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Artemin, a Novel Member of the GDNF Ligand Family, Supports Peripheral and Central Neurons and Signals through the GFRα3–RET Receptor Complex

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Summary

The glial cell line–derived neurotrophic factor (GDNF) ligands (GDNF, Neurturin [NTN], and Persephin [PSP]) signal through a multicomponent receptor system composed of a high-affinity binding component (GFRα1–GFRα4) and a common signaling component (RET). Here, we report the identification of Artemin, a novel member of the GDNF family, and demonstrate that it is the ligand for the former orphan receptor GFRα3–RET. Artemin is a survival factor for sensory and sympathetic neurons in culture, and its expression pattern suggests that it also influences these neurons in vivo. Artemin can also activate the GFRα1–RET complex and supports the survival of dopaminergic midbrain neurons in culture, indicating that like GDNF (GFRα1–RET) and NTN (GFRα2–RET), Artemin has a preferred receptor (GFRα3–RET) but that alternative receptor interactions also occur.

Introduction

Neurotrophic factors represent a heterogeneous group of signaling molecules that orchestrate multiple aspects of the development and maintenance of the central and peripheral nervous systems (CNS and PNS). The GDNF family of ligands, consisting of glial cell line–derived neurotrophic factor (GDNF; Lin et al., 1993), Neurturin (NTN; Kotzbauer et al., 1996), and Persephin (PSP; Milbrandt et al., 1998) comprise a subfamily of ligands within the TGF-β superfamily of signaling molecules that have neurotrophic properties. Interest in the GDNF family was initially fueled largely by the ability of all known members to support the survival of dopaminergic midbrain neurons and spinal and facial motor neurons in both in vitro survival and in vivo injury paradigms, identifying them as potential therapeutic agents in the treatment of neurodegenerative diseases (Lin et al., 1993; Henderson et al., 1994; Oppenheim et al., 1995; Horger et al., 1998; Milbrandt et al., 1998; reviewed by Grondin and Gash, 1998). However, the GDNF ligands also influence a broad spectrum of other neuronal populations in both the CNS and PNS. GDNF and NTN both support the survival of many peripheral neurons in culture, including sympathetic, parasympathetic, sensory, and enteric neurons (Buj-Bello et al., 1995; Ebendal et al., 1995; Trupp et al., 1995; Kotzbauer et al., 1996; Heuckeroth et al., 1998). In contrast, PSP does not share any of these peripheral activities but does support the survival of dopaminergic midbrain neurons and motor neurons (Milbrandt et al., 1998). Despite the fact that GDNF acts on many cell populations in vitro, analysis of GDNF knockout mice revealed that the major developmental importance of GDNF is in the enteric nervous system and in kidney organogenesis, both of which are lost in GDNF null mice (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996).

Although members of the TGF-β superfamily, the GDNF ligands utilize a receptor system that is more similar to that observed for many cytokines, in that a common signaling component is used by multiple ligands, and an additional protein binds to the ligand with high affinity and provides specificity. The common signaling component for the GDNF ligands is the RET receptor tyrosine kinase, which is expressed at all known sites of GDNF ligand action and is activated by GDNF or NTN stimulation in multiple in vitro paradigms (Durbec et al., 1996; Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996; Worby et al., 1996; Creedon et al., 1997). GFRα1-GFRα4 comprise a structurally novel family of glycosyl-phosphatidyl inositol– (GPI-) anchored proteins that function as high-affinity binding proteins for the GDNF ligands. Results from in vitro experiments by multiple groups together indicate that GFRα1–RET is the preferred receptor for GDNF, and GFRα2–RET is the preferred receptor for NTN; however, cross talk between the different receptors is possible (Jing et al., 1996; Treanor et al., 1996; Baloh et al., 1997; Buj-Bello et al., 1997; Jing et al., 1997; Klein et al., 1997; Sanicola et al., 1997; Suvanto et al., 1997). Recent analysis of GFRα1-deficient mice indicated that GFRα1 is the only physiologically critical GDNF coreceptor in kidney organogenesis and enteric nervous system development (Cacalano et al., 1998; Enomoto et al., 1998). However, GDNF-deficient mice have greater losses in peripheral ganglia than GFRα1-deficient mice, suggesting that GDNF can utilize another receptor to support the survival of peripheral neurons, likely GFRα2–RET (Cacalano et al., 1998; Enomoto et al., 1998). PSP cannot signal through either the GFRα1–RET or GFRα2–RET receptor complexes (Milbrandt et al., 1998), but a recent report indicated that PSP binds to GFRα4, a receptor currently only identified in the chicken (Enokido et al., 1998).

GFRα3 was first identified as an expressed sequence tag (EST) that is homologous to GFRα1 and GFRα2 (Jing et al., 1997; Baloh et al., 1998; Naveilhan et al., 1998; Widenfalk et al., 1998; Worby et al., 1998). However, analysis in transfected cells indicated that GFRα3 could

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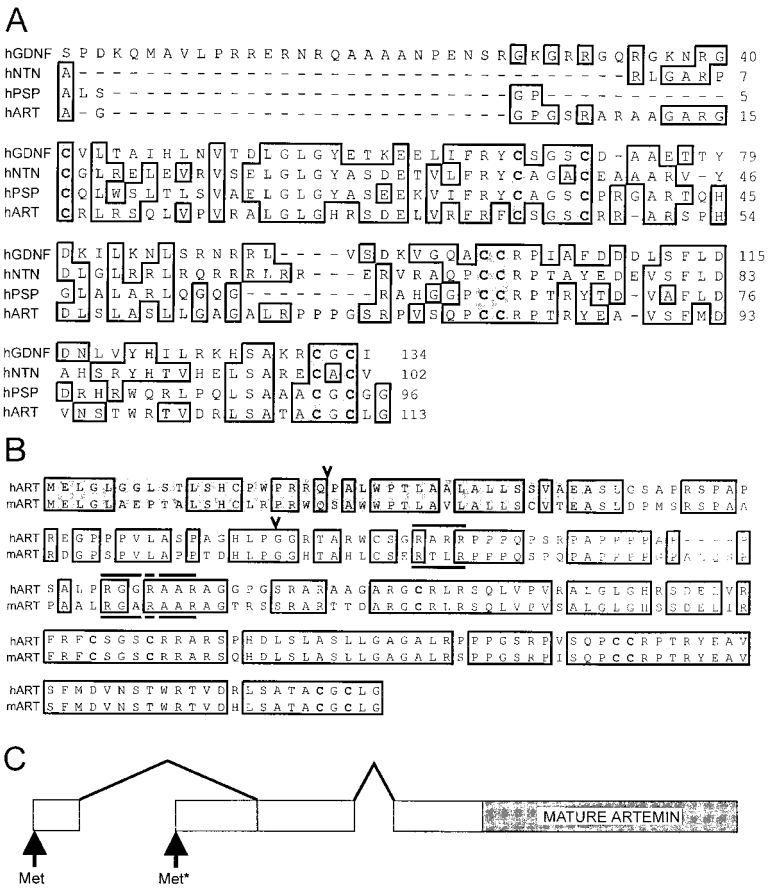

Figure 1. Artemin Is a Member of the GDNF Family of Ligands

(A) Alignment of predicted mature human Artemin (ART) with the other GDNF family ligands. Conserved cysteine residues are in bold type and shaded.

(B) Alignment of human and mouse prepro Artemin. The putative signal sequence is shaded, and putative RXXR cleavage sites are designated by thick lines. Arrowheads denote the location of the two introns in the *ARTEMIN* gene.

