HGF regulates the development of cortical pyramidal dendrites

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Summary

Although hepatocyte growth factor (HGF) and its receptor tyrosine kinase MET are widely expressed in the developing and mature central nervous system, little is known about the role of MET signaling in the brain. We have used particle-mediated gene transfer in cortical organotypic slice cultures established from early postnatal mice to study the effects of HGF on the development of dendritic arbors of pyramidal neurons. Compared with untreated control cultures, exogenous HGF promoted a highly significant increase in dendritic growth and branching of layer 2 pyramidal neurons, whereas inactivation of endogenous HGF with function-blocking, anti-HGF antibody caused a marked reduction in size and complexity of the dendritic arbors of these neurons. Furthermore, pyramidal neurons transfected with an MET dominant-negative mutant receptor likewise had much smaller and less complex dendritic arbors than did control transfected neurons. Our results indicate that HGF plays a role in regulating dendritic morphology in the developing cerebral cortex.

Key words: MET, c-MET, Neurons, Process growth, GFP, Slice cultures

Introduction

Hepatocyte growth factor (HGF), or scatter factor, is a secreted protein that exerts a variety of effects on many cell types by binding to the MET receptor tyrosine kinase (Birchmeier and Gherardi, 1998; Maina et al., 2001; Ponzzeto et al., 1994). HGF promotes the survival and proliferation of several cell types, stimulates migration and dissociation of epithelial sheets and plays a role in the metastasis of some tumors (Gherardi and Stoker, 1991; Tsarfaty et al., 1992). Studies of mice lacking either HGF or a functional MET receptor have shown that HGF and MET signaling are required for the development of the placenta, liver and skeletal muscle (Bladt et al., 1995; Maina et al., 1996; Schmidt et al., 1995; Uehara et al., 1995).

HGF plays a role in several aspects of neural development (Maina and Klein, 1999). Experiments in chick embryos have suggested that HGF might play a role in neural induction (Bonner-Fraser, 1995). Later in embryonic development and in the adult nervous system a variety of neurons and glial cells express HGF and MET (Andermarcher et al., 1996; Di Renzo et al., 1993; Jung et al., 1994; Krasnoselsky et al., 1994; Maina et al., 1997; Sonnenberg et al., 1993; Thewke and Seeds, 1999). HGF promotes the survival of a subset of motoneurons and has been implicated in guiding a subset of motor axons to their targets (Ebens et al., 1996; Wong et al., 1997; Yamamoto et al., 1997). HGF promotes the survival of sympathetic neuroblasts (Maina et al., 1998) and enhances the survival of subsets of parasympathetic and sensory neurons grown with ciliary neurotrophic factor (CNTF) and nerve growth factor (NGF), respectively (Davey et al., 2000; Maina et al., 1997; Yang et al., 1998). HGF also enhances neurite growth from these neurons cultured with neurotrophic factors, and mice possessing a non-functional MET receptor have shorter, less branched spinal sensory nerves in vivo than wild-type embryos (Maina et al., 1997). HGF also increases the number of calbindin D-expressing neurons in postnatal rat hippocampal cultures and increases neurite outgrowth from these neurons (Korhonen et al., 2000).

HGF and MET are widely expressed in the developing and mature mouse brain, with expression beginning as early as embryonic day 12 (E12) and E13, respectively (Achim et al., 1997; Jung et al., 1994; Thewke and Seeds, 1999). In the cerebral cortex, HGF is expressed in pyramidal neurons of layers IV and V, whereas MET is expressed in cortical neurons of layers II, III, IV and V. Other sites of HGF expression include the hippocampus, granule cell layer of the cerebellum, ependymal cells, chorioid plexus, and pineal body (Jung et al., 1994; Korhonen et al., 2000). MET is also expressed in the CA-1 area of the hippocampus, the septum and the pons (Thewke and Seeds, 1999).

Despite the extensive expression of HGF and MET in the central nervous system (CNS), only a handful of studies have begun to investigate the potential functions of MET signaling in the brain. HGF enhances the survival of tyrosine hydroxylase-positive midbrain neurons (Hamanoue et al., 1996) and hippocampal neurons (Honda et al., 1995) in culture. In vivo, HGF rescues hippocampal CA1 neurons following transient global ischemia (Miyazawa et al., 1998) and rescues cerebellar granule neurons following N-methyl-D-aspartate

(NMDA) and quinolinic acid-induced excitotoxicity (Zhang et al., 2000). HGF also promotes the migration of cortical interneurons from the ventral to the dorsal telencephalon in rodents (Powell et al., 2001), and MET signaling has been implicated in regulating the proliferation and differentiation of cerebellar granule cells (Ieraci et al., 2002).

To assess the possible role of HGF in regulating the morphology of CNS neurons, we examined the effect of exogenous HGF and blockade of endogenous HGF on the growth of postnatal cortical pyramidal dendrites in organotypic slice cultures of mouse somatosensory cortex. These cultures have the advantage of preserving the local three-dimensional environment of each neuron, the laminar organization of the cortex and the pattern of connections within and between these layers (Hayar et al., 1999; McAllister et al., 1995). Using particle-mediated gene transfer, a subset of pyramidal neurons were labeled with GFP and their morphology was examined by confocal microscopy. These studies revealed that manipulating the supply of HGF in these cultures markedly affects the dendritic arbors of layer 2 pyramidal neurons: HGF increasing, and anti-HGF decreasing, dendritic growth. These results demonstrate that HGF and MET signaling in the cortex stimulates dendritic growth and may play a role in regulating neuronal plasticity in the developing cerebral cortex.

Materials and methods

Slice cultures

We cut 300 μm vibrotome slices of postnatal day 6 (P6) or P7 CD1 mouse brains in the coronal plane between the tectum and the hippocampal commissure. Sectioning was made in cold (4°C) artificial cerebrospinal fluid (160 mM NaCl, 200 mM KH_2PO_4, 5 mM KCl, 1 mM $MgSO_4$, 33 mM glucose, 10 mM HEPES, 1 mM $CaCl_2$). The slices were cultured in 35 mm Petri dishes on 0.4 μm Millicel inserts (Millipore) floating on 1 ml of culture medium (50% Dubecco's Minimal Essential Medium, 25% heat-inactivated horse serum, 25% Hank's Balanced Salt Solution, 6.5 mg/ml glucose and 100 U/ml streptomycin and penicillin). The cultures were incubated in 5% CO_2 at 37°C.

