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Gene Targeting Reveals a Critical Role for Neurturin in the Development and Maintenance of Enteric, Sensory, and Parasympathetic Neurons

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Summary

Neurturin (NTN) is a neuronal survival factor that activates the Ret tyrosine kinase in the presence of a GPI-linked coreceptor (either GFRα1 or GFRα2). Neurturin-deficient ($NTN^{-/-}$) mice generated by homologous recombination are viable and fertile but have defects in the enteric nervous system, including reduced myenteric plexus innervation density and reduced gastrointestinal motility. Parasympathetic innervation of the lacrimal and submandibular salivary gland is dramatically reduced in $NTN^{-/-}$ mice, indicating that Neurturin is a neurotrophic factor for parasympathetic neurons. GFRα2-expressing cells in the trigeminal and dorsal root ganglia are also depleted in $NTN^{-/-}$ mice. The loss of GFRα2-expressing neurons, in conjunction with earlier studies, provides strong support for GFRα2/Ret receptor complexes as the critical mediators of NTN function in vivo.

Introduction

Neurturin (NTN) is a member of a family of neurotrophic factors that activate the Ret tyrosine kinase in the presence of a glycosylphosphatidylinositol- (GPI-) linked coreceptor (Kotzbauer et al., 1996). Closely related factors include glial cell line–derived neurotrophic factor (GDNF) (Lin et al., 1993), Persephin (PSP) (Milbrandt et al., 1998), and Artemin (ARTN) (Baloh et al., 1998b). Neurturin and GDNF share many biological properties in vitro including the ability to support the survival of rat sympathetic (Kotzbauer et al., 1996), midbrain dopaminergic (Lin et al., 1993; Horger et al., 1998), dorsal root ganglion (DRG) (Buj-Bello et al., 1995; Molliver et al., 1997), nodose ganglion (Kotzbauer et al., 1996), and enteric neurons (Hearn et al., 1998; Heuckeroth et al., 1998). Artemin has biological activities that are similar to GDNF and Neurturin in systems where it has been tested (Baloh et al., 1998b). In contrast, Persephin, which has similar neurotrophic actions on CNS neurons, does not support survival of peripheral neuron populations (Heuckeroth et al., 1998; Milbrandt et al., 1998). The difference in biological activity between Persephin and the other family members in vitro may reflect the differences in GFRα coreceptor specificity for these ligands.

GFRα1–3 (Jing et al., 1996; Treanor et al., 1996; Baloh et al., 1997, 1998a; Buj-Bello et al., 1997; Klein et al., 1997; Widenfalk et al., 1997; Naveilhan et al., 1998; Nomoto et al., 1998; Trupp et al., 1998; Worby et al., 1998) and (at least in avian systems) GFRα4 (Thompson et al., 1998) comprise a family of high-affinity GPI-linked coreceptors that are required for activation of the Ret kinase. In vitro studies have demonstrated that Neurturin preferentially activates Ret via GFRα2, whereas GFRα1 is the preferred coreceptor for GDNF (Baloh et al., 1997; Buj-Bello et al., 1997; Jing et al., 1997; Klein et al., 1997; Sanicola et al., 1997). Similarly, Artemin preferentially activates Ret through GFRα3 (Baloh et al., 1998a) and PSP can bind to the chick protein, GFRα4 (Enokido et al., 1998). These studies also demonstrated that some members of this ligand family can use alternate GFRα–Ret complexes at higher ligand concentrations. For example, NTN and Artemin can activate Ret via GFRα1 in vitro, whereas GDNF can activate Ret via GFRα2.

Neurotrophic factors are typically produced in limited quantities in vivo, therefore preferred ligand/coreceptor combinations identified in vitro are likely to be the most biologically relevant pairings. Thus, regions of paired expression of a particular ligand along with its preferred coreceptor will identify cell populations influenced by that ligand in vivo. This hypothesis is supported by the expression patterns of GDNF and GFRα1 during development. While GDNF is expressed in the mesenchyme of the developing kidney and developing intestine, Ret and GFRα1 are expressed in the ureteric bud and in neural crest cells that migrate into the gut and form the enteric nervous system (Nosrat et al., 1997; Widenfalk et al., 1997; Yu et al., 1998). As predicted by the in vitro data and expression patterns for GDNF and GFRα1, mice deficient in either GFRα1 (Cacalano et al., 1998; Enomoto et al., 1998) or GDNF (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) have remarkably similar defects in kidney and enteric nervous system development. Given the in vitro pairing between Neurturin and GFRα2 and the observation that GDNF and GFRα1 are closely paired in vivo, it is likely that Neurturin will be most important for GFRα2-expressing cell lineages, and paired expression of GFRα2 and Neurturin will identify those tissues influenced by Neurturin during development and in adulthood.

While Ret and members of the GFRα family are widely expressed in the developing embryo (Widenfalk et al., 1997; Golden et al., 1998; Yu et al., 1998), paired expression of Neurturin and GFRα2 occurs in a much more restricted pattern. Areas of paired Neurturin and GFRα2

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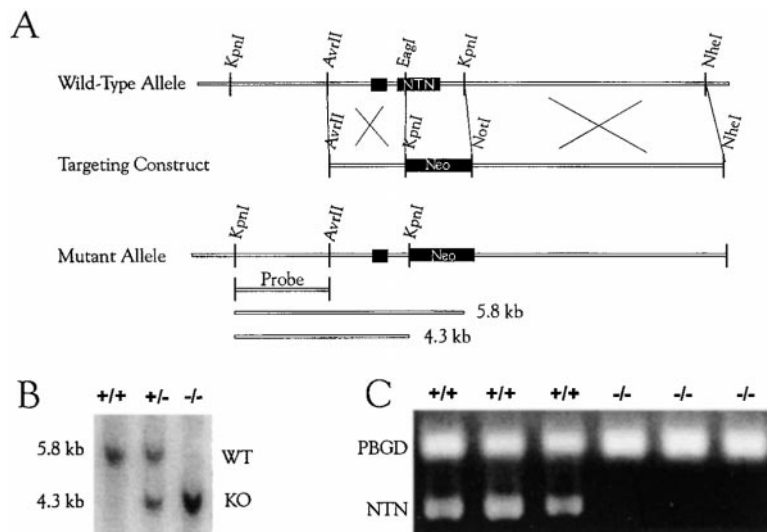

Figure 1. Targeted Inactivation of the Neurturin Gene

(A) Structure of the mouse Neurturin locus, targeting construct, and mutant allele. Homologous recombination replaces the entire coding sequence of the mature Neurturin protein with the pgk-Neo cassette.

(B) Southern Blot of mouse tail DNA after Kpn I digestion. The location of the external probe used to distinguish mutant and wild-type alleles is shown in (A). Wild-type band is 5.8 kb, while the targeted allele gives a 4.3 kb product.

(C) RT-PCR analysis of Neurturin expression. The expected Neurturin PCR product was readily amplified using cDNA prepared from adult heart of WT mice (n = 5) but was not observed in $NTN^{-/-}$ animals (n = 5). Porphobilinogen deaminase (PBGD) cDNA was amplified from each PCR reaction as an internal positive control for the reverse transcriptase reactions.

expression include the intestine, salivary gland, whisker pad, and testis. In the intestine, GFRα2 is expressed in the myenteric plexus, while Neurturin is produced in the circular muscle layer. In the salivary gland, GFRα2 is produced in parasympathetic ganglia, while Neurturin is produced by the gland parenchyma. In the whisker pad, GFRα2 is expressed by innervating nerve fibers from the trigeminal ganglion, while Neurturin is produced in and around vibrissae. These areas of NTN and GFRα2 coexpression are likely to be most affected by NTN deficiency.

To determine the role of Neurturin in vivo, Neurturin-deficient ($NTN^{-/-}$) mice were produced by homologous recombination. $NTN^{-/-}$ animals have a reduction in neuron fiber density and neuronal size in the enteric nervous system and have abnormal gut motility. The observed deficits in the enteric nervous system of $NTN^{-/-}$ mice demonstrate that Neurturin is critical as a trophic factor for postmitotic enteric neurons. $NTN^{-/-}$ mice also have a significant loss of GFRα2-expressing neurons from the dorsal root and trigeminal sensory ganglia. In addition, GFRα2-expressing parasympathetic neurons are lost from the ciliary ganglion and submandibular salivary gland, and parasympathetic innervation of the lacrimal gland is dramatically reduced in $NTN^{-/-}$ mice. Surviving salivary gland parasympathetic neurons and GFRα2-expressing trigeminal ganglion cells are smaller in $NTN^{-/-}$ mice than in wild-type littermates. These observations support the hypothesis that Neurturin is the major physiologic ligand for GFRα2 and demonstrate that Neurturin is a trophic factor for some parasympathetic neuron populations.

