MET signaling in GABAergic neuronal precursors of the medial ganglionic eminence restricts GDNF activity in cells that express GFRα1 and a new transmembrane receptor partner

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Summary

GDNF (glial cell line-derived neurotrophic factor) promotes the differentiation and migration of GABAergic neuronal precursors of the medial ganglionic eminence (MGE). These functions are dependent on the GPI-anchored receptor GFRα1, but independent of its two known transmembrane receptor partners RET and NCAM. Here we show that soluble GFRα1 is also able to promote differentiation and migration of GABAergic MGE neurons. These activities require endogenous production of GDNF. Although GDNF responsiveness is abolished in *Gfra1*$^{-/-}$ neurons, it can be restored upon addition of soluble GFRα1, a result that is only compatible with the existence of a previously unknown transmembrane signaling partner for the GDNF-GFRα1 complex in GABAergic neurons. The roles of two candidate transmembrane receptors previously implicated in GABAergic interneuron development – MET, a receptor for hepatocyte growth factor (HGF), and ErbB4, the neuregulin receptor – were examined. GDNF did not induce the activation of either receptor, nor did inhibition of MET or ErbB4 impair GDNF activity in GABAergic MGE neurons. Unexpectedly, however, inhibition of MET or HGF per se promoted neuronal differentiation and migration and enhanced the activity of GDNF on MGE neurons. These effects were dependent on endogenous GDNF and GFRα1, suggesting that MET signaling negatively regulates GDNF activity in the MGE. In agreement with this, *Met* mutant MGE neurons showed enhanced responses to GDNF and inhibition of MET or HGF increased *Gfra1* mRNA expression in MGE cells. In vivo, expression of MET and GFRα1 overlapped in the MGE, and a loss-of-function mutation in *Met* increased *Gfra1* expression in this region. Together, these observations demonstrate the existence of a novel transmembrane receptor partner for the GDNF–GFRα1 complex and uncover an unexpected interplay between GDNF–GFRα1 and HGF–MET signaling in the early diversification of cortical GABAergic interneuron subtypes.

Key words: MGE, GABAergic neuron, GFRα1, GDNF, Neurite outgrowth

Introduction

The inhibitory (GABAergic) interneurons of the cerebral cortex are born in transient neurogenic zones located in the ventral telencephalon known as the ganglionic eminences (Corbin et al., 2001; Levitt et al., 2004; Marín and Rubenstein, 2003; Wonders and Anderson, 2006). Both the medial (MGE) and caudal (CGE) ganglionic eminences are thought to contribute GABAergic neurons to the cortex and hippocampus. The lateral ganglionic eminence (LGE) produces interneurons destined to colonize the olfactory bulb. In mice, postmitotic GABAergic neuron precursors leave the mantle zone of the eminences between embryonic day 12.5 (E12.5) and E15.5 and migrate tangentially to the cortex. However, the mechanisms that promote and guide this migration and determine the allocation of interneurons to different cortical areas are not understood. Mature cortical interneurons exhibit a wide variety of molecular, morphological and electrophysiological properties. This functional heterogeneity manifests during their postnatal maturation in the cortex. To which extent this diversity is already encoded in the precursors generated in the MGE and CGE remains an open question. Combinatorial codes of transcription factors have been found to delineate different progenitor domains in the subventricular zone of the MGE (Flames et al., 2007; Fogarty et al., 2007; Liodis et al., 2007; Miyoshi et al., 2007). However, such domains can account for only a small fraction of the diversity that is known to be present among mature cortical interneurons.

Several extrinsic cues have been found to influence the development of GABAergic interneurons, including neurotrophins, hepatocyte growth factor (HGF), semaphorins and neuregulin-1 (NRG-1) (Flames et al., 2004; Hirschberg et al., 2010; Marín et al., 2001; Polleux et al., 2002; Powell et al., 2001). Whereas semaphorins restrict the migration of MGE-derived interneurons, NRG-1 has been found to function as a cortex-derived chemoattractant by activation of the ErbB4 receptor in MGE-derived neurons. On the other hand, the specific roles of neurotrophins and HGF in the MGE are less clear. We found in previous work that the neurotrophic factor GDNF and its receptor GFRα1 promote the differentiation and migration of immature GABAergic interneurons derived from the MGE (Pozas and Ibáñez,

2005). GFRα1 is expressed in a subset of postmitotic GABAergic precursors located in the ventro-medial portion of the MGE (Pozas and Ibáñez, 2005). Neurons isolated from the MGE of mutant mice lacking GFRα1 do not respond to GDNF (Pozas and Ibáñez, 2005), indicating the essential requirement of this receptor subunit. Using a mutant mouse line that circumvents the lethality of the null mutation of *Gfra1*, herein referred to as 'cis-only mice', we have recently found that GFRα1 is required for the correct allocation of parvalbumin (PV) expressing interneurons in the cortex (Canty et al., 2009). Cis-only mice displayed a striking loss of PV interneurons in discrete regions of the cerebral cortex (PV holes) interspersed amongst areas of normal density of PV-positive cells (Canty et al., 2009). Since mature PV interneurons do not express GFRα1, GFRα1 may be downregulated during development in GABAergic MGE precursors and/or function non-cell-autonomously. By guiding the development of a subset of PV-expressing interneurons populating discrete cortical regions, GFRα1 contributes to the diversification and allocation of distinct cortical interneuron subtypes.

GFRα1 is a GPI-anchored receptor and so lacks transmembrane and intracellular domains. Transmission of the GDNF signal is mediated by the association of GFRα1 with signaling receptor subunits such as the RET tyrosine kinase or the NCAM-140 isoform of the neural cell adhesion molecule NCAM (Airaksinen and Saarma, 2002; Paratcha et al., 2003). GFRα1 is also released from cells and able to function in soluble form by binding GDNF and activating RET receptors on responding cells (Ibáñez, 2010; Ledda et al., 2002; Paratcha et al., 2001). Intriguingly, neither RET nor NCAM are required for the effects of GDNF on migration or differentiation in GABAergic interneurons (Pozas and Ibáñez, 2005), indicating that either GFRα1 can somehow mediate GDNF signaling on its own, or else other transmembrane effectors may partner with GFRα1 to mediate the effects of GDNF on these cells. Ligand-mediated aggregation of GPI-anchored receptors may in principle lead to clustering of membrane microdomains and activation of intracellular signaling in the absence of transmembrane receptors. Many GPI-anchored receptors are able to function in soluble form together with ligand, but this mode of action strictly depends on their interaction with transmembrane receptor partners.

In this study, we found that soluble GFRα1 is biologically active in MGE GABAergic neurons, suggesting the existence of a previously unknown transmembrane receptor partner on these cells. We tested the roles of two candidate molecules previously implicated in GABAergic interneuron development – MET and ErbB4, the tyrosine kinase receptors for HGF and NRG-1, respectively. To our surprise, we found that HGF and MET negatively regulate GDNF responses in MGE cells, suggesting an unexpected interplay between GDNF–GFRα1 and HGF–MET signaling in MGE-derived GABAergic interneurons.