(C) A schematic diagram of the location of the two introns in the *Artemin* cDNA and their splicing. cDNAs containing a second starting methionine were also identified by RACE PCR from some human cDNA libraries (Met*); however, a similar message was not identified in mouse, and mouse genomic sequence does not contain a methionine in this position. Mature Artemin protein generated from either of the human messages would be identical.

not form a functional receptor with RET for any of the known GDNF ligands (Baloh et al., 1998; Worby et al., 1998). GFRα3 expression is much more restricted than GFRα1 or GFRα2, with high-level expression observed only in developing peripheral nerves and ganglia (Baloh et al., 1998; Naveilhan et al., 1998; Widenfalk et al., 1998; Worby et al., 1998). Furthermore, one report demonstrated that in sensory neurons of the trigeminal ganglion, GFRα3 is expressed in a population of neurons distinct from GFRα1 and GFRα2 and likely overlaps with RET (Naveilhan et al., 1998). Therefore, structural similarity, expression, and functional data together suggested that GFRα3 can interact with RET and that another member of the GDNF ligand family remained to be identified.

Here, we report the identification of a novel member of the GDNF ligand family, which we have named Artemin. Like GDNF and NTN, Artemin supports the survival of several peripheral neuron populations and can also support dopaminergic neurons of the ventral midbrain. However, Artemin appears to be the only member of the GDNF family that binds to and activates the GFRα3–RET receptor complex. Interestingly, Artemin can also activate the GFRα1–RET receptor complex, indicating that like GDNF (GFRα1–RET) and NTN (GFRα2–RET), Artemin has a preferred receptor (GFRα3–RET), but alternative receptor interactions are possible.

Results

Identification and Analysis of the *ARTEMIN* Gene and cDNA

In an effort to identify additional members of the GDNF family, we searched multiple databases using the BLAST 2.0 algorithm (Altschul et al., 1997), with mature NTN protein sequence as a query. A search of the high throughput genome sequences (HTGS) database revealed two human bacterial artificial chromosome (BAC) clones (AC005038, AC005051) containing a homologous open reading frame (hORF) similar but not identical to human GDNF, NTN, or PSP. A search of the EST database using the predicted 3′ untranslated region of this hORF revealed two ESTs (AA931637 and AA533512), suggesting that the hORF represents an expressed gene. We also identified by PCR a fragment from mouse genomic DNA corresponding to the same hORF. To isolate full length mRNA species encoded for by this hORF, we performed rapid amplification of cDNA ends (RACE) PCR on multiple human and mouse tissue cDNA libraries. Analysis of the full length cDNAs from human and mouse confirmed that the hORF represents a member of the GDNF ligand family, which we have named *ARTEMIN* (Figure 1).

Alignment of the predicted mature Artemin protein with the other GDNF ligands indicates that Artemin is

most similar to NTN and PSP (~45% identity) and somewhat more divergent from GDNF (~36% identity; Figure 1A). Analysis of the predicted full length Artemin protein revealed the presence of a signal peptide for secretion and a large proregion separated from the mature region by multiple conserved RXXR furin protease cleavage sites (Figure 1B). Comparison of the human mRNA sequence with the genomic locus revealed the presence of two introns in the *Artemin* coding region (Figures 1B and 1C), the second of which is positioned similarly to the introns found in the prodomains of the other GDNF family ligands.

Analysis of markers present on the *ARTEMIN*-containing BAC clones indicated that the *ARTEMIN* gene is located on chromosome 1p32–33, flanked by markers D1S190-*ARTEMIN*-D1S211 (telomeric to centromeric).

Artemin Is Expressed during Embryogenesis and in Adult Tissues

As an initial survey of Artemin expression in adult and embryonic human tissues, we probed a human master RNA blot (MRB) array using a fragment of the human *Artemin* cDNA (Figures 2A and 2B). Relatively low-level expression was observed in many peripheral adult tissues, with the highest level in the pituitary gland, placenta, and trachea. Among human fetal tissues, kidney and lung showed the highest level of expression. Artemin expression was low in the adult and fetal brain. Interestingly, low-level brain expression was present in structures of the basal ganglia (subthalamic nucleus, putamen, substantia nigra) and in the thalamus, suggesting that Artemin may influence subcortical motor systems. Expression was also observed in the spinal cord; however, this RNA preparation contains DRG material as well as spinal cord, which likely contributes to the observed expression (Clontech; see Figure 2C).

Artemin Is Expressed in Sites Consistent with a Role in Paracrine and/or Target Influence of Developing Peripheral Neurons

To determine more precise localization of Artemin expression during embryogenesis, we performed in situ hybridization on embryonic day 14 (E14) rats. No signal was observed in the brain or spinal cord at this embryonic age. The most striking expression was observed in the nerve roots, but not the developing neurons, of the dorsal root ganglia (Figure 2C). Furthermore, significant diffuse expression was also observed around the superior mesenteric artery, corresponding to either the surrounding mesenchyme or expression by cells of the artery itself (Figure 2D). These data suggest the possibility that Artemin may act as a survival/trophic factor for peripheral neurons either in a paracrine fashion for developing sensory neurons of the DRG or as a target-derived factor for autonomic innervation of the superior mesenteric artery. Interestingly, among potential GFRα receptors, expression of Artemin is most complementary with that of the orphan GFRα3, which is expressed at high levels in peripheral ganglia but which has no detectable expression in the developing CNS by in situ hybridization (Baloh et al., 1998; Trupp et al., 1998; Widenfalk et al., 1998; Worby et al., 1998; Yu et al., 1998).

The expression of Artemin in the developing nerve roots at E14 suggested that it is produced by Schwann cell precursors or immature Schwann cells. To investigate if Schwann cells express Artemin, we performed RT-PCR analysis on cDNA samples from adult sciatic nerve and from Schwann cells cultured from early postnatal rats (Figure 2E). Artemin is expressed at much higher levels in immature Schwann cells in culture than by mature myelinating Schwann cells of the adult sciatic nerve. Interestingly, Artemin expression is upregulated in the distal nerve segment after sciatic transection, a paradigm in which Schwann cells reacquire an immature state to support regenerating axons (reviewed by Scherer, 1997). These data indicate that Schwann cells produce Artemin and suggest that Artemin expression is regulated appropriately to influence developing and regenerating peripheral neurons.

Artemin Is a Survival Factor for Peripheral and Central Neurons In Vitro

GDNF and NTN, but not PSP, support the survival of several peripheral neuronal populations in vitro (Buj-Bello et al., 1995; Ebendal et al., 1995; Trupp et al., 1995; Kotzbauer et al., 1996; Milbrandt et al., 1998). As shown above, expression of Artemin suggests it may influence developing peripheral neurons, like other members of the GDNF family. Therefore, we cultured neurons from the dorsal root, trigeminal, nodose, and superior cervical ganglia (SCG) from neonatal rats to compare Artemin's ability to support their survival with that of GDNF, NTN, and PSP (Figure 3). Like GDNF and NTN, Artemin supported the survival of a subset of sensory neurons from both the dorsal root ganglion (DRG) and the trigeminal ganglion (TG), whereas PSP did not (Figures 3A and 3C). Interestingly, in both of these populations of sensory neurons, Artemin supported a larger number of neurons than GDNF and NTN and supported a similar (DRG) or a greater number (TG) of neurons than NGF. Dose–response analysis of Artemin's survival-promoting effect on DRG neurons revealed an EC_{50} of 1–3 ng/mL, $\sim 10^{-10}$ M (Figure 3B). We also examined Artemin's ability to support the survival of visceral sensory neurons of the nodose ganglion (NG), which consists of a large number of neurons responsive to the neurotrophin BDNF (Lindsay et al., 1985). Artemin, GDNF, and NTN supported similar numbers of NG neurons, and each factor yielded equal or greater survival promotion than BDNF, similar to previous reports (Trupp et al., 1995; Kotzbauer et al., 1996) (Figure 3D).

