Signalling by glial cell line-derived neurotrophic factor (GDNF) requires heparan sulphate glycosaminoglycan

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Summary

Glial cell line-derived neurotrophic factor, GDNF, is vital to the development and maintenance of neural tissues; it promotes survival of sympathetic, parasympathetic and spinal motor neurons during development, protects midbrain dopaminergic neurons from apoptosis well enough to be a promising treatment for Parkinson's disease, and controls renal and testicular development. Understanding how GDNF interacts with its target cells is therefore a priority in several fields. Here we show that GDNF requires glycosaminoglycans as well as the already-known components of its receptor complex, c-Ret and GFRα-1. Without glycosaminoglcyans, specifically heparan sulphate, c-Ret phosphorylation fails and GDNF cannot induce axonogenesis in neurons, in PC-12 cells, or scatter of epithelial cells. Furthermore, exogenous heparan sulphate inhibits rather than assists GDNF signalling. The involvement of heparan sulphates in GDNF signalling raises the possibility that modulation of heparan expression may modulate signalling by GDNF in vivo.

Key words: GDNF, Glycosaminoglycan, Heparan sulphate, c-Ret

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is vital to the development and maintenance of neural and other tissues. It was first identified as a survival factor for dopaminergic neurons of the mid-brain (Lin et al., 1993); these are precisely the neurons that degenerate in Parkinson's disease, and treatment of animal models of Parkinson's disease with exogenous GDNF promotes functional recovery (Gash et al., 1996). GDNF also protects these neurons against the neurotoxic drugs 6-hydroxydopamine and metamphetamine (Shults et al., 1996; Cass, 1996), and these neuroprotective features make the GDNF system a promising target for clinical manipulation. In normal development, GDNF promotes survival of sympathetic and parasympathetic neurons (Buj-Bello et al., 1995; Trupp et al., 1995) and spinal motor neurons (Zurn et al., 1994; Henderson et al., 1994). It even promotes regeneration of spinal motor neurons after spinal cord injury (Ramer et al., 2000). Outside the nervous system, GDNF induces the development of the urinary collecting duct system, without which kidneys fail to form (Pichel et al., 1996; Moore et al., 1996; Sainio et al., 1997), and it also induces spermatogonial differentiation in the testis (Meng et al., 2000). Understanding the details of how GDNF interacts with its receptors is therefore a priority in several fields of cell, developmental and neuro-biology.

The components of the GDNF receptor complex characterized so far comprise a high-affinity receptor tyrosine kinase, c-Ret, and a glycosylphosphatidylinositol- (GPI-) linked co-receptor, GFRα1 (Jing et al., 1996). Other members of the GDNF family, such as neurturin, persephin and artemin, also signal through c-Ret but do so in association with other co-receptors (GFRα2-4 respectively) (Airaksinen et al., 1999). GFRα1 is associated with membrane rafts, and promotes translocation of c-Ret to those rafts in the presence of GDNF (Tansey et al., 2000). Interactions between the receptor components are complex. c-Ret, for example, seems capable of initiating a pro-apoptotic signal in a manner that is independent of both its kinase activity and ligand binding, though association between c-Ret and GFRα1 inhibits this pro-apoptotic signal even in the absence of GDNF (Bordeaux et al., 2001).

The interactions between some other growth factors and their (generally simpler) receptors are facilitated by sulphated glycosaminoglycans. FGF-2, for example, requires heparan sulphate proteoglycans for activation of its receptor tyrosine kinase (Rapraeger et al., 1991; Yayon et al., 1991) and HGF requires dermatan sulphates to activate c-Met (Lyon et al., 2002). GDNF is known to bind to heparin, a fact which was used in its original purification (Lin et al., 1993), so it is likely to bind its commoner cell surface relative, heparan sulphate. Heparan sulphate is known to be required for development of renal collecting ducts in vivo and in culture (Davies et al., 1995; Bullock et al., 1998), and the effect of depriving kidneys of heparan sulphate is very similar to that of depriving them of GDNF or c-Ret. Furthermore, the development of collecting ducts deprived of heparan sulphates in culture can be rescued to a large extent by application of supraphysiological concentrations of exogenous GDNF (Sainio et al., 1997). We have therefore tested the hypothesis that heparan sulphate GAGs play a direct role in GDNF signalling, and find it to be correct in each of the systems we have examined.

Materials and Methods

Reagents

Porcine dermatan sulphate (C 3788), heparan sulphate (H9902) and chondroitin sulphate C (C4384), anti tyrosine hydroxylase, anti neurofilament 68, GDNF (recombinant human) and chondroitinase ABC (EC 4.2.2.4) were obtained from Sigma. Antibodies against c-Ret, phosphotryosine and GFRα1 were from Santa Cruz. Heparinase III (EC 4.2.2.8) was obtained from Sigma and from Calbiochem. Units of enzyme acitivity are as defined by Sigma; 1 U heparinase III liberates 0.1 μmol uronic acid from heparin per hour at 25°C.

Cell and tissue culture experiments

Cultures of midbrain neurons were made from E15 mouse embryos by the method of Engele and Franke (Engele and Franke, 1996), and were incubated for 7 days in the absence or presence of 100 ng/ml GDNF and 30 mM sodium chlorate and then stained with anti-tyrosine hydroxylase to detect dopaminergic neurons. Dorsal root ganglia were dissected from the thoracic region of E11 mice, plated on poly-ornithine-coated glass coverslips and cultured in Eagle's MEM (Sigma M5650) with 10% foetal calf serum and 2 mM glutamate, for 48-72 hours. Hindguts (the distal half of the length from stomach to cloaca) were isolated from E10 mouse embryos and cultured in the above medium for 96 hours. PC-12 cells were maintained in RPMI 1640 medium with 10% foetal calf serum, 2 mM glutamate, penicillin and streptomycin. For neurite outgrowth, they were plated on to 13 mm coverslips that had been coated for 1 hour in 60 μl of 10 μg/ml EHS laminin (Sigma) in RPMI 1640 medium with 10% foetal calf serum, 2 mM glutamate, 10 μg/ml GFRα1-Fc chimeara (R&D systems), penicillin and streptomycin, at the bottom of 24 well plates (the coating solution was removed before the cells were added). The cells were then incubated for 3-4 days in the presence or absence of 100 ng/ml GDNF, 10 ng/ml NGF, 30 mM sodium chlorate, 2 mM sodium sulphate, 0.3 U/ml heparinase III or 0.3 U/ml chondroitinase ABC (units as defined by Sigma). Responses were quantitified by counting the total numbers of cells in at least 12 fields of view, and also the number of cells that bore neurites at least twice the diameter of the cell body (most were significantly longer or very much shorter so the judgement of twice the body diameter was not critical and was done by eye). MDCK cells and RET/GFRα1-MDCK cells were maintained in the same medium as DRGs, with 0.2 mg/ml G418 in the case of RET/GFRα1-MDCK cells. Scatter experiments were performed on glass coverslips in 24-well plates. For staining with anti-phosphotyrosine, cells were fixed in cold methanol for 5 minutes, washed in PBS, incubated 40 minutes in 1/100 anti-phosphotyrosine at 4°C, washed in PBS, incubated in 1/100 Sigma FITC-anti mouse IgG for 40 mintues at 4°C, washed again and photographed on an epifluorescence microscope. For phosphotyrosine experiments, all photomicrographs were taken and digitised at the same exposure, and digital adjustments of brightness and contrast were performed on the grouped images so that the relationships between their relative brightnesses were maintained.

