Roles for GFRα1 receptors in zebrafish enteric nervous system development

Iain T. Shepherd[1,2,†], Jacy Pietsch[2], Stone Elworthy[3,*], Robert N. Kelsh[3] and David W. Raible[1]

[1]Department of Biological Structure, University of Washington, Box 357420, Seattle, WA 98195, USA
[2]Department of Biology, Emory University, Rollins Research Center, 1510 Clifton Road, Atlanta GA 30322, USA
[3]Centre for Regenerative Medicine and Developmental Biology Program, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

*Present address: Centre for Developmental Genetics, Department of Biomedical Science, University of Sheffield, Sheffield S10 2TN, UK
†Author for correspondence (e-mail: ishephe@emory.edu)

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Summary

Components of the zebrafish GDNF receptor complex are expressed very early in the development of enteric nervous system precursors, and are already present as these cells begin to enter the gut and migrate caudally along its length. Both *gfra1a* and *gfra1b* as well as *ret* are expressed at this time, while *gfra2* expression, the receptor component that binds the GDNF-related ligand neurturin, is not detected until the precursors have migrated along the gut. Gfra genes are also expressed in regions of the zebrafish brain and peripheral ganglia, expression domains conserved with other species. Enteric neurons are eliminated after injection with antisense morpholino oligonucleotides against *ret* or against both Gfra1 orthologs, but are not affected by antisense oligonucleotides against *gfra2*. Blocking GDNF signaling prevents migration of enteric neuron precursors, which remain positioned at the anterior end of the gut. Phenotypes induced by injection of antisense morpholinos against both Gfra orthologs can be rescued by introduction of mRNA for *gfra1a* or for *gfra2*, suggesting that GFRα1 and GFRα2 are functionally equivalent.

Key words: Zebrafish, GFRα1, GFRα2, Ret; GDNF, Enteric nervous system, Morpholino oligonucleotides

Introduction

The vertebrate enteric nervous system (ENS) consists of an integrated network of neurons that controls gut motility and function. Enteric neurons are principally derived from neural crest cells that migrate ventrally just posterior to the pharyngeal arches, enter the anterior gut primordium and proceed posteriorly to populate the gut tube (Newgreen and Young, 2002b). As they migrate, ENS precursors proliferate and then differentiate to form a wide variety of neuronal subtypes necessary for intestinal function (Furness, 1987; Gershon, 1994). In avian and mammalian species, a second population of neural crest from sacral regions also contributes to the distal gut (Burns and Douarin, 1998; Le Douarin and Teillet, 1973; Pomeranz et al., 1991; Serbedzija et al., 1991). If migration or proliferation of the vagal neural crest is impaired, varying lengths of the gut become aganglionic (Newgreen and Young, 2002a; Newgreen and Young, 2002b). The intestinal motility disorder Hirschprung's Disease results from this type of impairment. Individuals with this condition have aganglionosis of varying lengths of the distal portion of the colon (Kapur, 1999; Newgreen and Young, 2002a; Newgreen and Young, 2002b; Parisi and Kapur, 2000). The ENS therefore requires proper specification, migration and proliferative expansion of enteric precursors for correct development and function.

A key regulatory factor for ENS development is glial cell line-derived neurotrophic factor (GDNF), a potent trophic factor for many different types of CNS and PNS neurons (Arenas et al., 1995; Ebendal et al., 1995; Henderson et al., 1994; Mount et al., 1995; Trupp et al., 1995). GDNF is the founding member of a subgroup of the TGFβ superfamily, of which there are four members: GDNF, neurturin (Kotzbauer et al., 1996), persepehin (Milbrandt et al., 1998) and artemin (Baloh et al., 1998b). The biological activities of the GDNF family ligands are mediated through a multicomponent receptor complex that consists of a GPI-linked ligand binding component, the GDNF family receptor α (GFRα) subunit and a common transmembrane signaling component, the tyrosine kinase Ret (Jing et al., 1996; Treanor et al., 1996). There are four GFRα subunits; each preferentially binds to a different GDNF family member (Baloh et al., 1998a; Baloh et al., 1997; Baloh et al., 1998b; Buj-Bello et al., 1997; Jing et al., 1996; Jing et al., 1997; Klein et al., 1997; Masure et al., 2000; Naveilhan et al., 1998; Thompson et al., 1998; Trupp et al., 1998; Worby et al., 1998). GDNF preferentially binds to GFRα1 (Jing et al., 1996; Treanor et al., 1996) but can also bind to and mediate signaling in vitro through GFRα2, the α subunit that preferentially binds to neurturin (Baloh et al., 1997; Creedon et al., 1997; Sanicola et al., 1997).

The role of GDNF in ENS development has been studied extensively in vitro and in vivo. In cell culture, GDNF has been shown to promote survival, proliferation, differentiation and neurite out growth of enteric precursors (Chalazonitis et al., 1998; Hearn et al., 1998; Heuckeroth et al., 1998; Taraviras et

al., 1999; Worley et al., 2000). More recently in vitro studies have shown that GDNF can act as a chemoattractant for mouse enteric neural crest precursors (Young et al., 2001). The crucial role of GDNF in ENS development has been conclusively demonstrated by mouse in vivo studies. Mice deficient in GDNF or its receptor components die prior to birth and exhibit an absence of enteric neurons in the gut distal to the stomach, as well as agenesis of the metanephric kidney (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). The role of GDNF in ENS development has been conserved evolutionarily. We previously showed that blocking zebrafish GDNF function through injection of antisense morpholino oligonucleotides disrupted ENS neuronal differentiation (Shepherd et al., 2001).