Gene transfer

The dendritic arbors of cortical pyramidal neurons were visualized by transfecting these neurons with an enhanced green fluorescent protein (EGFP) expression plasmid (Clontech) using the hand-held gene-gun (Helios Gene-gun, BioRad) 1 hour after slice preparation. Gold particle cartridges were prepared beforehand using the manufacturer's protocol. Briefly, 20 mg of 1.6 μm gold particles were suspended in 100 μl of 50 mM spermidine and 20 μg of plasmid DNA (pEGFP, Clontech). The gold particles were then precipitated with 100 μl of 2M $CaCl_2$, washed three times with 100% ethanol, resuspended in 1.2 ml of 100% ethanol plus 0.01mg/ml poliviniylpirrolidone and loaded into Teflon tubing microcarriers. The gold particles in the microcarriers were shot into the slices at a pressure of 250-300 psi. A 70 μm nylon mesh screen was placed between the gun and the slice to protect the tissue from the shock wave. For MET kinase-dead transfections, cartridges were prepared by co-precipitating 20 μg of the pEGFP plasmid and 20 μg of the MET KD expression vector (kindly provided by Flavio Maina, Marseilles). Control transfections were carried out with cartridges carrying pEGFP and the corresponding empty vector.

Reagents

Recombinant human HGF (R&D) and BDNF (Genentech) were used at a concentration of 200 ng/ml, and function-blocking anti-HGF (polyclonal anti-human HGF antibody R&D) was used at concentrations of 1 and 3 μg/ml. In some experiments, HGF was pre-incubated with anti-HGF for 1 hour at 37°C before adding to the cultures. Reagents were added immediately after bombardment.

Dendritic analysis

Forty-eight hours after transfection, layer 2 pyramidal neurons of the somatosensory cortex were studied with an Axioplan Zeiss laser scanning confocal microscope. Thirty minutes fixation with 4% paraformaldehyde in PBS and DAPI counterstaining was used to confirm the laminar localization. For every experimental condition studied, the dendritic organization of between 50 and 60 neurons was reconstructed and analysed. GFP-expressing cells were selected for analysis only if they where in layer 2 of the somatosensory cortex and they had a pyramidal morphology with a single apical dendrite oriented toward the dorsal cortical surface and at least two basal dendrites. For each neuron, 15 and 20 optical sections were obtained using 20× and 40× water immersion objectives. Three-dimensional projections were generated by merging the resulting Z stacks, and the dendritic arbors were traced using LSM510 software. These traces were analyzed using a customized matlab script for the automatic counting of branching points, number of primary dendrites, dendritic length and other topological parameters. Sholl analysis was also carried out on the Z-stack images. For this analysis, concentric, digitally generated rings, 15 μm apart, were centered on the cell soma, and the number of dendrites intersecting each ring was counted (Sholl, 1953). Pair-wise comparisons were made using Student's *t*-test. For multiple comparisons, ANOVA was performed followed by Fisher's post-hoc test.

For the time course experiments, a total of 10 neurons per condition was scanned 18 hours after transfection. By carefully marking the culture plate orientation and recording the coordinates of the field (as measured with the XY scale of the microscope stage), the same cells were scanned 24 hours later (42 hours of incubation in total). The images were processed and analyzed as above.

MET and HGF RNA levels in cortical slice cultures

The somatosensory cortex was dissected from vibrotome slices and stored at –70°C. Total RNA was extracted, purified using the BIO101 RNAID kit (Biogen) and recovered in 40 μl of DEPC-treated water. The RNA was reverse transcribed for 1 hour at 37°C with MmuLV-reverse transcriptase, RNaseH in a 40 μl reaction containing the manufacturer's buffer supplemented with 0.5 mM dNTPs and 10 μM random hexanucelitides. A 5 μl aliquot of each reaction was then amplified in a 30 μl multiplex PCR reaction containing 1×PC buffer (Helena Biosciences), 0.1 mM dNTPs, 2 units of Taq supreme (Helena Biosciences), 20 μM of GAPDH primers and 200 μM of either HGF or MET primers. The forward and reverse assay primers for MET cDNA were 5′-CCAGRCCTATATTGATGTC-3′ and 5′-TTCGAAG-GCATGTATGTAC-3′, respectively. The forward and reverse primers for HGF were 5′-CCCATGAACACAGCTATCGC-3′ and 5′-TAAGCGTCCTCTGGATTGC-3′, respectively. The cDNAs were amplified using the following cycling conditions: 1 minute at 95°C, 1 minute at 50°C and 1 minute at 68°C. MET cDNA was amplified for 28 cycles and HGF cDNA for 26 cycles.

Results

Exogenous HGF enhances the growth of pyramidal neuron dendrites

To investigate the potential role of HGF in regulating the morphology and growth of pyramidal neuron dendrites in the developing cerebral cortex, we cultured postnatal mouse brain slices for up to 48 hours in the presence of either HGF or function-blocking anti-HGF antibodies and visualized dendritic morphology by transfecting the neurons with an

EGFP expression plasmid using gold particle-mediated gene transfer.

We investigated the potential influence of HGF on dendritic growth and morphology by studying the effect of exogenous HGF on layer 2 pyramidal neurons of the postnatal mouse somatosensory cortex. These neurons were chosen because they are rapidly growing and can be easily observed between P5 and P7 in this region. Stable, high quality labeling of these neurons was observed after 2 days of culture in the mouse brain slice cultures (see Fig. 1A). The dendritic arbors of these neurons were visualized by transfecting them with an EGFP expression plasmid by firing gold particles coated with this plasmid into 300 μm cortical slices. Labeling of layer 2 pyramidal neurons was clearly observed after 12 hours in culture (Fig. 1A), and was stable for at least 2 days. The dendritic morphology of these neurons was conveniently analyzed by constructing a Z stack projection from multiple confocal images taken throughout the dendritic arbor (Fig. 1B).

HGF and MET expression in the cortex has been reported during the first postnatal weeks. To ascertain whether expression of HGF and MET transcripts is sustained in the cortical mantle of slices throughout the period of culture, we used RT/PCR to detect HGF and MET RNAs in total RNA extracted from the cortical mantle dissected from slices before and after culture. Similar levels of RNA from uncultured and cultured cortical mantle were detected, as illustrated by the similar levels of RT/PCR product for mRNA encoding the house-keeping enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) (Fig. 1C). Similar levels of HGF and MET RT/PCR products were also obtained from uncultured slices of cortical mantle and from slices that had been cultured for 48 hours, suggesting that the expression of HGF and MET is sustained in the cortex throughout the period of culture used for these studies (Fig. 1). Because brain-derived neurotrophic factor (BDNF) is a potent promoter of the growth and branching of pyramidal neuron dendrites across all cortical layers (McAllister et al., 1995, 1997; Niblock et al., 2000), we were interested in contrasting the possible effects of HGF on dendrite growth with those observed with BDNF.