Immunoprecipitation and blotting

Approximately 2 million RET/GFRα1-MDCK cells per 60 mm dish were cultured overnight and placed in 5 ml serum-free medium for 2 hours and treated with 0.1-0.5 U/ml heparinase-III for 1 hour. 100 ng/ml GDNF was added for 30 minutes and cells homogenized in MB buffer (50 ml Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 10% glycerol, 100 μM sodium orthovanadate, 1% aprotenin, 1 mM PMSF). For immunoprecipitation, goat anti-c-Ret (Santa Cruz) was adsorbed to rabbit anti-goat IgG agarose beads (Sigma) by incubation on ice for 2 hours. The beads were washed with MB buffer and incubated with RET/GFRa1-MDCK cell homogenates at 4°C overnight. Beads were washed twice in MB buffer and bound c-Ret was eluted by boiling in Laemelli buffer containing 5% β-mercaptoethanol for 3 minutes. Eluate from each immunoprecipitation was divided into two equal fractions and subjected to SDS-PAGE analysis and transferred to Hybond nitrocellulose membranes (Amersham). Membranes were probed with mouse anti-phosphotyrosine (1/2000) (Upstate Biotechnology, UK) or with goat anti-c-Ret (1/1000). Secondary antibodies (Sigma) were used at a 1/2000 (anti-mouse-HRP) or at 1/50000 (anti-goat-HRP). Signals were detected using an ECL plus kit (Amersham).

^{125}I-GDNF binding

RET/GFRa1-MDCK cells were cultured directly on the plastic of 24-well plates, at approx 60,000 cells/well, and incubated for 20-40 hours in standard medium (no G418) with or without 30 mM sodium chlorate. They were then washed in ice cold binding buffer (PBS with 0.5% BSA) and then a range of dilutions in binding buffer of ice cold stock ^{125}I-GDNF (supplied by Amersham/Pharmacia 64.1 TBq/mmol, and diluted to 6.41 TBq/mmol using unlabelled GDNF to form the primary stock) were applied to the wells (total volume 200 μl/well). After 4 hours on ice, the ^{125}I solution was removed, the cells were washed twice, quickly, in binding buffer, and were then lysed in 500 μl 10% SDS. ^{125}I-GDNF was measured using a Packard-Bell gamma counter. Machine background was assessed using wells to which no ^{125}I-GDNF had been added, the counter was calibrated using a known quantity of ^{125}I-GDNF stock diluted in 10% SDS and added directly to a scintillation vial, and non-specific binding was measured by applying a 200-fold excess of unlabelled GDNF to some wells. Binding parameters (B_{max}, K_d) were calculated automatically using built-in functions of Prism (Graphpad software).

Results

GDNF fails to induce morphological changes in the absence of GAGs

GDNF activity can be detected by its effect on cell morphology and survival in a variety of culture systems. We have therefore tested whether cells in these systems would show normal responses to GDNF even in the absence of their glycosaminoglycans. Promotion of axon sprouting from cultured ganglia is a simple assay for GDNF (Ebendal et al., 1995). We found that E14 mouse thoracic dorsal root ganglia showed no axon sprouting in the absence of exogenous neurotrophic factors (Fig. 1a) but showed copious axon sprouting in the presence of 100 ng/ml GDNF (Fig. 1b). Inclusion in the culture medium of 20 mM sodium chlorate, a competitive inhibitor of sulphation that has been widely used to block synthesis of sulphated GAGs (Rapraeger et al., 1991; Davies et al., 1995), prevented GDNF-induced axonogenesis (Fig. 1c). It did not, however, inhibit axonogenesis elicited by 100 ng/ml NGF (Fig. 1d). NGF is a very powerful inducer of axonogenesis and it is one of the few growth factors that does not bind heparin. Its ability to elicit axonogenesis even in chlorate demonstrates that GAGs are not required for axonogenesis itself but rather for GDNF to be able to induce the process.

Studies of *gdnf*$^{-/-}$ transgenic mice have shown that GDNF, expressed naturally by the gut, is required for formation of most of the enteric nervous system (Pichel et al., 1996; Moore et al., 1996). This observation was the basis of another assay used by us to explore the requirement of the system for glycosaminoglycans. When hindguts were isolated from E10 mice and cultured for 96 hours, they developed a complex network of neurofilament 68-positive nerve fibres in response to their endogenous GDNF (Fig. 1e), but those cultured in 20

mM sodium chlorate developed few axons, and those cultured in 0.1 U/ml heparinase III developed fewer still (Fig. 1f,g). Early enteric neurons are not responsive to exogenous NGF, so the control of showing that cells can still produce axons in response to growth factors that do not require GAGs is not available in this system. The cultured hindguts could, however, be protected from the effect of sodium chlorate by the addition of 2 mM sulphate (Fig. 1h). This common control confirmed that the effects of chlorate were indeed due to it acting as a competitive inhibitor of GAG sulphation.

The physical geometry of the DRG and gut culture systems described above made the effect of GDNF difficult to quantify since axons were too tangled to count and the number of potential axon-forming cells was also uncountable. PC-12 pheochromocytoma cells will undergo neural differentiation in response to exogenous GDNF (Chen et al., 2001), and will do so as scattered single cells that are easily countable. With no GDNF, PC12 cells attached loosely to their substrate but remained rounded and extruded only tiny processes (Fig. 2a). 100 ng/ml GDNF caused approximately 13% of these cells to produce processes (neurites) at least twice as long as their cell diameter, and usually much longer (Fig. 2b). The presence of 30 mM chlorate inhibited this effect strongly (Fig. 2c) although 2 mM sulphate again achieved a substantial rescue (Fig. 2d). Even in the presence of chlorate, NGF elicited a massive response from PC-12 cells (Fig. 2e), again showing that GAGs are not required for neurite formation itself, but rather for cells to be able to respond to GDNF. The quantitative data from these experiments are shown in Fig. 2f (except for the NGF-treated controls, which were impossible to count because the neurite network was so dense that it was not clear to which cell each neurite belonged). In contrast to differentiation, total cell numbers were not affected significantly by the presence of GDNF or chlorate (Fig. 2g) so, in this system, the observed rates of axonogenesis are unlikely to reflect differences in proliferation or survival.