We further assessed Artemin's ability to support sympathetic neurons of the SCG, which are responsive to GDNF (Trupp et al., 1995) and NTN (Kotzbauer et al., 1996). Artemin also supported the survival of SCG neurons in culture, although fewer neurons were supported by Artemin than by GDNF or NTN, and in contrast to the sensory neurons examined above, none of the GDNF family ligands supported as many neurons as NGF (Figure 3E). Therefore, similar to GDNF and NTN, and consistent with its embryonic expression pattern, Artemin is

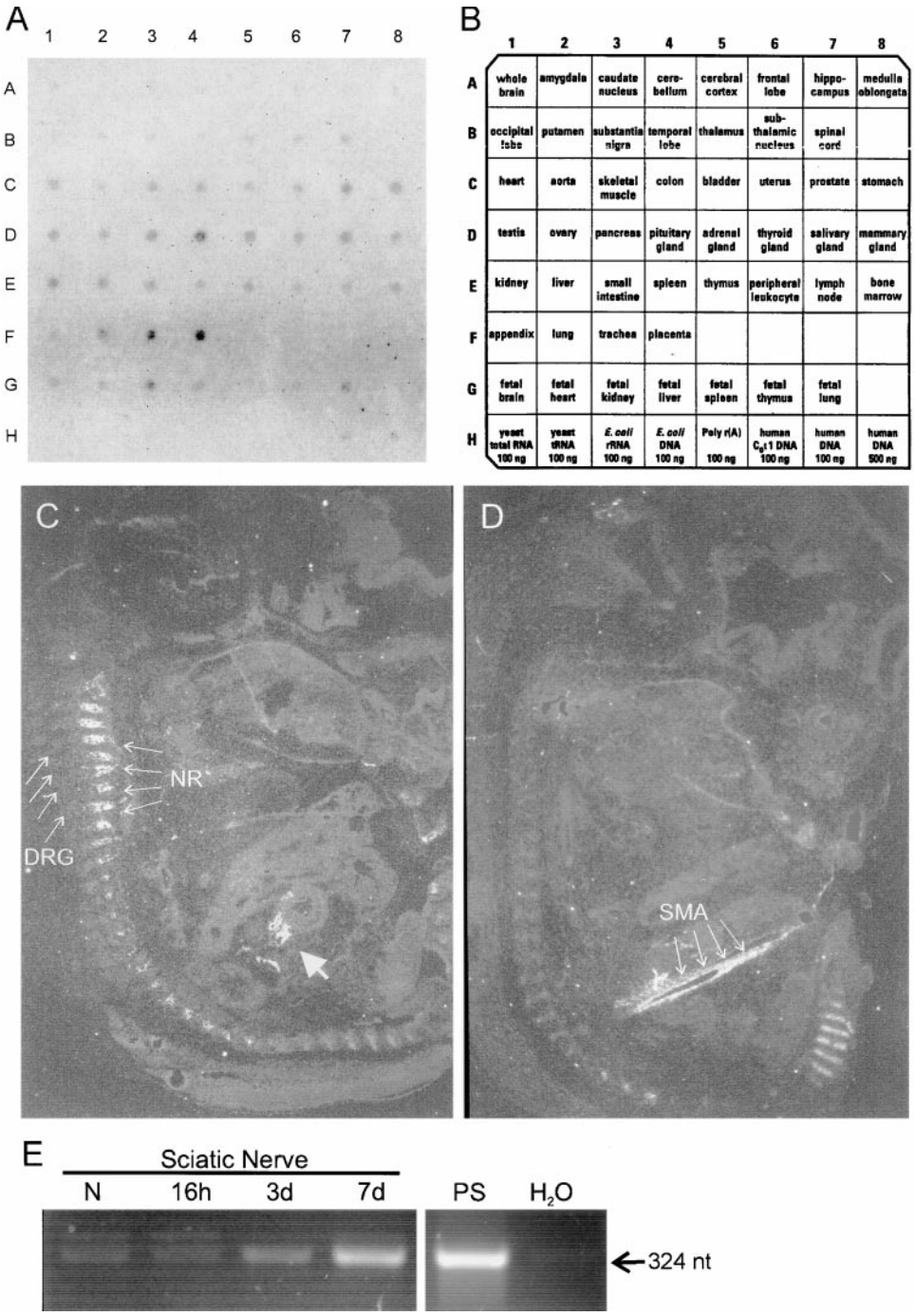

Figure 2. Analysis of *Artemin* RNA Expression in Developing and Adult Tissues

(A) MRB (Clontech) analysis of Artemin expression in human adult and fetal tissues. A ^{32}P-labeled fragment of the human *Artemin* cDNA was used as a probe. Artemin showed a broad expression pattern in peripheral tissues, with little signal visible in brain. Low-level signal is observed, however, in the adult substantia nigra, thalamus, and subthalamic nucleus of the CNS. The spinal cord sample contains DRG material, which may contribute to the signal observed in this tissue sample (Clontech).

(B) Location of human tissues on the MRB array.

(C) In situ hybridization analysis of Artemin expression in embryonic rat suggests a role in peripheral neuron development. Saggital section of an E14 rat. The orientation of the section is top, rostral and left, dorsal. While no signal is visible in the DRG themselves at this age, clear Artemin expression is observed in tissue representing the exiting nerve roots (NR). A sense version of the rat Artemin ^{33}P-labeled probe did not hybridize to any tissues (data not shown). The expression below the liver (large arrow) likely represents a lateral extension of the superior mesenteric artery (see [D]) but may also correspond to a pancreatic duct or the common bile duct.

(D) A parasaggital section of E14 rat in the same orientation as (A), showing high-level Artemin expression surrounding the developing superior mesenteric artery (SMA). Signal corresponding to what is likely developing cartilage is observed in the lower right of the photomicrograph. As in (A), no expression was visible in the brain or spinal cord of embryos at this age.

(E) Semiquantitative RT-PCR analysis demonstrates regulated Artemin expression by Schwann cells. All cDNAs were normalized to GAPDH levels as described in Experimental Procedures. Primary cultures of Schwann cells in neonatal rats (PS) express Artemin, whereas myelinating Schwann cells from the adult sciatic nerve (N) show much lower level expression. However, Artemin is upregulated in the distal segment of the sciatic nerve following nerve transection. Abbreviations: 16 h, 16 hours; 3d, 3 days; and 7d, 7 days).

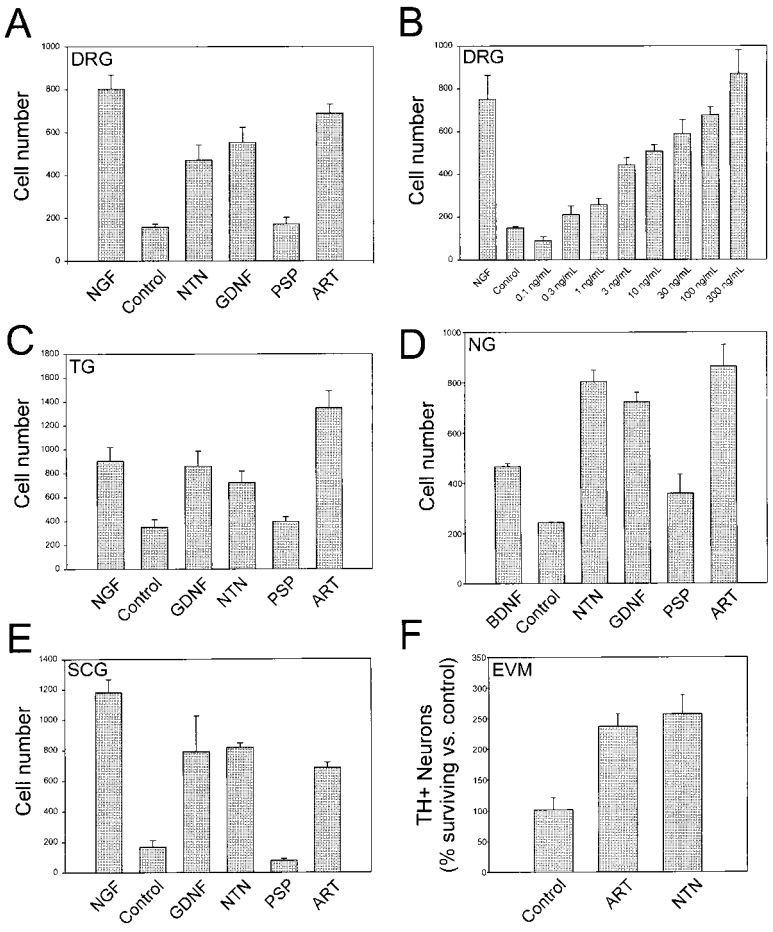

Figure 3. Artemin Supports the Survival of Peripheral and Central Neurons in Culture

(A) Histogram of the number of surviving DRG neurons cultured from P1 rats in the presence of the indicated factors. Neurons were plated directly into the presence of the indicated factors and cultured for 3 days. Artemin (ART) supported the survival of as many or more DRG neurons than GDNF or NTN. The mean and SEM are shown (n = 5–11).