Figure 2 shows a representative sample across the entire range of dendritic morphologies in four experimental conditions: control cultures (no growth factor supplements to the medium) and cultures supplemented with HGF, function-blocking anti-HGF antibody or BDNF. These images illustrate a clearly discernable increase in the size and complexity of pyramidal neuron dendritic arbors in cortical slices treated with either HGF or BDNF for 48 hours compared with control cultures, whereas dendritic arbors in cultures treated with anti-HGF were clearly smaller and less complex than those of control cultures. To quantify dendritic size and complexity, we determined the total number of branch points per arbor, total dendritic length and number of primary dendrites arising from the cell body. Table 1 shows data for total dendritic length and number of branch points for the different experimental conditions. Quantitative ANOVA comparisons showed statistically significant effects among groups in all three

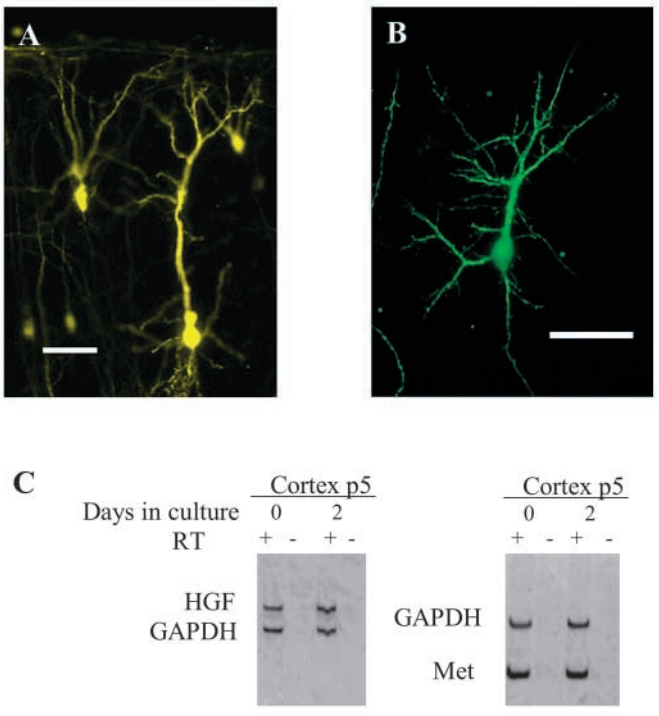

Fig. 1. (A) Epifluorescence photomicrograph of layer 2 and III pyramidal neurons showing stable labeling with GFP 24 hours after biolistic transfection with a GFP expression plasmid. (B) Z stack projection of a representative layer 2 pyramidal neuron after 48 hours in culture constructed from 10 confocal images of the neuron obtained at 10 μm intervals through the dendritic arbor. Scale bars: 50 μm. (C) RT-PCR analysis of HGF and MET expression in freshly prepared cortical slices and in cortical slices that had been cultured for 2 days. The PCR products for HGF, MET and GAPDH are indicated. No amplification products are observed in control reactions in which the reverse transcription (RT) step was omitted.

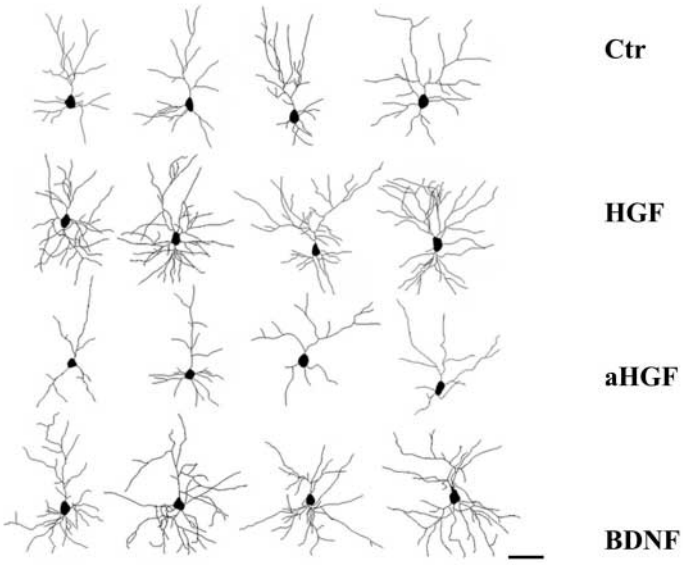

Fig. 2. Representative sample of reconstructed layer 2 pyramidal neurons after 48 hours in culture. The neurons shown correspond to percentiles 25, 50, 75 and 100 of the sampled populations in terms of total dendritic length. Control, no additives; HGF, cultures treated with 200 ng/ml HGF; anti-HGF, cultures treated with function blocking anti-HGF antibodies (1 μg/ml); BDNF, cultures treated with 200 ng/ml BDNF. Scale bar: 50 μm.

Table 1. Total length and number of branch points in the dendritic arbors of pyramidal neurons under different experimental conditions

	Total length (μm)	Number of branch points
Control	853.9±30.6	12.1±0.8
HGF	1085.8±56.0*	18.3±0.9**
Anti-HGF	660.6±42.1*	8.3±0.7*
HGF + anti-HGF1	918.5±64.0	11.7±0.8
HGF + anti-HGF2	685.7±61.7*	9.3±0.7*
BDNF	1200.3±47.9**	19.0±1.3**
BDNF + HGF	1093.3±74.3*	17.9±1.5**

Mean values±s.e.m. of total dendritic length (expressed in μm) and total number of branches. Statistical significance was tested by simple ANOVA followed by Fisher post-hoc analysis. Experimental conditions indicated are: Control (untreated), HGF (medium supplemented with 200 ng/ml HGF), anti-HGF (1 μg/ml anti-HGF), HGF + anti-HGF (200 ng/ml HGF + 1 μg/ml anti-HGF), HGF + anti-HGF2 (200 ng/ml HGF + 3 μg/ml anti-HGF), BDNF (200 ng/ml BDNF) and BDNF + HGF (both factors at 200 ng/ml each). *P<0.05 and **P<0.001 versus control.

morphological indexes. HGF promoted statistically significant increases in total dendrite length (P<0.05) and dendrite branching (P<0.001) compared with control cultures. BDNF promoted similar, statistically significant increases in dendrite growth and branching and, as shown in Fig. 3, additionally promoted a significant increase in the number of primary dendrites (P<0.05). There was also a tendency to an increased number of primary dendrites in the presence of HGF without reaching statistical significance (Fig. 3).