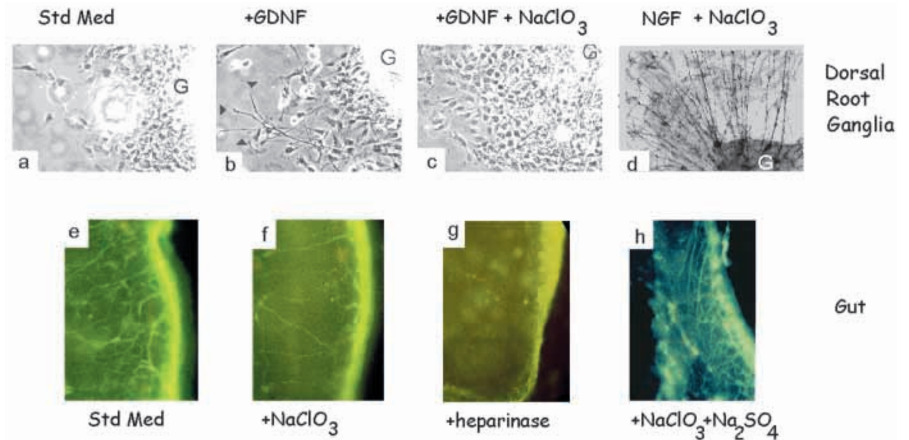

Fig. 1. GAGs are required for responses to GDNF in primary cultures. (a-d) Embryonic dorsal root ganglia form no neurites in the absence of GDNF (a), but form axons quickly in response to 100 ng/ml GDNF (b; arrowheads); inhibition of GAG sulphation with chlorate blocks this response to GDNF (c), but does not block the (stronger) axongenic response to 10 ng/ml NGF (d). (e-h) E10 embryonic intestine develops an extensive network of neurites in culture in response to endogenous GDNF (e); this is reduced by culture in chlorate (f) and inhibited still further by 0.3 U/ml heparinase III (g). Inclusion of 2 mM sulphate in the medium rescues cultures from the effect of chlorate (h) as expected, because chlorate and sulphate compete in the synthesis of the specific donor molecule involved in GAG sulphation.

Our final morphological assay was based on the behaviour of a cell line derived from canine collecting ducts and transfected with both c-Ret and GFRα1. Based on the well-known Madin-Darby Canine Kidney (MDCK) cell line, the transfected cell line is called RET/GFRα1-MDCK. That it does indeed express both c-Ret and GFRα1 can be seen in the Western blots in Fig. 5a,b In the absence of exogenous GDNF, RET/GFRα1-MDCK cells grew as normal MDCK cells; when grown on glass coverslips in sub-confluent culture, they formed separated islands with cell-free spaces between them (Fig. 3; spaces are marked with arrows). Addition of GDNF caused the cells to scatter so that the formerly clear inter-island spaces became filled with spindly cells; this response is similar to that

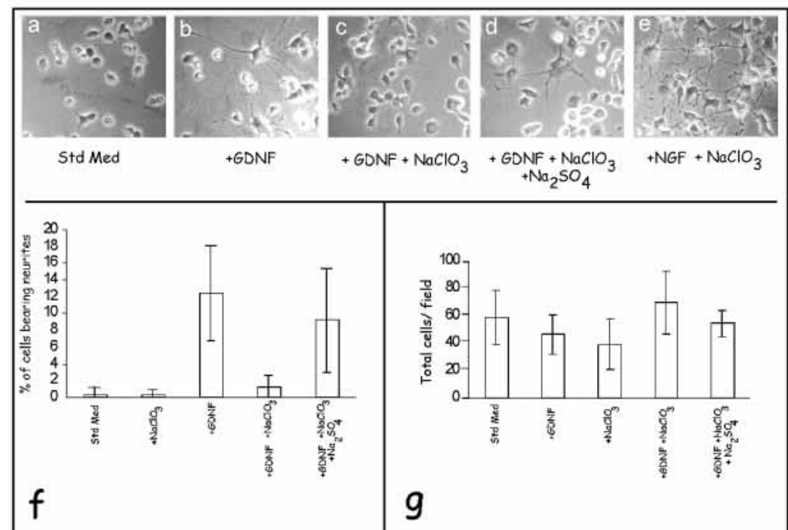

Fig. 2. GAGs are required for GDNF-induced axonogenesis in PC-12 cells. (a) PC-12 cells cultured in the absence of GDNF remain rounded and form no axons, whereas about 12% of cells form axons when treated with GDNF (b). 30 mM sodium chlorate, a competitive inhibitor of GAG sulphation, blocks this response to GDNF (c), unless sulphate is added to antagonise the effects of chlorate (d). PC-12 cells remain able to produce axons in response to another neurogenic factor, NGF, even in the presence of chlorate (e), showing that GAGs are not required for axon morphogenesis but merely for responsiveness to GDNF. The data from a-d are shown quantitatively in f (NGF-treated PC-12 cells form meshworks of neurites too complicated to count). Total numbers of cells, counted whether or not they bear axons, show no significant variation between these conditions (g).

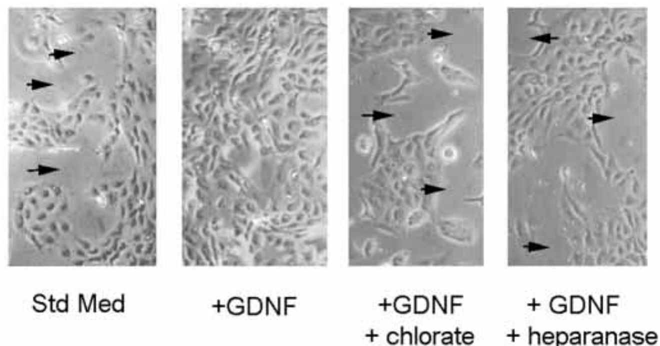

Fig. 3. Scatter of RET/GFRα1-MDCK cells in response to GDNF. Untreated cells (std med) remain as well-bounded islands that are separated by clear spaces (arrows). On addition of GDNF, the inter-island spaces are invaded by motile cells. Incubation of the cells in 30 mM chlorate for the preceding 24 hours and during the scattering period, or incubation in 0.3 U/ml heparanase III for the preceding 2 hours and during the scattering period, greatly reduces this effect of GDNF and clear spaces remain (arrows) although the edges of the islands do still lose their smoothness compared to controls.

of normal MDCK cells treated with HGF/scatter factor [for a review of HGF-induced scattering see Balkovetz (Balkovetz, 1998)]. Once again, treatment with 20 mM chlorate or with 0.1 U/ml heparinase III blocked the effects of exogenous GDNF and the islands of cells remained separated by clear space.