Results

Soluble GFR 1 stimulates differentiation and migration of GABAergic MGE cells

We investigated the biological activities of GDNF and soluble GFRα1 in dissociated cultures of the mouse E12.5 MGE. After 48 hours in culture under control conditions, 43.9±0.6% of the total number of cells were GABAergic. This proportion increased to 57.6±3.4% (a 30% increase; $P<0.01$) in the presence of GDNF, in agreement with earlier results (Pozas and Ibáñez, 2005). The fact that the total number of cells in culture remained constant suggests that GDNF affects the GABAergic differentiation of MGE precursors rather than their survival or proliferation, which also agrees with our earlier observations (Pozas and Ibáñez, 2005). To assess neurite outgrowth in GABAergic MGE neurons, MGE cultures were fixed, stained with anti-GABA antibodies and the proportion of cells displaying neurites longer than twice the cell diameter was determined (Fig. 1A). GDNF induced neurite outgrowth in 14.0±0.7% of GABAergic neurons (8.1±0.4% of all cells in the culture).

The effect of soluble, exogenous GFRα1 was tested using a purified chimeric protein of the GFRα1 ectodomain (minus GPI-anchoring sequences) fused to the Fc domain of a human immunoglobulin (GFRα1–Fc). This treatment stimulated robust neurite outgrowth in GABAergic MGE cells although at a lower level than that elicited by GDNF (Fig. 1B). The combined effects of GDNF and GFRα1–Fc showed some enhancement over single treatments but were not strictly additive. The activity of GFRα1–Fc was also tested in a migration assay by using dissociated MGE cells plated on Boyden chambers. After 2 days in culture with the tested substance in the lower chamber, cells remaining on the top of the filter were scraped away and migratory cells on the lower part were stained with DAPI for imaging and quantification (Fig. 1C). GDNF displayed a strong chemoattractive effect on dissociated cells from the E12.5 MGE but not on cells derived from the CGE or LGE (Fig. 1D), which is in agreement with the expression pattern of GFRα1 in the ganglionic eminences. The effect of GDNF on cell migration was only evident when the factor was added to the lower compartment of the chamber (Fig. 1E). The fact that GDNF had no effect when added to the upper compartment, or to both compartments, indicates that it functions as a chemoattractant rather than a generic cell motility factor. Interestingly, GFRα1–Fc was also able to stimulate migration of MGE cells although to a lesser extent than GDNF (Fig. 1F).

The effects of exogenous GFRα1 require endogenous production of GDNF

Both GDNF and GFRα1 are endogenously expressed by MGE cells (Pozas and Ibáñez, 2005), and so we tested whether the effects of exogenous GFRα1 required endogenous GDNF expression. For this purpose, we established cultures derived from wild type and *Gdnf* knockout (*Gdnf*$^{-/-}$) mouse embryos and tested the ability of GFRα1–Fc to induce differentiation and migration in E12.5 MGE cells. Although GDNF was equally active in wild type and mutant cells, GFRα1–Fc did not induce any noticeable biological effects in mutant cells (Fig. 2A and B). This defect could be rescued by addition of GDNF together with GFRα1–Fc. These data indicate that the effects of exogenous GFRα1 in MGE cells require endogenous production of GDNF.

Evidence for the existence of a new transmembrane receptor mediating the effects of GDNF and GFRα1 in GABAergic neurons of the MGE

We next investigated whether the effects of exogenous GFRα1 required endogenous production of this receptor by MGE cells using cultures derived from *Gfra1* knock–out (*Gfra1*$^{-/-}$) mouse embryos. In agreement with previous results (Pozas and Ibáñez, 2005), GDNF was unable to induce differentiation or migration of GABAergic MGE cells lacking GFRα1 (Fig. 2C and D). By contrast, GFRα1–Fc was equally active in wild type and mutant cells. Importantly, the activity of GDNF in *Gfra1*$^{-/-}$ MGE cells could be rescued upon addition of GFRα1–Fc (Fig. 2C and D). Thus, both components of the GDNF–GFRα1 complex can per se