(B) Dose-response histogram of Artemin survival promotion in P1 rat DRG neurons. Artemin promoted the survival of cultured DRG neurons with an EC_{50} of ~3 ng/mL (10^{-10} M). The mean and SEM are shown (n = 5).

(C) Trigeminal ganglion (TG) neurons cultured from P1 rats. Cells were plated directly into factors, and survival was assessed after 3 days in culture. Artemin's survival-promoting activity in the TG was significantly greater than GDNF, NTN, or NGF. The mean and SEM are shown (n = 4).

(D) NG neurons cultured from P1 rats. Neurons were plated directly into medium containing BDNF (100 ng/mL) or the GDNF family ligands. Artemin, like GDNF and NTN, also supports the survival of placodally derived visceral sensory neurons of the NG. The mean and SEM from a representative experiment are shown (n = 2).

(E) SCG from P1 rats. SCG neurons were isolated and maintained in the presence of NGF for 5 days. The culture medium was changed, and the indicated factors were added for an additional 3 days, after which the number of surviving neurons was counted. Artemin, GDNF, and NTN support a similar number of sympathetic neurons of the SCG. The mean and SEM are shown (n = 2–8).

(F) Dopaminergic ventral midbrain neurons from E14 rats (EVM). After 3 days in culture in the presence of the indicated factor, the number of TH^+ neurons was determined. Results from two independent experiments are shown as the percentage of surviving TH^+ neurons over control (mean and SEM, n = 9–15).

Note: factors were added to cultures at 50 ng/mL, unless otherwise indicated.

a survival factor for sensory and sympathetic peripheral neurons in culture.

As shown above, Artemin expression was not observed in the brain or spinal cord of embryonic rats at E14 but was present at very low levels in human fetal brain and in some adult brain regions by blot analysis. Therefore, to compare Artemin's ability to influence central neurons to the other GDNF ligands, and to contrast its peripheral survival-promoting activities, we cultured dopaminergic neurons from the rat embryonic ventral midbrain. Interestingly, although there is no apparent Artemin expression in the ventral midbrain at this age, Artemin also supported the survival of dopaminergic midbrain neurons (Figure 3F). Therefore, Artemin can influence central as well as peripheral neuronal survival, and receptors for Artemin are present in both of these populations.

Neuroblastoma cell lines are derivatives of peripheral sympathetic neuroblast tumors that respond to GDNF and NTN and display a variety of GFRα–RET receptor profiles (Hishiki et al., 1998; M. G. T., unpublished data). Therefore, we investigated Artemin's ability to influence several of these neuronal cell lines to determine if responsive cells had a consistent receptor profile. Artemin induced a strong differentiation response in the line SH-SY5Y, similar to retinoic acid treatment (Figures 4A–4C). Furthermore, like GDNF, Artemin stimulated proliferation in the NBL-S line, as measured by bromodeoxyuridine (BrdU) incorporation (Figure 4D). Lines that do not express RET (CHP126, CHP134, SAN) and do not respond to GDNF or NTN (M. G. T. unpublished data) also did not respond to Artemin (data not shown). Both the SH-SY5Y and NBL-S lines express GFRα1–GFRα3 and therefore did not suggest the usage of a particular coreceptor by Artemin.

In summary, all of the biological responses of Artemin in peripheral and central neurons and in neuronal cell lines suggest that Artemin utilizes receptor components similar to or overlapping with GDNF and NTN.

Artemin Signals through RET Using the Former Orphan GFRα3 as a Coreceptor

Because Artemin supports the survival of neuronal populations that overlap with GDNF and NTN, and because its activity correlated with RET expression in neuroblastoma cell lines, we first investigated Artemin's ability to activate RET in primary cultured SCG neurons and in the responsive neuroblastoma cell line NBL-S (Figures

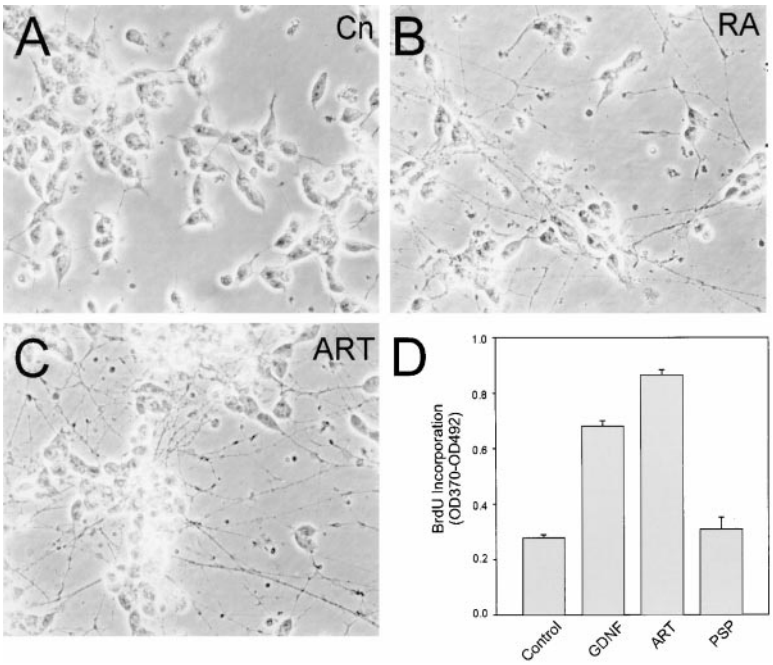

Figure 4. Artemin Induces Differentiation and Proliferation in Neuroblastoma Cell Lines

SH-SY5Y neuroblastoma cells were cultured in the presence of no factor (Cn; [A]), 10 μM all-*trans* retinoic acid (RA; [B]), or 50 ng/mL Artemin (ART; [C]). Like RA, a known inducer of differentiation in these cells, Artemin induced clear differentiation of SH-SY5Y cells, as illustrated by their robust neurite outgrowth and neuronal morphology.

(D) Histogram of BrdU incorporation by NBL-S neuroblastoma cells in the presence of 50 ng/mL of the indicated factor. Artemin, like GDNF, induced robust proliferation of NBL-S cells, whereas PSP did not.

5A and 5B). Like GDNF, Artemin induced tyrosine phosphorylation of RET in SCG neurons and in the NBL-S cell line, and this activation was effective in eliciting downstream signaling, as measured by activation of the mitogen-activated protein (MAP) kinase pathway. Furthermore, pretreatment of the NBL-S cell line with the enzyme PI-PLC, which specifically cleaves GPI-anchored proteins from the cell surface, abrogated Artemin's ability to activate RET and downstream MAP kinase signaling (Figure 5B). Therefore, these data indicate that like the other members of the GDNF family, Artemin signals through the RET receptor tyrosine kinase and requires a GPI-anchored coreceptor to do so.

