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GFRα1 Is Required for Development of Distinct Subpopulations of Motoneuron

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Glial cell-line derived neurotrophic factor (GDNF) and its relative neurturin (NTN) are potent trophic factors for motoneurons. They exert their biological effects by activating the RET tyrosine kinase in the presence of a glycosyl-phosphatidylinositol-linked co-receptor, either GFRα1 or GFRα2. By whole-mount *in situ* hybridization on embryonic mouse spinal cord, we demonstrate that whereas *Ret* is expressed by nearly all motoneurons, *Gfra*1 and *Gfra2* exhibit complex and distinct patterns of expression. Most motoneurons purified from *Gfra*1 null mutant mice had lost their responsiveness to both GDNF and NTN. However, a minority of them (~25%) retained their ability to respond to both factors, perhaps because they express GFRα2. Surprisingly, *Gfra2*$^{-/-}$ motoneurons showed normal survival responses to both GDNF and NTN. Thus, GFRα1, but not GFRα2, is absolutely required for the survival response of a majority of motoneurons to both GDNF and NTN. In accordance with the phenotype of the mutant motoneurons observed in culture we found the loss of distinct groups of motoneurons, identified by several markers, in the *Gfra1*$^{-/-}$ spinal cords but no gross defects in the *Gfra2*$^{-/-}$ mutant. During their natural programmed cell death period, motoneurons in the *Gfra1*$^{-/-}$ mutant mice undertook increased apoptosis. Taken together these findings support the existence of subpopulations of motoneuron with different trophic requirements, some of them being dependent on the GDNF family.

Key words: motoneuron subpopulations; motoneuron survival; neurotrophic factors; GDNF; NTN; Ret; Gfra1; Gfra2; in situ *hybridization; mutant mice*

Spinal motoneurons are organized into discrete longitudinal columns that contain pools of motoneurons distributed along the dorsoventral, rostrocaudal and mediolateral body axes according to their target muscles. In the embryo, motor columns can be distinguished by the combinatorial expression of members of a family of LIM homeodomain proteins, called the LIM code (Tsuchida et al., 1994). Recently, transcription factors belonging to the Ets family have been shown to be expressed by individual motor pools (Lin et al., 1998).

Because different subpopulations of motoneuron can also be distinguished by the receptors for neurotrophic factors they synthesize (Yamamoto et al., 1997), we hypothesized that these different subpopulations of motoneuron might have different trophic requirements. To test this hypothesis we focused on two members of the glial cell line-derived neurotrophic factor (GDNF) family: GDNF and neurturin (NTN). GDNF is the original member of a family distantly related to transforming growth factor-β, which at present includes three additional members: NTN, persephin (PSP), and artemin (ART) (Lin et al., 1993; Kotzbauer et al., 1996; Baloh et al., 1998; Milbrandt et al., 1998). Members of this family are potent survival factors for several populations of central and peripheral neurons. In particular, GDNF, NTN, and PSP have potent effects on the survival of motoneurons either *in vivo* or *in vitro* (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995; Klein et al., 1997; Milbrandt et al., 1998).

The actions of GDNF and its relatives are mediated by a receptor complex consisting of the tyrosine kinase receptor RET and a ligand-binding glycosyl-phosphatidylinositol (GPI)-linked protein (GFRα). There are multiple GFRα proteins: GFRα1, 2, 3 and 4. *In vitro* experiments indicate that GFRα1 and 2 are the favored receptors for GDNF and NTN, respectively. Comparison of the phenotypes of mutant mice lacking either the ligand or the GFRα receptor highlighted the preferential interaction between GDNF and GFRα1 and NTN and GFRα2 (for review, see Airaksinen et al., 1999). However, at high concentrations GDNF can signal after binding to GFRα2 (Baloh et al., 1997; Jing et al., 1997; Sanicola et al., 1997), and NTN can bind to GFRα1 (Creedon et al., 1997; Jing et al., 1997). In accordance with the biological activities of GDNF and NTN, *Gfra1* and *2* are widely distributed in both the PNS and CNS (Treanor et al., 1996; Baloh et al., 1997; Klein et al., 1997; Trupp et al., 1997; Widenfalk et al., 1997; Yu et al., 1998; Soler et al., 1999).