Results

Neurturin-Deficient Mice Lack Major Structural Anomalies

Neurturin-deficient mice were made by homologous recombination in embryonic stem (ES) cells. The construct was designed to replace the entire coding region for the mature NTN protein (Figure 1A) with a neomycin resistance cassette. Two independent ES cell lines that had undergone homologous recombination were injected into blastocysts to create chimeric mice, which were then bred to create Neurturin-deficient animals (Figure 1B). To confirm the loss of Neurturin expression, we used RT-PCR from adult heart mRNA, because low levels of Neurturin mRNA and protein preclude other approaches. The expected RT-PCR product was easily detected in mRNA prepared from five of five wild-type animals, but in none (zero of five) of the homologous recombinant mice (Figure 1C).

Unlike mice deficient in Ret (Schuchardt et al., 1994), GDNF (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), or GFRα1 (Cacalano et al., 1998; Enomoto et al., 1998), which die in the neonatal period due to renal agenesis and severe intestinal aganglionosis, survival of Neurturin-deficient mice ($NTN^{-/-}$) is comparable to wild-type littermates. $NTN^{-/-}$ animals are born at the expected frequency (156 wild-type [27%], 282 heterozygotes [50%], 133 $NTN^{-/-}$ [23%]), and both male and female $NTN^{-/-}$ mice are fertile. Unlike GFRα2-deficient mice, which have severe growth failure (30%–50% smaller than wild-type littermates [Rossi et al., 1998]), $NTN^{-/-}$ mice have normal growth rates (Figure 2). Major organs appear grossly and microscopically normal including the kidney, ureter, testes, and oviduct, where Neurturin is expressed. $NTN^{-/-}$ mice have normal renal function as evidenced by normal levels of blood urea nitrogen (wild-type, 24.4 ± 3.9; $NTN^{-/-}$, 21.0 ± 2.2) and creatinine (wild-type, 0.16 ± 0.05; $NTN^{-/-}$, 0.14 ± 0.05). Neurologic function of $NTN^{-/-}$ mice as assessed by gait, activity level, coordination, response to tail pinch, and ability to suckle also appears normal. Although Neurturin is present in the olfactory mucosa (Figure 7A) and GFRα2 is expressed in the olfactory bulb, the sense of smell of $NTN^{-/-}$ mice appears intact as they can find a buried scented pellet faster than an unscented pellet (scented pellet: wild-type, 19 ± 10 s; $NTN^{-/-}$, 26 ± 23 s; $p = 0.4$; unscented pellet: wild-type, 145 ± 36 s; $NTN^{-/-}$, 139 ± 14 s; $p = 0.7$). Motor neurons were present in apparently normal numbers in the anterior horn of the spinal cord, and nodose-petrosal ganglion profile counts are normal (wild-type, 6777 ± 665; $NTN^{-/-}$, 6363 ± 1060; $p = 0.5$).

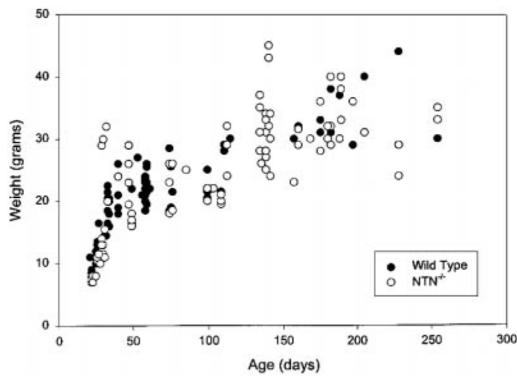

Figure 2. Neurturin-Deficient Mice Grow at a Normal Rate

(A) Weight versus age is plotted for wild-type (filled circles) and Neurturin-deficient (open circles) mice. Data represents weights for 64 wild-type and 79 Neurturin-deficient mice.

Hearing tests by brainstem auditory-evoked response testing were equivalent to matched wild-type animals. The brain of $NTN^{-/-}$ mice does not have major malformations either grossly or microscopically as discerned by thionin or acetylcholinesterase staining, GFRα1 or GFRα2 in situ hybridization, or by serotonin or tyrosine hydroxylase antibody staining.

Neurturin Is Critical for Proper Function of the Enteric Nervous System

Because of the absence of enteric neurons distal to the stomach in Ret-, GDNF-, and GFRα1-deficient animals, the enteric nervous system (ENS) of $NTN^{-/-}$ mice was examined (Figure 3). Whole-mount preparations of the ENS of adult mice demonstrated the presence of myenteric (Figure 3) and submucosal ganglion cells (data not shown) in normal numbers along the entire length of the bowel. Examination of the myenteric plexus by acetylcholinesterase histochemistry, however, revealed a striking decrease in small fiber density in the small intestine (Figures 3A and 3B) and in the colon. Fiber density in the duodenum (quantitated by counting fibers crossing a grid) is 40% lower in $NTN^{-/-}$ mice ($p < 0.001$). Since loss of a single large subpopulation of enteric neurons could result in reduced fiber density, staining of myenteric plexus whole-mount preparations for VIP (Figures 3E and 3F) and substance P (Figures 3G and 3H) was performed. VIP and substance P are each expressed in about 30% of the myenteric neurons and represent largely nonoverlapping populations. Neither of these populations of neurons is absent from $NTN^{-/-}$ mice, but both VIP- and substance P–expressing fibers appear to be less abundant in $NTN^{-/-}$ mice than in wild-type animals. To determine whether the decrease in fiber density reflected a more general neuronal loss, myenteric neurons were quantitated after staining with cuprolinic blue (Karaosmanoglu et al., 1996) (Figures 3C and 3D). Although there is a trend toward decreased enteric neuron number (15% loss in knockouts), the difference between wild-type and $NTN^{-/-}$ mice is not statistically significant ($p = 0.17$). However, myenteric ganglion cell size in the adult $NTN^{-/-}$ mice (mean cell area = $75 \pm 4\ \mu m^2$) is significantly smaller than in the wild-type

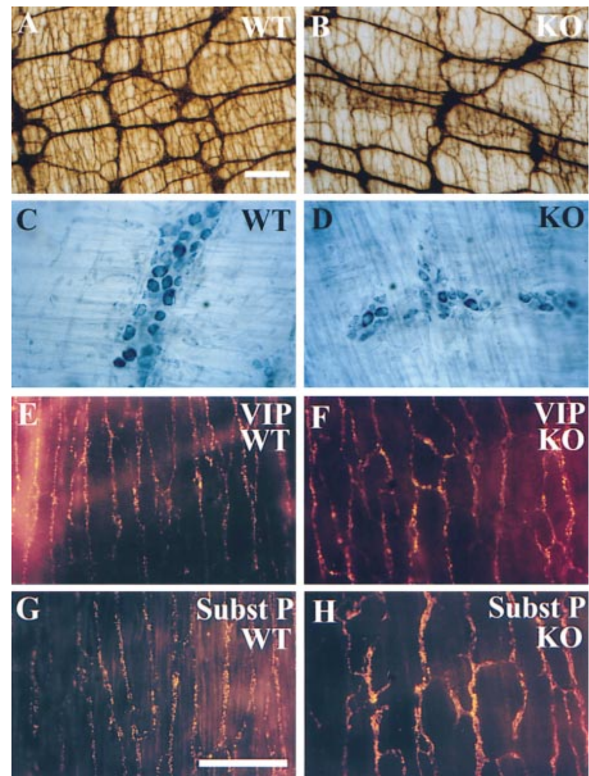

Figure 3. Neurturin-Deficient Adult Mice Have Smaller Nerve Cell Bodies and a Reduction in Small Fiber Density in the Myenteric Plexus

(A and B) Acetylcholinesterase staining of the duodenal myenteric plexus shows a reduced density of small fibers in the Neurturin-deficient mice (B) compared to wild-type littermates (A). The larger dark stained regions are myenteric ganglia connected by neuronal fiber bundles. Scale bar for (A) and (B) is 0.2 mm.

(C and D) Individual ganglion cells within the duodenal myenteric plexus of wild-type (C) and Neurturin-deficient mice (D) were visualized after cuprolinic blue staining. This staining technique was used for measuring neuron soma area and for determining neuron number in the myenteric plexus.

(E–H) Immunohistochemical staining for enteric neuron subpopulations that express VIP (E and F) and substance P (G and H) was performed on myenteric neuron laminar preparations from the adult duodenum of wild-type (E and G) and Neurturin-deficient (F and H) mice. Figures show fibers in the circular muscle layer of the gut. Both VIP- and substance P–expressing neuron fibers are readily seen in the Neurturin-deficient mice, although the neuron fiber density is lower for each in the $NTN^{-/-}$ mice. Scale bar for (E)–(H) is 0.1 mm.

mice (mean cell area = $96 \pm 4\ \mu m^2$, $p < 0.001$, $n = 100$ cells), suggesting that Neurturin provides crucial trophic support for these neurons and that the decreased fiber density may reflect smaller cell size and reduced axonal arborization.