Pyramidal neurons have two distinctive dendritic compartments. The basal compartment is comprised of shorter, denser dendrites congregated around the perikaryon, whereas the apical dendrites are longer and are orientated toward the pial surface of the cortex. Because these compartments receive different patterns of synaptic input, and because previous studies have revealed that neurotrophins generally have different effects on dendritic growth and complexity in each compartment, we analysed the effects of each treatment on the apical and basal dendritic compartments separately (Fig. 4). The data for each experimental condition are plotted as a percentage of control values. Quantitative ANOVA comparisons showed statistically significant increases in dendritic growth and branching in both compartments in both HGF-treated and BDNF-treated cultures. However, whereas HGF treatment promoted similar percentage increases in dendrite length and branching in both compartments, BDNF had a much greater effect on basal dendrites (3-fold greater increase in length of basal dendrites compared with apical dendrites and a 2-fold greater increase in basal dendrite branching compared with apical dendrites). Interestingly, in the basal compartment, the effect of HGF and BDNF in combination was similar to the effect of HGF alone, suggesting that HGF is able to modulate the growth promoting effect of exogenous BDNF.

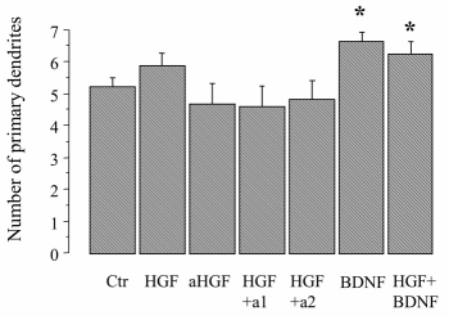

Fig. 3. Quantitative analysis of the effect of different treatments on the number of primary dendrites. Cortical slices were grown under control conditions (Ctr), or in medium supplemented with 200 ng/ml HGF (HGF), 1 μg/ml anti-HGF (aHGF), 200 ng/ml HGF plus 1 μg/ml anti-HGF pre-incubated for 1 hour before addition to the cultures (HGF + a1), 200 ng/ml HGF plus 3 μg/ml anti-HGF pre-incubated for 1 hour before addition to the cultures (HGF + a2), 200 ng/ml BDNF (BDNF) or HGF + BDNF. The bar charts illustrate the total number of primary dendrites. *P<0.05. Between 50 and 60 individual neurons were analysed under each condition.

Taken together, the above observations indicate that exogenous HGF and BDNF are each capable of increasing the growth and complexity of cortical pyramidal neuron dendritic arbors in the postnatal period. However, whereas HGF promotes a general increase in growth and branching, affecting apical and basal dendrites to a similar extent, the effect of BDNF on growth and branching was much more marked in the basal dendritic compartment. The finding that there was no additional dendritic growth and branching in cultures treated with both factors in combination compared with cultures treated with either factor alone (Fig. 4) suggests that these factors act largely on the same subset of pyramidal neurons.

The growth of pyramidal neuron dendrites is promoted by endogenous HGF

To investigate the physiological significance of endogenous HGF in regulating the growth and morphology of cortical dendrites, we treated cultures with a function-blocking anti-HGF antibody. This antibody caused a statistically significant decrease in the length and branching of pyramidal dendrites compared with control cultures (P<0.05), although there was no significant reduction in the total number of primary dendrites (Fig. 4). This suggests that endogenously produced HGF plays a role in regulating the growth and morphology of cortical pyramidal dendrites in the developing cerebral cortex. To ascertain the specificity of the anti-HGF antibody in this experimental paradigm, we pre-incubated 200 ng of HGF with either 1 or 3 μm of anti-HGF antibodies for 1 hour before adding to the cultures (HGF+a1 and HGF+a2, respectively, in Fig. 3). Pre-incubation with 1 μg/ml antibody greatly reduced the effects of exogenous HGF on dendrite growth and branching, and 3 μg/ml antibody blocked the effects of both endogenous and exogenously added HGF on these two morphological indexes.

Sholl analysis of dendritic arbors was consistent with the above results and provided complementary quantitative data on the morphological changes brought about by the different experimental conditions. Figure 5 plots the number of dendrite intersections on a series of concentric rings centered on the cell soma of neurons grown under different experimental conditions (HGF, BDNF, HGF + BDNF and anti-HGF) compared with neurons grown under control conditions (no additions). Under all experimental conditions, the number of dendritic intersections initially increased with distance from the cell body to reach a maximum at 45 μm from the cell body, which is indicative of dendritic branching over this distance. Beyond this distance, there was a gradual decrease in the

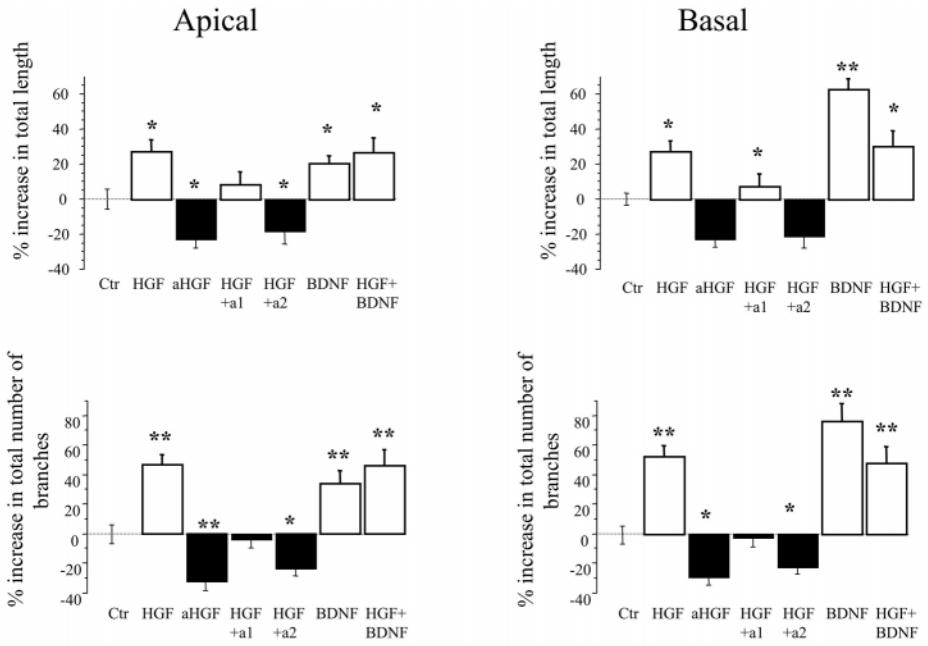

Fig. 4. Quantitative analysis of the effect of different treatments on the apical and basal compartment of dendritic arbors. Cortical slices were grown under control conditions (Ctr), or in medium supplemented with 200 ng/ml HGF (HGF), 1 μg/ml anti-HGF (aHGF), 200 ng/ml HGF plus 1 μg/ml anti-HGF pre-incubated for 1 hour before addition to the cultures (HGF+a1), 200 ng/ml HGF plus 3 μg/ml anti-HGF pre-incubated for 1 hour before addition to the cultures (HGF+a2), 200 ng/ml BDNF (BDNF) or HGF+BDNF. The bar charts illustrate percent change on either dendrite length or number of branches relative to untreated controls. **P*<0.05, ***P*<0.01. Between 50 and 60 individual neurons were analysed under each condition.

