Molecular Neurobiology

ISSN0893-7648/98/19(1): 43–59/$14.25

GDNF

A Novel Factor with Therapeutic Potential for Neurodegenerative Disorders

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Abstract

The identification of novel factors that promote neuronal survival could have profound effects on developing new therapeutics for neurodegenerative disorders. Glial cell line-derived neurotrophic factor (GDNF) is a novel protein purified and cloned based on its marked ability to promote dopaminergic neuronal function. GDNF, now known to be the first identified member of a family of factors, signals through the previously known receptor tyrosine kinase, Ret. Unlike most ligands for receptor tyrosine kinases, GDNF does not bind and activate Ret directly, but requires the presence of GPI-linked coreceptors. There are several coreceptors with differing affinities for the GDNF family members. The profile of coreceptors in a cell may determine which factor preferentially activates Ret. In vivo differences in localization of the GDNF family members, its coreceptors and Ret suggest this ligand/receptor interaction has extensive and multiple functions in the CNS as well as in peripheral tissues.

GDNF promotes survival of several neuronal populations both in vitro and in vivo. Dopaminergic neuronal survival and function are preserved by GDNF in vivo when challenged by the toxins MPTP and 6-hydroxydopamine. Furthermore, GDNF improves the symptoms of pharmacologically induced Parkinson's disease in monkeys. Several motor neuron populations isolated in vitro are also rescued by GDNF. In vivo, GDNF protects these neurons from programmed cell death associated with development and death induced by neuronal transection. These experiments suggest that GDNF may provide significant therapeutic opportunities in several neurodegenerative disorders.

Identification of GDNF

Glial cell line-derived neurotrophic factor (GDNF) was cloned as a factor secreted from the glial cell line B49 based on its ability to promote dopamine uptake in embryonic mesencephalic cultures (*1*). Although GDNF was shown subsequently to support the survival of both dopaminergic and motor neurons in vitro and in vivo, the receptor for GDNF remained unknown. The amino acid sequence of GDNF contained a cysteine-knot, as seen in several

other growth factors, such as the neurotrophins and the transforming growth factor-β (TGF-β) superfamily (*2*). The relative spacing of the cysteines closely resembled that of the TGF-β superfamily (*1*). Within this superfamily, which includes activins, bone-morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs), protein sequence similarities within the defined subfamilies range from 40% to >90% (*3*). The GDNF protein sequence was only 20% similar, suggesting that GDNF defined a new subfamily within this superfamily. This relationship to TGF-β implied that the GDNF receptor would be a serine/threonine kinase, like the other members of the TGF-β superfamily. Instead, the signaling receptor for GDNF was found to be a receptor tyrosine kinase and, furthermore, activation of this kinase required the presence of GPI-linked coreceptors. Thus, GDNF represents a new signaling system for the superfamily of cysteine-knot-containing proteins (*2*).

Knockouts

The technique of knocking out the expression of individual genes in mice and examining the effect on development and cellular function has clarified the roles of many proteins. For GDNF, knockouts demonstrated the relationship between an orphan receptor and this orphan ligand.

Ret

The orphan receptor tyrosine kinase Ret was first identified as a human proto-oncogene rearranged in a large proportion of thyroid papillary carcinomas (*4*). In addition, germline point mutations of Ret cause the dominantly inherited cancer syndromes, familial medullary thyroid carcinoma (FMTC) and the multiple endocrine neoplasia (MEN) types 2A and 2B (*5–7*). Loss-of-function mutations of Ret result in Hirschsprung's disease, characterized by a loss of autonomic ganglia in the hindgut (*8–11*). The expression of Ret in the developing CNS and PNS of mice suggested a role for this receptor in neuronal development. It was thought that Ret knockouts might help elucidate this role.

Although mice heterozygous for Ret appeared to be identical to wild-type animals, the homozygous Ret knockouts died within 24 h after birth (*12*). Upon dissection, the mice were found to have either rudimentary or absent kidneys. The rudimentary kidneys had a reduced number of nephric elements that were randomly distributed and had no recognizable medulla or cortex. Normal kidney development involves reciprocal signaling between the ureteric bud and the adjacent nephrogenic mesenchyme (*13–16*). Because Ret was expressed in the ureteric bud, but not in the mesenchyme, it was suggested that Ret in the ureteric bud transduced a signal from the mesenchyme necessary for bud development.

A second abnormality was detected in the homozygous Ret null pups. Milk did not progress from the stomach to the intestine, indicating a defect in gastrointestinal peristalsis. Histological analysis revealed that the neurons of the enteric nervous system were missing from the stomach and both the large and small intestines. Because Ret is normally expressed in these neurons, it appeared that Ret expression was also necessary for their development. Interestingly, the esophageal neurons were present, even though they also express Ret. The ligand(s) for Ret remained unidentified.

GDNF

GDNF was an orphan ligand that protected dopaminergic and motor neurons from degeneration both in vitro and in vivo (*17–23*). It also improved the symptoms of pharmacologically induced Parkinson's disease in both mice and monkeys (*24,25*). In addition, the expression pattern of GDNF as determined by *in situ* analysis suggested that it may have important roles in embryonic kidney and enteric nervous system (*26*). To further explore the role of GDNF in development, several investigators

created GDNF null mice (*27–29*). In all three cases, the homozygous null mice died approx 24 h after birth.