To determine whether the anatomic differences between wild-type and $NTN^{-/-}$ mice were functionally significant, muscle strips from the stomach (gastric fundus), small intestine (jejunum), and distal colon of adult mice were evaluated for release of neurotransmitters and muscle contraction (Figure 4). As might be predicted from the anatomic studies, release of VIP and substance P from unstimulated muscle (basal release) was reduced

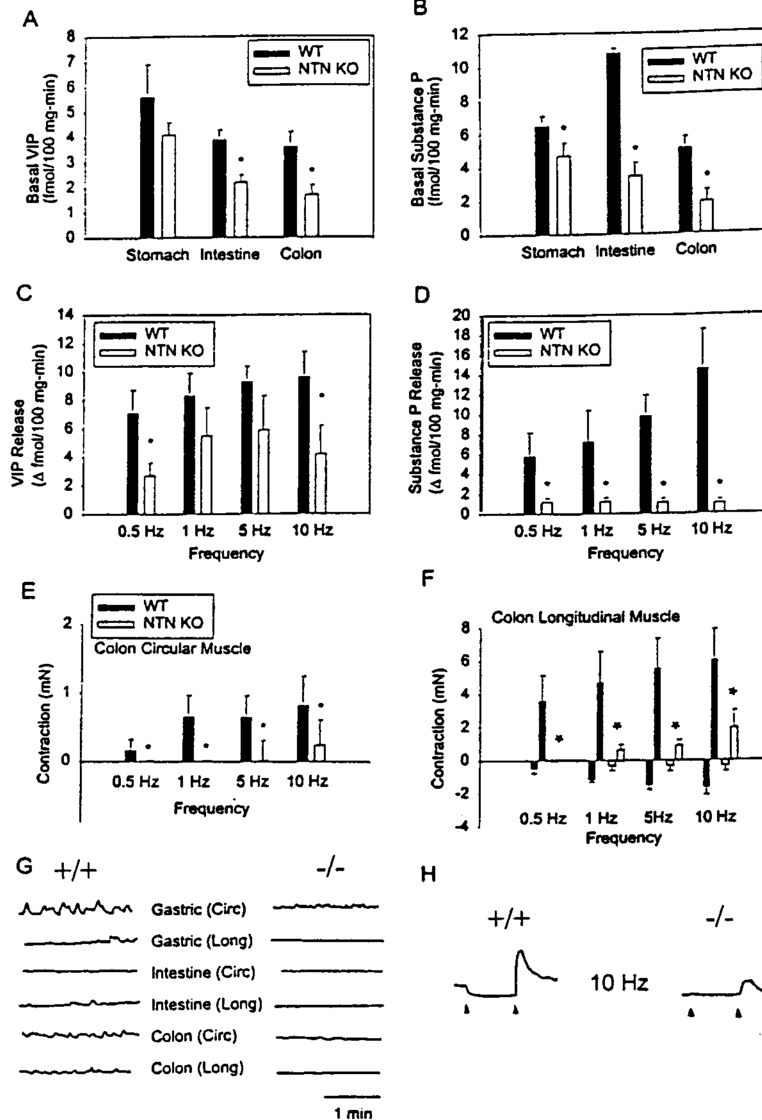

Figure 4. Neurturin-Deficient Adult Mice Have Abnormal Gut Motility

(A and B) VIP (A) and substance P (B) release from unstimulated muscle of the stomach (gastric fundus), small intestine (jejunum), and distal colon. For all panels, wild-type mice are shown in black bars and $NTN^{-/-}$ are shown in white bars. * indicates that values for $NTN^{-/-}$ mice are different from control at $p < 0.05$, $n = 24$.

(C and D) VIP (C) and substance P (D) release from colonic muscle after electric field stimulation at various frequencies (0.5 Hz, 1 Hz, 5 Hz, and 10 Hz). * indicates that values for $NTN^{-/-}$ mice are different from control at $p < 0.05$, $n = 6$.

(E and F) The strength of contraction of colonic circular (E) and longitudinal (F) muscle during and after electric field stimulation was quantitated for wild-type and $NTN^{-/-}$ mice. Note that for colonic longitudinal muscle, there is a relaxation that occurs during electric field stimulation followed by rebound contraction. The relaxation phase is represented by negative force values for contraction. * indicates $p < 0.05$.

(G and H) Basal contractility patterns (G) for different regions of the gut in wild-type (+/+) and Neurturin-deficient (-/-) mice. (H) Stimulated contractility patterns in colonic longitudinal muscle for wild-type (+/+) and Neurturin-deficient (-/-) mice exposed to 10 Hz electric field stimulation is shown. During application of the electric field (time between arrowheads), there is active relaxation, with a rebound contraction after the electric field has stopped.

in $NTN^{-/-}$ mice compared to wild-type animals (Figures 4A and 4B). The largest differences in transmitter release were seen in the distal gut. The differences in transmitter release between $NTN^{-/-}$ and wild-type mice were even more striking after electric field stimulation. Whereas stimulation led to a marked increase in VIP and substance P release from the colon of wild-type animals, transmitter release from the gut of $NTN^{-/-}$ mice increased to a smaller degree (Figures 4C and 4D). The reduction in substance P release from the gut of Neurturin-deficient animals was particularly dramatic.

To investigate the functional consequences of the decrease in transmitter release and reduced ENS fiber density of $NTN^{-/-}$ mice, in vitro assays to measure intestinal motility were performed. Muscle strips from all regions of the gut of wild-type mice (except intestinal circular muscle) developed spontaneous patterns of slow frequency, low-amplitude contractions (Figure 4G, left panel). In contrast, muscle strips from $NTN^{-/-}$ mice had significantly less spontaneous activity (Figure 4G, right panel). Contractile responses to electric field stimulation were also investigated. In most regions of the wild-type or $NTN^{-/-}$ gut, stimulation caused frequency-dependent contraction of the circular and longitudinal muscle. Quantitative analysis of muscle contractile strength in response to electric field stimulation demonstrated greatly reduced contractile strength in the $NTN^{-/-}$ mouse compared to wild-type controls (Figures 4E and 4F). In contrast to other regions of the gut, colonic longitudinal muscle relaxes during the 1 min period of electric field stimulation and then contracts at the end of the electrical stimulation (Figure 4H). Both the relaxation that occurs during stimulation and the rebound contraction at the end of stimulation are greatly reduced in NTN-deficient mice (Figures 4F and 4H). Together, these data demonstrate that Neurturin is required for maintaining proper function of the enteric nervous system.

Neurturin Is Essential for GFRα2-Expressing Sensory Neurons

The GFRα coreceptors and Ret are expressed in partially overlapping subsets of neurons within the trigeminal ganglion (Naveilhan et al., 1998), and Neurturin and

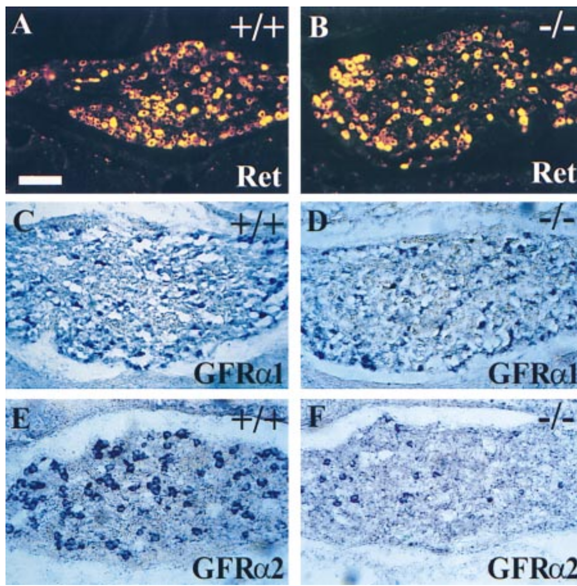

Figure 5. Neurturin Deficiency Results in Selective Loss of GFRα2-Expressing Cells from the Neonatal Trigeminal Ganglion

Trigeminal ganglia from wild-type (A, C, and E) and Neurturin-deficient (B, D and F) P0 mice were stained with antibodies to Ret (A and B), or by in situ hybridization for GFRα1 (C and D) and GFRα2 (E and F). Numbers of Ret- and GFRα1-positive neurons were similar in ganglia from wild-type and $NTN^{-/-}$ mice. There was a marked decrease in the number of GFRα2-stained cells in the $NTN^{-/-}$ animals versus wild-type littermates. Scale bar for (A)–(F) is 0.1 mm.