To determine the crucial steps of zebrafish ENS development regulated by GDNF and its receptors, we have isolated full-length clones of two Gfra1 genes and a *gfra2* ortholog. We have examined the temporal and spatial expression, along with the expression of *gdnf* and *ret*, within the developing zebrafish ENS, and have blocked their function using antisense morpholino oligonucleotides. We find that *gfra1a* and *gfra1b*, as well as *ret*, are expressed very early in the development of ENS precursors, and that interfering with their function blocks migration into the gut and subsequent proliferative expansion. However, precursors are initially specified, as identified by expression of *phox2b*, and are still present at the anterior end of the gut in morpholino-injected embryos after migration has substantially progressed in controls. Although *gfra2* is expressed later in ENS development in only a small subset of ENS neurons, introduction of mRNA for this gene is able to functionally compensate for loss of *gfra1*.

Materials and methods

Isolation of zebrafish *gfra1* and *gfra2* orthologs

We extended the partial sequences we had previously identified for the zebrafish *gfra1a* and *gfra1b* orthologs (Shepherd et al., 2001) by using RACE (rapid amplification of cDNA ends) (Frohman, 1993). RACE cDNA was isolated from 24 hours postfertilization (hpf) and 48 hpf embryos using a Smart RACE cDNA Amplification Kit (Clonetech). Genomic PCR was also carried out using Genome Walker genomic DNA libraries (Clonetech) constructed from genomic DNA isolated from *AB strain zebrafish. The continuity of full-length sequence assembled from RACE and Genome Walker sequences was confirmed by RT-PCR on single stranded cDNA isolated from 48 hpf embryos using the following primers: *gfra1a* forward, 5′-TGAGGAAGGATTATCCTTTTCATCGTC-3′; *gfra1a* reverse: 5′-GCGATTGACTTTGTACAAACTGTCTGTCC-3′; *gfra1b* forward: 5′-AGCTGGCGCGCAATGGATTTATTGTGG-3′; *gfra1b* reverse: 5′-TTGTCTCTTGCATGTCTTACAGCCCTG-3′.

PCR conditions used were 1 cycle of 94°C for 3 minutes; 39 cycles of 94°C for 1 minute, 60°C for 1minute and 72°C for 1.5 minutes; and 1 cycle of 94°C for 1 minute, 60°C for 1 minute, 72°C for 5 minutes.

Using probes generated to the previously published partial cDNAs for zebrafish *gfra1a* and *gfra1b* we screened a colony macroarrayed cDNA library filter set (RZPD library ICRFp524) that was generated from cDNAs isolated from late somitogenesis (~18-24 hpf) whole zebrafish embryos (Clark et al., 2001). We identified a single clone that on sequencing was shown to be a zebrafish *gfra2* ortholog.

Sequence data has been submitted to GenBank (Accession Numbers AY436320, AY436321 and AY436322).

Whole-mount in situ hybridization

Embryos were collected and processed for whole-mount in situ hybridization as previously described (Thisse et al., 1993). Digoxigenin-labeled riboprobes were synthesized from templates linearized with *Not*I using Sp6 RNA polymerase for *gfra1a*, *gfra1b* and *gfrα2* and using T7 RNA polymerase for *ret*. Other digoxigenin-labeled riboprobes used in this study were synthesized from templates linearized and transcribed as follows: *tyrosine hydroxylase* (*th*), *Hin*dIII and T7; *crestin*, *Sac*I and T7; *phox2b*, *Not*I and T7; and *islet2*, *Eco*RI and T7.

Immunocytochemistry

Embryos were processed for immunocytochemistry as previously described (Raible and Kruse, 2000). The pattern of primary motoneuron axon projection was revealed with anti-Znp1 (University of Oregon) (Melancon et al., 1997; Trevarrow et al., 1990). Posterior lateral axon projection was revealed by anti-acetylated tubulin (Sigma) (Raible and Kruse, 2000). The pronephric kidney was revealed by α6F immunoreactivity (Developmental Studies Hybridoma Bank) (Drummond et al., 1998; Takeyasu et al., 1988). Differentiated enteric neurons and cranial ganglia neurons were revealed with the anti-Hu mAb 16A11 (Molecular Probes) that labels differentiated neurons (Marusich et al., 1994). All mAbs were visualized using an Alexa Fluor 568 anti-mouse IgG antibody (Molecular Probes).

mRNA injection

gfra1a, *gfra1b* and *gfra2* mRNAs were synthesized using the mMessage mMachine kit (Ambion) and injected at a concentration of 50 ng/μl. The mRNAs were co-injected with *GFP* mRNA, also at a concentration of 50 ng/μl, to assess expression. Approximately 1 nl of diluted mRNA was injected into one- to two-cell embryos using a gas-driven microinjection apparatus (Model MMPI-2; Applied Scientific Instrumentation) through a micropipette.

gfra1a, *gfra1b* and *ret* antisense oligo injections

The antisense oligonucleotides used were 25-mer morpholino oligos (Gene Tools LLC) with the following the base composition: *gfra1a morpholino*, 5′-CGCTTTTATCCGTGGTAAGTTCGCT-3′; *gfra1b morpholino* 5′-TCATCGTCGCTTTATTCAGATCCAT-3′; *ret morpholino* 5′-GTCAATCATAAGTGTTAATGTCACAA-3′.

The oligos were resuspended in sterile filtered water and diluted to working concentrations in a range between 1-5 μg/μl. Approximately 1 nl of diluted morpholino was injected into one- to two-cell embryos using a gas-driven microinjection apparatus. In morpholino rescue experiments *gfra1a* or *gfra1b* morpholinos, at a concentration 1-5 μg/μl, were co-injected with modified *gfra1a* mRNA made from a plasmid encoding the modified *gfra1a* containing the following sequence modifications to *gfra1a*: 5′-ACCGACAAGTCTGCAatg-TTTATCGCCGCG-3′ (underlined bases were modified lower case is the translational start site); or *gfra1b* mRNA or *gfrα2* mRNA, at a concentration of 50 ng/μl, into one- to two-cell stage embryos.