The GDNF heterozygotes exhibited delayed and decreased ureteric branching in the kidney, suggesting a role for GDNF in kidney development (*28*). There was no ureteric bud formation in most of the GDNF null embryos, even though apparently normal mesenchyme was present. In addition, no enteric neurons were present in the stomach, small intestine or colon. Effects such as these were anticipated by the earlier *in situ* analysis showing GDNF expression in embryonic metanephric kidney mesenchyme and in the mesenchymal cells of the intestine (*26*). These phenotypes were strongly reminiscent of the defects in the Ret knockout mice. Additional losses of 20–40% of motor neurons in the trigeminal motor nucleus, superior cervical ganglia, and dorsal root ganglia were also noted (*27,29*). Interestingly, although GDNF rescued the dopaminergic neurons of the substantial nigra and the noradrenergic neurons of the locus coeruleus following lesion in adult rats, the factor was unnecessary for their development (*17,20,25,30,31*). These two regions developed normally in the GDNF null animals.

The remarkable similarities between the Ret and GDNF knockout mice did not go unnoticed. Several laboratories were working to identify the GDNF receptor. Although the data from the knockout mice correctly indicated that Ret was a receptor for GDNF, this was only part of the story.

Elucidation of the Tripartate Signaling System

Confirmation That Ret Mediates GDNF Signaling

Nearly coincident with the publication of the GDNF null mice and their similarity to the Ret null mice, results were presented revealing that GDNF activated Ret (*32–34*). GDNF induced mesoderm formation in *Xenopus* embryos injected with Ret RNA (*32*). GDNF also induced Ret autophosphorylation in the LA-N-5 neuroblastoma cell line (*35*). In addition, GDNF stimulated ureteric bud branching in kidney explants from wild-type mice, whereas neutralizing antibodies against GDNF inhibited branching (*34*). Furthermore, the factor had no effect on explants from Ret null mice (*36*).

Crosslinking studies using iodinated GDNF incubated with the MN1 motor neuron hybrid cell line identified a band of approx 180 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), corresponding to a 15-kDa GDNF monomer bound to a protein of 155 kDa (*33*). This band was found to be tyrosine phosphorylated, and its identity was confirmed using antibodies to Ret. The identification of Ret as the receptor for GDNF was further supported by showing that ^{125}I-GDNF could be crosslinked to Ret transfected into fibroblasts. Yet, in both MN1 cells and fibroblasts, additional crosslinked proteins of 70–95 kDa were observed.

Identification of Coreceptors

The additional bands from GDNF crosslinking suggested the presence of other receptors for GDNF. Expression cloning confirmed the existence of additional novel GDNF-binding proteins, which were anchored to the cell through glycosylphosphatidylinositol (GPI) linkages (*37–39*). One of these receptors, GFRα-1 (Table 1), bound to GDNF with low picomolar affinity. Because GFRα-1 was attached to the cell surface through a lipid linkage, an additional protein that could transduce a signal into the cell was needed, perhaps in a manner similar to that of the CNTF receptor (*40*). The Ret and GDNF knockout results suggested that GFRα-1 was a coreceptor for this signaling system.

GFRα-1 is a high-affinity receptor for GDNF, binding with a low picomolar K_d (*37,38,41*). The requirement for GFRα-1 in GDNF signaling was confirmed by treatment of cells with exogenous phosphoinositide phospholipase C

Table 1
GFRα Coreceptors and Preferred Ligands

Coreceptor	Alternative nomenclature[a]	Preferred ligand
GFRα-1	TrnR-1 (*39*) RETL1 (*48*) GDNFR-α (*37,38*)	GDNF
GFRα-2	TrnR-2 (*39*) NTNR-α (*41,46*) RETL2 (*48*) GDNFR-β (*45,47*)	Neurturin
GFRα-3		Artemin[b]
GFRα-4		Persephin

[a]Because several laboratories identified the coreceptors nearly simultaneously, a consistent nomenclature was adopted to avoid any confusion created by multiple publications (*95*). See text for details on the preferred ligands.

[b]Artemin was recently identified as a specific ligand for GFRα-3 (Balon R. H. et al. [1998] *Neuron* **21**, 1291–1302).

(PIPLC), which releases GFRα-1 from the cell surface (*37,38*). The Neuro-2a neuroblastoma normally express Ret, but no GFRα-1; high-affinity receptors for GDNF could not be detected in these cells. Transient transfection of a GFRα-1 expression vector into these cells resulted in the presence of high-affinity GDNF receptors and allowed GDNF to induce Ret phosphorylation. Furthermore, PIPLC treatment completely blocked GDNF-induced Ret phosphorylation. In addition, the ability of GDNF to rescue rat dopaminergic or motor neurons in vitro was attenuated by PIPLC treatment (*38*). In a manner similar to that demonstrated by other GPI-linked coreceptors, Ret could be activated when expressed in cells without any GFRα-1 if GDNF was applied in the presence of soluble GFRα-1.