GDNF signalling requires heparan sulphates, not chondroitin or dermatan sulphates

The experiments on enteric neurons and on scatter of RET/GFRα1-MDCK cells reported above suggest that heparan sulphate, the target for the heparinase III enzyme, is particularly important for GDNF signalling. We explored this further using both an immunohistochemical assay for GDNF signalling in RET/GFRα1-MDCK cells and a quantitative neuritogenesis assay using PC-12 cells. When fixed and stained with anti-phosphotyrosine, RET/GFRα1-MDCK cells showed only low levels of tyrosine phosphorylation at their plasma membranes after being cultured for 2 hours in serum-free medium (Fig. 4a). Tyrosine phosphorylation at the membrane increased markedly following treatment with 100 ng/ml GDNF (Fig. 4b), but this rise in tyrosine phosphorylation at the membrane was abolished in cells whose medium had been supplemented with 30 mM sodium chlorate overnight. Membrane-associated tyrosine phosphorylation was also abolished by treatment with 0.1 U/ml heparinase III for 1 hour before GDNF was added (Fig. 4c). It was not, however, abolished by treatment with 0.1 U/ml chondroitinase ABC (Fig. 4d). GDNF was able to induce significant neuritogenesis in PC12 cells either in the absence of any exogenous glycansases or in the presence of chondroitinase ABC, but not in the presence of heparinase III (Fig. 4e). These data strongly suggest that heparan sulphates (targets of heparinase III) rather than chondroitin/dermatan sulphates (both targets of chondroitinase ABC) are important to GDNF signalling.

Heparan sulphates are required for GDNF to activate c-Ret

In each of the morphological and survival assays above, responses to GDNF did not take place in the absence of glycosaminoglycans. These assays show that GAGs are required for a range of GDNF responses, but do not show whether this requirement is for GDNF to activate its c-Ret receptor tyrosine kinase or for events subsequent to that activation (such as events in cytoplasmic signal transduction). In order to establish whether GAGs are needed for GDNF to activate its c-Ret receptor tyrosine kinase, or merely for events downstream of Ret activation, we again made use of the RET/GFRα1-MDCK cell line. Phosphorylation of the c-Ret receptor tyrosine kinase was measured by immunoprecipitation for c-Ret followed by Western Blotting using anti-phospho-tyrosine as a probe (anti-c-Ret being used as a control probe to confirm equal recovery from samples during immunoprecipitation). Phosphorylation of c-Ret was low in cells cultured for 2 hours in serum-free medium, increased substantially when they were treated with GDNF, but failed to do so when the cells were treated for the preceding hour with 0.1-0.5 U/ml heparinase III (Fig. 5a). These observations were made in four independent runs of the experiment. Heparan sulphates are therefore required for GDNF to activate its c-Ret receptor tyrosine kinase.

GAGs are involved in binding GDNF to the cell surface

Now that we have shown GAGs to be required for efficient

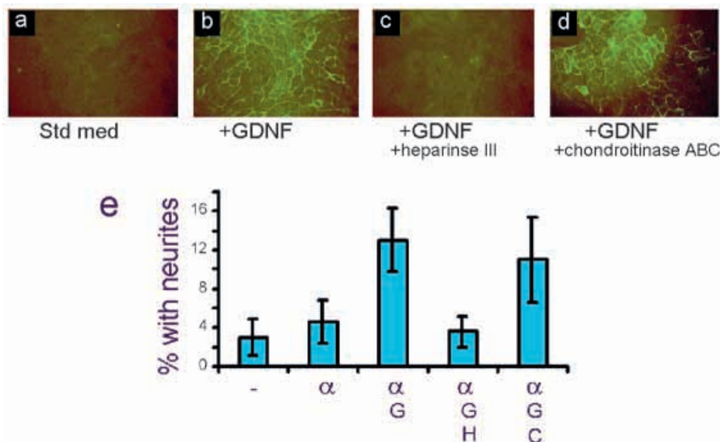

Fig. 4. GDNF signalling is blocked by heparinase III but not chondroitinase ABC; (a) RET/GFRα1-MDCK cells growing in serum-free medium show little phosphotyrosine at their plasma membranes, but treating them with 100 ng/ml GDNF increases the amount of this phosphotyrosine dramatically (b). Pre-treatment of the cells with 0.3 U/ml heparanase III blocks this effect of GDNF (c), although treating them with 0.3 U/ml chondroitinase ABC does not (d). GDNF-induced neuritogenesis by PC12 cells show a similar result (e); cells in plain medium (–) and in medium supplemented with only soluble GFRα1 (α) show only background neuritogenesis, those treated additionally with 100 ng/ml GDNF (αG) show significantly enhanced neuritogenesis unless treated throughout the culture period with 0.3 U/ml heparinase III (αGH); 0.3 U/ml chondroitinase ABC has no significant effect on neuritogenesis (αGC).

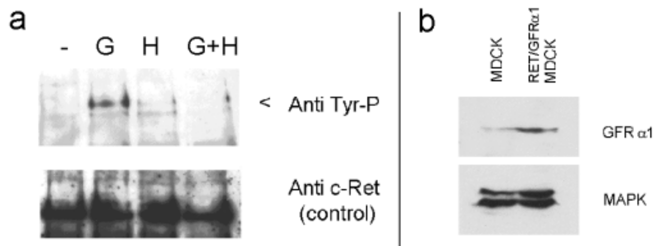

Fig. 5. Ret autophosphorylation demonstrated by western blotting. (a) Autophosphorylation of c-Ret is barely detectable in the absence of GDNF (–), is strongly induced by 100 ng/ml GDNF (G), but not if cells are pre-treated with 0.1 U/ml heparinase III (G+H). Track H shows control cells treated with just heparitinase III but no GDNF. The bottom panel showing c-Ret regardless of phosphorylation state confirms equal loading of the samples. (b) Confirmation of GFRα-1 expression by RET/GFRα1-MDCK cells. RET/GFRα1-MDCK cells express GFRα-1, as expected, in addition to c-Ret (see b), and even normal MDCK cells express a little. The bottom panel, probed for MAP-kinase, is again a loading control.