Results

Identification of the complete open reading frames for *gfra1a*, *gfra1b* and *gfra2*

We isolated clones representing the complete ORF for zebrafish *gfra1a*, *gfra1b* and *gfra2* (Fig. 1). We used 5′ and 3′ RACE and genomic RT-PCR to identify flanking coding regions to the partial cDNAs of *gfra1a* and *gfra1b* that we had previously reported (Shepherd et al., 2001). We also isolated a cDNA encoding a zebrafish *gfra2* ortholog by screening an arrayed zebrafish EST library (Clark et al., 2001) with probes for the partial *gfra1a* and *gfra1b* cDNAs. Sequence comparison

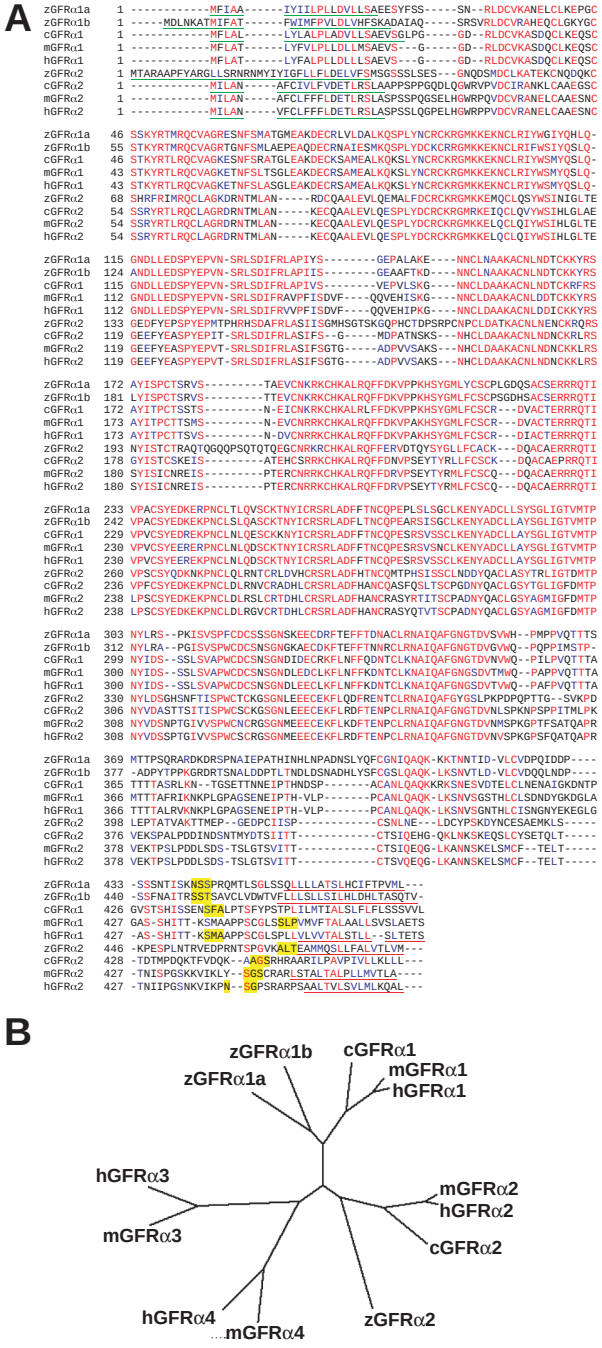

Fig. 1. The identification of the complete open reading frames of zebrafish *gfra1a*, *gfra1b* and *gfra2*. (A) Alignment of the predicted amino acid sequence of zebrafish *gfra1a*, *gfra1b* and *gfra2* with those of chick, mouse and human. Conserved amino acids between the different sequences are shown in red. The N-terminal, hydrophobic, putative signal peptides are underlined in green, the C-terminal hydrophobic domain is underlined in red, and the putative binding/cleavage sequences for GPI linkage are highlighted in yellow. (B) Unrooted phylogram of the Gfra protein family including the zebrafish orthologs. Multiple sequence alignments were made using the CLUSTALW program as part of the Biology Workbench package (http://workbench.sdsc.edu/) the unrooted phylogenetic tree was then generated from this alignment using the DRAWTREE application of the Biology Workbench that uses PHLYLIP version 3.5c.

of the complete ORFs of these genes by BLAST and phylogenetic analysis using CLUSTALW algorithm (Thompson et al., 1994) confirm that the zebrafish *gfra1a* and *gfra1b* genes were most similar to the mammalian Gfra1 orthologs previously identified (Fig. 1). This analysis also showed that the zebrafish *gfra2* gene was most similar to other *gfra2* genes rather than to *gfra3* or *gfra4* genes. Like all other Gfra family members, the zebrafish *gfra1* and *gfra2* orthologs have an N-terminal, hydrophobic, putative signal peptide for secretion and the characteristic features of GPI-linked proteins, namely a C-terminal hydrophobic domain separated by a hydrophilic linker region from a cleavage/binding consensus sequence for GPI linkage (Airaksinen et al., 1999).

Expression of gdnf receptor genes in the enteric nervous system

GDNF receptor component genes can be detected very early in ENS precursor development from just before the onset of their migration along the gut at 36 hpf (Fig. 2). *gfra1a*, *gfra1b* and *ret* are not expressed in all vagal neural crest cells. GDNF receptor components are only expressed in the subset of vagal crest cells that give rise to the ENS with expression of the receptor components beginning after the onset of their migration towards the gut. These enteric neural crest cells migrate ventrally just posterior to the pharyngeal arches to associate with the gut and express *crestin*, a pan neural crest marker (Rubinstein et al., 2000). These cells are identified as