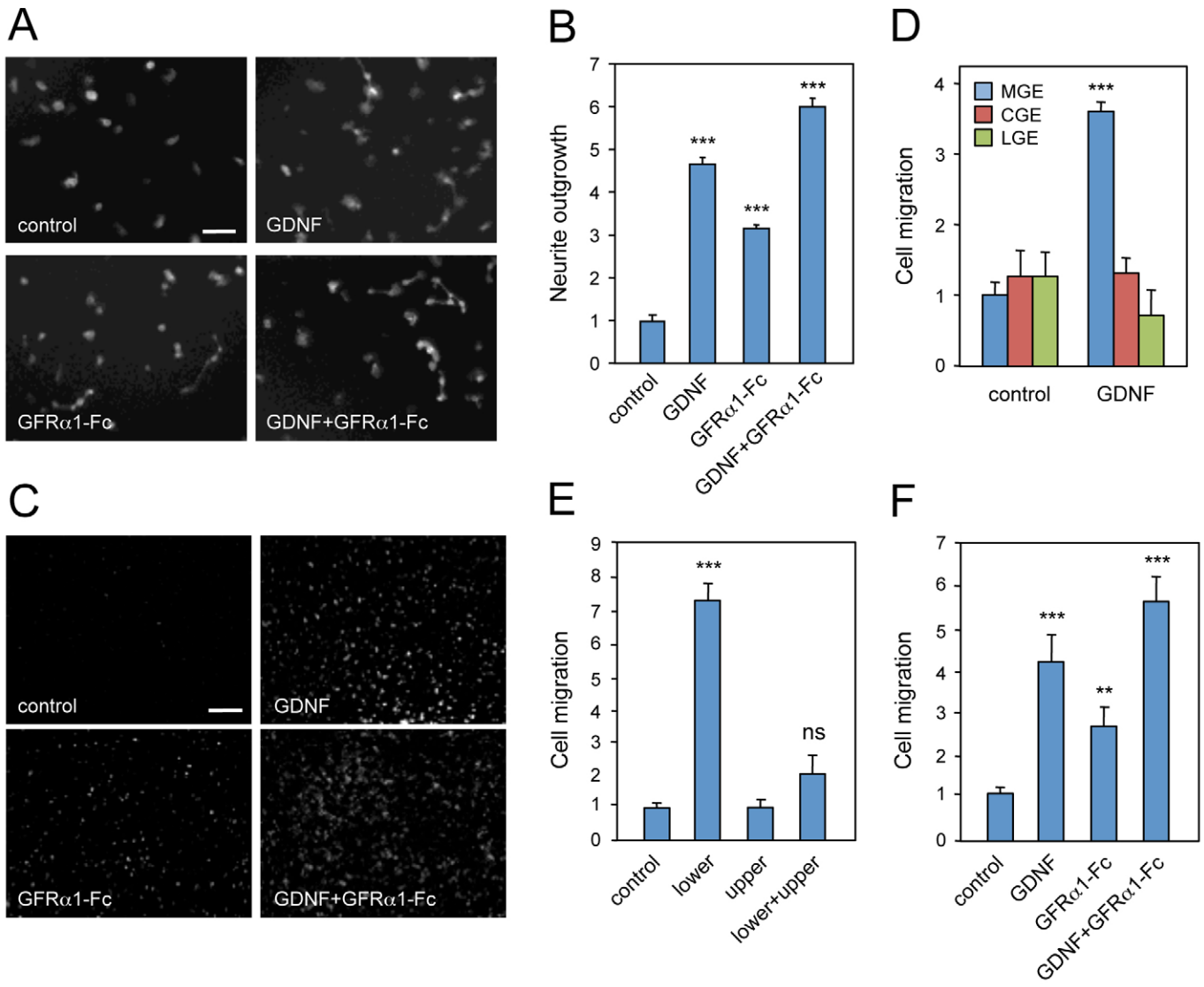

Fig. 1. Both GDNF and soluble GFRα1 can induce neurite outgrowth and migration of cultured MGE GABAergic neurons. (**A**) Micrographs of MGE neurons stained with anti-GABA antibodies grown for 48h under the indicated conditions. Scale bar, 25 µm. (**B**) Neurite outgrowth of MGE GABAergic neurons can be induced by both GDNF and exogenous soluble GFRα1 (GFRα1-Fc). Histogram shows average ± s.e.m. of three experiments each performed in triplicate. ***P<0.001 vs control. (**C**) Micrographs of migrating MGE neurons stained with DAPI grown for 48 hours under the indicated conditions. Scale bar, 100 µm. (**D**) Cell migration in Boyden chamber assays of GABAergic neurons from the MGE, LGE and CGE induced by GDNF. Histogram shows average ± s.d. of quadruplicate determinations. ***P<0.001 vs control. (**E**) Migration of MGE cells in Boyden chambers after application of GDNF to the lower, upper or both chamber compartments. Histogram shows average ± s.e.m. of three independent experiments each performed in quintuple. ***P<0.001 vs control; ns, non significant vs control (P=0.08). (**F**) Cell migration of MGE neurons can be induced in Boyden chambers by both GDNF and exogenous soluble GFRα1 (GFRα1-Fc). Histogram shows average ± s.e.m. of three experiments each performed in triplicate. ***P<0.001; **P<0.01 vs control.

elicit biological effects in GABAergic MGE neurons but each requires the endogenous expression of its respective complex partner.

To determine whether GDNF and GFRα1 function in MGE cells together in a complex or through independent pathways, we used a point–mutant of GDNF (GDNF E61/62A) that cannot bind GFRα1 but is otherwise structurally intact and expresses at normal levels (Eketjäll et al., 1999). Wild type GDNF and E61/62A mutant were produced in the conditioned medium of transiently transfected COS cells (see Materials and Methods; Fig. 3A). Unlike the wild type protein, the mutant GDNF was unable to elicit neurite outgrowth in *Gdnf*$^{-/-}$ MGE cells (Fig. 3B), indicating that binding to GFRα1 is required for GDNF activity. Moreover, wild type, but not mutant, GDNF was able to cooperate with exogenous GFRα1–Fc in GDNF-deficient neurons (Fig. 3B). These results indicate that GDNF and GFRα1 are likely to work in a complex and not through independent receptors in MGE cells. As we had previously shown that neither RET nor NCAM are expressed at significant levels in the MGE or required for the effects of GDNF on GABAergic interneurons (Pozas and Ibáñez, 2005), the ability of a soluble GDNF–GFRα1 complex to elicit biological effects on MGE cells lacking GFRα1 can only be explained by the existence of a novel transmembrane receptor partner on these cells.

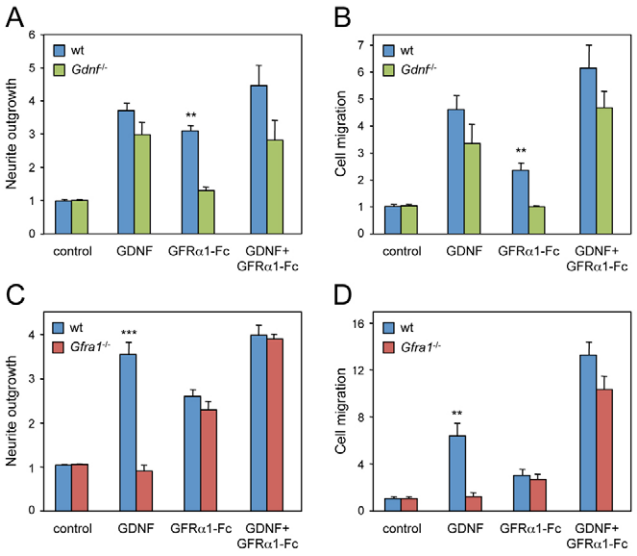

Fig. 2. The activities of GDNF and soluble GFRα1 on cultured MGE GABAergic neurons depend on endogenous expression of GFRα1 and GDNF, respectively. (**A**) Neurite outgrowth induced by GDNF, exogenous soluble GFRα1 (GFRα1-Fc) or their combination in wild type and *Gdnf*$^{-/-}$ mutant neurons. Histogram shows average ± s.d. of quadruplicate determinations. $^{**}P<0.01$ vs mutant. (**B**) Cell migration induced by GDNF, GFRα1-Fc or their combination in wild type and *Gdnf*$^{-/-}$ mutant neurons. Histogram shows average ± s.d. of quadruplicate determinations. $^{**}P<0.01$ vs mutant. (**C**) Neurite outgrowth induced by GDNF, GFRα1-Fc or their combination in wild type and *Gfra1*$^{-/-}$ mutant neurons. Histogram shows average ± s.d. of quadruplicate determinations. $^{***}P<0.001$ vs mutant. (**D**) Cell migration induced by GDNF, GFRα1-Fc or their combination in wild type and *Gfra1*$^{-/-}$ mutant neurons. Histogram shows average ± s.d. of triplicate determinations. $^{**}P<0.01$ vs mutant.

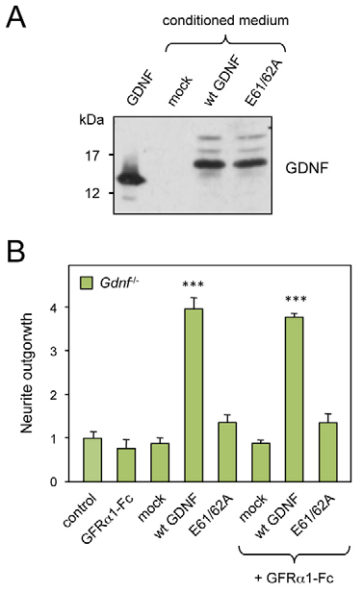

Fig. 3. A mutant GDNF deficient in GFRα1 binding can not cooperate with exogenous GFRα1 in morphological differentiation of MGE GABAergic neurons. (**A**) Immunoblot of commercially available purified GDNF (100 ng) and conditioned medium of COS cells transfected with either mock, wild type GDNF or E61/62A GDNF plasmid constructs. The blot was probed with anti-GDNF antibodies. (**B**) Neurite outgrowth induced by COS cell conditioned medium (mock, wt GDNF or E61/62A GDNF), exogenous soluble GFRα1 (GFRα1-Fc) or their combination in *Gdnf* mutant neurons. Histogram shows average of three independent experiments ± s.e.m. each performed in triplicate determinations. $^{***}P<0.001$ vs control.