Additional Ligand/Coreceptor Family Members

Two growth factors related to GDNF have been identified, neurturin and persephin, which are 63% and 53% similar to GDNF at the amino acid level, respectively (*42,43*). Neurturin promoted the survival of embryonic superior cervical ganglia (SCG) neurons in vitro and preferentially signals through Ret and GFRα-2 (*41*). Persephin promoted the survival of embryonic mesencephalic neurons and lumbar motor neurons in vitro and can signal through GFRα-4 (*43,44*).

A second GPI-anchored coreceptor, GFRα-2, was identified by both expression cloning and sequence similarity to GFRα-1 (*39,41,45–48*). GFRα-2 is about 60% similar to GFRα-1 and also mediates signaling via Ret. GFRα-2 demonstrated selectivity for neurturin, binding neurturin with a K_d value of 10 p*M* but GDNF with a K_d value of >1 n*M* (*41*). By contrast, GFRα-1 had greater affinity for GDNF, binding with a K_d value of 3 p*M*, while binding neurturin with a K_d value of >1 n*M* (*41*). In agreement with these studies, neurturin induced greater Ret phosphorylation when GFRα-2 was coexpressed with Ret than with GFRα-1. Although preferential Ret phosphorylation was not detected when GDNF was examined in this assay, survival of SCG neurons microinjected in vitro with expression plasmids for Ret and either GFRα-1 or GFRα-2 was more selective to either GDNF or neurturin, respectively (*46*). These results collectively support the conclusion that the receptors show preferential signaling of the factors.

A third putative coreceptor, GFRα-3, was not identified functionally, but solely by sequence similarity to GFRα-1 and GFRα-2 (*49–52*). GFRα-3 is the most divergent member of these receptors. The amino acid sequences of GFRα-1 and GFRα-2 are nearly 50% similar, whereas GFRα-3 is only about 35% similar to either. Unlike GFRα-1 and GFRα-2, direct binding of GDNF to GFRα-3 was not detected.

A fourth coreceptor, GFRα-4, was cloned by screening a chicken brain cDNA library with a probe for GFRα-1 (*53*). This coreceptor does not bind GDNF or neurturin; rather, it interacts with persephin with a K_d value of 1 n*M* (*44*). This affinity is not as high as the affinity shown by the other factors for their respective receptors, possibly because the receptor and ligand are from different species (chicken GFRα-4 and mouse persephin). Using expression plas-

mids microinjected into SCG neurons, the interaction of persephin with GFRα-4 is functional, in that it promotes neuronal survival, which is dependent on Ret coexpression (*44*).

Localization

In order to understand the functions of GDNF and its family of factors in vivo, it is important to know where both the factors and their receptors are expressed. Localization of Ret and each of the coreceptors may identify the cell populations preferentially responsive to either GDNF or neurturin. Because many of the tissues that express GDNF or Ret are apparently unaffected in the homozygous null animals, these proteins are apparently important in the development of only select tissues and must have alternative roles in the unaffected tissues. In addition, the presence of additional signaling molecules or signaling mechanisms is suggested by results identifying tissues in which only one of the receptors and/or ligands localize.

GDNF and Family Members

GDNF was detected by *in situ* analysis both centrally and peripherally in several tissues. In the CNS, GDNF was not detected in developing or adult dopaminergic neurons of the substantia nigra, but rather in the striatum, the target of the dopaminergic nigral projections (*47,54*). GDNF was also expressed in several other adult brain regions, including hippocampus, cerebellum, and thalamus (*47,54*). In embryonic rat, GDNF was detected at high levels in kidney, intestine, stomach, and testis (*26,47,55*). In kidney and testis, GDNF mRNA was present only during the embryonic or newborn stages, and not in the adult. Although GDNF expression in embryonic tissue was always higher than in adult, the development of most of these tissues was apparently unaffected in the GDNF null mice (*56*).

Neurturin expression was detected by *in situ* analysis in several regions of developing rat brain, including striatum, pineal gland, and brainstem (*47*). Analysis in the adult CNS found neurturin only in the intermediate lobe of the pituitary gland, a part of the endocrine system. Several peripheral tissues expressed neurturin during development, including olfactory mucosa, salivary glands, lung, intestine, testis, and kidney. None of these tissues expressed neurturin in the adult, except for testis (*47*). This finding is in dramatic contrast to the expression pattern of GFRα-2, the putative receptor for neurturin, which was detected in several regions of the adult CNS (*41,47*)(see below).

Persephin, as determined by reverse transcription-polymerase chain reaction (RT-PCR), was detected in heart, kidney, liver, and brain of both E18 and adult rat (*43*).