number of intersections with distance, reaching an average of a single process at a distance of 165 μm in control cultures. Figure 5A,B shows that there were significantly more intersections at almost all circles in cultures supplemented with HGF or BDNF compared with control cultures. The increase in number of dendrite intersections between 15 μm and 45 μm was greater in HGF- and BDNF-supplemented cultures compared with control cultures (8 versus 5), but with greater distances this difference between trophic factor-supplemented cultures and control cultures became much less pronounced, suggesting that the enhanced dendritic branching observed in HGF- and BDNF-supplemented cultures is restricted mainly to the proximal regions of the dendritic arbors of cortical neurons. A similar trend was observed in cultures supplemented with HGF plus BDNF, although no additive effect was apparent at any radial distance (Fig. 5C). Anti-HGF treatment resulted in a statistically significant reduction in the overall number of intersections at almost all distances from the soma (Fig. 5D), consistent with a role of endogenous HGF in promoting dendrite branching.

To determine whether the differences in dendritic morphology after HGF or anti-HGF treatment reflected differences in the rate of growth of dendritic processes during normal dendritic maturation we followed the growth of the same neurons at intervals in culture. Slice cultures were treated with either 200 ng/ml HGF or 1 μg/ml anti-HGF function-blocking antibodies; control cultures did not receive any treatment. In these experiments, 10 neurons per condition were each scanned after 18 and 42 hours incubation. Figure 6A shows images of representative of the same neurons at each of these time points in cultures maintained under the different experimental conditions. Clear differences in the rate of change

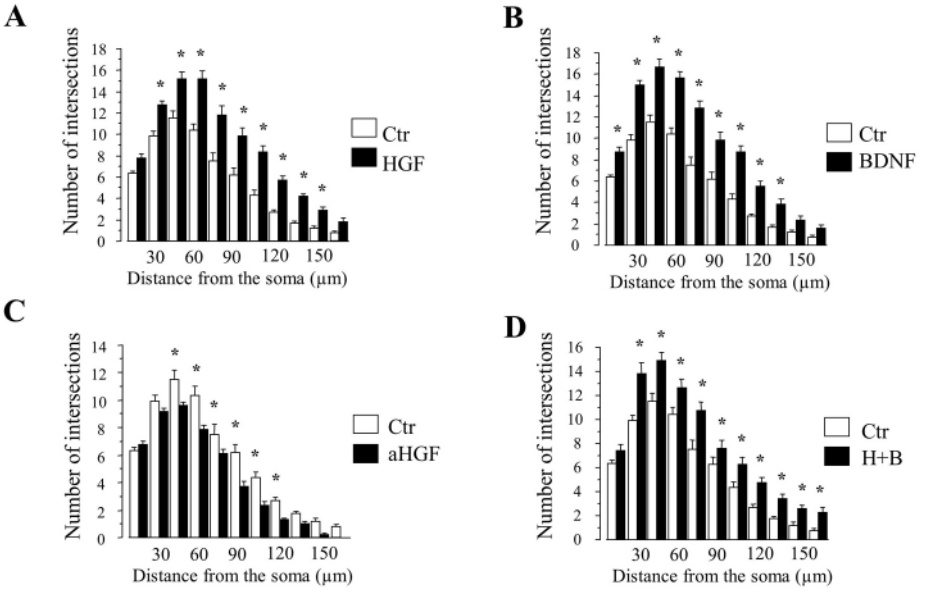

Fig. 5. Sholl analysis of dendritic morphology. The number of dendrite intersections on concentric rings spaced at 15 μm intervals from the cell soma are shown for neurons grown under control conditions compared with cultures grown with 200 ng/ml HGF (A), 200 ng/ml BDNF (B), 1 μg/ml anti-HGF (C) and HGF plus BDNF (D). Between 50 and 60 individual neurons were analysed under each condition.