Ret activation, it is natural to ask whether GAGs are involved in the binding of GDNF to the cell surface in the first place. The question is particularly pertinent because heparan sulphate would be an obvious candidate for being the uncharacterised, high abundance, low affinity GDNF receptor mentioned by Jing et al. (Jing et al., 1996) when they reported the discovery of the high-affinity GPI-linked receptor, GFR1α (as it is now called). If GAGs are involved in binding GDNF to cells, binding would be expected to be diminished in cells whose GAG synthesis has been inhibited. This is indeed the case (Fig. 6); while there is no significant difference in the ability of normal and chlorate-treated RET/GFRα1-MDCK cells to bind GDNF from very dilute solutions (<300 pM), the binding diverges significantly for higher concentrations of GDNF. The B_{max} (GDNF binding capacity) of the chlorate treated cells (0.38 pmol/well, s.e. 0.06 pmol/well) is only about a quarter that of the normal cells (1.52 pmol/well, s.e. 0.82 pmol/well); if GAGs play a

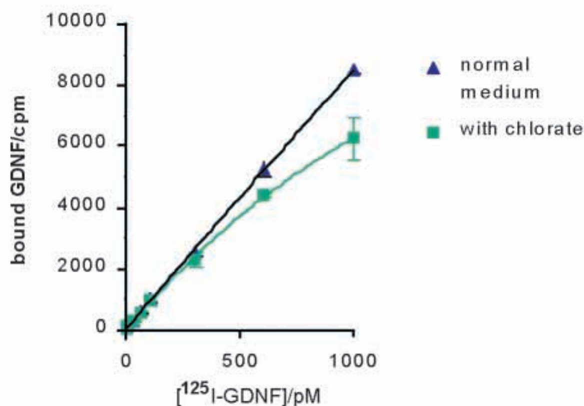

Fig. 6. Inhibition of GAG synthesis diminishes binding of ^{125}I-GDNF to the surface of RET/GFRα1-MDCK cells. The separation between the curves becomes statistically significant above 300 pM: the plotted points are means of triplicate wells, and error bars represent standard deviation; all point have error bars, but some are too small to see behind the squares and triangles themselves.

role in binding GDNF to the cell surface, we would expected B_{max} to be reduced when the synthesis of new GAGs has been inhibited and only a diminishing stock of pre-existing GAGs remains. The receptors that still persist on the surface of chlorate treated cells retain an affinity (K_d) of 2.2 nM (s.e. 0.5 nM) similar to that of controls. This is within an order of magnitude of the K_d of the uncharacterised abundant low-affinity GDNF receptor (0.33 nM) described by Jing et al. (Jing et al., 1996), but very different from that of GFRα1 itself (K_d=0.0015 nM).

GDNF signalling requires cell surface heparan sulphates

There are several ways in which GAGs might facilitate signalling by growth factors (discussed in more detail below). One possible role for GAGs, which are borne by abundant proteoglycans associated with the plasma membrane, is to bind quantities of growth factor with relatively low affinity and thereby increase the local concentration of growth factor at the plasma membrane where its high-affinity receptor tyrosine kinase is situated. This may or may not be combined with other roles such as stabilisation of the receptor complex. If GAGs do concentrate GDNF at the plasma membrane, exogenous soluble GAGs added by an experimenter would be expected to compete with the membrane-located GAGs and will therefore inhibit signalling, while if GAGs were only required for other purposes, such as stabilising ligand-receptor complexes, exogenous GAGs will not be detrimental and may even aid stabilization. We have tested this in our system by adding exogenous chondroitin, heparan and dermatan sulphates to the media of RET/GFRα1-MDCK cells treated simultaneously with 100 ng/ml GDNF. As little as 10 ug/ml heparan sulphate inhibited GDNF-induced tyrosine phosphorylation very effectively (Fig. 7a-i), whereas the same concentration of chondroitin and dermatan sulphates had no detectable effect and even 100 ug/ml dermatan and chondroitin sulphates failed to inhibit GDNF signalling completely. A similar effect can be seen in the response of PC-12 cells to 100 ng/ml GDNF, and as little as 100 ng/ml heparan sulphate is enough to reduce neuritogenesis to control levels (Fig. 7j, blue bars). Because these data showed a hint, albeit one not statistically significant at P=0.05, that low concentrations (10 ng/ml) of exogenous heparan sulphate may slightly potentiate signalling by GDNF, we tested whether exogenous heparan sulphate might be able to rescue the response of chlorate-treated PC-12 cells to GDNF. It was unable to do so at any concentration examined (Fig. 7j, red bars).

In earlier studies on the role of GDNF in kidney development, it was shown that very high concentrations of GDNF (1000 ng/ml) could begin to rescue renal morphogenesis even in kidneys deprived of GAGs by chlorate treatment (Sainio et al., 1997). One possibility, suggested by us in that paper, was that signalling by physiological concentrations of GDNF might require GAGs but that supraphysiological concentrations of GDNF might be able to signal even without them. We have tested that using RET/GFRα1-MDCK cells, and find that while GAGs are needed for robust signalling by 100 ng/ml GDNF, GDNF at the supraphysiological concentration of 1000 ng/ml elicits tyrosine phosphorylation even in cells treated with heparinase III (Fig. 8a-c).

