phosphorylation of MAP2 in the presence of LY294002 and Akt inhibitor X. These inhibitors blocked the ability of HGF to reduce MAP2 phosphorylation, consistent with increased activity by GSK-3 β (Fig. 6B). In addition, both inhibitors blocked the ability of HGF to increase the number of primary dendrites and the average primary dendritic length (Fig. 6C-E).

To further test whether GSK-3 β mediates the effects of HGF on dendrite elongation, we treated cultured hippocampal neurons with SB415286, a specific GSK-3 β inhibitor. Treatment of neurons with either HGF or SB415286 caused a similar reduction in phosphorylation of MAP2 (Fig. 7A). In addition, GSK-3 β inhibition by SB415286 caused an increase in dendritic number and length that was statistically the same as that induced by HGF. Treatment of neurons with a combination of HGF and SB415286 did not cause an additive effect on the number of dendrites or average dendritic length (Fig. 7B-D), which suggests that HGF and SB415286 may both affect dendritic growth by a common mechanism. Together, these results thus demonstrate the importance of c-Met activation and subsequent activation of the Akt/GSK-3 β pathway in dendritic maturation.

4. Discussion

Growth factors and their associated receptor tyrosine kinases (RTKs) play important roles in various neuronal functions such as neuronal polarization, neurite extension, and dendrite maturation during central nervous system (CNS) development [1–3,32]. Although many signals have been identified that modulate dendrite growth and maturation, relatively few have been identified that regulate the initial stages of dendrite elongation [34]. HGF and c-Met are expressed by specific classes of neurons from cerebral cortex, hippocampus, amygdala, and septum as well as by non-neuronal cells [35–37]. HGF initiates multiple intracellular signals to regulate protein expression, cytoskeletal rearrangement and motility, and intercellular adhesion in CNS neurons [12,38,39]. HGF can promote elaboration of dendrites in mature hippocampal neurons [5,16]. It can also stimulate formation of neurites in thalamic, dorsal root ganglion, neocortical, and spinal motor neurons [4,6,7,40]. In this study, we investigated the role of HGF and c-Met and the signals they initiate to regulate dendrite elongation during early stages of hippocampal neuron differentiation. Differentiation of these neurons *in vitro* can be divided into five stages during which neurites extend from the soma and differentiate into a single axon and multiple dendrites [31–33]. At stage 4, about 4 DIV after plating, new dendrites elongate, branch, and take on a tapered morphology [31,33]. We tested the effects of HGF and c-Met on dendrite maturation at this stage.

We show that endogenous HGF expression increases and c-Met phosphorylation becomes prominent starting about 4 DIV in cultured hippocampal neurons, the time point when neurites take on a dendritic morphology. Treatment of stage 4 cultured neurons with exogenous HGF increases both the number of primary dendrites and their average length. When signaling by c-Met is suppressed with either the specific c-Met inhibitor, PHA-665752, or by shRNAs against c-Met, the number of primary dendrites and their average lengths are reduced. We also demonstrate that activation of c-Met increases phosphorylation of Akt and GSK-3 β and reduces phosphorylation of MAP2, a GSK-3 β target, steps known to regulate microtubule

polymerization and dendrite elongation [19,41]. Inhibiting PI3-K, an activator of Akt, with LY294002 or directly inhibiting Akt with Akt Inhibitor X blocked the ability of HGF to promote elongation of dendrites. Inhibiting GSK-3 β with SB415286 mimicked the increase in the number of dendrites and dendritic length induced by HGF. This strongly suggests that the PI3-K/Akt/GSK-3 β pathway mediates the effects of HGF on dendrite elongation in neurons at this developmental stage.

Signaling proteins, such as Akt, MAPK, GSK-3 β and MAP2, which are downstream of c-Met and other growth factor receptors, are crucial regulators of dendritic growth and development [12–14]. PI3-K and Akt, which are activated downstream of c-Met, can regulate dendritic branching, axonal growth, neuronal differentiation, and migration [17,18,42,43]. Phosphorylation of Akt by PI3-K at threonine 308 activates it, and it can in turn phosphorylate several downstream targets, including GSK-3 β , which becomes inactivated when Akt phosphorylates it at serine 9 [13,33]. GSK-3 β is a serine/threonine kinase implicated in regulation of microtubule dynamics by phosphorylating MAPs, including adenomatous polyposis coli (APC), MAP1, or MAP2, and mediates the effects of Akt activation during growth factor-induced dendrite growth [17,18]. Phosphorylation of MAPs is key step to regulate neurite initiation and growth, neuronal polarity, and neurite branching in hippocampal neurons [19,20]. Decreased activity of GSK-3 β reduces phosphorylation of MAP2, which increases the interaction of MAP2 with microtubules to stabilize their structure and promote neuronal polarization and multiple neurite formation [13,33].