To investigate which if any of the known coreceptors Artemin can utilize to activate RET, we first assessed its ability to directly bind to soluble Fc-fusion forms of GFRα1–GFRα3 (Figures 5C–5E). In this assay, GFRα1–Fc was able to bind to both GDNF and NTN with similar affinity but not to PSP or Artemin. GFRα2–Fc bound to NTN but not GDNF or Artemin, consistent with a previous report that GDNF can only bind to GFRα2–Fc in the presence of RET (Sanicola et al., 1997). Interestingly, the former orphan receptor GFRα3–Fc was able to bind to Artemin but not to any of the other GDNF family members, consistent with the inability of GFRα3 to form a functional receptor complex for GDNF, NTN, or PSP with RET (Baloh et al., 1998; Worby et al., 1998). Each of the receptor bodies bound to their respective ligands with an apparent K_d of ~3 nM, which is similar to the previously reported affinity between GFRα1–Fc and GDNF in this assay (Sanicola et al., 1997). Therefore, Artemin can bind to the former orphan receptor GFRα3 with an affinity similar to that of GDNF and NTN binding to their cognate receptors.

The binding data above suggest that GFRα3–RET is the functional receptor for Artemin; however, direct binding studies alone have been unreliable in predicting all GDNF family receptor interactions because of the observation that RET can modulate receptor binding (Sanicola et al., 1997). To directly test which receptor combinations can form functional receptors for Artemin on cells, we transiently transfected the individual GFRα coreceptors together with the Gal4–Elk1/Gal4–luciferase reporter system into fibroblasts that stably express RET. This system, which utilizes the ability of the Gal4–Elk1 fusion protein to respond to MAP kinase activity and activate transcription of the Gal4–luciferase reporter, has been used previously to monitor NGF–TrkA activation of MAP kinase in PC12 cells (Vossler et al., 1997; York et al., 1998) and GDNF/RET activation of MAP kinase in neuroblastoma cell lines (Worby et al., 1996, 1998). Consistent with previous reports in multiple systems, GDNF can activate RET signaling in fibroblasts expressing GFRα1–RET or GFRα2–RET but not GFRα3–RET (Baloh et al., 1997, 1998; Jing et al., 1997; Sanicola et al., 1997; Suvanto et al., 1997; Worby et al., 1998) (Figure 6A). As predicted from the binding data, Artemin was able to activate GFRα3–RET but not GFRα2–RET receptor complexes. Interestingly, Artemin was also able to activate RET in cells expressing GFRα1–RET, although to a lesser degree than GDNF. RT-PCR analysis of cDNA from GFRα1–RET-expressing cells confirmed that expression of GFRα1 did not induce the expression of GFRα3, a possible alternative explanation for their responsiveness to Artemin (data not shown).

To confirm these results in a more neuronal cell line, we also transiently transfected receptor components into neuroblastoma cells from the NLF line that express GFRα2 and RET and natively respond to GDNF by RET and MAP kinase activation (M. G. T., unpublished data) (Figure 6B). NLF cells transfected with GFRα1 responded more intensely to GDNF and became responsive to Artemin, confirming the results observed in fibroblasts. Furthermore, transfection of GFRα3, but not GFRα2, into

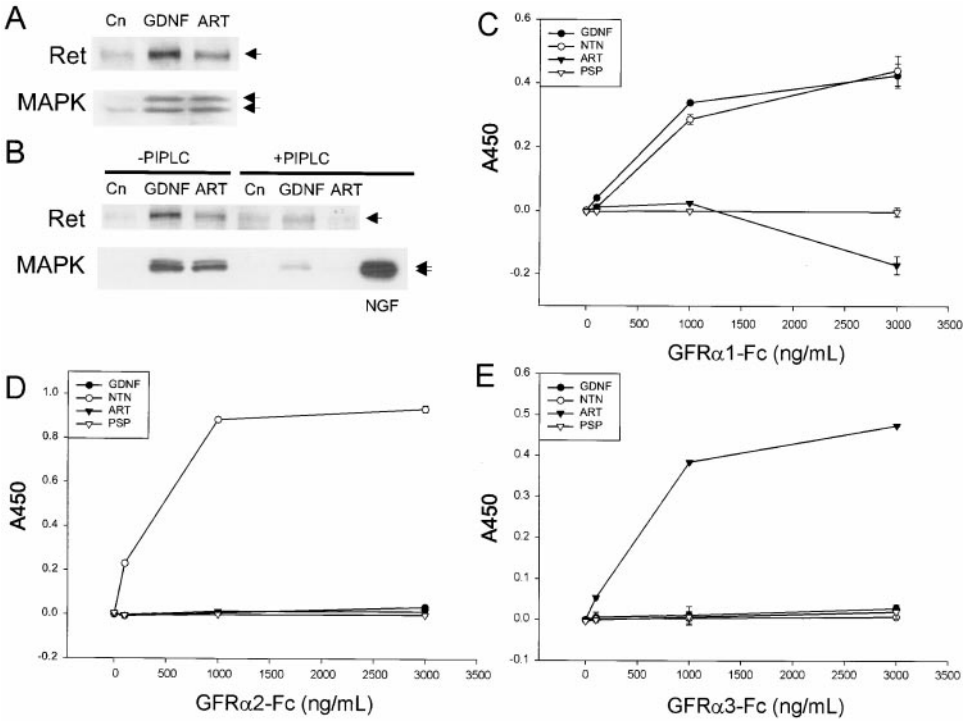

Figure 5. Artemin Activates RET in Neurons and Binds to GFRα3–Fc Receptor Bodies

(A) Artemin activates RET and downstream signaling in primary cultured SCG neurons. Neurons were treated with either no factor (Cn), GDNF, or Artemin (ART) at 50 ng/mL. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies and separated by SDS-PAGE, and immunoblot analysis was performed with anti-RET antibodies to determine the status of RET tyrosine phosphorylation (RET). A portion of the total lysate was removed before immunoprecipitation, run on an SDS-PAGE gel, and immunoblotted with anti-phospho-MAPK antibodies (MAPK). Stimulation of SCG neurons by either GDNF or Artemin led to activation of RET and the MAP kinase pathway downstream.

(B) Artemin activates RET in NBL-S neuroblastoma cells, and this activation is dependent upon a GPI-anchored protein. NBL-S cells were stimulated and analyzed as above (−PI-PLC), and a parallel set of cells was pretreated with the enzyme PI-PLC, which specifically cleaves GPI-anchored proteins from the cell surface (+PI-PLC). Removal of GPI-anchored proteins vastly depleted the ability of Artemin to activate RET or MAPK signaling. NGF activation of MAPK after PI-PLC treatment is shown as a control for cell viability (NGF).

(C–E) Direct binding of GFRα1–Fc (C), GFRα2–Fc (D), and GFRα3–Fc (E) receptor bodies to the GDNF ligands. Microtiter plates coated with GDNF, NTN, ART, or PSP were treated with increasing concentrations of soluble GFRα–Fc fusion proteins. Plates were then washed, and bound receptor bodies were detected with anti-human Fc antibodies conjugated to HRP. Binding was measured in a plate reader with the chromogenic HRP substrate 3,3′,5,5′-tetramethylbenzidine (measured at 450 nm; A450). Error bars in the figure represent the standard deviation of duplicates from a representative experiment. Both GDNF and NTN bound to GFRα1–Fc bodies in this assay with similar affinity; however, only NTN binding to GFRα2–Fc was detected. Artemin is the only ligand capable of direct binding to GFRα3–Fc.

NLF cells allowed them to respond to Artemin stimulation. Transfection of GFRα3 also decreased the ability of NLF cells to respond to GDNF, presumably because cytomegalovirus promoter– (CMV-) driven overexpression of GFRα3 decreases the relative amount of GFRα2–RET complexes on the cells, thereby decreasing the number of functional GDNF receptors.

In summary, direct binding data and receptor activation experiments in vitro together indicate that Artemin is the only known GDNF family ligand for the GFRα3–RET receptor complex, but like GDNF and NTN, it can also activate the GFRα1–RET receptor complex.