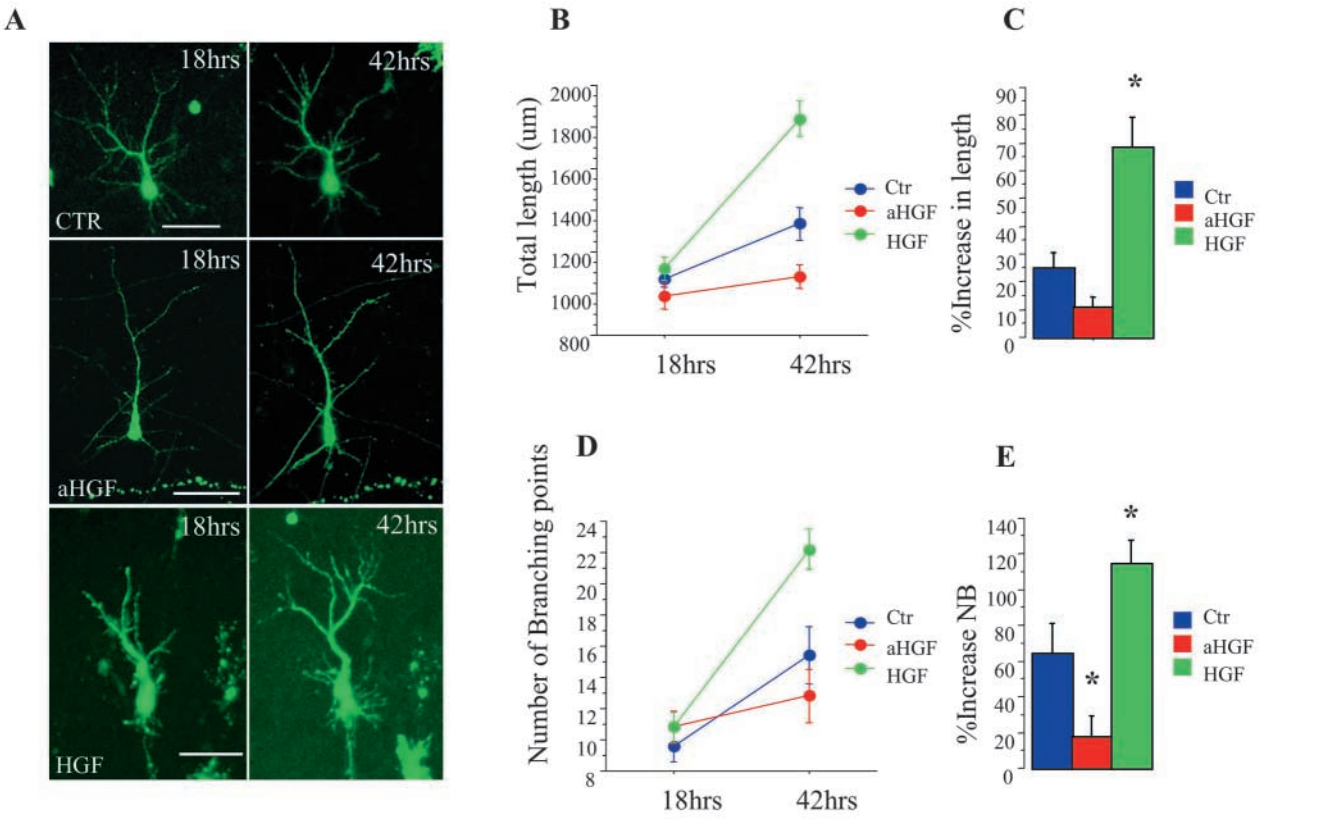

Fig. 6. Effects of HGF and anti-HGF treatment on the growth rate of the same layer 2 pyramidal neurons imaged at two time points in culture. Cultures were treated with either 200 ng/ml HGF or 1 μg/ml anti-HGF function-blocking antibodies. A number of sampled neurons per condition were each scanned at two different time points: 18 and 42 hours of incubation in either treatment. Control cultures did not receive any treatment. (A) Pictures of representative cells taken from each condition at each time point. Within this time frame an apparent difference in the rate of change of both the total dendritic length and number of branching points was observed (5B,D, respectively). (C,E) The differences in the actual rate expressed as percentage of increase in length and number of branching points, respectively, with respect to the measurements taken at 18 hours (lgt $F(2,27)=15.86$, $P<0.0001$; NB $F(2,27)=11.51$, $P=0.0002$).

of both the total dendritic length and number of branching points were observed within this time frame (Figs 6B,D, respectively). Figure 6C,E shows these differences expressed as percentage of increase in length and number of branching points by 42 hours with respect to the measurements taken at 18 hours ($F(2,27)=15.86$ and 11.51; $P<0.0001$ and $P<0.0002$, respectively). HGF treatment resulted in a significantly faster increase in both morphological indexes relative to control cells. By contrast, anti-HGF treatment led to a significant reduction in the rate of change in the number of branching points relative to controls and a trend toward reduction in total dendritic length. Taken together, these results suggest that the opposing effects of exogenous HGF or endogenous HGF blockade on dendritic morphology result from a change in the growth rate and branching of the developing dendritic arbors.

HGF acts directly on pyramidal neurons

Although blockade of endogenous HGF leads to a significant reduction in the overall size of the dendritic arbor of layer 2 pyramidal neurons, the question remains as to whether endogenous HGF exerts its effect on dendrite growth by acting directly on these neurons or influences dendritic growth as an indirect consequence of its effect on some other cell type. To address this question, we examined the consequences of expressing a kinase-dead MET receptor (MET KD) in layer 2 pyramidal neurons that acts as a dominant-negative receptor, selectively impairing MET signaling in these neurons and no other cell types. This MET KD receptor possesses the equivalent aspartic acid to asparagine point mutation present in the W42 c-kit mutation that abolishes kinase activity and acts as a dominant-negative in c-kit signal transduction (Tan et al., 1990). Kinase assays have demonstrated that the mutated MET receptor is likewise kinase dead (Flavio Maina, personal communication).

Slice cultures were bombarded with gold particles coated with the GFP-expressing plasmid plus either an MET KD-expressing vector (gift of Flavio Maina) or an empty control vector. Figure 7 shows a representative sample across the entire range of dendritic morphologies of layer 2 pyramidal neurons expressing the MET KD receptor or transfected with the control vector. These images illustrate a clear decrease in the size and complexity of pyramidal neuron dendritic arbors in neurons expressing the MET KD receptor. Quantification of total dendritic length and total branch number (Table 2) revealed highly statistically significant reductions in MET KD-expressing neurons compared with control transfected neurons ($P<0.001$). The kinase-dead receptor also significantly reversed the growth-promoting effects of exogenous HGF (Table 2).

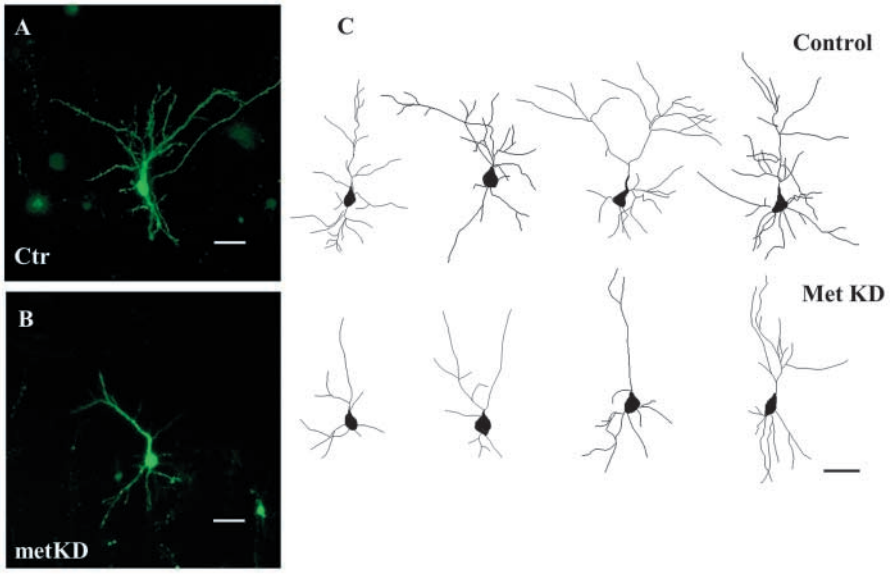

Fig. 7. Representative confocal images of GFP expressing layer 2 pyramidal neurons 48 hours after biolistic co-transfection with either an empty vector (A) or the MET KD vector (B). (C) A representative sample of reconstructed control transfected neurons (top row) and MET KD-transfected neurons (bottom row) corresponding to percentiles 25, 50, 75 and 100 of the sampled populations in terms of total dendritic length. Scale bar: 50 μm.