In the chick, *Gfra* receptors are expressed in different subpopulations of motoneuron (Soler et al., 1999) in accordance with the complementary and sometimes overlapping patterns of expres-

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sion of *Gfra1* and *Gfra2* we have observed in the rat embryonic spinal cord (our unpublished observations). In particular, in the rat lumbar region there are subpopulations of motoneuron that express mRNA coding for one receptor but not for the other. These results raised the possibility that there might be two classes of lumbar motoneurons: one sustained by GDNF acting through GFRα1 and the other by NTN acting through GFRα2. The best way of testing this hypothesis is by genetic analysis in the mouse. We therefore chose to study the patterns of GDNF family receptor expression in mouse spinal cord and to analyze the effects of null mutations in *Gfra1* and *Gfra2* on survival of specific motoneuron subpopulations.

It has been indeed reported that *Gdnf* and *Gfra1* knock-out mice show a loss of 25% of motoneurons in lumbar spinal cord, but the missing population has not been identified (Moore et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998). We show that GFRα1 is required for survival signaling by both GDNF and NTN in spinal motoneurons. Furthermore, prominent motoneuron groups that strongly express *Gfra1* (but not *Gfra2*) are lost in the *Gfra1*$^{-/-}$ mutant, whereas other motoneurons are apparently unaffected. Thus, GFRα1 is critical for the survival of specific groups of motoneuron during development, and different motoneurons have clearly different trophic requirements *in vivo*.

MATERIALS AND METHODS

Animals and genotype analysis. Embryos were collected from either wild-type or mutant mice. Vaginal positive plug was recorded as embryonic day 0.5 (E0.5). *Gfra2*$^{-/-}$ mice, previously described (Rossi et al., 1999), were bred as homozygous mice like C57Bl/6, their wild-type counterparts. Heterozygous *Gfra1*$^{+/-}$ mice were generously provided by A. Rosenthal (Genentech, South San Francisco). They were back-crossed to C57Bl/6 mice for at least four generations and were mated to obtain homozygous embryos; wild-type embryos from the same litters were used as controls.

Genotype analysis of the *Gfra*1 allele was performed using a previously described PCR technique (Arce et al., 1999). The primers CAGCTTCCTACCTAATCTG, GTTGTAGAGAGACTTCTGC and GGAGCAAAGCTGCTATTGG were used to amplify the targeted allele (422 bp band) and the *Gfra1* wild-type allele (342 bp band). When used for motoneuron purification, embryos were kept during genotyping at 4°C in Hybernate E medium supplemented with B-27 (Life Technologies, Cergy Pontoise, France).

Motoneuron purification and culture. Ventral spinal cords from E12.5 or E13.5 mouse embryos were dissected and dissociated, and motoneurons were isolated as described previously (Arce et al., 1999). Briefly, motoneurons were purified by a combination of metrizamide density-gradient centrifugation and indirect magnetic cell sorting with an antibody that recognizes the p75 low-affinity NGF receptor, a specific marker for motoneurons at this stage. A rat anti-mouse p75 purchased from Chemicon (Temecula, CA) was used. We used either E12.5 motoneurons purified by this procedure or E13.5 motoneurons purified only by a metrizamide density gradient. Identical results were obtained with motoneurons purified by both methods, irrespective of stages (data not shown). Purified motoneurons (800 or 1000 neurons per well) were plated in four-well tissue culture dishes (Nunc, Roskilde, Denmark). Wells had been previously coated with polyornithine/laminin (Henderson et al., 1995). Culture medium (basal medium) was Neurobasal, supplemented with the B-27 supplement (Life Technologies), horse serum (2% v/v), L-glutamine (0.5 mM), L-glutamate (25 μM), and 2-mercaptoethanol (25 μM).

To evaluate motoneuron survival after 3 d in culture, wells were filled with warm L-15 medium, and the cover of the dish was replaced. The number of large phase-bright neurons with long axonal processes was counted in duplicate either in the total area of the well or across two diameters of the well. To allow for comparison of values from different experiments or from different sets of embryo, survival values were corrected for the value in basal medium (taken as 0%) and expressed relative to the survival in 1 ng/ml BDNF (taken as 100%).

Neurotrophic factors. Recombinant neurotrophic factors were added at the time of seeding. Rat GDNF was purchased from Sigma (St. Louis, MO), and human BDNF was purchased from R & D Systems (Minneapolis, MN). Human NTN was either generously provided by Genentech or purchased from Peprotech (London, UK). Neurotrophic factors were prepared as stock solutions (1–100 μg/ml) in PBS supplemented with 0.5% BSA (Sigma) and kept in aliquots at −70°C. Once thawed, aliquots were kept at 4°C and used within 1 week.