Discussion

We have reported the identification of Artemin, a novel member of the GDNF family of ligands. Artemin supports the survival of neurons from all peripheral ganglia examined thus far, including sympathetic neurons (SCG) and neural crest (DRG, TG) and placodally derived (NG) sensory neurons. Furthermore, Artemin can also support the survival of at least one population of CNS neurons (dopaminergic midbrain neurons). Artemin signals through the RET receptor tyrosine kinase and represents the sought after ligand for the former orphan coreceptor GFRα3, which together with RET represents the preferred multicomponent receptor for Artemin signaling.

Artemin/GFRα3–RET Signaling in the PNS

Expression analysis of Artemin in adult human tissues indicated that it is found at low levels in many peripheral tissues. Little expression was observed in the CNS, and in situ hybridization analysis of developing rat embryos at E14 indicated that by far, the highest level of expression corresponded to regions that are likely to influence the development of peripheral neurons. Furthermore, Artemin is expressed by cultured Schwann cells and is upregulated after nerve injury, suggesting that glia are

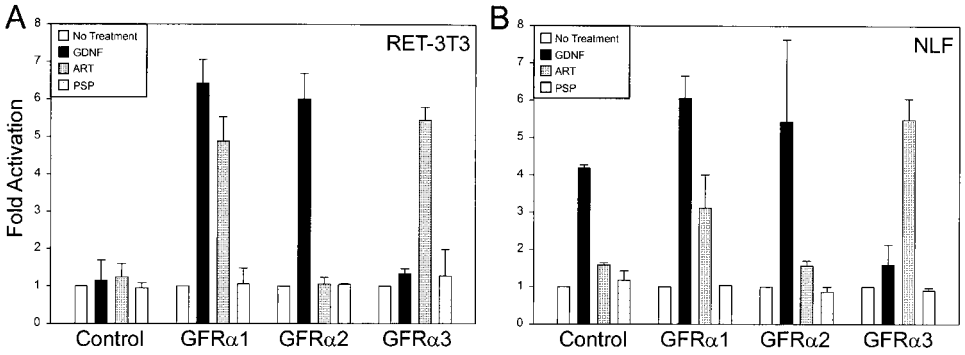

Figure 6. Artemin Activates GFRα3–RET and GFRα1–RET Receptor Complexes on Transfected Cells

Receptor activation in the presence of defined coreceptor components. MG87 fibroblasts stably expressing RET (RET-3T3; [A]) or NLF neuroblastoma cells (NLF; [B]) were transiently transfected with an expression plasmid for the indicated coreceptor or the CMV plasmid with no insert (Control), together with the Gal4–Elk/Gal4–luciferase reporter system. Data are presented as fold activation by dividing luciferase activity in the treatment conditions by the no treatment control, and error bars represent the standard deviation of duplicate measurements from a representative experiment. In RET-expressing fibroblasts, GDNF was able to activate RET/MAPK signaling in cells transfected with GFRα1 or GFRα2 but not GFRα3. Neurturin acted similarly to GDNF with all receptors (data not shown). Artemin activates cells expressing GFRα1–RET or GFRα3–RET but not GFRα2–RET. NLF neuroblastoma cells express GFRα2–RET and therefore natively respond to GDNF but not Artemin. Transfection of GFRα1 or GFRα3, but not GFRα2, into NLF cells allowed it to respond to Artemin stimulation.

a major source of peripheral Artemin expression. This general expression pattern is strikingly complementary to that observed for GFRα3, which is expressed at high levels only in the developing and adult sensory and sympathetic ganglia of the PNS (Baloh et al., 1998; Naveilhan et al., 1998; Widenfalk et al., 1998; Worby et al., 1998). Given the complementary expression patterns, the demonstration that Artemin is the only ligand for GFRα3–RET, and the ability of Artemin to support developing peripheral neurons in culture, we believe that the Artemin/GFRα3–RET system represents an important neurotrophic influence in PNS development.

Studies of the neurotrophins using knockout mice and ligand–receptor expression analysis in developing peripheral ganglia have elegantly established that specific subsets of PNS sensory neurons are supported by particular members of the neurotrophin family (reviewed by Lewin and Barde, 1996; Snider and Wright, 1996). However, even in the DRG, where this analysis has been most extensive, there still remain neurons whose developmental and/or mature trophic factor dependence is unclear. A large percentage of adult DRG neurons (~60%–70%) express RET, and these are largely distinct from TrkA- and parvalbumin-expressing neurons (markers for NGF- and NT-3-supported neurons, respectively), suggesting that they are not influenced by neurotrophins (Molliver et al., 1997; Bennett et al., 1998). Recently, it was shown that GDNF is capable of supporting the survival of a group of neurotrophin-independent neurons (small neurons that bind the lectin IB4) and that these neurons express either one or both of the competent GDNF receptors (GFRα1–RET or GFRα2–RET) (Molliver et al., 1997; Bennett et al., 1998). However, ~30%–40% of the RET-expressing DRG neurons are not IB4 binding, indicating that many additional DRG neurons exist that may be supported by Artemin or other GDNF ligands. Although a comparison of GFRα1–GFRα3 expression has not been performed in the DRG, analysis in the trigeminal ganglion revealed that GFRα3 appears to be expressed in a population of neurons distinct from those expressing GFRα1 and GFRα2 (Naveilhan et al., 1998). Because Artemin is the only ligand that can activate the GFRα3–RET receptor complex, these neurons may represent neurotrophin-independent cells that critically depend on Artemin for their proper development and/or postnatal survival. It will be interesting to determine if the different members of the GDNF ligand family, like the neurotrophins, support the development/maturation/survival of distinct subsets of peripheral neurons that are either spatially or temporally independent from neurotrophin influence.

Artemin and Central Neurons

Artemin was also able to support the survival of dopaminergic ventral midbrain neurons in culture. Since GFRα3 expression was not observed in this area during development or in adulthood by several groups (Baloh et al., 1998; Trupp et al., 1998; Widenfalk et al., 1998; Worby et al., 1998; Yu et al., 1998), this survival-promoting effect is presumably due to Artemin's ability to also activate the GFRα1–RET receptor complex, which is highly expressed in these neurons during development (Treanor et al., 1996; Baloh et al., 1997; Horger et al., 1998). No Artemin expression was detected by in situ hybridization in the developing rat brain at a time when these neurons are responsive to Artemin in vitro (E14), suggesting that this effect is only pharmacologically relevant. However, we cannot exclude that at other embryonic ages, Artemin may be expressed in the developing ventral midbrain or other regions of the CNS. Furthermore, blot analysis showed low-level Artemin expression in the adult substantia nigra, suggesting that Artemin could influence these neurons in adulthood. This raises the possibility that although GFRα3–RET is the preferred receptor for Artemin, it may utilize the alternative GFRα1–RET receptor in vivo, particularly to influence neurons in the CNS.

Specificity and Cross Talk in the GDNF Ligand–GFRα System

A schematic interaction diagram of the GDNF ligands and the GFRα receptors is presented in Figure 7. Similar

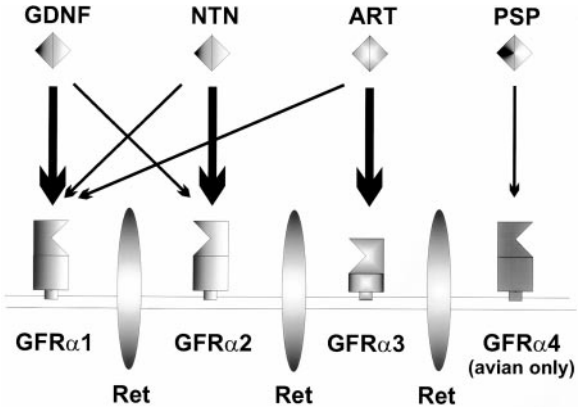

Figure 7. Schematic Diagram of Ligand–Receptor Interactions in the GDNF Ligand Family Deduced from Multiple Experimental Paradigms In Vitro