Quantitative analysis of the effect of MET KD on the apical and basal compartments (Fig. 8) revealed highly significant reductions in length and branch number in both compartments compared with control transfected neurons. This result was observed with and without HGF in the culture medium. However, the reduction in length and branching observed in MET KD-expressing neurons in the presence of exogenous HGF did not reach the same level observed in MET KD-expressing neurons grown without HGF. This is probably due to some residual MET signaling via normal MET receptors that cannot be fully blocked by the co-expressed dominant-negative MET receptor in the presence of an experimental excess of HGF. The overall reduction in the size and complexity of the dendritic arbors of layer 2 pyramidal neurons expressing the kinase dead MET receptor was further confirmed by Sholl analysis (Fig. 9). Taken together, these results further confirm that endogenous HGF plays a key role in regulating the normal growth and morphology of layer 2 cortical pyramidal neuron dendrites, and demonstrate that HGF exerts its effects by a direct action on MET receptors expressed on these neurons.

Table 2. Total length and number of branch points in the dendritic arbors of pyramidal neurons transfected with Met KD plasmid or control plasmid

	Total length (μm)	Total number of branches
Control	1006.6±47.7	18.6±1.1
Met KD	598.2±40.2**	11.9±0.8**
Control + HGF	1263.5±65.1**	27.0±1.7**
Met KD + HGF	855.1±58.2***	15.9±1.2***

Mean values±s.e.m. of total dendritic length (expressed in μm) and total number of branches. Statistical significance was tested by simple ANOVA followed by Fisher post-hoc analysis. Experimental conditions indicated are: control (neurons transfected with empty plasmid), Met KD (neurons transfected with the Met KD plasmid), control + HGF (control transfected neurons in cultures supplemented with 200 ng/ml HGF) and Met KD + HGF (neurons expressing Met KD in cultures supplemented with 200 ng/ml HGF). **P<0.001 versus control. ***P<0.001 between control + HGF and Met KD + HGF.

Discussion

We have used particle-mediated gene transfer in organotypic slice cultures of the somatosensory cortex of postnatal mice to study the effects of HGF on the growth and morphology of layer 2 pyramidal neurons. Whereas treatment with exogenous HGF enhanced dendritic length and branching, inactivation of endogenous HGF with a function-blocking anti-HGF antibody reduced the size and complexity of the dendritic arbors compared with untreated controls. Furthermore, layer 2 neurons transfected with the kinase-dead MET receptor also exhibited markedly smaller and less complex dendritic arbors. These findings indicate that HGF plays a role in stimulating dendritic growth and complexity in the cerebral cortex during an early critical period of development when extensive synaptogenesis is taking place.

Several other neurotrophic factors have been shown to affect the growth and complexity of the pyramidal neuron dendritic arbors in cortical slice cultures, including BDNF, NT-3, NGF, NT4 and IGF (Horch et al., 1999; McAllister et al., 1995, 1997; Niblock et al., 2000). Because the TrkB ligands BDNF, NT3 and NT4 have been shown to have a particularly pronounced effect on dendritic growth in the developing cerebral cortex (McAllister et al., 1995), we undertook a direct comparison of the effects of exogenous BDNF and HGF on the growth of layer 2 pyramidal neuron dendrites. Although both factors were found to promote similar overall increases in dendritic length and branching, analysis of the effects of these factors in the basal and apical dendritic compartments separately revealed that they display clear differences in their effects on dendritic morphology. Whereas HGF promotes a generalized increase in growth and branching, affecting the apical and basal compartments equally, BDNF has a more pronounced effect on growth and branching of basal dendrites. In addition, BDNF promotes a modest, though significant, increase in the total number of primary dendrites. In a study of layer 2 pyramidal neurons in somatosensory cortical slices of postnatal day 10 rat pups, BDNF was also found to have a marked effect on the growth and branching of basal dendrites (Niblock et al., 2000),

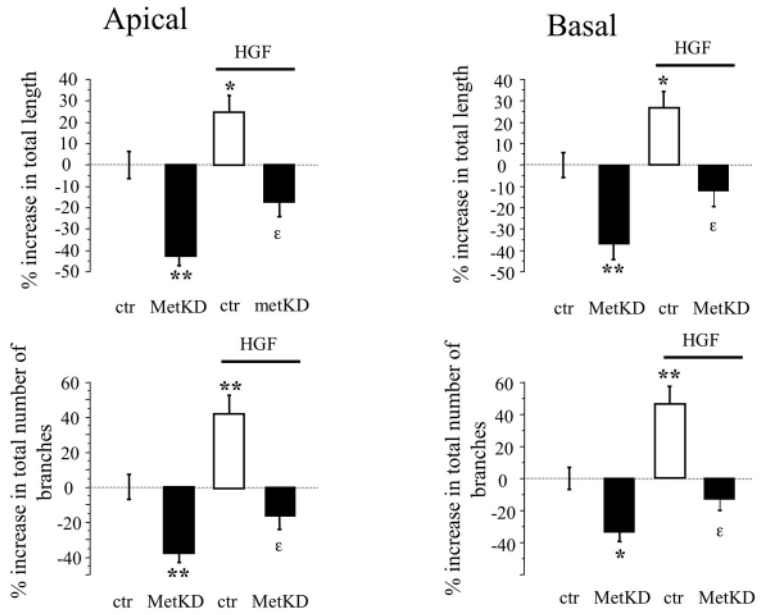

Fig. 8. Quantitative analysis of the effect of MET KD transfection on the apical and basal compartment of dendritic arbors. Cortical slices were bombarded with gold particles carrying a combination of either pEGFP and a control empty vector (Ctr) or pEGFP and a vector expressing the kinase-dead MET receptor (METKD). We additionally tested the effect of the dominant negative transfection on cultures supplemented with 200 ng/ml HGF (HGF). The bar charts illustrate percent change in dendrite length and branch number relative to control-transfected neurons. **P*<0.05, ***P*<0.01 versus control transfections; ε *P*<0.001 post-hoc significance between control and MET KD transfections in HGF-treated cultures. Between 40 and 50 individual neurons were analysed under each condition.

but in contrast to our study, no effect of BDNF on apical dendrites was observed. Also, in this study, the number of primary dendrites of layer 2 neurons was unaffected by BDNF. The differences in the effects of BDNF on layer 2 pyramidal dendrites in this study of the postnatal rat cortex and our study of the postnatal mouse cortex may be because our study was undertaken at an earlier stage in development when primary dendrites are still sprouting; it is possible that some aspects of dendritic architecture, such as the number of primary dendrites, are still plastic and responsive to extracellular signals early in the differentiation of cortical neurons. Like the generalized effect of HGF on dendritic growth and branching observed in the present work, IGF was reported to promote similar increases in dendritic growth and branching in both apical and basal compartments of layer 2 pyramidal neurons in the postnatal rat somatosensory cortex (Niblock et al., 2000). Our finding that there is no additional overall dendritic growth and branching in cultures treated with HGF and BDNF in combination compared with cultures treated with either factor alone suggests that these factors exert their actions on the same subset of pyramidal neurons. Taken together, these findings indicate that HGF and BDNF can influence the growth and morphology of layer 2 pyramidal neurons of the developing somatosensory cortex in distinctive ways. Our finding that anti-HGF suppresses dendritic growth and branching to a similar extent in the apical and basal dendritic compartments suggests that endogenous HGF exerts a similar influence on the dendritic arbors of these neurons.