Ret

Mutations of the *ret* gene in humans can result in an inherited syndrome of multiple endocrine neoplasia as well as multiple peripheral neuronal abnormalities. The wild-type expression of Ret during development correlated with the tissues affected by the mutated protein (*13,57*). In the mouse peripheral nervous system, *in situ* hybridization analysis showed Ret was present at high levels in subsets of cells of the sensory, autonomic, and enteric neuronal lineages during development. High levels of Ret in the CNS were detected in many regions, including the dopaminergic neurons of the substantia nigra, the somatic and visceral motor neuron groups of the hindbrain, hippocampus, and spinal cord. No Ret was detected in the striatum. As noted earlier, the localization of Ret to the nephric duct and the ureteric bud epithelium supports a role for Ret in kidney development (*13,16,56*). Ret is expressed in many of the same areas in both chick and zebrafish (*58,59*). The critical role of Ret during development and the conservation of its expression pattern throughout vertebrates suggest that its functions are conserved as well.

Coreceptors

Both GFRα-1 and GFRα-2 were detected by *in situ* hybridization analysis at high levels in many regions of the developing and adult CNS (*39,47,54*). In the dopaminergic neurons of the substantia nigra of adult rat, GFRα-1 was expressed at high levels, but no GFRα-2 was detected (*47,54*). Many other regions of the adult CNS expressed both receptors, such as hippocampus, cerebellum, and thalamus. Two regions that expressed GFRα-2, but not GFRα-1, are the subthalamic nucleus and the glomerular layer of the olfactory bulb (*49*).

GFRα-3 expression was highest in embryonic d 11 rat or mouse brain and rapidly decreased thereafter (*49–52*). No GFRα-3 was detected in adult brain (*49*). By contrast, GFRα-3 was expressed in several peripheral neuronal ganglia, i.e., the dorsal root ganglia, trigeminal ganglia and superior cervical sympathetic ganglia. These results indicate a role for this receptor in peripheral neuronal function. GFRα-4 expression increases in developing chicken brain from E6 to E18, becoming as prominent as that in any other tissue (*53*). In the adult, although its level in brain appears to be much lower, it is expressed at high levels in spinal cord.

The coreceptors were also expressed in many non-neuronal tissues. In fetal rat, although GFRα-1 was detected in many brain regions, the highest levels were in intestine and kidney (*48*). GFRα-1 was also detected in adult rat ovary, heart, lung, and kidney at levels similar to levels found in brain (*48*). GFRα-2 was detected in fetal human lung and kidney at levels similar to those found in fetal brain (*45*). In human adult, GFRα-2 was expressed in small intestine, heart, testis, and placenta at levels lower than those found in brain (*45,47*). There may be some differences in tissue expression between human and rat: GFRα-2 was expressed in fetal rat liver at levels higher than any other tissue examined while no expression could be detected in fetal human liver (*45,46*). GFRα-3 was detected in several tissues in rat, including embryonic skeletal muscle and adult heart, lung, liver, kidney, and duodenum (*49–52*). GFRα-4 is expressed in most tissues during chicken development (*53*). In the adult, however, expression is predominantly limited to spinal cord and kidney.

Circuitry of the Tripartate Signaling Complex

Comparison of GDNF, GFRα-1, and Ret expression in adult rat brain suggests a role for GDNF in the maintenance of neuronal circuits (*54*). High levels of both Ret and GFRα-1 were detected in the dopaminergic neurons of the substantia nigra, but no GDNF was detected in these neurons (*47,54*). Instead, GDNF was expressed by the striatum, the synaptic target of the dopaminergic nigral projections. These data suggest that GDNF may be an important maintenance factor for nigral DA neurons (*47*).

The role of neurturin and its putative receptor GFRα-2 in the brain is unclear. Neurturin was virtually undetectable in the adult brain but GFRα-2 was expressed in multiple brain regions (*47*). This finding suggests that either signaling of GDNF by GFRα-2 may be physiologically important or that there are additional ligands for GFRα-2.

The existence of additional receptors may be indicated by the several tissues in which either one of the co-receptors or Ret were expressed, but not both. For example, *in situ* hybridization indicated that Ret, but no GFRα-1, was detected in the granular layer of the cerebellum and the subthalamic nucleus (*54*). Conversely, GFRα-1, but no Ret, was detected in the lateral geniculate nucleus, in extensive regions of the cerebral cortex, and perhaps in the hippocampus (*54,60*). In addition, GDNF is expressed by the sensory hair cells of the rat cochlea and GFRα-1 is expressed by the auditory neurons that innervate the hair cells (*61*). Lesion models that induce loss of the hair cells result in a secondary loss of the auditory neurons. Although the auditory neurons do not express Ret detectable by *in situ* hybridization GDNF promoted neuronal survival in these lesion models. These results strongly suggest

Table 2
Primary Activities of GDNF In Vitro and In Vivo

Tissue/cell type	Comments	References
In vitro		
Kidney ureteric bud	Enhances bud growth	*(36)*
Dopaminergic neurons (mesencephalic cultures)	Protects against trophic withdrawal and MPP^+	*(1,64,65)*
Motor neurons (cranial and spinal cord)	Protects against trophic withdrawal	*(19,23)*
Sympathetic ganglia (nodose, superior cervical, trigeminal)	Protects against trophic withdrawal; sensitivity is developmentally regulated	*(19,42,55,66)*
In vivo		
Dopaminergic neurons (axonal transection, 6-OHDA, MPTP)	Responsiveness determined by cell counts, TH measurements, and behaviorally	*(17,20,24,30,67,68,71–73,75)*
Motor neurons (embryonic programmed cell death, axonal transection)	Both newborn and adult motor neurons respond after transection	*(18,19,21,22,76–78)*

either the existence of a novel Ret homolog or a novel signaling mechanism by the co-receptors.