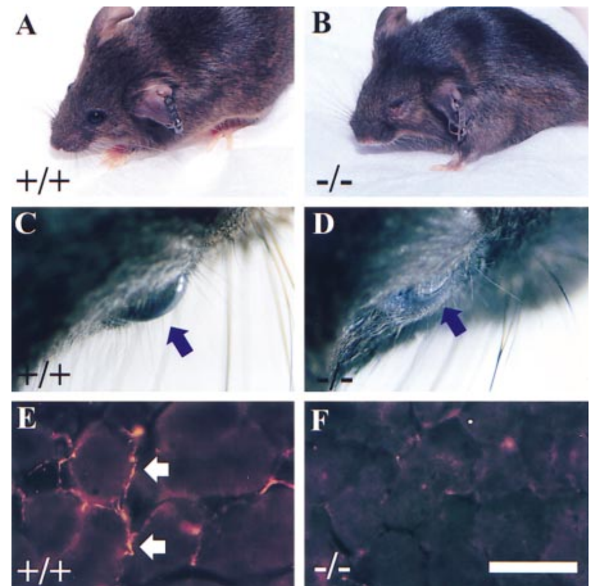

Figure 6. Neurturin-Deficient Adult Mice Have Reduced Lacrimal Gland Innervation and Depression of the Eyeball within the Orbit

(A and B) Neurturin-deficient mice are comparable in size to wild-type littermates. Obvious periorbital abnormalities distinguish wild-type from Neurturin-deficient mice.

Eyeball of the wild-type mouse (C) protrudes from the bony orbit, but the eyeball does not protrude from the orbit in Neurturin-deficient animals (D).

(E and F) Immunohistochemical analysis of lacrimal gland innervation with PGP 9.5 antibody demonstrates nerve fibers around individual gland acini of wild-type mice (E) (arrows). Neuronal fibers are rare around the acini in Neurturin-deficient mice (F). Scale bar for (E) and (F) is 0.1 mm.

GDNF are expressed in the target fields for trigeminal ganglion cells. In GDNF-deficient mice, physiologic pairing between GDNF and GFRα1 was clearly demonstrated in the trigeminal ganglion by the selective loss of GFRα1-expressing neurons (Naveilhan et al., 1998). To test for physiologic pairing of Neurturin and GFRα2 in vivo, the neonatal trigeminal ganglion was first stained with antibodies to Ret, but no obvious difference between wild-type and $NTN^{-/-}$ animals was observed (Figures 5A and 5B). Expression of the GFRα1 and GFRα2 was then examined in the trigeminal ganglion of neonatal wild-type and $NTN^{-/-}$ mice using in situ hybridization (Figures 5C–5F). Quantitative analysis of trigeminal ganglion neurons expressing each of the coreceptors demonstrated a marked reduction in the number of trigeminal neuron profiles expressing GFRα2 in the $NTN^{-/-}$ mice (wild-type, 567 $\pm$ 76; $NTN^{-/-}$, 181 $\pm$ 42; $p = 0.002$) but no change in the number of GFRα1-expressing cell profiles (wild-type, 605 $\pm$ 73; $NTN^{-/-}$, 556 $\pm$ 96; $p = 0.5$). In addition, the surviving GFRα2-expressing cells that are present in $NTN^{-/-}$ mice were significantly smaller than in wild-type animals (wild-type, 260 $\pm$ 12 μm^2; $NTN^{-/-}$, 214 $\pm$ 10 μm^2; $p = 0.004$), suggesting that Neurturin is providing trophic support necessary for maintaining cell size.

To determine whether Neurturin is required by other GFRα2-expressing sensory neurons, L4 dorsal root ganglia from adult mice were stained for GFRα2 by in situ hybridization. GFRα2-expressing cells were readily detected in the wild-type and $NTN^{-/-}$ DRG but appeared less abundant in the $NTN^{-/-}$ mice. Quantitation of $GFR\alpha2^+$ neuron profiles demonstrated a 45% reduction in GFRα2-expressing cells in the $NTN^{-/-}$ mouse compared to age-matched wild-type animals (wild-type, 333 $\pm$ 28; $NTN^{-/-}$, 188 $\pm$ 34; $p = 0.005$). Thus, Neurturin also provides important trophic support for a subset of GFRα2-expressing DRG neurons. The reduction in GFRα2 expressing cells observed in the DRG and trigeminal ganglion of $NTN^{-/-}$ mice is likely due to neuronal losses; however, it is also possible that expression of GFRα2 is reduced in the absence of Neurturin. In either case, these observations indicate that Neurturin and GFRα2 are physiologically paired in vivo and that GDNF does not substitute for Neurturin to support these neurons.

Neurturin Is Critical for Parasympathetic Innervation of the Lacrimal and Salivary Glands

By two months of age, Neurturin-deficient mice can be easily identified by the presence of periorbital abnormalities. Most of the mice have discernable eyelid droopiness, and many of the animals have crusting drainage around the eye with eyelid thickening (Figures 6A and 6B). Since Neurturin promotes the in vitro survival of superior cervical ganglion (SCG) sympathetic neurons that innervate the eyelid tarsal muscle, it was first suspected that this phenotype was the result of defects in the SCG; however, the SCG from $NTN^{-/-}$ mice is grossly and microscopically normal. In addition, neuron profile counts and ganglion volume from the SCG of newborn

WT and $NTN^{-/-}$ mice were not statistically different (profile counts: wild-type, 26705 ± 1935; $NTN^{-/-}$, 28156 ± 3664; p = 0.5; volumes: wild-type, 32 ± 4 × 10^6 μm^3; $NTN^{-/-}$, 35 ± 6 × 10^6 μm^3; p = 0.5). Even in adult animals, the size of SCG neurons was normal in $NTN^{-/-}$ mice (cell size: wild-type, 380 ± 25 μm^2; $NTN^{-/-}$, 429 ± 30 μm^2; p = 0.3). Since ptosis could also result from a defect in the third cranial nerve, which innervates the levator palpebrae, axons of this nerve from adult mice were examined. Nerve cross-sectional area (wild-type, 2.8 ± 0.3 × 10^4 μm^2; $NTN^{-/-}$, 2.8 ± 0.3 × 10^4 μm^2; p = 0.9) and axon number (wild-type, 779 ± 72; $NTN^{-/-}$, 718 ± 51; p = 0.2) were not statistically different between Neurturin-deficient and wild-type mice. Defects in eyelid innervation therefore seem an unlikely explanation for the periorbital appearance of NTN-deficient animals.

Upon further inspection, it was evident that one major component of the apparent eyelid droopiness of $NTN^{-/-}$ mice is related to the position of the eyeball within the eye socket (Figures 6C and 6D). Wild-type mice have eyes that protrude beyond the bony rim of the orbit, whereas in the Neurturin-deficient animals, the eyeballs are set deeper in the eye socket. The size of the eyeball is comparable in wild-type and Neurturin-deficient animals and does not explain the difference observed in eye protrusion. While this difference in eyeball protrusion could explain the apparent droopiness of the eyelids, the crusting discharge around the eyes suggested additional deficits.

Expression of GFRα2 in the pterygopalatine ganglion (data not shown) and of Neurturin in the lacrimal gland (Rossi et al., 1998; J. P. G., unpublished data) suggested that $NTN^{-/-}$ mice might have defective lacrimal gland innervation. Staining of the lacrimal gland of adult mice with PGP 9.5 antibody revealed readily identifiable nerve fibers within the gland of the wild-type mouse (Figure 6E), but these fibers were almost completely absent in the lacrimal gland of the $NTN^{-/-}$ mouse (Figure 6F). Reduced tear production as a result of defects in lacrimal gland innervation is therefore a likely cause for the periorbital appearance of $NTN^{-/-}$ mice.

Defects in parasympathetic innervation to the lacrimal gland and the reduction in other GFRα2-expressing neurons in $NTN^{-/-}$ mice prompted further evaluation of the parasympathetic nervous system. Strong Neurturin expression in the developing submandibular salivary gland (Figure 7A) and expression of GFRα2 within the parasympathetic ganglion cells innervating the gland suggested that these neurons might also be affected in $NTN^{-/-}$ mice. GFRα2-stained neurons were easily seen in the neonatal wild-type submandibular ganglion (Figure 7B) but could not be found in the $NTN^{-/-}$ gland (Figure 7C). Parasympathetic ganglia were, however, readily identified in $NTN^{-/-}$ submandibular gland by thionin staining (Figures 7C and 7D). Quantitation of parasympathetic ganglion neuron profiles in newborn $NTN^{-/-}$ mice demonstrated a 45% reduction in thionin-stained profiles compared to wild-type controls (wild-type, 370 ± 129; $NTN^{-/-}$, 207 ± 72; p = 0.007). Nerve cell areas were also significantly smaller in $NTN^{-/-}$ than in wild-type mice (wild-type, 100 ± 5 μm^2; $NTN^{-/-}$, 68 ± 3 μm^2; p = 0.001). Although nerve fibers were still readily apparent within the submandibular gland of $NTN^{-/-}$