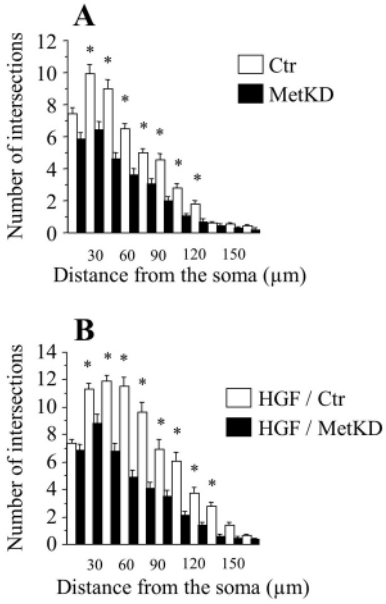

Fig. 9. Sholl analysis of dendritic morphology of MET KD-transfected pyramidal neurons. (A) Number of dendrite intersections on concentric rings spaced at 15 μm intervals from the cell soma for neurons transfected with the control vector compared with cells transfected with the MET KD vector. (B) The same analysis carried out on cultures grown in the presence of 200 ng/ml HGF. **P*<0.05, ***P*<0.001 in post-hoc comparisons between control and MET KD transfections at each ring.

The observed differences in the response of layer 2 pyramidal neurons to HGF and BDNF in terms of numbers of primary dendrites and morphological changes in apical and basal dendritic compartments suggests a specific role for HGF in particular aspects of dendritic development and function. However, it is also possible that HGF only participates in the general support of dendritic growth instead of being specifically involved in the fine-tuning of dendritic architecture. Further work comparing the responses to HGF and those triggered by neurotrophins and their combinations, as well as comparisons across different cortical layers, will be needed to address this issue. In addition to promoting dendrite growth, neurotrophins can also exert negative effects on the size and complexity of dendritic arbors, and the same factors can exert opposing actions in different cortical layers. For example, in layer 4 of the developing ferret visual cortex BDNF stimulates growth of pyramidal dendrites that is inhibited by NT3, whereas in layer 6 NT3 stimulates growth

that is inhibited by BDNF (McAllister et al., 1997). In future studies it will be of interest to ascertain whether HGF has markedly different effects on neurons in different cortical layers, including negative effects on dendritic growth.

During development of the cerebral cortex, both HGF and MET are expressed as early as E14 and continue to be expressed as the cortical plate matures and thickens. HGF expression seems to be more intense in the ventral aspects of the early cortical plate, whereas MET concentrates more in the outermost layers, a pattern of expression that continues into the postnatal period (Achim et al., 1997; Jung et al., 1994; Thewke and Seeds, 1999). In the adult cortex, neuronal expression of both HGF and MET has been observed in a layer-specific manner: HGF is expressed in layers 4 and 5, and MET in layers 2, 3, 4 and 5 (Thewke and Seeds, 1999), suggesting that cortical neurons are able to respond to endogenous HGF signaling. Here, we observed a morphological change in pyramidal neurons by manipulating the endogenous availability of HGF, suggesting a possible direct modulatory role for HGF on dendritic growth. One possibility is that layer 2 pyramidal neurons may be exposed to HGF synthesized by neurons with cell bodies that reside in deeper cortical layers by several possible routes in vivo. Anterograde axonal transport and release of neurotrophins is now well established (reviewed by Davies, 2003), although it is as yet unknown whether HGF can be transported and released in this manner. In addition to axonally derived HGF, layer 2 pyramidal neurons could potentially obtain HGF released by the apical dendrites of pyramidal neurons with cell bodies that reside deeper in the cortex, or could potentially respond to endogenous HGF diffusing from deeper cortical layers. An alternative interpretation is that the regulatory influence of HGF and anti-HGF on dendrite branching reported here could be indirect, either by influencing the release of other factors that act directly on pyramidal dendrites or by influencing the number of presynaptic inputs that are well-characterized regulators of dendrite growth and branching. Studies of pyramidal neurons transfected with a kinase-dead, dominant-negative MET receptor showed that the influence of HGF on dendritic morphology is the result of a direct response of layer 2 pyramidal neurons to endogenous HGF, demonstrating that HGF directly regulates the dendritic morphology of these neurons. By contrast to our result, dendrites of dissociated E18 cortical neurons have been observed not to respond to exogenous addition of HGF (Whitford et al., 2002). Although the experimental model used in the present study corresponds to a later stage of development, it could also mean that a more complex set of regional interactions in the intact cortex is needed for HGF to exert its normal influence on dendritic development.

HGF has been shown to enhance the growth and branching of neuritic processes of NGF-dependent sensory and sympathetic neurons in the developing peripheral nervous system (Maina et al., 1998; Maina et al., 1997; Yang et al., 1998), a response that requires the presence of NGF. In our current study we have provided evidence for a new role for HGF: promoting dendritic growth and branching in the central nervous system. Whether this response also requires the cooperation of another neurotrophic factor remains to be ascertained. Interestingly, it has been shown that cortical slices need to be electrically active in order to respond to the growth-promoting effects of BDNF (McAllister et al., 1996). It will be important to ascertain in future work whether the modulatory effects of HGF in the cortex also require neural activity. Studies of neurons of transgenic mice in which the multifunctional docking sites of the MET receptor that bind phosphatidylinositol-3 kinase (PI3K), SRC, GRB2 and GAB1 have been converted into optimal binding motifs for either PI3K, SRC, or GRB2 have shown that the neurite growth-promoting effects of HGF are dependent on binding and activation of PI3K (Maina et al., 2001). Whether this is the case for the effects of HGF on dendritic growth in the cerebral cortex remains to be ascertained, as the use of these mutants is hampered by their failure to survive until birth.

The present findings demonstrate that HGF plays a role in regulating the morphology of cortical pyramidal dendrites in the early postnatal period and that endogenous levels of HGF are necessary for the normal development of these neurons. Our findings provide further support for the notion that HGF enhances neural maturation throughout the entire neuraxis.

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