Regarding the coreceptors, an alternative mechanism could account for their absence from Ret-expressing cells. GFRα-1 shed from a cell surface could present GDNF to an apposing cell (*54,62*). This scenario is supported by the regulation of Ret, GDNF and GFRα-1 mRNA expression following rat or chick sciatic nerve transection (*54,55,63*). In rat, Ret mRNA expression is detected by *in situ* hybridization in the sacrolumbar spinal motor neurons which contribute axons to the sciatic nerve. After transection, there is an increase of Ret expression in these neurons. GFRα-1 and GDNF mRNA expression also increased after transection, but distal to the lesion. It is possible that the GFRα-1 is cleaved from the expressing cells and functions to present the soluble GDNF to the regenerating axons, which would be growing through an increasing concentration gradient of the activating GFRα-1/GDNF complex.

Activity of GDNF In Vitro

Several in vitro models using primary cells or tissue have been used to examine GDNF function (Table 2). Because GDNF was isolated as a supportive factor for dopaminergic neurons, this interaction has been extensively studied. The expression of both Ret and GDNF mRNA in motor neurons resulted in many studies with these cells, as well. However, the dramatic effect of the Ret and GDNF knockouts on kidney development presented a strong rationale for studying the function of these proteins in embryonic kidney.

Kidney Ureteric Bud Branching

In the embryo, the kidney initially develops as a bud from the wolffian duct into the nephrogenic mesenchyme (*16*). The elongating bud induces epithelial transformation of the mesenchyme, followed by branching of the bud. This process is repeated hundreds of thousands of times to establish the network of

collecting ducts. Ret is expressed only at the tip of the elongating bud (*13*). GDNF-soaked beads applied to kidney rudiments placed in vitro expanded the diameter of the nearby branches, with most growing buds directed toward the beads (*36*). In the Ret null mice, the embryonic kidney rudiments did not respond to GDNF-soaked agarose beads (*36*).

In spite of this increase in bud branching, GDNF itself is not mitogenic (*36*). BrdU-labeling of ureteric buds was not detected when they are incubated with GDNF in isolation from the mesenchyme. When isolated pairs of buds are grown without a supportive matrix (hanging drop culture), they remained small, cells were shed and the epithelial morphology became disrupted. Addition of GDNF to these cultures resulted in bud fusion, few cells shed and retention of epithelial morphology. Furthermore, addition of exogenous GDNF had no effect on ureteric branching if the kidney mesenchyme was replaced with that from tooth, salivary, limb, or gut. These results suggest that GDNF increased adhesion between cells and that additional factors, specific to kidney mesenchyme, are necessary to stimulate bud branch development. Yet, it is clear that the role of GDNF is critical, as the effects of the GDNF knockout are devastating to kidney development.

Interestingly, GDNF may also affect adhesion of neurons. A dramatic difference was detected between the neurite outgrowth induced by NGF and GDNF in paravertebral ganglia explants. GDNF elicited fasciculated outgrowth, whereas NGF produced thin, long individual neurites (*55*). The fasciculated appearance of the GDNF-induced neurites may be caused by increased adhesion among the neurites, much like the effect GDNF appears to have in embryonic kidney bud development.

Dopaminergic Neurons

Mesencephalic cultures from embryonic d 14 (E14) rats are used to study dopaminergic neurons. These cells are characterized by being tyrosine hydroxylase positive (TH^+) and comprise about 5% of the cells in culture. If exogenous factors, such as neurotrophins, are not added to the cultures, both the total neuronal cell number and the TH^+ neuronal cell number decrease by 70% over a period of 3 wk (*1*). At least a portion of the TH^+ neurons die by apoptosis. The addition of GDNF to the culture media completely rescued the TH^+ neurons without measurably altering the total neuronal population loss (*1,64*). GDNF also affected neuronal function of the TH^+ cells: dopamine uptake increased by nearly threefold, neurite outgrowth was induced and cell body size increased (*1,65*). GDNF had no effect on the survival of γ-aminobutyric acid (GABA)-ergic or serotonergic neurons in these cultures (*1,64*).

The toxin MPTP and its active derivative, MPP^+, are toxic to dopaminergic neurons both in vitro and in vivo and are used to injure and kill these neurons selectively. The effect of GDNF on MPP^+ induced death in embryonic mesencephalic cultures depended on when it is added relative to toxin. GDNF completely rescued the TH^+ neurons if added prior to MPP^+. When the cells were first treated with MPP^+ alone, GDNF had no effect on the loss of TH^+ neurons (*65*). However, the remaining TH^+ neurons showed an increase in DA uptake, suggesting that survival and enhanced DA uptake are separatable properties of GDNF.