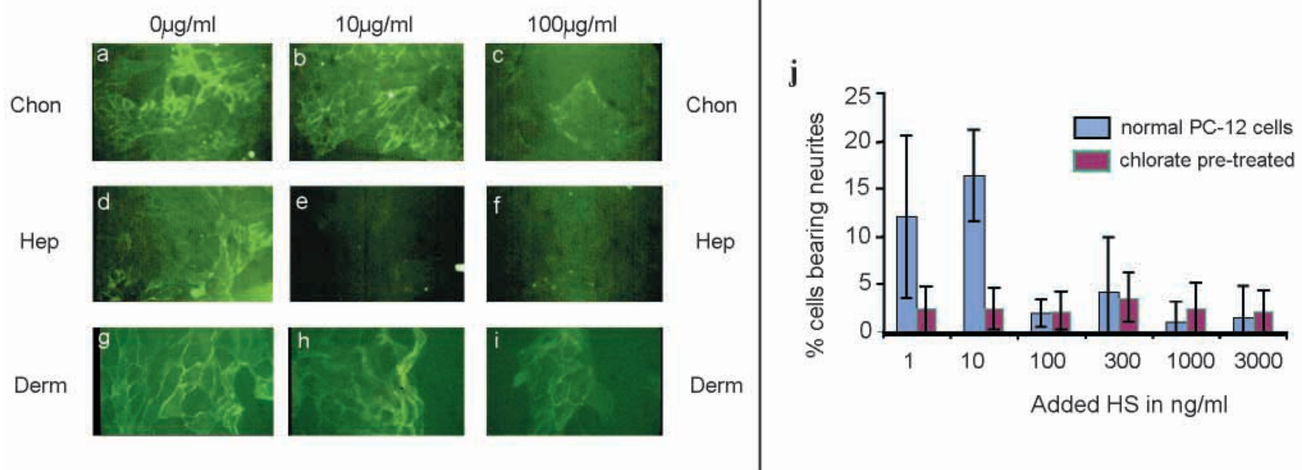

Fig. 7. Sensitivity of GDNF signalling to exogenous GAGs. GDNF-induced tyrosine phosphorylation of RET/GFRα1-MDCK cells takes place in the presence of 0 (a,d,g), 10 μg/ml (b) and even 100 μg/ml (c) exogenous chondroitin sulphate, although in 100 μg/ml it is somewhat reduced. Heparan sulphate at just 10 μg/ml (e), as well as 100 μg/ml (f) blocks the response. Dermatan sulphate has little effect at 10 μg/ml, although it does, like chondroitin sulphate, reduce the response somewhat at 100 μg/ml. GDNF-induced neuritogenesis in PC-12 cells is also inhibited by exogenous heparan sulphate, even at concentrations as low as 100 ng/ml (j, blue bars); the addition of exogenous heparan sulphate to chlorate-treated cells fails to rescue their response to GDNF at any concentration examined (j, red bars). Error bars represent 95% confidence intervals.

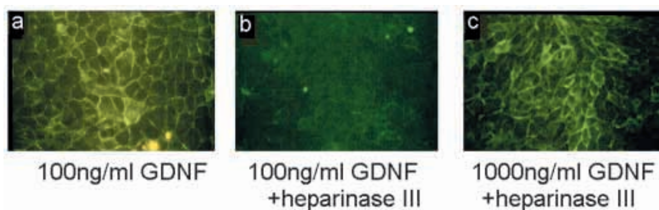

Fig. 8. Heparinase III blocks signalling by 100 ng/ml GDNF (b; compare with the control a). GDNF at the supraphysiological concentration of 1000 ng/ml, however, manages to signal even to cells treated with heparinase III (c)

Discussion

The data we have presented make the receptor system for GDNF more complicated than has been supposed. It should now be regarded as including heparan sulphate as well as GFRα1 and c-Ret, and the range of growth factors known to depend on GAGs for signalling, such as FGF and HGF, can be extended to include at least this member of the GDNF family of neurotrophic factors.

Our enzyme data shows that GDNF signalling requires heparan but not dermatan or chondroitin sulphates, and addition of exogenous GAG chains shows that exogenous dermatan and chondroitin sulphates are much less effective than is heparan sulphate at inhibiting GDNF signalling. The specificity of GDNF for heparan sulphate is a common but not universal feature of growth factors. FGFs, for example, use only heparan sulphates but HGF can use heparan or dermatan sulphates (Ashikari et al., 1995; Lyon et al., 2002). Of these classes of glycosaminoglycan, heparan sulphates show the greatest range of structural variations in vivo, differing in amounts and positions of deacetylation, N-sulphation, O-sulphation and uronic acid epimerisation. Where specificity has been examined, it is clear that heparan-sulphate binding growth factors show specificity for a very limited range of possible heparan sulphate sequences. Furthermore, these differ subtly between different proteins. FGF-1, for example, requires heparan sulphate domains composed of a cluster of IdoA($2SO_4$)-GlcNSO$_3$($6SO_4$)-IdoA($2SO_4$)-trisaccharide motifs, FGF-2 binds with highest affinity to a IdoA($2SO_4$)-GlcNSO$_3$-IdoA($2SO_4$) trisaccharide, while HGF binds clusters of IdoA($2SO_4$)-GlcNSO$_3$($6SO_4$), or three repeats of IdoA-GalNAc($4SO_4$) when it binds to dermatan sulphate instead (Ashikari et al., 1995; Lyon et al., 1998; Kreuger et al., 2001). As yet we know nothing about the binding specificity of GDNF for different types of heparan sulphate, except for the fact that some sulphated residues must be involved (or chlorate ions, which competitively block sulphation, would not have had an effect in our experiments); presumably, however, GDNF too has a specificity for particular heparan sequences.

If GDNF does indeed require specific types of heparan sulphate, the involvement of these GAGs might add another layer of regulation to the GDNF signalling system in vivo. Tissues in which GDNF is active, such as developing kidney and brain, are rich in heparan sulphate proteoglycans such as syndecans and glypicans, and each member of these families has its own distinct spatiotemporal distribution (reviewed by Bovolenta and Fernaud-Espinosa, 2000; Bandtlow and Zimmerman, 2000; Davies et al., 2001). Crucially, the specific composition of heparan sulphate chains borne by these molecules is also developmentally controlled, changing in both length and 6-O-sulphation during development of the mouse brain (Brickman et al., 1998). Indeed, expression of different heparan sulphate sulphotransferase enzymes, important in heparan sulphate synthesis, is itself regulated during brain development (Guimond et al., 2001). This raises the possibility that changes in expression of heparan sulphates in development may exert a powerful modulatory effect on cells' sensitivity to

GDNF, or on their relative sensitivity to different growth factors, even when they bear the high-affinity receptor tyrosine kinases at a constant level. Certainly at the crude level of whole tissues, it is known that different sources of heparan sulphates can have quite different affinities for a specific growth factor; pig liver heparan sulphate has a strong binding affinity for HGF while pig aorta heparan sulphate has none (Ashikari et al., 1995).

Since interactions between growth factors and GAGs were first identified, there have been a number of models for how these interactions aid signalling. In the best-studied example, the FGF1 signalling system, interactions with heparan sulphate perform several tasks simultaneously. To begin with, two FGF1 monomers are linked together into a biologically active complex by means of their interaction with a shared heparan sulphate chain; crystallography this $FGF1_2HS_1$ heterotrimer reveals that the FGF1 monomers share no protein-protein interface, and are kept together solely by the heparan sulphate (DiGabriele et al., 1998). The same chain of heparan sulphate also binds to one molecule of the FGF receptor tyrosine kinase, though the other FGFR monomer, which is recruited to make a 'dimer' of receptor tyrosine kinases, is apparently recruited through protein interactions alone; the whole complex therefore has the structure $FGF1_2FGFR_2HS$ (Pellegrini et al., 2001). A heparan decasaccharide is sufficient to promote assembly of this complex in vitro.