Large arrows indicate preferred ligand–receptor interactions, whereas smaller arrows indicate alternate receptor interactions. GFRα1–RET is the most promiscuous member of the receptor family, with the ability to interact with three of the four ligands, whereas GFRα3–RET is the least, in that it can only interact with Artemin. Although the importance of the cross talk between alternate ligand–receptor pairs is not fully understood, studies of GFRα1-deficient mice indicate that at least one of these interactions (GDNF–GFRα2–RET) is likely to occur in vivo (Cacalano et al., 1998; Enomoto et al., 1998). As indicated, a receptor named GFRα4 has been isolated from the chicken; however, a mammalian homolog has not yet been reported.

to many other ligand–receptor systems, including the neurotrophins, the system is characterized by having preferred ligand–receptor pairs; however, cross talk between the different ligands and receptors is apparent. The addition of Artemin to this diagram reveals several new features. First, both direct receptor binding experiments and receptor activation experiments presented here indicate that Artemin is the only GDNF family ligand capable of utilizing the GFRα3–RET receptor complex. One additional study indicated that GDNF was able bind to GFRα3 in the presence of RET (Trupp et al., 1998); however, this interaction was of low affinity, in that it required cross-linking to be observed, and its relevance is unclear, because it does not lead to RET activation in multiple experimental paradigms in vitro (Baloh et al., 1998; Trupp et al., 1998; Worby et al., 1998; Figure 6). Second, the GFRα1–RET receptor complex is highly promiscuous, and although results from most in vitro paradigms agree that it is the preferred receptor for GDNF, it is possible that NTN and Artemin may also utilize the GFRα1–RET receptor complex in some systems in vivo. Recent observations regarding differences in peripheral neuron losses between GDNF and GFRα1-deficient mice suggest that GDNF can use GFRα2–RET as a receptor in vivo and confirm that alternative ligand–receptor interactions may have biological importance in the GDNF family (Cacalano et al., 1998; Enomoto et al., 1998). Furthermore, a recent paper analyzing neurotrophin knockout mice concluded that NT-3 can signal through TrkB in vivo, an interaction observed many years earlier in vitro but that was not believed to be relevant in vivo (Ip et al., 1993; Farinas et al., 1998). Therefore, consideration of all possible ligand–receptor interactions identified in vitro is often necessary to understand results of in vivo analysis of neurotrophic factor influences.

Results from direct binding and RET activation experiments presented here further suggest that like the GDNF–GFRα2 interaction, the Artemin–GFRα1 interaction appears to be dependent on the presence of RET, as direct binding of Artemin to GFRα1–Fc or GDNF to GFRα2–Fc receptor bodies was not observed (Sanicola et al., 1997; Figures 6C and 6D). This may be due to the nature of the binding assay, which utilizes soluble receptor bodies binding to immobilized ligand. Alternatively, this may reflect an additional level of specificity available to the GDNF ligand–GFRα system, in cases where the GFRα coreceptors are expressed in the absence of RET (Baloh et al., 1997; Trupp et al., 1997; Golden et al., 1998; Yu et al., 1998). In these situations (i.e., GFRα1 in injured peripheral nerve and GFRα2 expression in cerebral cortex), in which coreceptors are expressed in *trans* and are hypothesized to secrete or present ligand–coreceptor complexes to cells or axons expressing RET, only the RET-independent subset of binding interactions would be possible.

With the data rapidly accumulating from the Human Genome Project, complete lists of the members of neurotrophic gene families will soon be available, allowing consideration of an extensive number of potential influences on neurons. This will facilitate more rapid identification of those influences that are most important in neuronal development/maintenance and those that can be manipulated advantageously for therapeutic intervention in diseases of the nervous system.

Experimental Procedures

Identification and Cloning of the Human and Mouse Artemin

BAC clones containing sequence from the human *ARTEMIN* gene were identified by searching (BLAST 2.0 algorithm; Altschul et al., 1997) the HTGS database, with mature human NTN protein sequence as a query. Sequence from these clones was used to design primers that were then used to PCR amplify the mature Artemin protein coding sequence from human genomic DNA, and these products were blunt cloned into the EcoRV site of pBluescript (Stratagene) and sequenced completely on both strands. Primers designed from this sequence were also used to PCR amplify a fragment of mouse Artemin from mouse genomic DNA that was cloned as above, and subsequently, mouse BAC genomic libraries were screened by this PCR reaction to identify a BAC clone containing the mouse *Artemin* gene.

Primers were made from human and mouse Artemin genomic sequence to perform RACE PCR to obtain the 5′ end of the *Artemin* cDNA from several tissue libraries. PCR fragments were blunt cloned as above and sequenced entirely. Human RACE products were obtained from Marathon RACE cDNA libraries (Clontech) from pituitary, placenta, and kidney. Mouse RACE products were obtained from an E18 mouse MARATHON RACE library. Complete double stranded sequence of cloned PCR fragments from genomic DNA and cDNA was performed to generate contigs of the human and mouse cDNAs. At least two independent clones of each PCR fragment were sequenced. Genomic sequence of mouse BAC clone restriction fragments (subcloned into pBluescript) and direct sequence of the human BAC clone were used to confirm the cDNA sequence. Intron locations in the cDNA and splicing were confirmed by sequencing PCR-amplified fragments from human and mouse cDNA libraries that cross both introns.

Production of Recombinant Artemin Protein

A PCR fragment was generated corresponding to the predicted mature human Artemin coding sequence (Figure 1A), with an NdeI

site and an 8-histidine tag on the 5′ end and a KpnI site on the 3′ end, and then cloned directly into the corresponding sites of the pET30a(+) bacterial expression vector (Novagen). Recombinant human Artemin protein was produced and purified in the *E. coli* strain BL21 as described previously for NTN (Creedon et al., 1997). Recombinant GDNF, NTN, and PSP were obtained from Genentech.

RNA Blot, RT-PCR, and In Situ Hybridization Analysis of Artemin Expression

The human MRB containing normalized samples of poly(A) RNA was used as described by the manufacturer's instructions (Clontech). The blot was probed with a random hexamer ^{32}P-labeled cDNA probe (nucleotides 478–793), and signals were visualized with a PhosphorImager (Molecular Dynamics). In situ hybridization analysis of fresh frozen E14 rat embryos was performed as described (Araki and Milbrandt, 1996). A fragment of the rat cDNA (nucleotides 359–642) was generated by PCR, cloned into pBluescript, and confirmed by sequence. Sense and antisense ^{33}P-labeled RNA probes were generated from the rat *Artemin* cDNA fragment. No signal was detected with the sense probe on any tissue samples.

Cultures of purified Schwann cells from neonatal rats were performed as described (Brockes et al., 1979). Sciatic nerve transection in adult rats and generation of reverse transcribed cDNA libraries are also described elsewhere (Araki and Milbrandt, 1996; Baloh et al., 1997). The cDNA samples were normalized to levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used were Fwd, 5′-TCG CGA CGG TCC CTC ACC GGT CTT-3′; and Rev, 5′-GCA CGA GCC GCT GCA GAA GCG GAA-3′. RT-PCR was performed using the following conditions: 95°C for 2 min; (95°C for 20 s; 54°C for 30 s; 72°C for 30 s) × 35 cycles; and 72°C for 2 min. Products were separated on a 3% agarose gel and stained with ethidium bromide.

Neuron Survival Assays

Cultures were performed by standard methods and are described elsewhere (Kotzbauer et al., 1994; Kotzbauer et al., 1996; Milbrandt et al., 1998). All peripheral neuron cultures were derived from postnatal day 1 (P1) Sprague-Dawley rats (Harlan Sprague-Dawley, IN). For SCG neurons, after dissection and dissociation, neurons were plated on collagen-coated 24-well tissue culture plates and maintained in NGF-containing medium (AM50) for 5 days, after which they were either kept in AM50 or switched to medium containing neutralizing anti-NGF antibodies plus GDNF, NTN, PSP, ART (50 ng/mL where not noted otherwise), or no factors. Cultures were maintained for 3 days, after which they were fixed in 4% paraformaldehyde and stained with toluidine blue, and surviving neurons were counted. For NG and DRG neuron cultures, dissociated neurons were plated directly into NGF (for dorsal root ganglia), BDNF (for nodose ganglia, 100 ng/mL), or one of the GDNF ligands in serum-containing medium (AM50 with anti-NGF in antibodies), and cells surviving after 3 days in culture were counted as above. Trigeminal ganglia were dissected, dissociated, and plated directly into medium containing NGF or the indicated GDNF ligands, and the number of surviving cells was assessed after 3 days in vitro. For all cultures systems, 2–3 independent experiments were performed.