Neither ErbB4 nor MET mediate the effects of GDNF and GFRα1 in MGE cells

NRG-1 promotes migration of GABAergic interneuron precursors from the MGE through the epidermal growth factor (EGF) receptor family member ErbB4 (Flames et al., 2004). The similarity of NRG-1 and GDNF activities prompted us to investigate whether ErbB4 is involved in the effects of GDNF in GABAergic MGE cells. ErbB4 is a receptor tyrosine kinase and its activation leads to tyrosine autophosphorylation. As expected, NRG-1 elicited robust ErbB4 tyrosine phosphorylation in cultures of E12.5 MGE cells. In contrast, no phosphorylation could be detected in response to GDNF at any time point tested (Fig. 4A). AG1478 is a selective pharmacological inhibitor of ErbB4. NRG-1 elicited strong phosphorylation of ERK kinases (ERKs) in cultures of MGE cells and this could be blocked by treatment with AG1478 (Fig. 4B). However, AG1478 was not able to inhibit the biological activities of GDNF in GABAergic MGE neurons, neither in morphological differentiation nor migration (Fig. 4C and D). We note that, in our hands, NRG-1 stimulated the migration of these cells to a lower extent than GDNF (Fig. 4D).

Another interesting candidate for mediating the effects of GDNF on MGE neurons is the HGF receptor MET. HGF has been reported to be a motogenic factor for neuronal precursors of the MGE (Powell et al., 2001). Like GDNF (Pozas and Ibáñez, 2005) HGF has been reported to be expressed by cells in the subventricular zone of the ganglionic eminences (Powell et al., 2001). Although the scatter and motogenic effects of HGF are well documented in a variety of cell types, its direct actions on MGE cells are less well understood. Mice lacking the urokinase-type plasminogen activator receptor (u-PAR), required for the processing of HGF, display defects in cortical GABAergic interneurons (Powell et al., 2001), but to which extent those phenotypes can be attributed to HGF signaling is not clear. Expression of the receptor tyrosine kinase MET in the ganglionic eminences has been detected at the mRNA level by in situ hybridization and RT-PCR (Powell et al., 2001). Interestingly, MET has also been shown to play a role in GDNF signaling via an indirect mechanism involving Src family kinases (Popsueva et al., 2003). We found that HGF can induce MET autophosphorylation and Erk phosphorylation in MGE cells (Fig. 5A). Although less potent than HGF, GDNF was also able to induce ERKs phosphorylation in MGE neurons (Fig. 5A), in agreement with previous results (Pozas and Ibáñez, 2005). In contrast to HGF, however, GDNF was unable to induce activation of MET under the same conditions (Fig. 5A). Phosphorylation of ERKs in response to HGF could be blocked by two different pharmacological inhibitors of Met, namely SU11274 (Fig. 5B) and PHA665752 (Fig. 5C). However, SU11274 was unable to block the effects of GDNF on neurite outgrowth in GABAergic MGE neurons (Fig. 5D). Unexpectedly, SU11274 stimulated per se neurite outgrowth in these cells and this effect was additive to the effect of GDNF (Fig. 5D). The second MET inhibitor, PHA665752, was also able to promote morphological

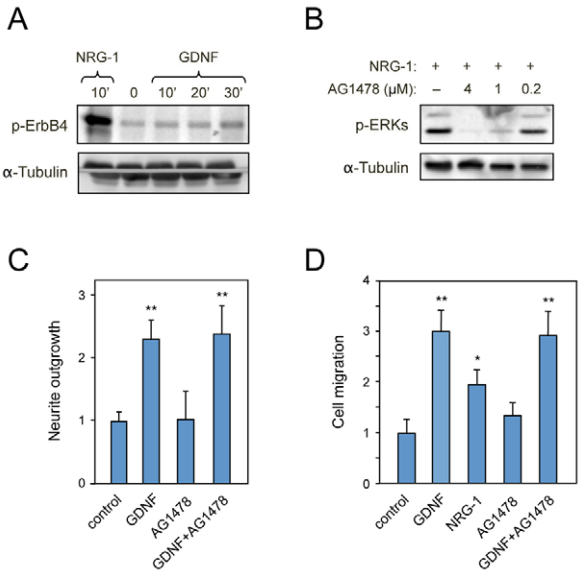

Fig. 4. The activities of GDNF on cultured MGE GABAergic neurons are not mediated by the receptor tyrosine kinase ErbB4. (**A**) Tyrosine phosphorylation of ErbB4 in response to treatment with 50 ng/ml neuregulin-1 (NRG-1) or GDNF for the indicated time periods (in minutes) analyzed by Western blotting. Reprobing with antibodies to α-tubulin is shown as loading control. (**B**) Phosphorylation or ERKs in response to NRG-1 can be blocked by the ErbB4 inhibitor AG1478. (**C**) Inhibition of ErbB4 does not interfere with the ability of GDNF to induce neurite outgrowth in MGE neurons. Histogram shows average ± s.d. of quadruplicate determinations. $^{**}P<0.01$ vs control. (**D**) Inhibition of ErbB4 does not interfere with the ability of GDNF to promote cell migration in MGE neurons. Histogram shows average ± s.d. of quadruplicate determinations. $^{**}P<0.01$; $^{*}P<0.05$ vs control.

differentiation of GABAergic MGE neurons (Fig. 5E). In summary, we conclude from these experiments that neither ErbB4 nor METcan be considered as direct mediators of the biological effects of GDNF in MGE cells. Unexpectedly, however, our studies revealed that pharmacological inhibition of MET stimulates neurite outgrowth from GABAergic MGE neurons and that this effect synergizes with that of GDNF.

HGF–MET signaling restricts the biological activities of GDNF–GFRα1 in GABAergic MGE neurons

We decided to explore further the effects of inhibition of MET signaling in MGE cells by investigating the involvement of HGF using an antibody that blocks HGF. We found that anti-HGF antibodies stimulated both morphological differentiation and cell migration of GABAergic MGE neurons (Fig. 6A and B). The magnitude of the effects of the anti-HGF antibody was comparable with that elicited by GDNF. These results indicated that endogenous HGF signaling may exert negative effects on the differentiation and migration of MGE neurons. Addition of HGF to MGE cultures had no significant effects, neither per se or in combination with GDNF, suggesting that the levels of endogenous HGF signaling normally present in MGE cells are sufficient to dampen neurite outgrowth and cell migration. Interestingly, treatment with anti-HGF antibody significantly enhanced the biological responses of MGE cells to GDNF (Fig. 6A and B). This prompted us to investigate whether the effects of anti-HGF were dependent on endogenous GDNF signaling. To this end, we used anti-GDNF blocking antibodies. In control experiments, these antibodies proved to be very effective at inhibiting the ability of exogenous GDNF to induce neurite outgrowth in GABAergic MGE neurons (Fig. 6C). We found that anti-GDNF antibodies completely cancelled the biological effects of anti-HGF on MGE neurons (Fig. 6D), suggesting that these were mediated by endogenous GDNF signaling. As a control, we verified that anti-GDNF antibodies had no effect on their own nor did they inhibit the effects of another trophic substance, brain-derived neurotrophic factor (BDNF) (Fig. 6D).