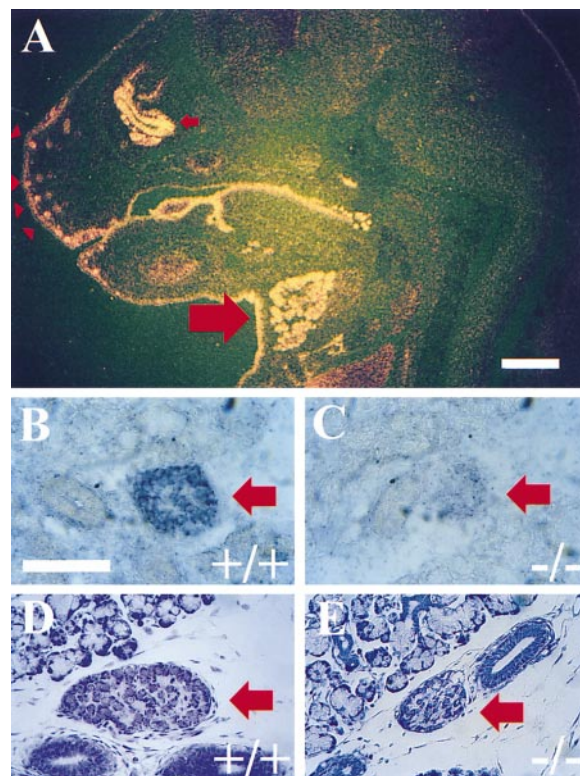

Figure 7. Neurturin-Deficient Mice Have Reduced Parasympathetic Innervation of the Submandibular Salivary Gland

In situ hybridization of the E16 mouse head (A) demonstrates that Neurturin is strongly expressed within the parenchyma of the developing submandibular gland (large arrow), around developing whisker follicles (arrowheads), as well as in the olfactory epithelium (small arrow). GFRα2 is expressed in the parasympathetic ganglion cells (arrow), which innervate the wild-type P0 mouse submandibular gland (B), but GFRα2 staining is virtually absent in the ganglia (arrow) of Neurturin-deficient animals (C). Parasympathetic ganglia (arrow) can be seen within the salivary gland of P0 $NTN^{-/-}$ mice by thionin staining (D), but they are smaller than in wild-type mice (C). Scale bar for (A) is 1 mm; for (B)–(E) it is 0.1 mm.

adult mice, fiber bundles revealed by PGP 9.5 immunostaining were thinner in $NTN^{-/-}$ than in wild-type animals (data not shown). This reduction in nerve fiber staining would be consistent with the moderate, but not complete, loss of parasympathetic neurons observed by cell profile counts.

To determine whether Neurturin signaling provides critical trophic support for other parasympathetic ganglia, the ciliary ganglion that innervates the pupillary constrictor muscle of the eye and the otic ganglion that innervates the parotid gland were examined. $NTN^{-/-}$ mouse pupils constrict in response to bright light, and the pupils are not markedly dilated in dim light. Despite this apparently normal function, there is a reduction in the number of ciliary ganglion neuron profiles in adult mice (wild-type, 81 ± 11; $NTN^{-/-}$, 42 ± 10; p = 0.04), but no significant change in the size of surviving ciliary ganglion cells (wild-type, 263 ± 10 μm^2; $NTN^{-/-}$, 255 ± 14 μm^2; p = 0.15). This implies that Neurturin is important for the survival of a subset of these neurons but that

other trophic factors also support ciliary ganglion neurons. In the otic ganglion of neonatal mice, parasympathetic neurons strongly express GFRα2, but in contrast to the submandibular gland, GFRα2 staining is not lost from the otic ganglion of neonatal $NTN^{-/-}$ animals. In addition, neuron profile counts in the neonatal otic ganglion were equivalent in wild-type and Neurturin-deficient animals (wild-type, 345 ± 36; $NTN^{-/-}$, 383 ± 62; $p = 0.4$) suggesting that these neurons are not solely dependent on Neurturin for survival prior to birth. In contrast to the preservation in neuron profile number, the size of individual otic ganglion cells was reduced in $NTN^{-/-}$ mice compared to wild-type controls (wild-type, 90 ± 5.5 μm^2; $NTN^{-/-}$, 61 ± 2 μm^2; $p = 0.001$) indicating that Neurturin provides trophic support for otic ganglion cells. The defects observed in the ciliary ganglion, otic ganglion, lacrimal gland, and submandibular salivary gland of $NTN^{-/-}$ mice demonstrate the importance of Neurturin for the survival and maintenance of parasympathetic neurons.

Discussion

The Ret tyrosine kinase is the signaling component of receptor complexes for four distinct ligands (Artemin, GDNF, Neurturin, and Persephin). The binding component of these receptor complexes comprise a structurally novel family of glycosylphosphatidylinositol- (GPI-) anchored proteins (GFRα1, GFRα2, GFRα3, and, in the avian system, GFRα4), which preferentially interact with specific ligands in vitro. For the NGF family of neurotrophins, each trophic factor and its cognate receptor are critical for survival and development of distinct populations of neurons. While GDNF and GFRα1 are essential for kidney and enteric nervous system development, the physiologic roles of the other GDNF family members have not been defined. Since neuron survival factors are typically made in limiting quantities in vivo, physiologic pairing may occur either because of preferential receptor activation or because of pairing of expression patterns for ligand and receptor during development. Characterization of Neurturin-deficient mice demonstrates the essential physiologic role for Neurturin in enteric and parasympathetic nervous system function and for the survival of GFRα2-expressing cells in sensory ganglia.

Superior Cervical Ganglion

Although Neurturin was first identified based on its ability to promote the survival of rat sympathetic (SCG) neurons, the SCG of $NTN^{-/-}$ mice appears normal. This was particularly surprising because the SCG is completely absent in $Ret^{-/-}$ mice (Schuchardt et al., 1994), but only 35% smaller than wild type in $GDNF^{-/-}$ (Moore et al., 1996) animals, suggesting that other Ret ligands play essential roles in SCG development. Neurturin was thought to be the critical factor supporting SCG neurons and the likely explanation for differences in SCG survival between Ret- and GDNF-deficient animals. It now seems more likely that other Ret ligands will provide the critical survival signals for SCG neurons in vivo. Persephin does not support SCG survival in vitro, but the recently identified factor Artemin is trophic for SCG neurons (Baloh et al., 1998b) and is likely to be important for SCG sympathetic neuron survival. Further support for this idea is the observation that Artemin's preferred coreceptor GFRα3 is expressed in the SCG (Worby et al., 1998; Yu et al., 1998). It remains intriguing that the SCG of $GFR\alpha1^{-/-}$ mice is well preserved (Cacalano et al., 1998; Enomoto et al., 1998), implying that GDNF may be acting primarily through an alternate GFRα to support SCG neurons.

Enteric Nervous System

The anatomic and functional deficits observed in the enteric nervous system of $NTN^{-/-}$ mice demonstrate the importance of Neurturin as a trophic factor for postmitotic enteric neurons. To form the enteric nervous system, neural crest cells migrate from the vagal and sacral regions of the developing spinal cord (Le Douarin and Teillet, 1973; Gershon et al., 1992). While migrating, these cells undergo many rounds of proliferation to produce enough neurons and glia to populate the ENS. The postmitotic neurons extend axonal processes to create a network of interacting cells that regulate motility, sensation, blood flow, and mucosal secretion in the gut (Costa and Brookes, 1994; Sang et al., 1997). In culture, developing enteric neuron precursors respond to GDNF (Chalazonitis et al., 1997; Hearn et al., 1998) and Neurturin (Heuckeroth et al., 1998) by continued proliferation and by extensive process formation. Cells grown in the absence of Neurturin or GDNF have smaller cell bodies and reduced axonal processes. Although ENS precursor cells cultured in vitro in GDNF and Neurturin are indistinguishable, the physiologic role of Neurturin and GDNF is quite distinct. GDNF-deficient mice have extensive intestinal aganglionosis, presumably as a result of insufficient proliferation of ENS precursor cells and increased cell death. Neurturin-deficient mice have a reduction in the density of nerve fibers in the ENS, smaller enteric neuron cell bodies, and abnormal gastrointestinal motility, but only a minimal reduction in enteric neuron cell number. The observed deficits in the enteric nervous system of $NTN^{-/-}$ mice imply that Neurturin is most important as a trophic factor for postmitotic myenteric neurons whereas GDNF is crucial for proliferation of enteric neuron precursors. The requirement for neurturin for maintenance of the adult enteric nervous system is demonstrated by the decrease in neuron soma diameter, decreased neuropeptide release, and decreased intestinal motility of $NTN^{-/-}$ mice. It is also clear that, although GDNF can activate Ret via either GFRα1 and GFRα2 in vitro, the presence of GDNF cannot substitute for the loss of Neurturin as an ENS trophic factor. The striking similarity of the ENS in Neurturin- and GFRα2-deficient mice (Rossi et al., 1998) indicates that in vivo, Neurturin exerts its effects in the enteric nervous system via the GFRα2/Ret receptor complex. Although the specific cell types within the ENS that express NTN, GDNF, and the GFRα coreceptors are not yet well established, differences in cell localization of receptors and the timing of ligand expression may contribute to differences between GDNF- and Neurturin-deficient mice.