We suppressed signaling by c-Met by two independent methods, both of which blocked phosphorylation of Akt and GSK-3 β , increased phosphorylation of MAP2, and reduced dendritic elongation. First, we used the selective c-Met inhibitor, PHA-665752. PHA-665752 has an IC₅₀ against c-Met that is 300-fold lower than other RTKs, such as epidermal growth factor receptor or fibroblast growth factor receptor [24,27]. PHA-665752 effectively inhibits c-Met phosphorylation in a variety of cells [24,27], and our study confirms that it also blocks activation of c-Met in neurons. Treatment of neurons with this inhibitor reduced the ability of HGF to stimulate phosphorylation of Akt and GSK-3 β . PHA-665752 reduced dendrite length compared to control-treated neurons, which suggests that endogenous signaling by c-Met participates in dendritic elongation under basal conditions. The inhibitor also completely blocked exogenous HGF from enhancing dendritic length. Second, we reduced the expression of c-Met with two distinct shRNAs against the 3'-UTRs of rat c-Met. The 305 and 612 shRNA constructs each reduced c-Met expression. These two shRNAs each reduced phosphorylation of Akt and GSK-3 β , increased phosphorylation of MAP2, and reduced dendritic length. The effectiveness by which they reduced expression of c-Met paralleled their effectiveness in reducing phosphorylation of Akt and GSK-3 β , increasing phosphorylation of MAP2, and reducing dendritic length. The similar biological effects of these two independent shRNA constructs strongly suggest that their effects are due to reduced signaling by Met rather than by off-target effects. Together, these results indicate that signaling by HGF and c-Met promotes elongation of dendrites in stage 4 neurons.

Although the functions of HGF and c-Met in the CNS are just beginning to be described, it is becoming increasingly clear that these powerful signaling molecules are important for many aspects of neural development and plasticity. A mutation in the promoter region of the c-Met gene that may reduce its expression was recently linked to autism [44,45]. Since autism likely arises from defects in neural circuits, understanding the effects of c-Met on development of circuits or synaptic function is likely important in the etiology of autism. As we demonstrate in this study, HGF and c-Met can regulate the early stages of dendritogenesis. They can also regulate changes in the dendritic arbor of mature neurons [5,16], promote axonal growth [4, 7], and alter expression and clustering of synaptic proteins [11]. HGF can modulate migration of cortical neurons and interneurons [18,46,47]. In addition, c-Met becomes activated in

response to neural activity [10]. HGF and c-Met thus play an important role in regulating many aspects of neural circuitry, and deficits in signaling by c-Met may lead to defects in neural development.

In conclusion, we demonstrate that activation of c-Met with HGF leads to increased elaboration of dendrites, but that inhibition of c-Met, either with an inhibitor or by shRNA, leads to decreased growth of dendrites. HGF treatment leads to phosphorylation of Akt and GSK-3 β , and inhibitors of PI3-K or Akt block the ability of HGF to stimulate dendritic growth. Furthermore, HGF treatment causes decreased phosphorylation of MAP2, a step that can enhance microtubule polymerization and dendritic growth. Together, these studies demonstrate that HGF and c-Met can regulate dendritic growth via the Akt/GSK-3 β pathway.

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Fig. 1.