To investigate the relationship between HGF and GDNF signaling in MGE cells, we tested the effects of anti-HGF antibody in MGE cells derived from *Gfra1* mutant mice. We found that MGE neurons lacking GFRα1 were completely unresponsive to anti-HGF antibody, as they were to GDNF, both in terms of morphological differentiation as well as migration (Fig. 7A and B). This confirmed that the biological effects of anti-HGF antibody are dependent on endogenous GDNF-GFRα1 signaling and suggested that tonic levels of endogenous HGF signaling in GABAergic MGE neurons, presumably through MET, restrict the biological effects of GDNF and GFRα1 in these cells. We tested this

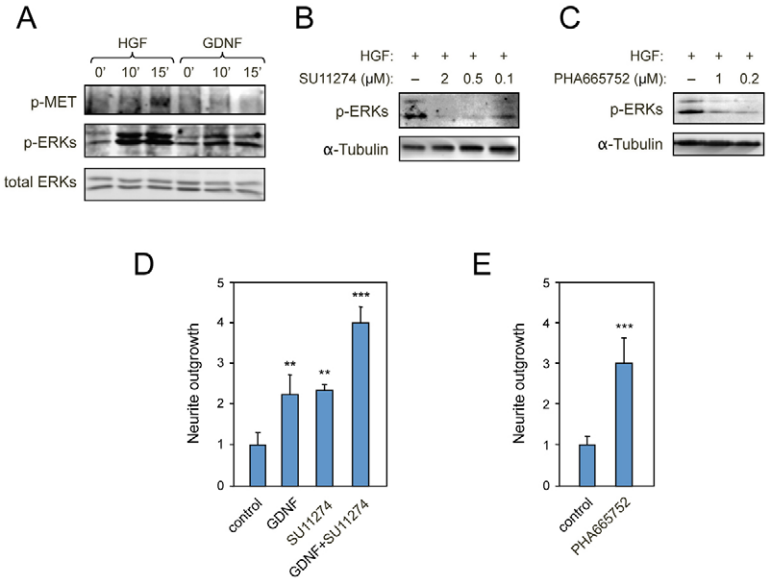

Fig. 5. The activities of GDNF on cultured MGE GABAergic neurons are not mediated by the receptor tyrosine kinase MET. (**A**) Tyrosine phosphorylation of MET in response to treatment with 50 ng/ml hepatocyte growth factor (HGF) or GDNF for the indicated time periods (in minutes) analyzed by Western blotting. Phosphorylation of ERKs by either treatment demonstrates the ability of both ligands to induce signaling events in MGE GABAergic neurons. Reprobing with antibodies to total ERKs is shown as loading control. (**B**) Phosphorylation of ERKs in response to HGF can be blocked by the MET inhibitor SU11274. Reprobing with antibodies to α-tubulin is shown as loading control. (**C**) Phosphorylation of ERKs in response to HGF can be blocked by the MET inhibitor SU11274. Reprobing with antibodies to α-tubulin is shown as loading control. (**D**) Inhibition of MET does not interfere with the ability of GDNF to induce neurite outgrowth in MGE neurons. SU11274 can promote neurite outgrowth on its own and potentiate the effects of GDNF. Histogram shows average ± s.d. of quadruplicate determinations. $^{***}P<0.001$; $^{**}P<0.01$ vs control. (**E**) PHA665752 can promote neurite outgrowth on its own in MGE GABAergic neurons. Histogram shows average ± s.d. of quadruplicate determinations. $^{***}P<0.001$ vs control.

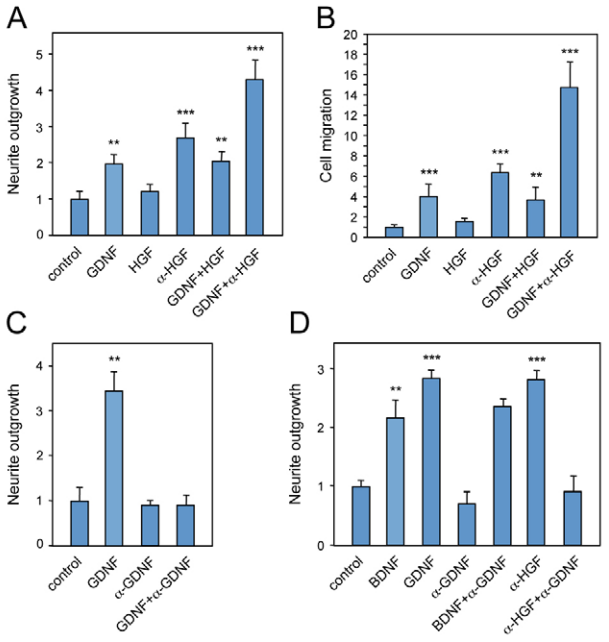

Fig. 6. Inhibition of HGF per se induces neurite outgrowth and cell migration of cultured GABAergic MGE neurons in a GDNF-dependent manner. (**A**) Treatment with antibody that blocks HGF (α-HGF) induces neurite outgrowth and synergizes with GDNF. Histogram shows average ± s.d. of quadruplicate determinations. ***P<0.001; **P<0.01 vs control. (**B**) Anti-HGF blocking antibody induces cell migration and synergizes with GDNF. Histogram shows average ± s.d. of sextuplicate determinations. ***P<0.001; **P<0.01 vs control. (**C**) Blocking anti-GDNF antibody (α-GDNF) inhibits the effects of GDNF on neurite outgrowth. Histogram shows average ± s.d. of triplicate determinations. **P<0.01 vs control. (**D**) The effects of anti-HGF antibody on neurite outgrowth can be inhibited by the anti-GDNF blocking antibody. Histogram shows average ± s.d. of triplicate determinations. ***P<0.001; **P<0.01 vs control.

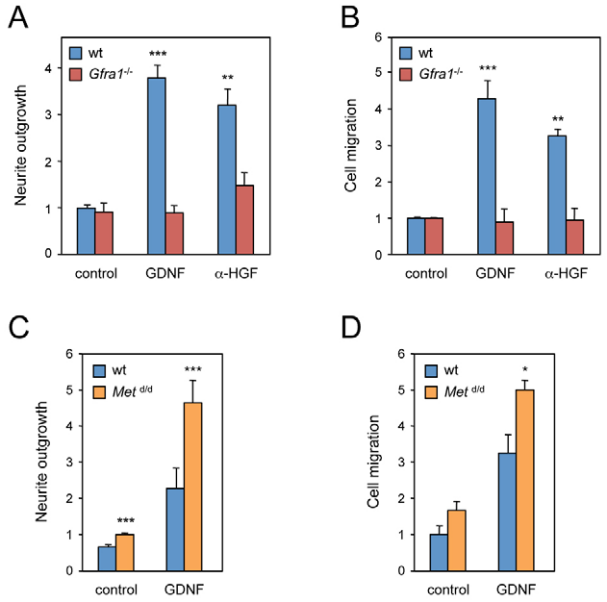

Fig. 7. Mutant GABAergic MGE neurons that lack GFRα1 are unresponsive to treatment with anti-HGF antibody and those neurons that lack MET signaling display enhanced responsiveness to GDNF. (**A**) Neurite outgrowth induced by GDNF and anti-HGF antibody (α-HGF) in wild type and *Gfra1*$^{-/-}$ mutant neurons. Histogram shows average ± s.d. of triplicate determinations. ***P<0.001; **P<0.01 vs mutant. (**B**) Cell migration induced by GDNF and anti-HGF in wild type and *Gfra1*$^{-/-}$ mutant neurons. Histogram shows average ± s.d. of triplicate determinations. ***P<0.001; **P<0.01 vs mutant. (**C**) Neurite outgrowth induced by GDNF in wild type and *MET*$^{d/d}$ mutant neurons. Histogram shows average ± s.d. of quintuple determinations. ***P<0.001 vs mutant. (**D**) Cell migration induced by GDNF in wild type and *MET*$^{d/d}$ mutant neurons. Histogram shows average ± s.d. of quintuple determinations. *P<0.05 vs mutant.