Probes. Plasmid cDNA clones were linearized and transcribed with T7 or T3 polymerase using digoxigenin (DIG)-labeling reagents (Roche Diagnostics, Meylan, France). Probes were used at a concentration between 10 and 500 ng/ml. The *Gfra1* and *Ret* clones were as previously described (Arce et al., 1998). The rat *Gfra*2 probe corresponded to nt 1–1297 (GenBank accession number AF005226). The rat *Islet1* probe (Pfaff et al., 1996) and a 600 bp fragment of the mouse *Raldh2* cDNA were kindly provided by T. M. Jessell. The mouse *EphA4* corresponded to nt 652–1834 and was generously given by P. Charnay.

In situ *hybridization.* Whole-mount *in situ* hybridization (ISH) was performed as described by Henrique et al. (1995). Spinal cords were dissected, and fixed overnight at 4°C in 4% (w/v) paraformaldehyde in PBT (0.1% Tween 20 in PBS), progressively dehydrated in increasing concentrations of ethanol/PBT, stored at −20°C, then rehydrated in decreasing concentrations of ethanol/PBT and treated with proteinase K (10 μg/ml in PBS). They were then post-fixed for 20 min in 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.1% Tween 20 and prehybridized 1 hr at 70°C in 1.3× SSC, 50% formamide, 2% Tween 20, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 5 mM EDTA, and 50 μg/ml yeast RNA. Hybridization was performed overnight with DIG-labeled riboprobes in the same buffer. Washes with hybridization buffer were followed by RNase A treatment (10 μg/ml in 0.5 M NaCl, 10 mM Tris, pH 7.5, and 0.1% Tween 20, 1 hr at 37°C), and subsequent washes with hybridization buffer at 65°C. Spinal cords were then blocked in MABT (0.1 M maleate, 0.15 M NaCl, and 0.1% Tween 20, pH 7.5) containing 20% sheep serum, and incubated overnight at 4°C with anti-DIG-alkaline phosphatase (AP)-conjugate (Roche Diagnostics) diluted 1: 2000 in MABT with 2% sheep serum. After extensive washes with MABT, revelation was performed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics) in 0.1 M NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, and 0.1 M Tris pH 9.5. After staining, the spinal cords were flat-mounted as open-book preparations in 80% glycerol and examined by at least two observers to whom the genotype of the embryos was unknown. Some preparations were subsequently embedded in 30% albumin, 0.5% gelatin, and 1.5% glutaraldehyde in 0.12 M phosphate buffer, and 30 μm sections were cut with a Leica (Nussloch, Germany) vibratome.

Whole-mount terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling. Whole-mount terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) and double TUNEL/Islet labeling were performed essentially as described by Yamamoto and Henderson (1999). Spinal cords were fixed, dehydrated, and rehydrated through graded ethanol concentrations into PBS and then stained with the ApopTag kit (Oncor). Briefly, spinal cords were incubated in ApopTag equilibration buffer for >5 min at room temperature and transferred to the working strength TdT enzyme solution for 12 hr at 4°C, followed by 2 hr at 37°C. The reaction was stopped by incubating the spinal cords in ApopTag stop solution for 40 min at 37°C. After washing in TBST (0.14 M NaCl, 10 mM KCl, 25 mM Tris, pH 7.0, and 0.1% Tween 20), endogenous AP was inactivated by incubating the spinal cords in TBST for 20 min at 65°C. The spinal cords were then incubated in blocking solution (10% goat serum, 1% BSA in PBS) followed by incubation with anti-DIG-AP conjugate (Roche Diagnostics; diluted 1:2000 in blocking solution) overnight at 30°C. They were then extensively washed in MABT, stained as described for the whole-mount ISH, and examined under transillumination. For double TUNEL/Islet staining, spinal cords were blocked and then incubated with biotin-SP-conjugated anti-digoxigenin antibodies (Jackson ImmunoResearch, West Grove, PA) diluted; 1:1000) and Islet 1/2 monoclonal antibodies 2D6 and 4D5 (Developmental Hybridoma Bank, Baltimore, MD). After several washes, the spinal cords were incubated with Cy3-conjugated anti mouse antibodies (Jackson ImmunoResearch; 1:1000) and Cy2-conjugated streptavidin (Amersham Pharmacia Biotech, Orsay, France; 1:500) and washed with MABT. The spinal cords were flattened into open-book preparations and analyzed with a Zeiss LSM 400 confocal microscope. At least two spinal cords from each genotype were examined for each stage. For each analysis, wild-type and mutant tissues were dissected from embryos belonging to the same littermate and processed in parallel.