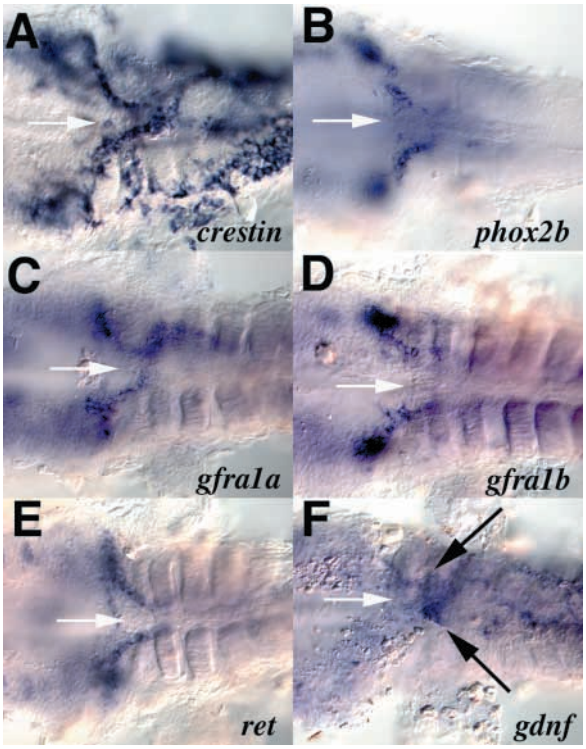

Fig. 2. Expression of *gdnf* receptor components in enteric precursors. Shown are ventral views of the vagal region of 36 hpf embryos after the yolk has been removed. Anterior is towards the left. In situ hybridization was performed for *crestin* (A), *phox2b* (B), *gfra1a* (C), *gfra1b* (D), *ret* (E) and *gdnf* (F). White arrows (A-E) indicate the anterior end of the gut tube. Black arrows in F indicate the mesendodermal expression of *gdnf* at the anterior end of the gut tube.

enteric precursors by the expression of the transcription factor *phox2b* (S.E., J. Pinto, L. Cancela and R.N.K., unpublished). Zebrafish enteric precursors are negative for *tyrosine hydroxylase* (not shown), in contrast to previous reports (Guo et al., 1999; Holzschuh et al., 2001); *tyrosine hydroxylase*-positive cells are probably sympathetic precursors. Enteric precursor cells are however positive for *gfra1a* (Fig. 2C), *gfra1b* (Fig. 2D) and *ret* (Fig. 2E) at 36 hpf. Importantly, *gdnf* is also expressed in this region in mesendodermal cells at the anterior end of the gut tube (Fig. 2F), suggesting that GDNF signaling may play an important role in enteric neuron development at this stage.

GDNF receptor genes continue to be expressed as the precursor cells migrate along the gut (Fig. 3). At 48 hpf, there are two streams of *gfra1a*-expressing cells either side of the gut (Fig. 3A). A similar pattern of expression can be seen for the transcription factor *phox 2b* (Fig. 3B) and *ret* (not shown). The stream of *gfra1a*-expressing cells extends almost the complete length of the gut at this age, while *gfra1b*-expressing cells are more restricted to the anterior end (data not shown). By 96 hpf, *gfra1a*- and *gfra1b*-positive cells can be seen located all around the circumference of the gut and along its complete length (Fig. 3C,E and data not shown), as previously reported (Shepherd et al., 2001). *gfra2* expression is seen in the gut beginning at 72 hpf in a dispersed group of cells along the length of the gut (data not shown). The same pattern of expression is maintained through 96 hpf although the number of expressing cells increases (Fig. 3D). At both 72 and 96 hpf there are far fewer *gfra2*-expressing cells than *gfra1*-expressing cells.

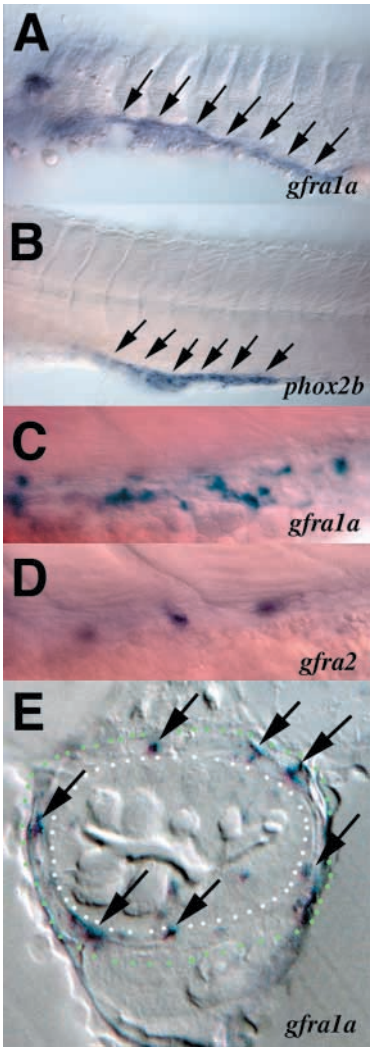

Fig. 3. Expression of *gfra1a*, *gfra2* and *phox2b* message in the enteric nervous system. Lateral views of the trunk of 48 hpf embryos, somites 1-11, with the yolk removed (A,B), and the trunk of 96 hpf embryos from somites 10-14 (C,D) that have been hybridized with riboprobes for *gfra1a* (A,C), *phox2b* (B) and *gfra2* (D). (E) Transverse section taken through a 96 hpf embryo at the level of somite 14 that has been hybridized with probe for *gfra1a*. Arrows in A,B indicate gene expression in the gut tube. Arrows in E indicate enteric neurons expressing *gfra1a* message around the circumference of the gut tube. The broken white line in E indicates the border of the mucosa while the broken green line indicates the gut wall. (A-D) Anterior is towards the left.

Expression in brain and peripheral ganglia

In addition to ENS expression, zebrafish Gfra orthologs are found in the embryonic central and peripheral nervous system (Fig. 4), as in mouse and chicken (Golden et al., 1999; Homma et al., 2000). *gfra1a* (Fig. 4A,B) and *gfra1b* (not shown) are expressed in cranial ganglia, including the anterior and