HGF and c-Met expression is developmentally regulated. **(A)** Dissociated hippocampal neurons were lysed at the indicated DIV and probed by immunoblot against HGF, phospho-c-Met (Y1230/1234/1235), c-Met, or tubulin as a loading control. Molecular masses in kDa are shown on the left. The ratio of pixel densities of each protein relative to tubulin was calculated for each time point from three independent blots and the means $\pm$ SD are shown. **(B)** Dissociated hippocampal neurons at 4 DIV were fixed and immunostained against HGF, c-Met, or phospho-c-Met (Y1230/1234/1235), and then labeled with Alexa 488-conjugated goat anti-rabbit or mouse IgG. Scale bar, 30 μ m. **(C)** Dissociated hippocampal neurons at 4 DIV were fixed and triple-labeled with use of antibodies against c-Met, MAP2, and Alexa 555-conjugated phalloidin to stain actin as described in Materials and Methods. Scale bar, 30 μ m.

Fig. 2.

Exogenous HGF treatment induces phosphorylation of c-Met and elongation of primary dendrites. **(A)** Hippocampal neurons at 4 DIV were pre-incubated with either the indicated concentrations of PHA-665752, a specific c-Met inhibitor, or an equal volume of DMSO for 2 hr, and treated with 50 ng/ml HGF for 10 min and then lysed. Fifty μ g of protein was separated by SDS-PAGE and probed with antibodies against phospho-c-Met (Y1230/1234/1235), c-Met, or α -tubulin as a loading control. Changes in phosphorylation and expression of c-Met relative to α -tubulin were quantified from three independent immunoblots and are shown as mean $\pm$ SD. **(B)** Cultured neurons were treated with the indicated doses of PHA-665752 and HGF for 24 hr, and cell viability was tested with use of a Cell Counting Kit-8 as described in Materials and Methods. Error bars denote SEM. *, $P < 0.05$. **(C)** Dissociated hippocampal neurons at 3 DIV were either untreated or treated for 24 hr with 50 ng/ml HGF (HGF), 50 ng/ml HGF plus 1 μ M PHA-665752 (HGF+PHA1), or only 1 μ M PHA-665752 (PHA1), fixed, immunostained against MAP2, and viewed by confocal microscopy. Scale bar, 30 μ m. **(D–F)** The numbers of

MAP2-positive primary dendrites directly emerging from cell somas were quantified, and the total length of primary dendrites and average length of each dendrite were measured using Image J software. $n=40$ neurons from three independent experiments. *, $P<0.05$.

Fig. 3.

Knock-down of c-Met by shRNA alters dendrites. **(A)** RT-PCR against c-Met and GAPDH was conducted with total RNA isolated at 4 DIV from hippocampal neurons transfected with control, c-Met shRNAs, or scrambled shRNA plasmids at 1 DIV. Ethidium bromide stains of the PCR products were scanned and densitometric analysis was performed with Image J software. Results were quantified from three independent experiments. Error bars represent $\pm$ SD. *, $P < 0.05$. **(B)** Hippocampal neurons were transfected with the indicated shRNA plasmids at 1 DIV and lysed at 4 DIV. One-hundred μ g of protein was probed by immunoblot against c-Met or α -tubulin. The sum of both bands of c-Met was normalized to α -tubulin expression. Error bars show $\pm$ SD. *, $P < 0.05$. **(C)** Neurons transfected with EGFP and the indicated c-Met

shRNAs, scrambled shRNA, or empty control plasmid were immunostained with mouse anti-c-Met and chicken anti-MAP2, and then Alexa 568-conjugated goat anti-mouse IgG and Cy5-conjugated goat-anti chicken IgG. Scale bar, 30 μm . **(D–F)** Primary dendrite number, total dendrite length per cell, and average dendritic length were quantitatively analyzed with MAP2-stained images from three independent experiments. $n=40$ cells/condition. Error bars represent SEM. *, $P<0.05$. *Con*, Control; *Scrm*, Scrambled.

Fig. 4.

Inhibition of c-Met suppresses MAP2 expression and increases its phosphorylation. **(A)** Dissociated hippocampal neurons incubated for 24 hr without or with 50 ng/ml HGF, 50 ng/ml HGF plus 0.25 or 1 μ M PHA-665752, with only 1 μ M PHA-665752, or first treated for 12 hr with 1 μ M PHA-665752 and then another 12 hr with 50 ng/ml HGF were lysed and probed against phospho-MAP2 (T1620/1623), MAP2, and α -tubulin. The staining intensity of bands from three independent trials was quantified relative to α -tubulin and graphed as mean $\pm$ SD. **(B)** Immunoblots against phospho-MAP2 (T1620/1623) and MAP2 were conducted after equalizing the amount of MAP2 protein in each lane. The relative band intensity of phospho-MAP2 was quantitatively analyzed from three independent experiments. Error bars represent SEM. *, $P < 0.05$. **(C)** Dissociated hippocampal neurons were transfected with shRNAs against c-Met or scrambled shRNA at 1 DIV and lysed at 4 DIV. The relative amount of phospho-MAP2 (T1620/1623) to total MAP2 was quantified from three independent immunoblots. Error bars represent SEM. *, $P < 0.05$. *Con*, Control; *Scrm*, Scrambled.