Motor Neurons

GDNF is a potent survival factor for motor neurons. Embryonic rat cranial motor neurons represent about 1% of the neurons in ventral mesencephalon cultures and are positive for choline acetyltransferase (ChAT) (*23*). The addition of GDNF to the culture media resulted in a threefold increase in the number of surviving motor neurons and a concomitant sixfold increase in ChAT activity (*23*). GDNF also increased the number and length of the motor neuron neurites, in a manner similar to that observed with the embryonic dopaminergic neurons. The survival of enriched motor neurons from E14 rat spinal cord was sup-

ported by GDNF with a half-maximal effective concentration (EC_{50}) of 7 f*M* (0.2 pg/mL). GDNF was 75–, 650–, and 2500-fold more potent than BDNF, CNTF and CDF-LIF, respectively (*19*). Because these cultures consisted almost entirely of motor neurons, it is likely that GDNF directly affected the motor neurons, rather than acting through an indirect pathway involving other cells in the culture.

Developmental Changes in Sensitivity

Several neuronal populations have varying sensitivity to GDNF, depending on developmental stage. The EC_{50} value of GDNF for survival of embryonic rat nodose ganglia (NG) neurons increased with age, from 0.12 ng/mL in E6 neurons to 6.1 ng/mL in E12 (*66*). GDNF also promoted survival of rat E14 trigeminal ganglia (TG) neurons (*66*). Although few TG neurons were responsive at E8, the response to GDNF increased over time, reaching a maximum by E12. However, another report indicated that GDNF had no effect on E18 rat TG neurons, suggesting that these neurons may become insensitive again as they mature (*19*).

Several studies report varying degrees of GDNF-mediated survival of sympathetic neurons from rat SCG, perhaps because of differences in developmental stage. While one report indicated GDNF had no effect on E18 rat SCG neuronal survival, E21 SCG neurons were rescued to a similar degree by GDNF and NGF (*42,66*). As the neurons matured further, GDNF again appeared to lose efficacy, because it maximally rescued only 10% of postnatal d 1 SCG neurons vs 80% rescued by NGF. Paravertebral sympathetic neurons may also rapidly alter their sensitivity to GDNF during development. GDNF rescued 70% of these neurons from E10 chick, whereas cells from E8 were barely responsive (*66*). It may be necessary to examine receptor expression in the different neuronal populations to determine whether differences in responsiveness are attributable to receptor expression or to downstream effectors.

Activity of GDNF In Vivo

To examine the effects of GDNF on the CNS in vivo, most studies focused on two cell types: mesencephalic dopaminergic neurons and motor neurons (Table 2). Studies examining the activity of GDNF on dopaminergic lesions in vivo strongly support the current interest in using this factor as a therapeutic for Parkinson's disease.

Dopaminergic Neurons

Several lesion techniques were used to test potential therapeutics for their ability to promote dopaminergic (DA) neuronal survival in vivo. GDNF was effective to some degree in all of these.

Axonal Transection

The DA neurons of the substantia nigra project to the striatum via the nigrostriatal tract. Cutting the medial forebrain bundle, resulting in transection of the nigrostriatal tract, induces loss of DA neurons in the substantia nigra. The dopaminergic neurons of the ventral tegmental area (VTA) are also lost in this model, since their projections to the frontal cortex are also cut when the medial forebrain bundle is transected. These VTA neurons are unaffected in the 6-OHDA model since the toxin is applied directly to the neurons in the substantia nigra or their terminals in the striatum. MPTP also does not affect the VTA neurons. In one series of experiments, 50% of the TH^+ neurons in the SN and VTA were lost 2 wk after transection (*20*). By contrast, 85% of TH^+ neurons remained after 2 wk when treated with daily GDNF injections adjacent to the substantia nigra. DA neurons in both the substantia nigra and ventral tegmental area were rescued to the same extent by GDNF. By contrast, TGF-α, bFGF, or NT-4/5 had no significant effect on neuronal survival in the substantia nigra when administered in the same manner.

The effects of a unilateral nigrostriatal lesion can be tested behaviorally because the striatal postsynaptic receptors on the lesioned side

become supersensitive to DA agonists. Parenteral administration of drugs, such as apomorphine, elicits a stable pattern of rotation that reflects the extent of DA degeneration (*67*). GDNF, administered by implantation of recombinant BHK cells expressing GDNF adjacent to the substantia nigra, diminished rotational behavior after medial forebrain bundle unilateral transection (*68*). Although this GDNF treatment also rescued the TH^+ cells in the substantia nigra, it did not prevent the loss of striatal dopamine, indicating that the nerve terminals were lost. The investigators suggested instead that GDNF increased dendritic sprouting in the substantia nigra, altering the feedback loop between the SN pars compacta neurons and the SN pars reticulata neurons. These results suggest GDNF can access alternative neuronal mechanisms for recovery from axotomy other than direct reinnervation into the striatum.