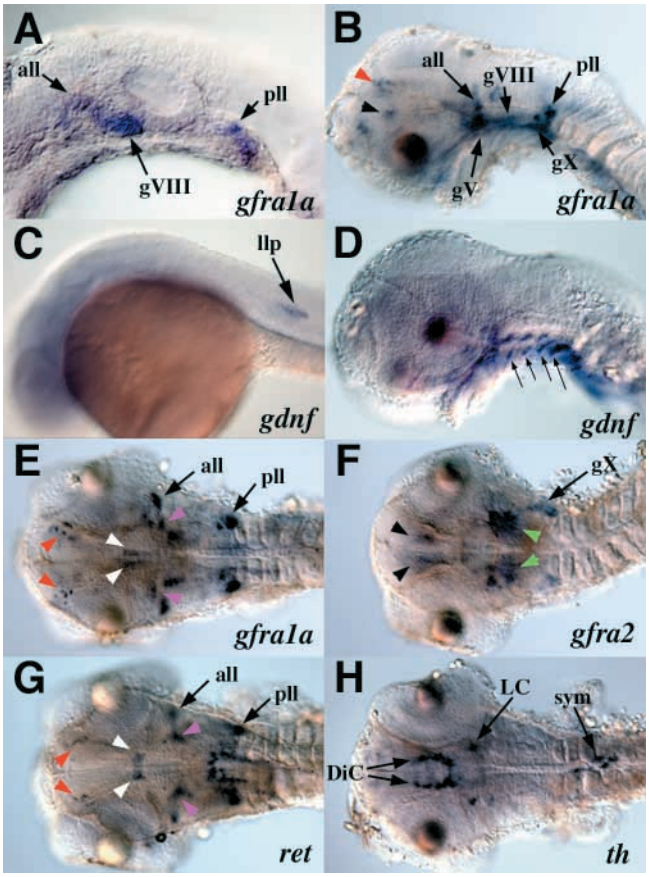

Fig. 4. Expression of *gdnf* receptor components in cranial ganglia and brain. Lateral views (A-D) and dorsal views (E-H) of 24 hpf (A,C) and 48 hpf (B,D-H) embryos. At 24 hpf, *gfra1a* (A) is expressed in lateral line ganglia, while *gdnf* (C) is expressed in the migrating lateral line primordium (arrow). At 48 hpf, *gfra1a* (B) is expressed in epibranchial ganglia, while *gdnf* (D) is expressed in pharyngeal pouches (arrows). *gfra1a* (E), *gfra2* (F), *ret* (G) and *th* (H) are shown at 48hpf. Black arrowheads indicate the ventral diencephalic neurons, red arrowheads indicate dorsal diencephalic neurons, white arrowheads indicate the ventral midbrain neurons, pink arrowheads indicate trigeminal motor nucleus, green arrowheads indicate anterior hindbrain domain. all, anterior lateral line ganglia; pll, posterior lateral line ganglia; llp, lateral line primordium; gV, trigeminal ganglia; gVI, facial ganglion; gVIII, octaval/statoacoustic ganglion; gIX, glossopharyngeal; gX, vagal ganglion; DiC, diencephalic catecholaminergic cell cluster; LC, locus coeruleus; sym, sympathetic neurons.

posterior lateral line ganglia, and epibranchial ganglia. *gfra2* is expressed in vagal and epibranchial ganglia but not others (Fig. 4F and data not shown). *gdnf* mRNA is expressed in the targets of these peripheral neurons, including the posterior lateral line primordium (Fig. 4C) and pharyngeal pouches (Fig. 4D). Within the central nervous system, Gfra genes are expressed in dorsal and ventral midbrain as well as in cranial motoneurons in the hindbrain (Fig. 4E,F). These expression patterns largely overlap with *ret* expression (Fig. 4G). However, expression does not overlap with *th* (Fig. 4H), a marker of neurons thought to be the equivalent of mammalian midbrain dopaminergic neurons (Wullimann and Rink, 2002). The lack of expression of GDNF receptor components in zebrafish midbrain dopaminergic neurons contrasts with their expression in midbrain dopaminergic neurons in the embryonic mouse (Golden et al., 1999).

Functional inactivation of gdnf receptor components

The early expression of GDNF receptor genes in enteric neuron precursors suggests that they have a role in the colonization of the gut. To determine whether *gfra1a* and *gfra1b* are necessary for enteric nervous system development, we used morpholino antisense oligonucleotides to block their function (Nasevicius and Ekker, 2000). One-cell stage embryos were injected with morpholino oligonucleotides complimentary to the region of the translation initiation site of the *gfra1a* and *gfra1b* mRNAs. *gfra1a* and *gfra1b* morpholinos were injected alone or together at concentrations of 1-10 μg/μl when injected alone or 1-5 μg/μl when injected together.

At all concentrations and in all combinations tested, *gfra1a* and *gfra1b* morpholino-injected embryos were morphologically indistinguishable from control embryos. In addition, no defects were observed in: the pattern of CaP/VaP primary motoneuron (PMN) differentiation, as determined by *islet2* expression; the pattern of PMN axon projection, as determined by ZNP-1 immunoreactivity; the pattern of expression *tyrosine hydroxylase* in the midbrain; the projection pattern of the posterior lateral line ganglion cell axons in the lateral line, as determined by acetylated-tubulin immunoreactivity; the development of the pronephric kidney, as determined by α6F immunoreactivity; and the pattern of cranial ganglia differentiation, as determined by Hu immunoreactivity (data not shown). These structures were also unaffected after injection of *ret* morpholino oligonucleotide (data not shown).

By contrast, interfering with GDNF receptor components had strong effects on ENS development. In control embryos, enteric neurons are distributed all along the complete length of the gut (Fig. 5A). Injection of the *gfra1a* morpholino alone resulted in a reduction in the total number of enteric neurons at 96 hpf, with the loss of neurons particularly apparent at the distal end (Fig. 5B). Injection of the *gfra1b* morpholino had no effect on the total number of enteric neurons (Fig. 5C); however, co-injection of both *gfra1a* and *gfra1b* morpholinos resulted in their nearly total elimination (Fig. 5D). A similar loss of enteric neurons was seen after injection of a *ret* morpholino oligonucleotide (Fig. 5F). By contrast, there was no statistically significant loss of neurons after injection of *gfra2* morpholinos, either alone (Fig. 5E) or additional effects in combination with *gfra1* morpholinos (data not shown).