Sensory Neurons

Examination of the trigeminal and dorsal root ganglia of $NTN^{-/-}$ mice indicates that Neurturin is important for GFRα2-expressing sensory neurons. In the trigeminal ganglion, there is a substantial loss of GFRα2-expressing cells with preservation of GFRα1-expressing cells. This directly mirrors the loss of GFRα1-expressing cells observed in $GDNF^{-/-}$ animals (Naveilhan et al., 1998) and demonstrates that even though GDNF and Neurturin are coexpressed in the terminal field for the trigeminal ganglion neurons, GDNF cannot substitute for Neurturin's ability to support GFRα2-expressing cells. The loss of GFRα2-expressing neurons from the DRG of adult mice is consistent with findings in the trigeminal ganglion and reinforces the evidence for physiologic pairing between Neurturin and GFRα2. This is further supported by the observation that Neurturin and GDNF undergo retrograde transport into distinct populations of DRG sensory neurons (M. Leitner et al., submitted). Since GFRα1 and GFRα2 are coexpressed in about half of $GFR\alpha2^{+}$ DRG neurons (Bennett et al., 1998), it is likely that the surviving $GFR\alpha2^{+}$ cells in the adult DRG of $NTN^{-/-}$ mice are maintained through activation of GFRα1 by GDNF or Artemin.

Parasympathetic Neurons

While the trophic factor requirements for many sensory and sympathetic neuron populations have been well defined, trophic factors important for parasympathetic neuron development are less well understood. The striking loss of parasympathetic innervation to the lacrimal and submandibular salivary glands of $NTN^{-/-}$ mice indicates that Neurturin is a parasympathetic neuron survival factor. The importance of Neurturin for parasympathetic neurons is supported by the reduction in ciliary ganglion neuron profiles and the smaller size of otic ganglion cells in $NTN^{-/-}$ mice. The observation that Neurturin/Ret signaling is important for development of parasympathetic neurons is consistent with defects in parasympathetic neurons in mice deficient in Phox2a, a transcription factor that regulates Ret expression (Morin et al., 1997). The normal numbers of the otic ganglion cells and normal size of surviving ciliary ganglion cells, however, suggests that other factors also provide trophic support for some parasympathetic neuron populations. In the otic ganglion, Neurturin may be primarily responsible for maintenance of postnatal parasympathetic neurons, rather than for their prenatal development.

Physiologic Pairing of Neurturin and GFRα2

The analysis above, based on knowledge of the expression patterns of GDNF, Neurturin, GFRα1, and GFRα2 suggests that Neurturin and GFRα2 are tightly physiologically coupled in vivo. Comparison of Neurturin-deficient and GFRα2-deficient animals (Rossi et al., 1999 [this issue of *Neuron*]) provides even stronger evidence of this ligand/coreceptor pairing. Both $NTN^{-/-}$ and $GFR\alpha2^{-/-}$ mice are viable, fertile, and appear to have normal kidneys. Both Neurturin- and GFRα2-deficient animals have a comparable reduction in myenteric plexus fiber density and disordered gastrointestinal motility. In addition, substance P–producing myenteric neurons appear more dramatically affected than VIP-expressing neurons in both the $NTN^{-/-}$ and $GFR\alpha2^{-/-}$ mice. Both animals have reduced innervation of the lacrimal and salivary glands. Both animals have similar periorbital anomalies, without defects in the superior cervical ganglion or third cranial nerve innervation of the eyelid. Trigeminal ganglia from developing GFRα2-deficient mice have reduced fiber outgrowth in response to Neurturin, while Neurturin-deficient mice have a reduction in the number of trigeminal ganglion GFRα2-expressing cells. Thus, for many GFRα2-dependent cells, Neurturin is the critical neurotrophic factor.

Although Neurturin and GFRα2 are tightly coupled in vivo, there are several areas of Neurturin and GFRα2 expression where phenotypic changes are not obvious in $NTN^{-/-}$ mice. For example, no striking abnormalities were observed in the central nervous system of $NTN^{-/-}$ mice, but this is also true for the $Ret^{-/-}$, $GDNF^{-/-}$, and $GFR\alpha1^{-/-}$ animals. While this may reflect the tendency of CNS neurons to rely on multiple trophic factors simultaneously (Snider, 1994), it is possible that more detailed analysis of the central nervous system will reveal subtle deficits. Although Neurturin is strongly expressed in the olfactory mucosa and GFRα2 is expressed in the olfactory bulb, the ability of $NTN^{-/-}$ mice to detect a scented pellet appears normal. While olfactory neurons, like other CNS neuron populations, may rely on multiple neurotrophic factors, it is also possible that Neurturin signaling is needed by only a subset of olfactory neurons, which respond to specific odorant molecules. Neurturin and GFRα2 are both expressed in the developing kidney, but grossly, microscopically, and functionally, the kidneys of $NTN^{-/-}$ mice appear normal. In addition, despite Neurturin expression in the testis and oviduct, male and female $NTN^{-/-}$ animals are fertile. While the role of Neurturin in these tissues may be related to innervation of these structures that would not have been detected with the current analysis, Neurturin deficiency could also be compensated for by the presence of other related trophic factors.

Despite the similarity of Neurturin- and GFRα2-deficient animals, there is one important difference. GFRα2-deficient mice have striking growth failure, while Neurturin-deficient mice cannot be distinguished from littermates based on size. Given the remarkable similarity between these animals, the etiology of growth failure in one animal, but not the other, remains unclear. It suggests that there may some areas where GDNF can substitute for Neurturin by activating the GFRα2 receptor and that at least one of these areas is important for growth. Alternatively, it is possible that the growth differences between these animals reflect differences in strain genetic background, severity of common defects, or animal husbandry. Differences in diet might be particularly important since parasympathetic innervation may influence salivary gland secretions and gastrointestinal motility. If genetic background differences explain the discrepancy in growth between $NTN^{-/-}$ and $GFR\alpha2^{-/-}$ mice, then there are likely to be modifier genes that may be particularly relevant for phenotypic expression of NTN and GFRα mutations in human populations.

The defects seen in Neurturin-deficient mice occur in human disorders of the autonomic nervous system. Although Sjogren's syndrome with xerostomia (dry mouth) and keratoconjunctivitis sicca (damage to the cornea because of decreased tear production) is usually associated with autoimmune disease, it also occurs in the context of primary neuropathy (Denislic and Meh, 1998). In addition, autonomic dysfunction accompanies systemic diseases like diabetes and multiple sclerosis. Autonomic dysfunction has also been reported in the context of functional gastrointestinal disease (Raethjen et al., 1997) including patients with uncoordinated peristalsis or hypoperistalsis (Camilleri and Fealey, 1990) and in patients with irritable bowel syndrome (Aggarwal et al., 1994). Functional studies indicate that $NTN^{-/-}$ mice have abnormal intestinal motility similar to that which occurs in human motility disorders. These observations raise the possibility that Neurturin may be useful to slow the progression of autonomic neuropathy in systemic disease.

Experimental Procedures

Generation of a Targeted Mutation in Neurturin

The murine (strain 129/SvJ) Neurturin P1 clone was obtained from Genome Systems. A 1.5 kb EagI/KpnI fragment extending from the start of the coding sequence for the mature Neurturin protein into the 3′ untranslated region was replaced by a 1.8 kb NotI/EcoRI fragment from pPNT (Tybulewicz et al., 1991) containing a pgkNEO cassette to generate the targeting vector, which has a 5′ short arm (2.0 kb) and a 3′ long arm (6.5 kb). The linearized targeting construct was electroporated into the 129/SvJ embryonic stem (ES) cell line RW4 (obtained from the ES Cell Core Laboratory, Washington University). After growth in G418, homologous recombinants were screened by Southern blotting using an external probe. The targeted ES clones were injected into C57BL/6 blastocysts and transferred into pseudopregnant female mice. High-percentage male chimeras were obtained and mated to C57BL/6 females. Germline transmission was obtained with two independent ES cell lines. Mice heterozygous for the mutant Neurturin allele were mated to obtain Neurturin-deficient animals. Analyses were performed on mice with a hybrid 129/SvJ:C57BL/6 background. The genotype of each mouse was determined by Southern blot analysis after digestion of tail DNA with KpnI. The absence of mRNA encoding the mature Neurturin protein was demonstrated by RT-PCR from adult heart RNA. The multiplex PCR reaction included primers for both Neurturin and an internal positive control (porphobilinogen deaminase [Fink et al., 1998]) (NTN primers: 5′-CAGCGGAGGCGCGTGCGCAGAGAGCG, 5′-TAGCGGCTGTGCACGTCCAGGAAGGACACCTCGT; porphobilinogen deaminase primers: 5′-TGTTGAGGTTTCCCCGAATACT, 5′-GATGCTGTTGTCTTTCACCCAAA) and produced fragments of 100 nt (Neurturin) and 154 nt (porphobilinogen deaminase). The reaction conditions were 2.5 mM $MgCl_2$, 10× Fisher Taq buffer B, 8% glycerol, and the cycling parameters were 94°C for 30 s, 60°C for 30 s, and then 72°C for 60 s × 40 cycles.