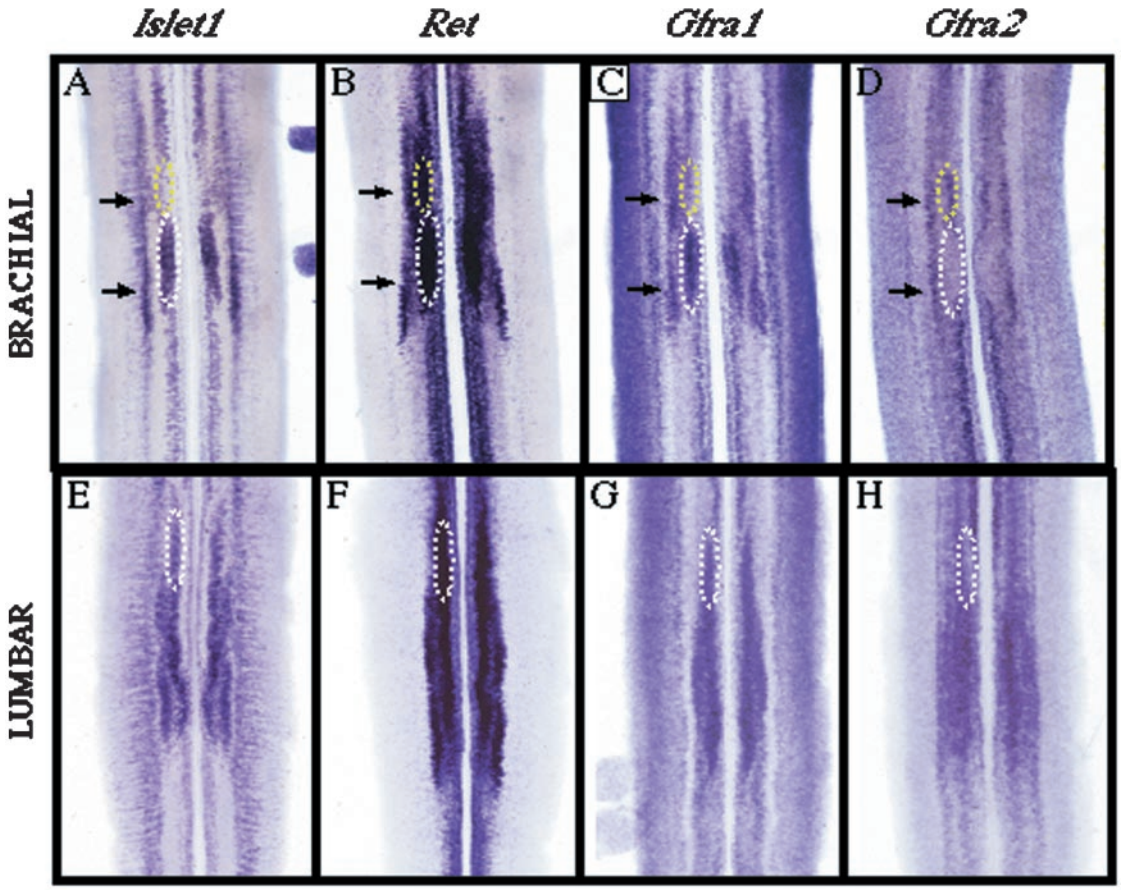

Figure 1. Distinct expression patterns of *Gfra1* and *Gfra2* in the E13.5 mouse spinal cord. Whole-mount ISH performed using probes to *Islet1* (*A, E*), *Ret (B, F)*, *Gfra1* (*C, G*), or *Gfra2* (*D, H*) on brachial (*A-D*) and lumbar (*E-H*) regions shows that *Ret* is widely expressed, whereas the GFRα-subunits are expressed in different subpopulations of motoneuron. The arrangement of the different motor columns is shown: *circled* in *white* are the groups of motoneurons that strongly express *Gfra1*, and in *yellow* are those that expresses *Gfra2. Arrows* point to motoneurons that express both *Gfra1* and *Gfra2*. The *white-circled* column belongs to lateral motor column (LMC); however, flattening of the cord in this preparation leads to it appearing close to the midline.

RESULTS

Distinct expression patterns of *Gfra1* and *Gfra2* within the mouse spinal cord

To get an overall view of the expression of GDNF family receptor components in different subpopulations of mouse motoneuron, whole-mount ISH was performed on predissected E13.5 or E14.5 spinal cords using *Ret*, *Gfra1,* and *Gfra2* probes. Spinal cords were then flattened into an "open-book" configuration, in which the ventral midline lies medial and the dorsal edges of the neural tube lie lateral. This allows the complex array of motor columns and pools to be visualized in the stereomicroscope. Different regions of the spinal cord were then examined. Identical results were obtained at both stages, thus only E13.5 stages are presented.