We quantified the loss of enteric neurons by counting Hu-positive cells along a 10-somite segment (Table 1). We observed a 40% reduction in enteric neurons after injecting *gfra1a* morpholino alone at maximal doses, and saw no effect after injecting *gfra1b* morpholinos. However, we saw a synergistic effect of injecting both Gfra1 morpholinos together; injecting as little as 2 ng of each morpholino eliminated almost all enteric neurons. A similar loss was observed after injection of *ret* morpholinos.

The combined effect of injecting both Gfra1 morpholinos suggests that there is some redundancy between these two

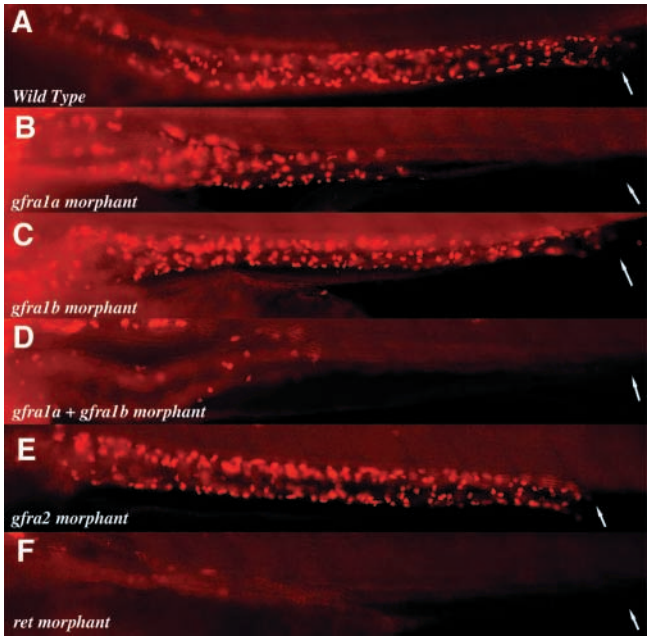

Fig. 5. Effect of *gfra1a*, *gfra1b* and *ret* antisense morpholino oligonucleotide injection on enteric neuron development. Shown are lateral views of the guts of 96 hpf embryos stained with anti-Hu antibody, anterior is towards the left. Arrows indicate the end of the gut. (A) Control embryo. (B) 5 ng *gfra1a* morpholino injected embryo. (C) 5 ng *gfra1b* morpholino injected embryo. (D) 5 ng *gfra1a* plus 5 ng *gfra1b* morpholino injected embryo. (E) 10 ng *gfra2* morpholino injected embryo. (F) 10 ng *ret* morpholino injected embryo.

Table 1. Loss of enteric neurons after morpholino oligonucleotide injection

	Morpholino amount (ng)			
Morpholino	0	1	5	10
gfra1a	100±6.3	97.6±5.1	63.5±3.9*	60.8±3.8*
gfra1b	100±6.6	99.5±4.8	98.1±4.7	99.5±4.7
gfra2	100±6.1	99.2±4.3	100±4.4	96.2±4.3
ret	100±2.9	92.3±2.1	N.D.	1.8±0.6*
Morpholino	0	1	2	5
gfra1a+gfra1b	100±4.8	95±3.4	6.9±0.7*	4.7±0.5*

Embryos were injected with morpholino oligonucleotides, fixed and stained with anti-Hu antibody at 96 hpf. For injection of *gfra1a+gfra1b* morpholinos, the amount of each oligonucleotide is shown. Total neurons over a 10-segment length were counted. Numbers represent percent of control±s.e.m. for at least five separate embryos per condition. *Significantly different from control (P<0.0005).

N.D., not determined.

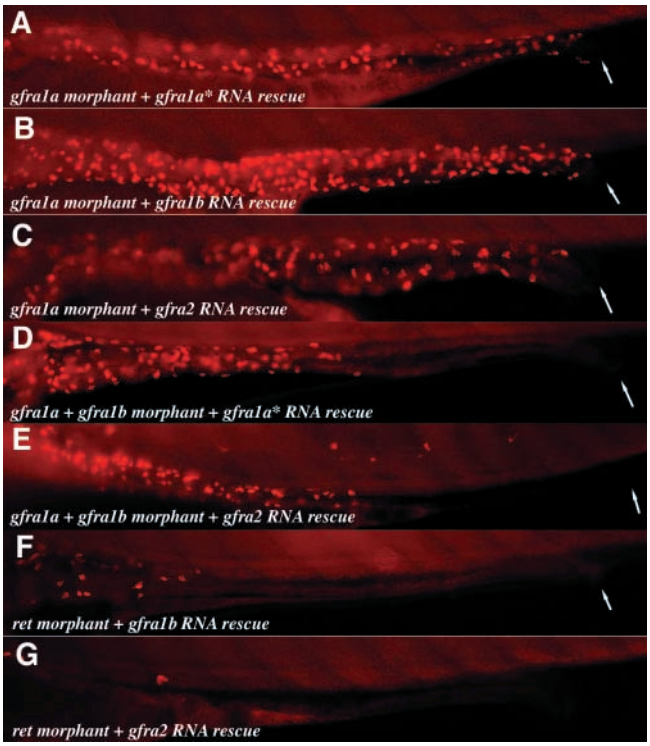

Fig. 6. mRNA rescue of morpholino injections. Shown are lateral views of the guts of 96 hpf embryos stained with anti-Hu antibody, anterior is towards the left. Arrows indicate the end of the gut. Embryos were injected with 5 ng *gfra1a* morpholino (A-C), 5 ng *gfra1a* plus 5 ng *gfra1b* morpholino (D,E) or 10 ng *ret* morpholino (F,G), combined with 50 pg *gfra1a** mRNA (A,D), 50 pg *gfra1b* mRNA(B,F) or 50 pg *gfra2* mRNA (C,E,G).

genes because together the phenotype is stronger than after injection of maximal doses of the individual morpholinos. We tested this potential redundancy by restoring function by reintroducing mRNA for each Gfra1 sequence. As expected, co-injection of *gfra1a* morpholinos along with *gfra1a* mRNA that has a modified 5′ UTR not recognized by the morpholino rescued enteric neuron development (Fig. 6A). Co-injection of *gfra1a* morpholinos along with *gfra1b* mRNA also restored enteric neuron development (Fig. 6B), confirming that these receptors are functionally equivalent. By contrast, *gfra1b* mRNA did not alter the effects of *ret* morpholinos (Fig. 6F).