hypothesis using MGE cultures derived from *Met* mutant mice homozygous for the *Met*^{d} allele, a mutation that blunts all downstream signaling from the Met receptor and phenocopies the effects of the *Met* null mutation (Maina et al., 1996). MGE neurons derived from *Met*$^{d/d}$ mutant mice showed enhanced responses to GDNF, both in neurite outgrowth as well as migration (Fig. 7C and D). Even in the absence of GDNF, *Met*$^{d/d}$ mutant neurons grew significantly longer neurites than wild type cells, and a tendency (albeit not statistically significant) to increased migration was also observed (Fig. 7C and D). Together, these results indicate that endogenous HGF–MET signaling restricts the effects of GDNF–GFRα1 on morphological differentiation and migration of GABAergic MGE neurons.

Endogenous HGF–MET signaling represses the expression of *Gfra1* mRNA in the MGE

To clarify the mechanism by which inhibition of endogenous HGF–MET signaling potentiates the biological effects of GDNF on MGE cells, we investigated its effect on the expression of the GFRα1 receptor. First, we verified the expression of *Met* mRNA in the mouse E12.5 MGE by reverse transcriptase (RT)-PCR (Fig. 8A). We then established the expression of MET protein in the mantle zone of the mouse MGE by immunohistochemistry followed by laser confocal microscopy. This analysis indicated that MET and GFRα1 overlap in this area of the MGE (Fig. 8B). Next, we used quantitative real-time PCR (Q-PCR) to assess the level of *Gfra1* mRNA expression in cultured MGE cells. We found that dissociated MGE cells cultured for 48 hours under control conditions displayed significantly reduced levels of *Gfra1* mRNA compared to freshly dissected MGE tissue (Fig. 9A). Incubation in the presence of GDNF restored *Gfra1* mRNA expression in MGE cultures to a similar level to the one found in MGE tissue without affecting the total amount of RNA recovered (Fig. 9A), which argues against a simple survival effect. Treatment with GFRα1–Fc had also a positive effect on *Gfra1* mRNA expression in MGE cell cultures. In addition to its effect on expression of *Gfra1* mRNA, treatment with GDNF increased the expression levels of several known markers of GABAergic MGE precursors, including those of *Gad65*, *Lhx6* and *Nkx2.1* (Fig. 9B), in agreement with the effect of GDNF on GABAergic differentiation. However, GDNF had no effect on *Met* mRNA levels in MGE cells (Fig. 9B).

Interestingly, treatment with anti-HGF antibodies or the MET inhibitor SU11274 inhibitor increased *Gfra1* mRNA levels in cultured MGE cells to an extent similar to that following treatment with GDNF (Fig. 9C). As the trophic effects of HGF–MET inhibition were found to be mediated by endogenous GDNF signaling (see above), we wondered whether the effects observed on *Gfra1* mRNA expression were also dependent on GDNF or, alternatively, were a direct consequence of HGF–MET blockade.

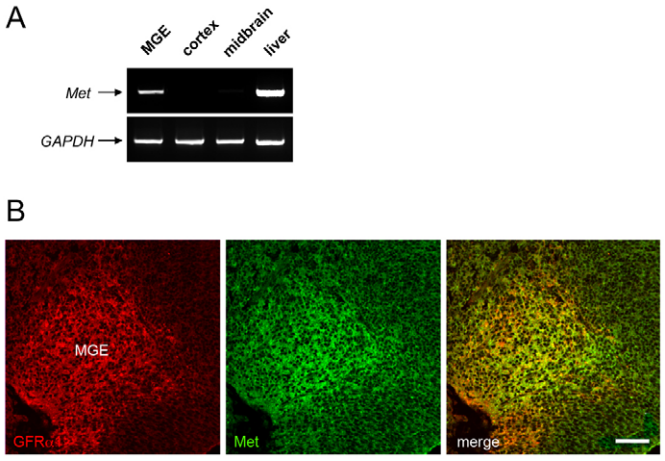

Fig. 8. Expression of MET and GFRα1 overlap in the mouse MGE. (**A**) RT-PCR analysis of *MET* mRNA expression in E12.5 MGE, cerebral cortex, midbrain and liver. Amplification of *Gapdh* mRNA was used as loading control. (**B**) Overlapped expression of GFRα1 (red) and MET(green) in the mouse E12.5 MGE visualized by immunofluorescence and confocal microscopy. Scale bar, 50 µm.

To address this, we combined anti-HGF and anti-GDNF antibody treatments and assessed the levels of *Gfra1* mRNA expression. Unlike its effect on neurite outgrowth (see Fig. 6D), anti-GDNF antibody did not impair the ability of anti-HGF to increase *Gfra1* mRNA levels in cultured MGE cells (Fig. 9D). Moreover, BDNF, which also has trophic activities on these cells (see Fig. 6D), did not affect the levels of *Gfra1* mRNA expression (Fig. 9D). Finally, we dissected E12.5 MGE from wild type and *Met*$^{d/d}$ mutant embryos and assessed the level of endogenous *Gfra1* mRNA expression by Q-PCR. We found that MET mutants displayed a two- to three-fold higher level of *Gfra1* mRNA expression in the MGE compared to that of wild type (Fig. 9E). Together, these results indicate that endogenous HGF–MET signaling represses *Gfra1* mRNA expression in the MGE, thereby restricting the biological activity of GDNF in cortical GABAergic neuron precursors.

Discussion

The GABAergic interneurons of the cerebral cortex play essential roles in information processing by regulating the function and output of excitatory neurons. Several major cognitive disorders in humans have been linked to defects in or malfunction of cortical inhibitory interneurons, including epilepsy, schizophrenia and autism (Cossart et al., 2005; Eyles et al., 2002; Rubenstein and Merzenich, 2003; Tabuchi et al., 2007). The realization that many of these disorders may have a strong developmental component has directed the focus of current research towards the understanding of those signals and mechanisms that control the development of cortical GABAergic interneurons. GDNF has emerged as an important signaling molecule guiding the differentiation and migration of GABAergic neuron precursors in the MGE (Canty et al., 2009; Pozas and Ibáñez, 2005). In addition to defects in cortical PV interneurons, adult 'cis-only' mice also show abnormal social behavior (Canty et al., 2009), suggesting a possible role for GFRα1 in human cognitive disorders. It has therefore become important to understand how GFRα1 mediates GDNF signaling in the MGE and elucidate the mechanisms that regulate the activities of this pathway.