Fig. 5.

HGF treatment increases phosphorylation of Akt and GSK-3 β . **(A)** Cultured hippocampal neurons at 4 DIV were pre-incubated with the indicated concentrations of PHA-665752 or an equal volume of DMSO for 2 hr. The cells were then incubated for 10 min with 50 ng/ml HGF and lysed. Fifty μ g of protein was separated by SDS-PAGE and probed with antibodies against phospho-Akt (T308), Akt, phospho-GSK-3 β (S9), GSK-3 β , or α -tubulin as a loading control. Relative phosphorylation changes of Akt and GSK-3 β were quantitatively analyzed from three independent trials. Error bars represent $\pm$ SD. **(B)** Dissociated hippocampal neurons transfected with empty pmU6pro plasmid or indicated shRNAs plasmids against c-Met at 1 DIV were lysed at 4 DIV. The lysates were analyzed by immunoblot as described above. Relative phosphorylation changes of Akt and GSK-3 β were quantitatively analyzed with blots from three independent trials. Error bars represent SEM. *, $P < 0.05$. *Con*, Control; *Scrm*, Scrambled.

Fig. 6.

Inhibitors that reduce Akt activity increase phosphorylation of MAP2 by GSK-3 β and suppress dendrite elongation in response to HGF. **(A)** Hippocampal neurons were incubated for 6 hr without or with 50 ng/ml HGF, 50 ng/ml HGF plus 20 μ M LY294002, 20 μ M LY294002, 50 ng/ml HGF plus 2.5 μ M Akt inhibitor X, or 2.5 μ M Akt inhibitor X. The cells were lysed, and the lysates were probed against phospho-GSK-3 β (S9), GSK-3 β , and α -tubulin as a loading control. The relative phosphorylation of GSK-3 β was quantitatively analyzed with blots from three independent trials. Error bars represent $\pm$ SD. **(B)** Hippocampal neurons were treated as described above and probed against phospho-MAP2 (T1620/1623) after equalizing the amount of MAP2 protein. Relative phosphorylation of MAP2 was quantitatively analyzed with blots from three independent trials. Error bars represent $\pm$ SD. **(C)** Neurons treated as above were fixed and immunostained with anti-MAP2 antibody and labeled with Alexa 568-conjugated goat anti-mouse IgG. The numbers refer to the concentration of inhibitors in μ M. Scale bar, 30 μ m. LY, LY294002; Inh X, Akt inhibitor X. **(D and E)** Primary dendrite number and average dendritic length were quantitatively analyzed from 40 neurons derived from three independent experiments. Error bars represent SEM. *, $P < 0.05$.

Fig. 7.

GSK-3 β inhibition decreases MAP2 phosphorylation and increases dendrite elongation similar to HGF. **(A)** Hippocampal neurons were incubated for 6 hr without or with 50 ng/ml HGF, 50 ng/ml HGF plus 10 μ M SB415286 or 20 μ M SB415286, or 10 μ M SB415286. The cell lysates were probed against phospho-MAP2 (T1620/1623) and MAP2 after equalizing the amount of MAP2 protein. The relative phosphorylation change of MAP2 was quantitatively analyzed with blots from three independent trials. Error bars represent $\pm$ SD. **(B)** Neurons treated as above were fixed and immunostained with anti-MAP2 antibody and then labeled with Alexa 568-conjugated goat anti-mouse IgG. The numbers indicate the concentration of SB415286 in μ M. Scale bar, 30 μ m. *SB*, SB415286. **(C and D)** Primary dendrite number and average dendrite length were quantitatively analyzed from 40 neurons derived from three independent experiments. Error bars represent SEM. *, $P < 0.05$.