Histopathological Analysis and Immunohistochemistry

Tissues were prepared as described (Enomoto et al., 1998). Paraffin-embedded newborn pups and adult tissues were sectioned at 6 μm and stained with thionin or hematoxylin and eosin. Immunohistochemistry was performed on either frozen or paraffin embedded sections as described (Enomoto et al., 1998).

In Situ Analysis of GFRα1 and GFRα2 Expression

Digoxigenin-labeled or ^{33}P-labeled riboprobes were synthesized from plasmids containing mouse cDNA sequences of GFRα1 and GFRα2; nucleotide numbers of GFRα1 and GFRα2 cDNAs are 574–1069 (GenBank accession number U59826) and 1002–1417 (AF002701), respectively. Slides were prepared as described (Golden et al., 1998) except that in some cases digoxigenin labeled probes were used instead of ^{33}P-labeled RNA. Digoxigenin labeled slides were developed with alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer Mannheim), NBT, and BCIP according to manufacturer's instructions.

Acetylcholinesterase Staining

Acetylcholinesterase staining was performed as described (Enomoto et al., 1998). For adult animals, the gut was opened along the mesenteric border and pinned to Sylgard plates without stretching. The pinned gut was then fixed in 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.4) (PB) for 1–2 hr. The gut was then transferred to saturated sodium sulfate and stored at 4°C until ready for analysis. The adult gut was dissected to separate muscle layers from the submucosa so that the myenteric and submucosal plexus could be stained independently. A readily apparent decrease in fiber density in the $NTN^{-/-}$ myenteric plexus was observed in tissues prepared as above, tissues from perfused animals, or in tissues allowed to reach resting length before fixation (Karaosmanoglu et al., 1996).

Quantitative Analysis of the Enteric Nervous System

Quantitative analysis of cell number and cell size in the myenteric plexus was performed after staining with cuprolinic blue (Karaosmanoglu et al., 1996). For these analyses, ganglionic volume and cell number per unit ganglionic area were determined from 14 randomly selected 200× fields from three wild-type and three Neurturin-deficient mice in the myenteric plexus of the duodenum using the UTHSCSA Image Tool program (developed at the University of Texas Health Science Center at San Antonio, Texas; see http://ddsdx.uthscsa.edu/dig/itdesc.html for information). Enteric neuron cell size was determined by measuring the area of 100 randomly selected cuprolinic blue-stained neurons from the duodenum using Image Tool software. Neuronal fiber densities in these duodenal myenteric neuron preparations were quantitated by counting the number of fibers that crossed a standardized grid. Sixteen randomly selected fields from three wild-type and three Neurturin-deficient animals were analyzed.

Contractility and Neurotransmitter Release

Gastric fundus, jejunum, and distal colon from adult mice were cut into strips parallel to the circular and longitudinal muscles. After removing the mucosa by blunt dissection, strips were mounted at 0.5 g preload in 37°C organ baths containing Krebs-bicarbonate buffer (pH 7.4) with 0.1% bovine serum albumin, 10 μM amastatin, and 1 μM phosphoramidon. After a 1 hr equilibration period, field stimulation was applied by parallel platinum electrodes (80 V strength, 1 ms duration and 0.5 to 10 Hz frequency for 1 min train lengths). Individual stimulation periods were separated by 1 hr equilibration periods. The bathing medium was collected for the 5 min period immediately before stimulation (basal period) and for 1 min during which stimulation was applied. Samples were stored at −80°C for subsequent radioimmunoassay of VIP and substance P as described previously (Grider, 1986; Grider and Makhlouf, 1986). VIP, substance P, and antiserums to VIP and substance P were from Peninsula Laboratories (Torrence, CA). ^{125}I VIP and ^{125}I substance P were from New England Nuclear (Boston, MA).

Neuronal Counting and Cell Size Analysis

The heads of P0 mice were embedded in paraffin, sectioned in the coronal plane at 6 μm, and stained with thionin. Ganglion volumes and neuronal profile counts were determined and the mean and standard deviation were calculated as described previously (Silos-Santiago et al., 1995; Enomoto et al., 1998). Volume and profile number determinations used four animals of each genotype for the SCG, nodose, submandibular gland, ciliary ganglion, and optic nerve counts, three animals of each genotype for the trigeminal gland and DRG in situ counts, and otic ganglion profile counts. Profiles of GFRα1- and GFRα2-stained cells in the trigeminal and ganglion were determined by counting stained cells in every eighth section (18 μm for each section) while DRG counts were performed on every fourth section. Because the nucleolus of the in situ stained cells could not be identified, all stained cells were counted. For cell size

analysis in the SCG, otic ganglion, ciliary ganglion, and submandibular ganglion, cell area was measured for 50 wild-type and NTN$^{-/-}$ thionin-stained cells with visible nucleoli using Image Tool software. For the analysis of cell size in GFRα2-expressing trigeminal ganglion cells, area of 50 wild-type and 50 NTN$^{-/-}$ cells was determined after in situ hybridization.

Behavioral Analysis

Food-restricted mice were tested for their ability to locate a cherry-scented food reward buried 2.5 cm beneath wood shavings (Alberts and Galef, 1971). The location of the scented food varied randomly, and animals were timed for ability to locate the food. On the seventh day of testing, an unscented food morsel was buried to determine whether the animals were using cues other than cherry odor to locate the food. Locomotor activity testing was performed as described (Bardgett et al., 1997, 1998) to measure breaks in beams of infrared light over a 75 min period.

Cranial Nerve III Analysis

Adult wild-type and Neurturin-deficient mice were perfused with 2% paraformaldehyde/2% glutaraldehyde in phosphate-buffered saline (pH 7.4). Animals were postfixed overnight, then washed thoroughly with PBS. The right oculomotor nerve was removed, washed in PBS, fixed in 2% osmium tetroxide, and embedded in Epon 812 (Electron Microscopy Sciences). The nerves were sectioned at 1 μm on a Reichert Ultracut E ultramicrotome and stained with toluidine blue. Sections were photographed at 1000×, and images were reconstructed for digital image processing. Nerve cross-sectional area and axon number was determined using the Image Tool program.

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References

Aggarwal, A., Cutts, T.F., Abell, T.L., Cardoso, S., Familoni, B., Bremer, J., and Karas, J. (1994). Predominant symptoms in irritable bowel syndrome correlate with specific autonomic nervous system abnormalities. Gastroenterology *106*, 945–950.

Alberts, J.R., and Galef, B.G., Jr. (1971). Acute anosmia in the rat: a behavioral test of a peripherally-induced olfactory deficit. Physiol. Behav. *6*, 619–621.

Baloh, R.H., Tansey, M.G., Golden, J.P., Creedon, D.J., Heuckeroth, R.O., Keck, C.L., Zimonjic, D.B., Popescu, N.C., Johnson, E.M.J., and Milbrandt, J. (1997). TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. Neuron *18*, 793–802.

Baloh, R.H., Gorodinsky, A., Golden, J.P., Tansey, M.G., Keck, C.L., Popescu, N.C., Johnson, E.M.J., and Milbrandt, J. (1998a). GFRα3 is an orphan member of the GDNF/neurturin/persephin receptor family. Proc. Natl. Acad. Sci. USA *95*, 5801–5806.

Baloh, R.H., Tansey, M.G., Lampe, P.A., Fahrner, T.J., Enomoto, H., Simburger, K.S., Leitner, M.L., Araki, T., Johnson, E.M.J., and Milbrandt, J. (1998b). Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRα3-Ret receptor complex. Neuron *21*, 1291–1302.

Bardgett, M.E., Jacobs, P.S., Jackson, J.L., and Csernansky, J.G. (1997). Kainic acid lesions enhance locomotor responses to novelty, saline, amphetamine, and MK-801. Behav. Brain Res. *84*, 47–55.

Bardgett, M.E., Jackson, J.L., Taylor, B.M., and Csernansky, J.G. (1998). The effects of kainic acid lesions on locomotor responses to haloperidol and clozapine. Psychopharmacology *135*, 270–278.

Bennett, D.L.H., Michael, G.J., Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, S.B., and Priestley, J.V. (1998). A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. J. Neurosci. *18*, 3059–3072.

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

Buj-Bello, A., Adu, J., Pinon, L.G., Horton, A., Thompson, J., Rosenthal, A., Chinchetru, M., Buchman, V.L., and Davies, A.M. (1997). Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature *387*, 721–724.

Cacalano, G., Farinas, I., Wang, L.-C., Hagler, K., Forgie, A., Moore, M., Armanini, M., Phillips, H., Ryan, A.M., Reichardt, L.F., Hynes, M., Davies, A., and Rosenthal, A. (1998). GFRα1 is an essential receptor component for GDNF in the developing nervous system and kidney. Neuron *21*, 53–62.