In this study, we found that either component of the GDNF–GFRα1 complex was capable of stimulating neurite outgrowth and cell migration in MGE neurons. These effects were dependent upon endogenous production of the respective member of the complex. As neither RET or NCAM are substantially expressed in the MGE, nor required for GDNF to have an effect (Pozas and Ibáñez, 2005), the ability of *Gfra1*$^{-/-}$ MGE neurons to respond to exogenous GDNF–GFRα1 indicates the existence of a previously unknown transmembrane receptor partner in these cells. The biological assays used in this study essentially recorded numbers

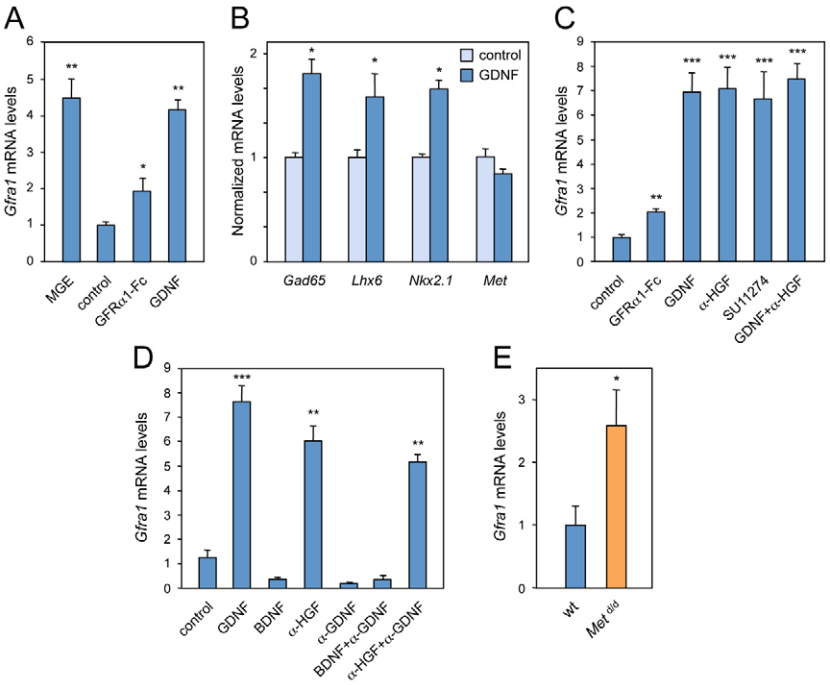

Fig. 9. Endogenous HGF-MET signaling negatively regulates *Gfra1* mRNA expression in MGE cells. (**A**) *Gfra1* mRNA expression in freshly dissected E12.5 MGE (MGE) and in dissociated MGE cells after 48 hours in culture with the indicated treatments. Results are shown as average ± s.d. of triplicate determinations. **P*<0.05; ***P*<0.01 vs control. (**B**) Expression of *Gad65, Lhx6, Nkx2.1* and *Met* mRNAs in dissociated MGE cells after 48 hours in culture in the presence and absence of GDNF. (**C**) *Gfra1* mRNA expression in dissociated MGE cells after 48 hours in culture with the indicated treatments. Results are shown as average ± s.d. of triplicate determinations. **P*<0.05; ****P*<0.001 vs control. (**D**) *Gfra1* mRNA expression in dissociated MGE cells after 48 hours in culture with the indicated treatments. Results are shown as average ± s.d. of triplicate determinations. ***P*<0.01; ****P*<0.001 vs control. (**E**) In vivo *Gfra1* mRNA expression in E12.5 MGE isolated from wild type and *Met*$^{d/d}$ mutant embryos. Histogram shows average ± s.e.m. *n*=6 (wt); 9 (*Met*$^{d/d}$). **P*<0.05 vs wt.

of GDNF-responsive cells, i.e. cells showing neurite outgrowth above a certain level or cells migrating towards the lower compartment of a Boyden chamber. The fact that the effects of GDNF and soluble GFRα1 were not strictly additive suggests that the same cell subpopulation responds to either treatment. In other words, soluble GFRα1 did not recruit any significant number of additional cells, i.e. cells that do not normally express GFRα1, to the subpopulation of GDNF–responsive cells. This suggests that the transmembrane component that partners with GFRα1 is not widely expressed in the MGE but, rather, is restricted to the subpopulation of cells that express GFRα1. Thus, these cells would appear to be different from other MGE neurons in more aspects than just their expression of GFRα1, which underlies a degree of diversity not previously appreciated in postmitotic GABAergic precursors of the MGE. While the present study was undergoing revision, Bespalov et al. reported that the heparan sulfate proteoglycan syndecan-3 is able to bind GDNF and transmit GDNF signals independently of GFRα1 (Bespalov et al., 2011). However, the effects of GDNF documented in our study are strictly dependent on GFRα1, suggesting that they are not mediated by syndecan-3. Moreover, micromolar concentrations of GDNF were required to enhance neuronal migration through syndecan-3 (Bespalov et al., 2011), an amount that is several orders of magnitude greater than the one required for the effects reported in our study.

In an effort to identify candidate transmembrane mediators, we tested the roles of ErbB4 and Met, two receptor tyrosine kinases that have previously been implicated in GABAergic interneuron development. However, neither of these receptors was found to mediate the effects of GDNF–GFRα1 in MGE GABAergic neurons. We confirmed that the ErbB4 ligand NRG-1 is able to stimulate cell migration in MGE neurons as previously reported (Flames et al., 2007; Fogarty et al., 2007; Liodis et al., 2007; Miyoshi et al., 2007). However, addition of HGF had no significant effects in either neurite outgrowth or cell migration of these cells, so our results do not support a direct role for this factor in GABAergic interneuron development. Surprisingly, however, inhibition of endogenous HGF or MET signaling by the use of blocking antibodies, pharmacological inhibitors or genetic mutation did promote the morphological differentiation and migration of MGE neurons. The fact that these activities can be abolished in *Gfra1*$^{-/-}$ neurons or by anti–GDNF antibodies indicates that they are mediated by endogenous GDNF–GFRα1 signaling. On the basis of these results, we postulate that endogenous HGF–MET signaling restricts the activity of GDNF–GFRα1 in neuronal precursors of the MGE and propose that earlier models implicating HGF–MET signaling in the development of cortical GABAergic interneurons are reconsidered. We note that, despite many reviews dedicated to this topic, the biological activities of HGF on MGE neurons have not been rigorously tested. The in vitro evidence that has previously been presented in support of a role for HGF in the development of MGE neurons was primarily based on the ability of MGE-derived HGF to induce scattering of kidney-derived MDCK cells (Powell et al., 2001). Although those observations demonstrated the presence of biologically active HGF in the MGE, they did not explain its endogenous function on MGE neurons. In explant cultures, addition of HGF was found to disrupt the pattern of migration of MGE cells (Powell et al., 2001), which we now suggest to be mediated through interference with endogenous GDNF signaling. Based on our findings, we propose that HGF–MET signaling in the MGE influences cortical GABAergic interneuron development by restricting the activities of GDNF–GFRα1 in a subset of MGE cells. As null mutations in *Gfra1* and *MET* are lethal, testing epistatic interactions between these genes in animal models will be challenging.