We also sought to test whether *gfra2* could substitute for loss of Gfra1 function. We found that injection of *gfra2* mRNA was as effective as *gfra1b* mRNA at rescuing the development of enteric neurons after *gfra1a* morpholino treatment (Fig. 6C) or after *gfra1a+ gfra1b* morpholino treatment (Fig. 6E). As expected, *gfra2* mRNA did not alter the effects of *ret* morpholinos (Fig. 6G). We never saw any effects of injecting either Gfra1 or *gfra2* mRNAs in control embryos (not shown). We counted Hu-positive cells in a 10-somite segment of the gut to quantify the combined effects of morpholinos and mRNA injections (Table 2).

The early expression of GDNF and its receptor components suggested that the loss of enteric neurons might be due to the failure of precursors to proliferate and migrate posteriorly along the gut. The initial migration of neural crest cells to enter

Table 2. Rescue of enteric neuron development by mRNA injection

	Injected mRNA				
Morpholino	None	*gfra1a*	*gfra1b*	*gfra2*	Control
gfra1a	64.2±2.8	93.1±3.5*	95.6±3.3*	96.6±3.5*	100±4.8
gfra1a+gfra1b	4.9±0.7	58.5±1.7*	N.D.	60.8±3.5*	100±2.5
ret	0.9±0.4	N.D.	1.6±0.5	0.6±0.4	100±5.7

Embryos were injected with morpholino oligonucleotides and co-injected with 50 pg of indicated mRNA, fixed and stained with anti-Hu antibody at 96 hpf. Control represents uninjected embryos. Total neurons over a 10-segment length were counted. Numbers represent percent of control±s.e.m. for at least five separate embryos per condition.

*Significantly different from morpholino injection alone (P<0.0005).

N.D., not determined.

the gut just posterior to the pharyngeal arches is not affected in morpholino-injected embryos (Fig. 7A,B), suggesting that ENS precursors are initially specified. However, enteric neuron precursors revealed by *phox2b* expression are conspicuously absent along the gut after morpholino injection (Fig. 7C,D). Similar results were seen at 48 hpf using *ret* and *crestin* as markers for enteric precursors (data not shown). However, some *phox2b+* enteric precursors remain at the anterior end of the gut at this stage (Fig. 7E,F), suggesting that they cannot progress further after reaching the gut mesendoderm.

Discussion

Our results suggest that GDNF signaling is required at the earliest stages of zebrafish enteric precursor migration along the intestine. Both ligand and receptor components are expressed very early in a subset of neural crest cells as they enter the anterior gut primordium consistent with our hypothesis that that GDNF plays a crucial role in the precursor migration along the intestine. GDNF receptor components are specifically expressed in a subset neural crest cells that generates the ENS precursors and is expressed only after that subset of vagal neural crest cells have begun migrating towards the intestine. This pattern of expression is consistent with previous descriptions of *ret* expression in zebrafish but contrasts with the pattern of *ret* expression reported in chick (Robertson and Mason, 1995). The lack of *th* expression in the zebrafish enteric precursors is consistent with the *th* expression pattern reported in chick but contrasts to that reported in rat and mouse which express *th* in enteric precursors (Cochard et al., 1978; Teitelman et al., 1979; Young and Newgreen, 2001).

Zebrafish enteric precursors can still reach the anterior gut in embryos when GDNF receptor components have been blocked by morpholino injection, but cannot migrate posteriorly or expand in number and instead remain in the same position. These results suggest that GDNF is not involved in the initial specification of ENS precursors, because they are still able to express *phox2b* when Gfra or *ret* expression is blocked. Alternatively, Gfra or *ret* morpholinos might block later differentiation of ENS neurons after precursor migration; however, we do not see *crestin*-positive cells in the distal part of the intestine after morpholino injection, suggesting that this is not the case. Our results are consistent with in vitro studies suggesting that GDNF acts as a chemoattractant for ENS neural crest precursors in their migration along the intestine