6-Hydroxydopamine Lesion

Nigral dopaminergic cell loss is induced by injection of the dopaminergic-specific toxin, 6-hydroxydopamine (6-OHDA), directly into the substantia nigra or into the medial forebrain bundle, which disrupts the nigrostriatal pathway. Both lesions result in a rapid loss of 90% of the DA neurons (*69*). Rats given 6-OHDA directly into the medial forebrain bundle showed apomorphine-induced rotational behavior equivalent to >95% loss of striatal DA and a biochemically determined striatal DA depletion of 70% (*25,70*). Four weeks following the 6-OHDA lesion, GDNF was administered adjacent to the substantia nigra. The GDNF-treated animals demonstrated decreased apomorphine-induced rotational behavior, which was maintained for ≤5 wk after dosing. GDNF treatment resulted in nigral DA levels 80% of the contralateral control, whereas vehicle-treated animals were only 20% of control. A single application of GDNF into the substantia nigra, 24 h before 6-OHDA injection at the same site, resulted in significant survival of DA neurons in the substantia nigra two weeks later as measured by TH^+ neuronal cell counts (*17*). These results demonstrated that a single application of GDNF is efficacious over an extended period.

Alternatively, 6-OHDA can be administered unilaterally into the striatum, thereby lesioning the dopaminergic terminals. This approach produces a delayed and progressive degeneration of the ipsilateral nigral dopamine neurons with onset 1 wk after injection (*71*). This delayed, slow mode of onset may more accurately reflect the loss of neurons in Parkinson's disease. In addition, the neurons that specifically project to the lesion site can be identified by fluorogold labeling. At 4 wk postlesion, the number of nigral neurons that projected specifically to the striatal lesion site, as identified by fluorogold labeling, were reduced by 65%, compared with the contralateral sham-lesioned control and the remaining fluorogold-labeled neurons, which were significantly atrophied (*72*). Application of GDNF directly into the substantia nigra every other day for 4 wk resulted in essentially complete protection of both neuronal number and size. Intrastriatal injection of replication deficient adenovirus, engineered to express GDNF, was nearly as effective in this model as direct injection of purified factor (*72,73*).

MPTP Lesion

Peripheral administration of another toxin, MPTP, results in Parkinson's like symptoms and lesions the DA neurons in man and monkey (*74*). MPTP also lesions DA neurons in mice. Treatment of mice with MPTP results in a 90% decrease of DA levels in the striatum within 6 d after injection (*30*). Injection of GDNF directly into the substantia nigra 24 h before MPTP administration resulted in the preservation of striatal TH^+ nerve terminals and partially maintained DA levels in both the striatum and the substantia nigra. Injection of GDNF 1 wk after application of MPTP also reduced the decrease in DA levels, as well as maintaining TH^+ nerve terminals, but to a lesser degree. Behavioral tests supported these results because GDNF-treated animals exhibited greater motor activity than did vehicle treated.

Of perhaps greater import with regard to human drug therapy are the effects of GDNF on induced Parkinson's disease in primates. In one study, the animals were pretreated with MPTP 3 mo before GDNF treatment (*24*). A single injection of GDNF, given either intranigral, intracaudate, or intracerebroventricular (ICV), resulted in significant improvements in the parkinsonian features of bradykinesia, rigidity, and postural instability by 2 wk after dosing. These effects continued for at least 4 wk after dosing. By contrast, the behavioral effects of a single administration of the standard anti-parkinsonian drug, L-DOPA, lasts only several hours. The ICV-treated animals were then maintained on GDNF, dosing once every 4 wk. This was sufficient to maintain the behavioral improvements. Histological and biochemical analysis of the brains showed an increased number of TH^+ neurons and an increase in dopamine levels in the substantia nigra of the treated animals.

A second study examined the dose response of GDNF in parkinsonian monkeys (*75*). The animals were dosed four times, once every 4 wk, and then followed for 4 mo following the last dose. Although the animals dosed at 100 and 1000 µg had their parkinsonian features increase in severity by the end of the 4 mo, the animals dosed at 300 µg retained their functional improvements. Clearly, GDNF has dramatic long-term effects on this model of Parkinson's disease.

Motor Neurons

GDNF promoted the survival of embryonic motor neurons undergoing programmed cell death *in ovo* and in lesion models in newborn and adult animals. After transection of the facial nerve in neonatal rats, direct cell counts showed that 6–30% of motor neurons survive 7 d later (*19,22,76*). Direct application of GDNF in Gelfoam at the site of transection resulted in nearly 100% neuronal survival. Lesioned adult facial motor neurons were also responsive to GDNF applied in the same manner, attenuating the loss of ChAT immunoreactivity. Administration of GDNF was effective in this model whether administered locally or subcutaneously. GDNF expressed by viral vectors and injected into the facial muscles also rescued axotomized facial neurons in the neonatal rat (*77,78*).

During development of the chick embryo, the main period of normal spinal cord motor neuron programmed cell death is from embryonic d 6 to d 9. GDNF applied directly to the chorioallantoic membrane rescued about 25% of the motor neurons that would have normally died (*21*). GDNF also rescued adult rat spinal cord motor neurons following spinal root avulsion of the seventh cervical (C7) segment (*18*). After 3 wk, only 30% of the C7 spinal cord motor neurons remained in control animals. GDNF, applied in Gelfoam directly in contact with the lesioned spinal cord, preserved 65% of the motor neurons, whereas neither NGF, BDNF, nor IGF-1 applied in the same manner was found to increase survival significantly.