How similar might be the role of heparan sulphate in signalling by GDNF? Though we do not yet have direct evidence for binding of heparan sulphate by GDNF, there are several good reasons to assume that such binding takes place. First, GDNF binds well to heparin, a close structural relative of heparan sulphate; this fact was used for GDNF's initial isolation (Lin et al., 1993). Second, the binding of GDNF to cells that have GFRα1 but no Ret shows binding with two distinct affinities, a high affinity binding $K_d=1.5\times10^{-12}$ and a low affinity one with $K_d=3.30\times10^{-10}$ (Jing et al., 1996). It is likely that the low affinity interaction reflects binding to heparan sulphate, especially since the dissociation constants are so similar to that of HGF for its receptor tyrosine kinase and for heparan sulphate [$K_d=4.6\times10^{-12}$ and $K_d=2.8\times10^{-10}$, respectively (Arakaki et al., 1992)]. We now report a very similar high abundance, low-affinity GDNF receptor, and show that its abundance is significantly decreased when cells are pre-incubated in sodium chlorate, an inhibitor of heparan sulphate synthesis. Third, GDNF has a good consensus heparan sulphate binding sequence [by the criteria of Hileman et al. (Hileman et al., 1998)] G**K**G**RR**G at amino acids 28-33 and a weaker consensus S**R**S**RR**L at 87-93. Neither of these sites is part of the GDNF-GFRα/Ret interface (Baloh et al., 1999), so might be available for binding to heparan sulphate. The first of these sites is known from homologue-scanning mutagenesis studies to be uneccesary for GDNF function, but removal of the second reduces the activity of GDNF by 40-60%, depending on the assay system used (Baloh et al., 1999). This second site is near the end of an alpha helix that spirals up away from the GDNF-GFRα/Ret interface, and may therefore be easily accessible. Even assuming that GDNF does bind heparan sulphate, however, the interaction cannot be responsible for GDNF dimerisation, for that is achieved by a covalent linkage via a disulphide bridge (Hui et al., 1999). In that respect, the GDNF and FGF systems are certainly different.

Might heparan sulphate cross-link GDNF to its receptors, and help to stabilise the interaction? GDNF is thought to assemble into a complex first with GFRα1, and only then to associate with Ret; indeed, there is good evidence that the GDNF/GFRα1 complex can initiate signalling via the Src-like-kinase pathway in its own right even in the absence of Ret (Poteryaev et al., 1999) and presence or absence of Ret on cells that possess GFRα1 has no effect on the observed affinities of GDNF for those cells (Jing et al., 1996). GFRα1 has a possible heparan sulphate binding consensus [by the criteria of Hileman et al. (Hileman et al., 1998)] N**RRK**CH**K**A at amino acids 188-196 at the N terminal boundary of helix 5, a much weaker consensus M**KK**E**K**N at amino acids 93-98 at the N-terminal boundary of helix 3, and a few other clusters of R&K elsewhere. Loss of the N-terminal domain of GFRα1, including the MKKEKN consensus, greatly reduces the ability of GFRα1 to bind GDNF, but it is not clear whether the MKKEKN site is the important part of this domain (Scott and Ibanez, 2001). Neither of these sites is itself the GDNF binding site, as identified by mutagenesis studies (Scott and Ibanez, 2001); it is therefore possible, in principle, that GFRα1 binds heparan sulphate and might therefore be cross-linked to GDNF by their binding the same oligosaccharide, as well as by direct protein-protein interactions. Determining whether this is so awaits detailed structural studies.

How else might heparan sulphate assist GDNF signalling? Since heparan sulphates are borne by proteoglycans of the cell surface and the pericellular matrix, one function of GDNF binding with low affinity to heparan sulphate might simply be to concentrate the growth factor in the vicinity of its high affinity receptors. Our finding that exogenous heparan sulphate inhibits GDNF signalling supports this model, for it suggests that that heparan sulphates are most useful to the system when they are located at the plasma membrane rather than diffusing freely. The model of concentration at the plasma membrane is also supported by our observation that very high concentrations of GDNF can include Ret phosphorylation even in cells depleted of GAGs, for if the concentration of GDNF in bulk medium is high enough, there will be no need for heparan-mediated concentration of GDNF near the plasma membrane. These observations do not, however, exclude the possibility that heparan sulphate also acts by the complex-stabilising model discussed above. They do, however, suggest that it cannot act only by stabilising complexes, for in that case soluble heparan sulphate would be expected to perform as well as proteoglycan-linked material on the surface of the cell. An important caveat to this last remark must be borne in mind, though; the exogenous heparan sulphate added by us might differ significantly (in precise patterns of sulphation, epimerisation etc) from the heparan sulphate with which GDNF normally interacts, and in that case our exogenous heparan might be able to compete with natural heparan sulphate but be unable to promote signalling for reasons of internal structure rather than its location.

In summary, we have shown that cell surface-associated heparan sulphate plays an important role in signalling by GDNF. It will be interesting to determine, in future work, the specific types of heparan involved, how they are involved, and whether expression of these types of heparan sulphate is modulated in a biologically meaningful way during development, disease and regeneration.

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References

Airaksinen, M. S., Titievsky, A. and Saarma, M. (1999). GDNF family neurotrophic factor signaling: four masters, one servant? *Mol. Cell Neurosci.* **13**, 313-325.

Arakaki, N., Hirono, S., Ishii, T., Kimoto, M., Kawakami, S., Nakayama, H., Tsubouchi, H., Hishida, T. and Daikuhara, Y. (1992). Identification and partial characterization of two classes of receptors for human hepatocyte growth factor on adult rat hepatocytes in primary culture. *J. Biol. Chem.* **267**, 7101-7107.

Ashikari, S., Habuchi, H. and Kimata, K. (1995). Characterization of heparan sulfate oligosaccharides that bind to hepatocyte growth factor. *J. Biol. Chem*. **270**, 29586-29593.

Balkovetz, D. F. (1998). Hepatocyte growth factor and Madin-Darby canine kidney cells: in vitro models of epithelial cell movement and morphogenesis. *Microsc. Res. Tech.* **43**, 456-463.

Baloh, R. H., Tansey, M. G., Johnson, E. M., Jr and Milbrandt, J. (1999). Functional mapping of receptor specificity domains of glial cell line-derived neurotrophic factor (GDNF) family ligands and production of GFRalpha1 RET-specific agonists. *J. Biol. Chem.* **275**, 3412-3420.

Bandtlow, C. E. and Zimmermann, D. R. (2000). Proteoglycans in the developing brain: new conceptual insights for old proteins. *Physiol. Rev*. **80**, 1267-1290.

Bordeaux, M. C., Forcet, C., Granger, L., Corset, V., Bidaud, C., Billaud, M., Bredesen, D. E., Edery, P. and Mehlen, P. (2001). The RET proto-oncogene induces apoptosis: a novel mechanism for Hirschsprung disease. *EMBO J.* **19**, 4056-4063.

Bovolenta, P. and Fernaud-Espinosa, I. (2000). Nervous system proteoglycans as modulators of neurite outgrowth. *Prog. Neurobiol*. **61**, 113-132.