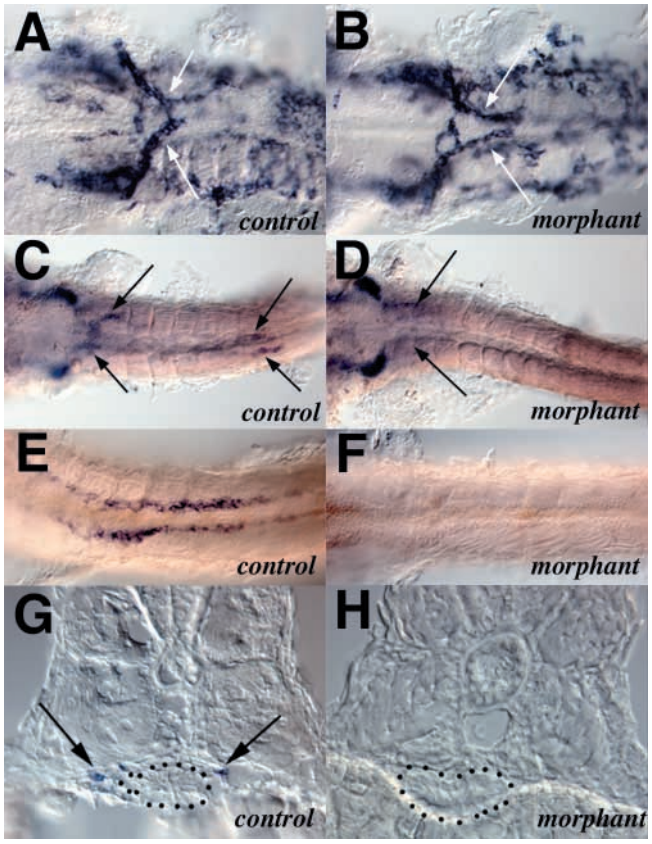

Fig. 7. Co-injection of *gfra1a* and *gfra1b* morpholinos causes the failure of enteric precursor migration along the gut but does not perturb the initial migration of vagal neural crest to the anterior gut. (A,C,E,G) Control embryos and (B,D,F,H) embryos co-injected with *gfra1a* plus *gfra1b* morpholinos. Arrows indicate the migrating enteric precursors. (A,B) Ventral view of the vagal region of 36 hpf embryos that have been hybridized with riboprobes for *crestin*. (C,D) Ventral view of the vagal region of 48 hpf embryos showing persistence of *phox2b*-expressing cells at the anterior end of the gut. (E,F) Ventral view of somites 3-10 of 48 hpf embryos that have been hybridized with a riboprobes for *phox2b*. (G,H) Cross-sections taken through 48 hpf embryos at the level of somite 8. The embryos had been hybridized with riboprobes for *phox2b* prior to sectioning. Broken outline indicates the border of the gut endoderm. The yolk has been removed from the embryos (A-F). Anterior is towards the left (A-F).

(Natarajan et al., 2002; Young et al., 2001). These studies demonstrated that GDNF and Ret are involved in promoting the directed migration of enteric neural crest precursors along the gut but not in directing the neural crest's initial migration from the post-otic region of the hindbrain to the anterior end of the gut. Our results are also consistent with a role for GDNF in controlling enteric precursor proliferation (Gianino et al., 2003) because we do not see an increased number of enteric neurons at the anterior end of the intestine.

Despite extensive analysis, we have not detected any changes in apoptotic cell death by TUNEL analysis (Shepherd et al., 2001). These results contrast with studies of mice that harbor a null mutation in *ret* in which there is an increase in the number of apoptotic cells at the anterior end of the gut as compared with wild-type siblings (Taraviras et al., 1999), but are consistent with recent findings that GDNF does not affect ENS cell death (Gianino et al., 2003). However, injection of morpholino oligonucleotides are best considered to cause only a partial loss of function, and thus lack of apoptosis observed in our system might result from residual GDNF signaling.

Blocking GDNF receptor function had no other effects besides altering enteric neuron development. Similarly, overexpression of Gfra mRNA had no effect. These results extend our previous observations that depletion of GDNF only affected the ENS (Shepherd et al., 2001). One possibility is that eliminating Gfra or *gdnf* function could be compensated for by other GFRα subunits or by other GDNF ligands such as neurturin. However, injection of *ret* morpholinos also only affected the ENS, making this scenario less likely as Ret is the common signaling component for these other ligands and receptors. Deficits are not observed in every expression domain after inactivation of receptors and ligand in mouse. Although GDNF was originally isolated as a neurotrophic factor (Lin et al., 1993), mice deficient in GDNF and its receptor components exhibit comparatively minor deficits in the majority of the central and peripheral nervous system excluding the ENS (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996).

We propose that there are two predominant GDNF-dependent precursor populations within the migrating ENS precursors in zebrafish. One population of ENS precursors expresses *gfra1a* and *ret*, while the second expresses both *gfra1a* and *gfra1b* as well as *ret*. As we have been unable to carry out successful double label in situ hybridization experiments with probes for both zebrafish Gfra1 genes, we cannot formally prove this hypothesis. It is also possible that there is a third population of precursors that do not express either Gfra1 subunit but express another Gfra subunit and *ret*. In embryos injected with both *gfra1* morpholinos there is always a small percentage (3-5%) of remaining enteric neurons, while in embryos injected with *ret* morpholinos there is consistently closer to 1% of cells remaining. Although this putative Gfra subunit might be *gfra2*, injecting morpholinos against all three identified zebrafish Gfra subunits still did not bring the number of neurons down to levels observed after *ret* morpholino injection (I.T.S., unpublished). However, some caution is needed in the interpretation of phenotypes as morpholinos may not completely eliminate gene function and small differences among the morpholino injection experiments may instead reflect differences in efficacy. The small number of enteric neurons remaining after *ret* morpholino injection may represent a population of cells that differentiate independently of *ret* function, as reported in the *ret* knock-out mouse (Durbec et al., 1996).

Our finding that zebrafish *gfra2* is expressed in a small subset of ENS precursors, and occurs later in development than that of *gfra1* expression, is consistent with expression patterns previously described for *gfra2* in mouse (Baloh et al., 1997; Golden et al., 1999; Widenfalk et al., 1997). In mice, *gfra2* has been shown to be required for the development of cholinergic neurons in the duodenum (Rossi et al., 1999). In addition our finding that *gfra2* expression occurs later in development than that of *gfra1* expression is consistent with that described for *gfra2* in mouse (Gianino et al., 2003). It will be interesting in the future to determine whether the small subset of cells expressing *gfra2* in zebrafish corresponds to a cholinergic population of enteric neurons.

Phenotypic rescue by co-injection of mRNAs along with morpholino oligonucleotides suggests that there is functional redundancy among *gfra1a*, *gfra1b* and *gfra2*. These results are consistent with previously described experiments in vitro demonstrating that GDNF can bind to *gfra2* and signal through Ret when *gfra1* is not present (Baloh et al., 1997; Creedon et al., 1997; Sanicola et al., 1997). If our interpretation is correct, this study would be the first to show that *gfra2* can functionally replace *gfra1* in vivo. However, we cannot determine if we have changed the cell fates of the ENS precursors by rescuing them with *gfra2* mRNA, so that they differentiate as another type of enteric neuron. The development of markers that recognize different subsets of neurons in zebrafish will help clarify this issue. It is also possible that the rescue we observe by overexpression of *gfra2* is not due GDNF signaling through this receptor but may be instead due to endogenous neurturin present in the intestine that signals through the overexpressed GFRα2 receptor. Isolation of zebrafish neurturin orthologs(s) and identifying patterns of expression during embryogenesis will help resolve this issue.

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