A unique model of motor neuronal death is presented in the *pmn/pmn* mice, which develop weakness in the hindlimbs during the third week of life and die at about 6 wk of age (*79,80*). Animals implanted with encapsulated GDNF-expressing fibroblasts did not live significantly longer than untreated *pmn/pmn* mice (*81*). Even though the facial nucleus motor neurons become demyelinated in both control and GDNF-treated animals, control animals lost 30% their facial motor neurons, whereas GDNF-treated animals lost only 16% of these neurons (*81*). Thus, GDNF supported survival of the motor neuronal cell bodies without affecting axonal degeneration.

Additional Targets

GDNF promoted survival of several other neuronal types in vivo. Axotomy of the fimbria/fornix results in severing the cholinergic neuronal projections of the medial septum to the ipsilateral hippocampus. Within 2 wk, these cells downregulate expression of ChAT, both the p75 and trkA NGF receptors, and become morphologically unrecognizable as neurons

(*82,83*). GDNF treatment by ICV administration completely prevented the loss of p75-positive neurons, which decreased by 40% in untreated animals (*84*). Injection of 6-OHDA adjacent to the locus ceruleus (LC) resulted in 50% loss of the TH^+ cells in the LC within 6 d (*31*). Grafting of GDNF-expressing fibroblasts near the LC before injection of toxin resulted in 100% survival of the TH^+ cells. GDNF increased the level of TH expression, in addition to inducing sprouting. Curiously, the effects of GDNF were potentiated by the lesion, suggesting upregulation of GDNF receptors, as has been noted in several lesion models (*54,55,85,86*).

Therapeutic Potential

The dramatic effects of GDNF on dopaminergic neurons in vitro and its ability to ameliorate DA lesions both in rats and in monkeys has raised hopes that it can be used as a therapeutic in Parkinson's disease. Clinical trials sponsored by Amgen, Inc. are currently in progress testing whether GDNF dosed by intrathecal application, i.e., directly into the cerebrospinal fluid, can alter progression of Parkinson's disease in humans. Since intracerebroventricular injections of GDNF reversed the effects of an MPTP lesion in monkeys, simply bathing the CNS in GDNF may produce clinically significant results. However, previous clinical trials in which NGF was administered to the CSF resulted in both loss of appetite and pain associated with movement (*87*). These side effects were not noticed when NGF was administered directly into the putamen of patients in a Parkinson's disease trial (*88*). Similarly, it may be necessary to administer GDNF directly to the DA neurons.

Alternative techniques are being explored to express GDNF in the CNS at its sight of action. Virus constructed specifically to express GDNF, injected into the rat striatum, was effective in limiting the effects of the progressive 6-OHDA lesion model as measured by the number of TH^+ cells and amphetamine-induced rotational behavior (*89*). By contrast, virally expressed GDNF injected into the substantia nigra resulted in survival of DA neurons, but no reduction in rotational behavior, suggesting that the nerve terminals were not preserved (*90*). This finding indicates that GDNF expressed locally in the striatum may maintain the nigral projections in the striatum, whereas GDNF expressed locally in the substantia nigra may not provide sufficient support of these projections. Thus, in therapies in which GDNF is administered locally, it may be necessary to overexpress GDNF mainly in the target of the neuronal projections.

GDNF may also be effective as a therapeutic for neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS). Effective systemic administration of growth factors, however, can be difficult. There were few adverse advents in the ALS clinical trials of insulin-like growth factor-1 (IGF-1), which is found at high levels in the circulation (*91–93*). By contrast, subcutaneous administration of ciliary neurotrophic factor (CNTF) for ALS induced multiple adverse events, including anorexia, weight loss, and cough (*94*). These were of sufficient intensity as to limit its dosing in many patients. Conceptually, the adverse events from CNTF are likely the result of high circulating levels of a factor that is normally present at very low levels, activating additional receptors. The efficacy of GDNF may similarly be limited by its effects on receptors outside the therapeutic target. Because receptors for GDNF are distributed both centrally and peripherally, whereas GDNF expression itself is mostly limited to the CNS in the adult, high peripheral levels of GDNF could be a concern.

Regarding the two IGF-1 trials for ALS, only one study showed a robust effect in slowing the progression of ALS (*92*). The CNTF trial showed no significant effect on either mortality or progression of the disease (*94*). GDNF is not currently in use in human trials for ALS.

Therapies that take advantage of GDNF are very attractive, owing to the striking effects of the factor on cell types important in several neurodegenerative disorders. The ongoing pre-

clinical and clinical experiments with GDNF will demonstrate whether this factor can be exploited for therapeutic use. However, the novel signaling system of GDNF, employing a receptor tyrosine kinase and nonsignaling co-receptors, may provide as yet unidentified targets that lead to novel therapeutics for PD, ALS, and other neurodegenerative diseases.

Acknowledgments

I thank C. Ibanez, J. Ylikoski, and U. Pirvola for communicating unpublished results and A. Camoratto, N. Neff, M. Saporito, M. Airaksinen, and M. Saarma for their critical reading of the manuscript.

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