Camilleri, M., and Fealey, R.D. (1990). Idiopathic autonomic denervation in eight patients presenting with functional gastrointestinal disease. A causal association? Dig. Dis. Sci. *35*, 609–616.

Chalazonitis, A., Rothman, T.P., and Gershon, M.D. (1997). Age- and cell type-dependence of the responses of crest-derived cells immunoselected from the fetal rat gut to GDNF and NT-3. (Summary) Neuroscience *23*, 1430.

Costa, M., and Brookes, S.J.H. (1994). The enteric nervous system. Am. J. Gastroenterol. *89*, S129-S137.

Denislic, M., and Meh, D. (1998). Sjogren's syndrome in patients with chronic idiopathic axonal polyneuropathy. J. Neurol. Neurosurg. Psychiatry *65*, 138–139.

Enokido, Y., de Sauvage, F., Hongo, J.-A., Ninkina, N., Rosenthal, A., Buchman, V.L., and Davies, A.M. (1998). GFRα4 and the tyrosine kinase Ret form a functional receptor complex for persephin. Curr. Biol. *8*, 1019–1022.

Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R.O., Snider, W.D., Johnson, E.M.J., and Milbrandt, J. (1998). GFRα1 deficient mice have deficits in the enteric nervous system and kidneys. Neuron *21*, 317–324.

Fink, L., Seeger, W., Ermert, L., Hanze, J., Stahl, U., Grimminger, F., Kummer, W., and Bohle, R.M. (1998). Real-time quantitative RT-PCR after laser-assisted cell picking. Nat. Med. *4*, 1329–1333.

Gershon, M.D., Chalazonitis, A., and Rothman, T.P. (1992). From neural crest to bowel: development of the enteric nervous system. J. Neurobiol. *24*, 199–214.

Golden, J.P., Baloh, R.H., Kotzbauer, P.T., Lampe, P.A., Osborne, P.A., Milbrandt, J., and Johnson, E.M. (1998). Expression of neurturin, GDNF, and their receptors in the adult mouse CNS. J. Comp. Neurol. *398*, 139–150.

Grider, J.R. (1986). Tachykinins as transmitters of the ascending contractile component of the peristaltic reflex. Am. J. Physiol. *257*, G709-G714.

Grider, J.R., and Makhlouf, G.M. (1986). Colonic peristaltic reflex: identification of VIP as a mediatior of descending relaxation. Am. J. Physiol. *251*, G40-G45.

Hearn, C.J., Murphy, M., and Newgreen, D. (1998). GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. Dev. Biol. *197*, 93–105.

Heuckeroth, R.O., Lampe, P.A., Johnson, E.M.J., and Milbrandt, J. (1998). Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. Dev. Biol. *200*, 116–129.

Horger, B.A., Nishimura, M.C., Armanini, M.P., Wang, L.-C., Poulsen, K.T., Rosenblad, C., Kirik, D., Moffat, B., Simmons, L., Johnson, E.J.,

et al. (1998). Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. J. Neurosci. *18*, 4929–4937.

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

Jing, S., Yu, Y., Fang, M., Hu, Z., Holst, P.L., Boone, T., Delaney, J., Schultz, H., Zhou, R., and Fox, G.M. (1997). GFRα-2 and GFRα-3 are two new receptors for ligands of the GDNF family. J. Biol. Chem. *272*, 33111–33117.

Karaosmanoglu, T., Aygun, B., Wade, P., and Gershon, M. (1996). Regional differences in the number of neurons in the myenteric plexus of the guinea pig small intestine and colon: an evaluation of markers used to count neurons. Anat. Rec. *244*, 470–480.

Klein, R.D., Sherman, D., Ho, W.H., Stone, D., Bennett, G.I., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J.A., et al. (1997). A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature *387*, 717–721.

Kotzbauer, P.T., Lampe, P.A., Heuckeroth, R.O., Golden, J.P., Creedon, D.J., Johnson, E.M., and Milbrandt, J.D. (1996). Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature *384*, 467–470.

Le Douarin, N.M., and Teillet, M.A. (1973). The migration of neural crest cells to the wall of the digestive tract in avian embryo. Embryol. Exp. Morphol. *30*, 31–48.

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

Milbrandt, J., Sauvage, F.J., Fahrner, T.J., Baloh, R.H., Leitner, M.L., Tansey, M.G., Lampe, P.A., Heuckeroth, R.O., Kotzbauer, P.T., Simburger, K.S., et al. (1998). Persephin, a novel neurotrophic factor related to GDNF and Neurturin. Neuron *20*, 245–253.

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q., and Snider, W.D. (1997). IB4-Binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron *19*, 849–861.

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

Morin, X., Cremer, H., Hirsch, M.-R., Kapur, R.P., Goridis, C., and Brunet, J.-F. (1997). Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene *Phox2a*. Neuron *18*, 411–423.

Naveilhan, P., Baudet, C., Mikaels, A., Shen, L., Westphal, H., and Ernfors, P. (1998). Expression and regulation of GFRα3, a glial cell line-derived neurotrophic factor family receptor. Proc. Natl. Acad. Sci. USA *95*, 1295–1300.

Nomoto, S., Ito, S., Yang, L.X., and Kiuchi, K. (1998). Molecular cloning and expression analysis of GFR alpha-3, a novel cDNA related to GDNFR alpha and NTNR alpha. Biochem. Biophys. Res. Commun. *244*, 849–853.

Nosrat, C., Tomac, A., Hoffer, B., and Olson, L. (1997). Cellular expression of GDNF mRNA predicts multiple functions in and outside the nervous system. Cell Tissue Res. *286*, 191–207.

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

Raethjen, J., Pilot, M.A., Knowles, C., Warner, G., Arand, P., and Williams, N. (1997). Selective autonomic and sensory deficits in slow transit constipation. J. Auton. Nerv. Syst. *66*, 46–52.

Rossi, J., Luukko, K., Poteryaev, D., Laurikainen, A., Sun, Y.F., Laakso, T., Eerikainen, S., Tuominen, R., Lakso, M., Rauvala, H., et al. (1998). Retarded growth and deficits in the parasympathetic nervous system in mice lacking GFRα2, a functional neurturin receptor. Neuron *22*, this issue, 243–252.

Sanchez, M.P., Silos-Santiago, I., Frisen, J., He, B., Lira, S.A., and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature *382*, 70–73.

Sang, Q., Williamson, S., and Young, H.M. (1997). Projections of chemically identified myenteric neurons of the small and large intestine of the mouse. J. Anat. *190*, 209–222.

Sanicola, M., Hession, C., Worley, D., Carmillo, P., Ehrenfels, C., Walus, L., Robinson, S., Jaworski, G., Wei, H., Tizard, R., et al. (1997). Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. Proc. Natl. Acad. Sci. USA *94*, 6238–6243.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Constantini, F., and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature *367*, 380–383.

Silos-Santiago, I., Molliver, D.C., Ozaki, S., Smeyne, R.J., Fagan, A.M., Barbacid, M., and Snider, W.D. (1995). Non-TrkA-expressing small DRG neurons are lost in TrkA-deficient mice. J. Neurosci. *15*, 5929–5942.

Snider, W.D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell *77*, 627–638.

Thompson, J., Doxakis, E., Pinon, L.G., Strachan, P., Buj-Bello, A., Wyatt, S., Buchman, V.L., and Davies, A.M. (1998). GFRalpha-4, a new GDNF family receptor. Mol. Cell. Neurosci. *11*, 117–126.

Treanor, J.J.S., Goodman, L., Sauvage, F., Stone, D.M., Poulsen, K.K.T., Beck, C.D., Gray, C., Armanini, M.P., Pollock, R.A., Hefti, F., et al. (1996). Characterization of a multicomponent receptor for GDNF. Nature *382*, 80–83.

Trupp, M., Raynoschek, C., Belluardo, N., and Ibanez, C.F. (1998). Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase. Mol. Cell. Neurosci. *11*, 47–63.

Tybulewicz, V.L., Crawford, C.E., Jackson, P.K., Bronson, R.T., and Mulligen, R.C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell *65*, 1153–1163.

Widenfalk, J., Nosrat, C., Tomac, A., Westphal, H., Hoffer, B., and Olson, L. (1997). Neurturin and Glial Cell Line-Derived Neurotrophic Factor Receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. J. Neurosci. *17*, 8506–8519.

Worby, C.A., Vega, Q.C., Chao, H.H.-J., Seasholtz, A.F., Thompson, R.C., and Dixon, J.E. (1998). Identification and characterization of GFRα-3, a novel co-receptor belonging to the glial cell line-derived neurotrophic receptor family. J. Biol. Chem. *273*, 3502–3508.

Yu, T., Scully, S., Yu, Y., Fox, G.M., Jing, S., and Zhou, R. (1998). Expression of GDNF family receptor components during development: implications in the mechanisms of interaction. J. Neurosci. *18*, 4684–4696.