Brickman, Y. G., Ford, M. D., Gallagher, J. T., Nurcombe, V., Bartlett, P. F. and Turnbull, J. E. (1998). Structural modification of fibroblast growth factor-binding heparan sulfate at a determinative stage of neural development. *J. Biol. Chem.* **273**, 4350-4359.

Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A. and Davies, A. M. (1995). GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* **15**, 821-828.

Bullock, S. L., Fletcher, J. M., Beddington, R. S. and Wilson, V. A. (1998). Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev.* **12**, 1894-1906.

Cass, W. A. (1996). GDNF selectively protects dopamine neurons over serotonin neurons against the neurotoxic effects of methamphetamine. *J. Neurosci.* **16**, 8132-8139.

Chen, Z., Chai, Y., Cao, L., Huang, A., Cui, R., Lu, C. and He, C. (2001). Glial cell line-derived neurotrophic factor promotes survival and induces differentiation through the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathway respectively in PC12 cells. *Neuroscience* **104**, 593-598.

Davies, J., Lyon, M., Gallagher, J. and Garrod, D. (1995). Sulphated proteoglycan is required for collecting duct growth and branching but not nephron formation during kidney development. *Development* **121**, 1507-1517.

Davies, J. A., Fisher, C. E. and Barnett, M. W. (2001). Glycosaminoglycans in the study of mammalian organ development. *Biochem. Soc. Trans.* **29**, 166-171.

DiGabriele, A. D., Lax, I., Chen, D. I., Svahn, C. M., Jaye, M., Schlessinger, J. and Hendrickson, W. A. (1998). Structure of a heparin-linked biologically active dimer of fibroblast growth factor. *Nature* **393**, 812-817.

Ebendal, T., Tomac, A., Hoffer, B. J. and Olson, L. (1995). Glial cell line-derived neurotrophic factor stimulates fiber formation and survival in cultured neurons from peripheral autonomic ganglia. *J. Neurosci. Res.* **40**, 276-284.

Engele, J. and Franke, B. (1996.) Effects of glial cell line-derived neurotrophic factor (GDNF) on dopaminergic neurons require concurrent activation of cAMP-dependent signaling pathways. *Cell Tissue Res*. **286**, 235-240.

Gash, D. M., Zhang, Z., Ovadia, A., Cass, W. A., Yi, A., Simmerman, L., Russell, D., Martin, D., Lapchak, P. A., Collins, F. et al. (1996). Functional recovery in parkinsonian monkeys treated with GDNF. *Nature* **380**, 252-255.

Guimond, S., Turner, K., Kita, M., Ford-Perriss, M. and Turnbull, J. (2001). Dynamic biosynthesis of heparan sulphate sequences in developing mouse brain: a potential regulatory mechanism during development. *Biochem. Soc. Trans.* **29**, 177-181.

Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., Simmons, L., Moffet, B., Vandlen, R. A. and Simpson, L. (1994). GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* **266**, 1062-1064.

Hileman, R. E., Fromm, J. R., Weiler, J. M. and Linhardt, R. J. (1998). Glycosaminoglycan-protein interactions: definition of consensus sites in glycosaminoglycan binding proteins. *Bioessays* **20**, 156-167.

Hui, J. O., Woo, G., Chow, D. T., Katta, V., Osslund, T. and Haniu, M. (1999). The intermolecular disulfide bridge of human glial cell line-derived neurotrophic factor: its selective reduction and biological activity of the modified protein. *J. Protein Chem.* **18**, 585-893.

Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R. et al. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **85**, 1113-1124.

Kreuger, J., Salmivirta, M., Sturiale, L., Gimenez-Gallego, G. and Lindahl, U. (2001). Sequence analysis of heparan sulfate epitopes with graded affinities for fibroblast growth factors 1 and 2. *J. Biol. Chem*. **276**, 30744-30752.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S. and Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130-1132.

Lyon, M., Deakin, J. A., Rahmoune, H., Fernig, D. G., Nakamura, T. and Gallagher, J. T. (1998). Hepatocyte growth factor/scatter factor binds with high affinity to dermatan sulfate. *J. Biol. Chem*. **273**, 271-278.

Lyon, M., Deakin, J. A. and Gallagher, J. T. (2002). The mode of action of heparan and dermatan sulphates in the regulation of hepatocyte growth factor/ scatter factor. *J. Biol. Chem.* **277**, 1040-1046.

Meng, X., Lindahl, M., Hyvonen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M. et al. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287**, 1489-1493.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver Moore, K. and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-79.

Pellegrini, L. (2001). Role of heparan sulfate in fibroblast growth factor signalling: a structural view. *Curr. Opin. Struct. Biol.* **11**, 629-634.

Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J. et al. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73-76.

Poteryaev, D., Titievsky, A., Sun, Y. F., Thomas-Crusells, J., Lindahl, M., Billaud, M., Arumae, U. and Saarma, M. (1999). GDNF triggers a novel ret-independent Src kinase family-coupled signaling via a GPI-linked GDNF receptor alpha1. *FEBS Lett.* **463**, 63-66.

Ramer, M. S., Priestley, J. V. and McMahon, S. B. (2000). Functional regeneration of sensory axons into the adult spinal cord. *Nature* **403**, 312-316.

Rapraeger, A. C., Krufka, A. and Olwin, B. B. (1991). Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**, 1705-1708.

Sainio, K., Suvanto, P., Davies, J., Wartiovaara, J., Wartiovaara, K., Saarma, M., Arumae, U., Meng, X., Lindahl, M., Pachnis, V. and Sariola, H. (1997). Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. *Development* **124**, 4077-4087.

Scott, R. P. and Ibanez, C. F. (2001). Determinants of ligand binding specificity in the glial cell line-derived neurotrophic factor family receptor alpha S. *J. Biol. Chem.* **276**, 1450-1458.

Shults, C. W., Kimber, T. and Martin, D. (1996). Intrastriatal injection of GDNF attenuates the effects of 6-hydroxydopamine. *Neuroreport* **7**, 627-631.

Tansey, M. G., Baloh, R. H., Milbrandt, J. and Johnson, E. M. (2000).

GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. *Neuron* **25**, 611-623.

Trupp, M., Ryden, M., Jornvall, H., Funakoshi, H., Timmusk, T., Arenas, E. and Ibanez, C. F. (1995). Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *J. Cell Biol.* **130**, 137-148.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991). Cell surface heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**, 841-848.

Zurn, A. D., Baetge, E. E., Hammang, J. P., Tan, S. A. and Aebischer, P. (1994). Glial cell line-derived neurotrophic factor (GDNF), a new neurotrophic factor for motoneurones. *Neuroreport* **6**, 113-118.