For E14 ventral mesencephalon cultures, the entire mesencephalon was removed into cold Leibovitz's L15 + 6 mg/ml glucose, and the tissue was kept on ice throughout the dissection. Following dissection, the tissue was digested in a mixture of dispase (1 mg/ml; Sigma) and collagenase (1 mg/ml; Worthington Biochemical) for 25 min. Tissue was then washed twice with modified N2 media and triturated 35 times. Cell density and viability were assessed with a hemocytometer to count trypan blue– excluding cells. Cells were plated at 20,000 cells/well on 8-well chamber slides (coated with 125 ng/mL poly-D-lysine and 25 ng/mL laminin) in serum-free medium consisting of Dulbecco's Modified Eagle Medium/Hams F12 (1:1), 1 mg/mL bovine serum albumin (BSA), 5 μM insulin, 10 nM progesterone, 100 μM putrescine, 30 nM selenium, 10 ng/mL rat transferrin, 100 U/mL penicillin, and 100 U/mL streptomycin. Factors were added within 15 min of plating. After 3 days in culture, cells were fixed and stained for tyrosine hydroxylase, and the number of tyrosine hydroxylase staining– (TH^+) neurons was counted.

Proliferation and Differentiation of Neuroblastoma Cell Lines

For differentiation of SH-SY5Y cells, cells were plated at 2×10^5 cells/ml/well in 12-well tissue culture plates. After 1 day in culture, cells were either left untreated or stimulated with 50 ng/mL Artemin or 10 μM retinoic acid (RA). Three days after factor addition, differentiation was assessed, and cells were photographed. For the NBL-S proliferation assay, cells were plated at 5×10^4 cells/well on 48-well plates in standard medium, either without treatment or in the presence of 50 ng/mL of GDNF, Artemin, or PSP. Actively dividing cells undergoing DNA synthesis 30 hr after factor addition were detected with the BrdU (colorimetric) cell proliferation assay kit according to the manufacturer's instructions (Boehringer-Mannheim).

Functional Analysis of Ret Phosphorylation and MAP Kinase Activation

Western blot analysis of Ret phosphorylation and MAP kinase activation were performed as described (Baloh et al., 1997; Creedon et al., 1997). SCG neurons were dissected from P1 rats and maintained in NGF-containing medium for 5 days, after which they were deprived of NGF by switching to NGF-free medium in the presence of anti-NGF antibodies. After 2 hr without NGF, neurons were switched to medium containing NGF, GDNF, or Artemin at 50 ng/mL for 20 min. Cells were washed in cold PBS and collected in immunoprecipitation buffer. The lysates were incubated with 30 μL agarose-conjugated anti-phosphotyrosine antibodies (Calbiochem) at 4°C for 1 hr. Beads were then washed with immunoprecipitation buffer, resuspended in SDS sample buffer, and boiled for 5 min. Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 hr at 25°C in TBS containing 5% dry milk, incubated overnight at 4°C with a 1:300 dilution of anti-Ret antibody (C-19; Santa Cruz), and then washed (three times in TBS containing 0.1% Tween-20) and incubated in a 1:10,000 dilution of anti-rabbit antibodies conjugated to horseradish peroxidase (HRP) (Jackson Immunoresearch). Membanes were washed three times, incubated with SuperSignal ULTRA (Pierce) for 5 min, and then exposed to film. NBL-S cells were plated at 3×10^5 cells/well in 6-well plates and after 46 hr switched to low-serum (0.5%) medium for 2 hr, and then stimulated with factors (50 ng/mL) for 20 min. Cells were then collected and analyzed as above. For MAP kinase assays, a portion of the total lysate was removed before immunoprecipitation, run on out by SDS-PAGE, and immunoblotted with an anti-phospho-MAP-kinase antibody (New England Biolabs). For PI-PLC treatment, a parallel set of NBL-S cells was plated as above but treated with 500 mU/mL of PI-PLC (Boehringer-Mannheim) for 1 hr and then washed with PBS and stimulated with factors as above.

Analysis of Direct Binding of GFRα–Fc Receptor Bodies to GDNF Ligands

GFRα1–Fc, GFRα2–Fc and GFRα3–Fc fusion proteins were obtained from R & D Systems. Binding assays were performed similarly to those previously described (Sanicola et al., 1997). Recombinant GDNF, NTN, Artemin, PSP, or BSA (Pierce) was coated to Nunc-Immuno MaxiSorp microtiter plates at 325 ng/mL in TBS (10 mM Tris-HCl [pH 7.5] and 150 mM NaCl) for 1 hr at 25°C. Plates were washed (three times in TBS-0.03% Tween-20) and then blocked (blocking solution: TBS-1% BSA) for 1 hr at 25°C. Receptor bodies diluted in blocking solution were added and incubated for 2 hr at 25°C, washed five times, and then incubated with a 1:10,000 dilution of HRP-conjugated anti-human Fc antibodies in blocking solution (Jackson Immunoresearch) for 45 min at 25°C. Finally, wells were washed five times, and the presence of HRP was assayed by the addition of the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine for 5 min. The reaction was stopped by adding an equal volume of 0.5 M H_2SO_4, and color was measured in a plate reader at 450 nm. Nonspecific binding of receptor bodies to the plates was measured in BSA-coated wells, and this binding was subtracted from all other measurements. All experiments were performed in duplicate.

Transient Transfections and Luciferase Assays

The generation of the expression plasmids for rat GFRα1 and human GFRα2 and of MG87 fibroblasts stably expressing human RET (RET-3T3) were described in detail previously (Baloh et al., 1997; Creedon et al., 1997). For generation of the GFRα3 expression plasmid, a

fragment containing the complete coding region of human GFRα3 was PCR amplified from a human pituitary cDNA library, and this fragment was directly cloned into the KpnI and BamHI sites of pCB6 (Brewer, 1994) by using sites engineered into the oligonucleotides. The Gal4–Elk1 chimera expression plasmid was a gift of P. Stork (Vollum Institute, Oregon Health Sciences University). Transfections were performed with the Superfect reagent (Qiagen) according to the manufacturer's instructions. Cells were plated at 85,000 cells/well in 12-well plates and transfected with the reporter plasmids (250 ng/well Gal4–luciferase, 50 ng/well Gal4–Elk), CMV-lacZ (50 ng/well) for transfection normalization, a CMV-GFRα (500 ng/well) expression plasmid, and 650 ng/well pBluescript as carrier, for a total of 1.5 μg DNA/well. RET-3T3 fibroblasts were exposed to DNA/Superfect mixtures in an incubator at 37°C overnight, washed and placed in low-serum (0.5%) medium, and stimulated with 50 ng/mL factor for 6–8 hr before collection (48 hr posttransfection). NLF neuroblastoma cells were exposed to DNA/Superfect mixtures for 2 hr, placed in full-serum medium overnight for recovery, and then switched to low-serum (0.5%) medium containing 50 ng/mL of factor for 24 hr before collection (48 hr posttransfection). Measurements of luciferase and β-galactosidase activity were made with a luminometer and performed as described (Svaren et al., 1998). The average luciferase activity of duplicate samples was normalized to β-galactosidase activity of the cotransfected lacZ reporter, and fold activation was calculated by dividing the treatment conditions by the no treatment control.

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GenBank Accession Numbers

The sequence of human *Artemin* cDNA has been submitted to GenBank under accession number AF109401; the sequence of mouse *Artemin* cDNA has been submitted under accession number AF109402.