Inhibition of HGF–MET signaling was found to increase *Gfra1* mRNA expression in MGE cells in vitro in culture and in vivo in *MET*$^{d/d}$ mutant MGE, suggesting that endogenous HGF–MET signaling negatively regulates *Gfra1* mRNA expression in MGE neurons. These results provide a mechanism by which HGF–MET may restrain GDNF–GFRα1 signaling in MGE precursors of cortical GABAergic interneurons. The overlapping protein expression of MET and GFRα1 in the MGE suggests that this to be a mechanism that operates in all GDNF-responsive cells of this structure. By gating GDNF responsiveness in a subset of MGE cells, HGF–MET signaling may contribute to the early diversification of cortical GABAergic interneurons. In addition to regulating GFRα1 expression, HGF–MET signaling might also antagonize intracellular pathways utilized by GDNF and GFRα1 to exert their effects in MGE cells. A better understanding of those pathways is likely to emerge from the identification of the transmembrane mediators of GDNF–GFRα1 signaling in the MGE.

Materials and Methods

Primary culture of MGE cells

Medial, lateral and caudal ganglionic eminences were dissected from E12.5 C57/B6 mouse embryos, trypsinized and cultured in Neurobasal medium supplemented with B27, glutamax and penicillin/streptomycin (Invitrogen). Mutant *Gfrα1*$^{-/-}$ mice (Enomoto et al., 1998), *Gdnf*$^{-/-}$ mice (Pichel et al., 1996) and *MET*$^{d/d}$ mice (Maina et al., 1996) have been described previously. Tissue culture dishes were coated with poly-D-Lysine (Sigma) for 2 hours before plating the cells. For biochemical assays, 4×10^5 cells were plated per well in six-well plates, cultured for 2 days, starved 2×4 hours in Neurobasal medium supplemented with glutamax and then stimulated with GDNF (50 ng/ml), HGF (0.5–50 ng/ml), NRG-1 (20 ng/ml) (all three from R&D). For immunostaining and neurite outgrowth assays, 3×10^4 cells were plated per well in 48-well plates and grown for 2 days either untreated or in the presence of GDNF (100 ng/ml), GFRα1-Fc (150 ng/ml, R&D), HGF (50 ng/ml), NRG-1 (20 ng/ml), in the presence or absence of the ErbB4 inhibitor AG1478 (Calbiochem) or MET kinase inhibitors SU11274 and PHA665752 (Calbiochem). Blocking antibodies anti-HGF (R&D) and anti-GDNF (R&D) were used at a final concentration of 1 μg/ml.

Western blotting

Primary cultures were lysed for 30 minutes in lysis buffer (PBS, 10% glycerol, 1% NP-40, protease and phosphatase inhibitors). Lysates were centrifuged at 10000 ***g*** for 5 min and protein content in cleared total cell lysates was measured with the BCA kit (Pierce). Samples were separated by SDS-PAGE and transferred to PVDF membranes (GE Healthcare). Membranes were blocked on BSA and then incubated overnight with anti-phospho-ErbB4, anti-phospho-Met, anti-phospho-ERKs, anti-ERKs (Cell Signaling) or anti-α-tubulin (Sigma) antibodies. Blots were developed using alkaline phosphatase conjugated secondary antibodies and enhanced chemifluorescence development reagent (GE Healthcare), and then scanned on a Storm phosphorimager (Molecular Dynamics).

COS cell conditioned medium

COS cells were transfected with expression plasmids for EGFP (mock), wild type GDNF or double point mutant E61/62A GDNF (Eketjäll et al., 1999). After transfection, cells were grown in serum-free Neurobasal medium for 3 days before conditioned medium was harvested, concentrated 20 times by ultrafiltration through Centriprep 10 cartridges (Millipore) and quantified by immunoblotting with anti-GDNF antibodies (Santa Cruz) against standards of commercial GDNF (R&D). It was used at a final concentration equivalent to 100 ng/ml.

Immunocytochemistry and neurite outgrowth

Dissociated cultures were fixed in 4% paraformaldehyde (PFA) and 0.05% glutaraldehyde. Cells were permeabilized with 0.1% Triton X-100 and, after blocking, cultures were stained with anti-GABA antibodies (1:5000, Sigma), and counterstained with DAPI. GABA staining was visualized with Cy2-conjugated secondary antibodies (Jackson) and photographed in an Axiovert 200M inverted fluorescence microscope (Zeiss). Neurite outgrowth counts were performed using ImageJ software (NIH) setting cell perimeter threshold above 18,000 and cell circularity below 0.5. This mask enabled the detection of differentiated GABAergic cells bearing neurites longer than twice the cell body diameter excluding undifferentiated circular cells. Cell counts were normalized to DAPI nuclear counterstaining and plotted relative to

neurite outgrowth level in the control condition. Statistical analysis of the results was performed using the Student's *t*-test.

Cell migration

Dissociated MGE, CGE or LGE neurons were plated on a PDL pre-coated filter of a Boyden ChemoTx (Neuroprobe) chamber with a 5 μm pore size in a 96-well format. 5×10^4 cells were resuspended in Neurobasal medium supplemented with B27 and plated in the top chamber in a 50 μl drop. Cell migration was induced by adding growth factors or blocking antibodies – at the concentrations described above – in the lower compartment of the chamber. Cultures were incubated for 48 hours after which cells that had remained on the top of the filter were gently scrapped away with a cotton tip. The filters were then fixed in 4% PFA and stained with DAPI. The lower side of the filters were imaged on an Axiovert 200M inverted fluorescence microscope (Zeiss) in the DAPI channel and counted using ImageJ software (NIH). Cell migration is displayed as relative to the control condition and statistical analysis was performed using the Student's *t*-test.

Immunohistochemistry

Perfusion, fixation and preparation of cryo-sections followed standard procedures. Immunohistochemistry was conducted in free floating sections according to standard protocols. Primary antibodies used were: goat anti-GFRα1 (Neuromics), and rabbit anti-MET (Santa Cruz Biotech). Immunofluorescence was imaged by laser confocal microscopy.

Quantitative real-time PCR

MGE neurons were plated at the same density as for biochemical assays and cultured for 48 hours with GDNF (100 ng/ml), GFRα1–Fc (150 ng/ml), BDNF (20 ng/ml, from R&D) anti-HGF (1 μg/ml), anti-GDNF (1 μg/ml) and SU11274 (1 μM) prior to lysis for RNA extraction. For comparison, E12.5 MGE tissue was also lysed directly for RNA extraction. Total RNA was isolated using RNA-easy columns (Qiagen) according to the manufacturer's instructions. Single stranded cDNA was synthesized using the Multiscribe reverse transcriptase and random hexamers (Perkin Elmer) and amplified using a StepOnePlus (Applied Biosystems) cycler with the SYBR Green PCR mix and specific primers as indicated in supplementary material Table S1.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/16/2797/DC1

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