Within the brachial region, most motor columns expressed *Islet1*, *Ret,* and *Gfra*1 (Fig. 1*A–C*). The expression of *Gfra2* was more restricted and found only in some columns. The most lateral one was the most widespread along the rostrocaudal axis (Figs. 1*D,* 2*C*, *arrows*) and was one of the columns positive for *Islet1*, *Ret,* and *Gfra1* (Figs. 1*A–C,* 2*A*,*B*). Because the whole-mount spinal cords were flattened, some columns appearing medial are actually lateral columns. This is the case for the *Gfra2*-positive column that was positive for *Ret* and *Islet2* but negative for *Islet1* with few cells positive for *Gfra1* (Figs. 1*A–D*, *yellow lines,* 2A–C, *circle;* data not shown). Immediately caudal to this column was another column that was negative for *Gfra2* but positive for the other probes (Figs. 1*A–D, white lines,* 2*D–F, circle*).

In the thoracic region, expression of *Ret*, *Gfra,1* and *Gfra2* was detected in the median motor column (MMC) (Fig. 1*B–D*), which can be further subdivided into lateral and medial columns (MMCl and MMCm, respectively). A careful analysis of sections from whole-mount spinal cord revealed that both columns were stained using the *Gfra*1 and the *Ret* probes, whereas only very few cells in the MMCl were positive for *Gfra*2 (Fig. 2*G–I*). Comparison of the staining observed with the *Ret* probe (Fig. 2*G*) and the *Gfra1* probe (Fig. 2*H*) revealed that only part of the MMCl was positive for *Gfra1*.

It was more difficult to analyze the lumbar motor columns because of their relatively compact organization in the mouse. However, whereas *Ret* was expressed in most motoneurons (Fig. 1*F*), the pattern of expression of *Gfra1* and *Gfra2* was different (Fig. 1*G,H*). For example, a group of motoneurons localized in the rostral part of the lumbar region were positive for *Gfra*1, *Ret,* and *Islet1* (Fig. 1*E–G, white lines*) and negative for *Gfra*2 (Fig. 1*H*). Sections performed at the lumbar spinal cord levels after whole-mount *in situ* hybridization confirmed the distinct patterns

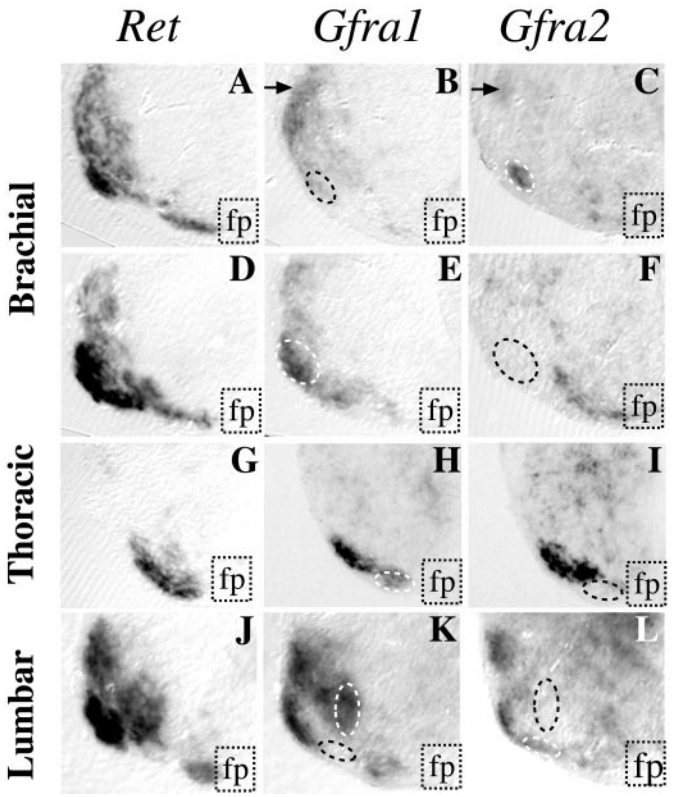

Figure 2. *Gfra1* is more widely expressed in motoneurons than *Gfra2*. Whole-mount ISH was performed on E13.5 spinal cords that were subsequently sectioned. Transverse sections were performed at brachial (*A–F*), thoracic (*G–I*), or lumbar (*J–L*) levels. In the brachial region sections were performed either at the level of the *yellow circle* (*A–C*) or the *white circle* (*D–F*) drawn on Figure 1. Panels show half-ventral horn of each section; *fp* indicates the floor plate. Nearly all motoneurons are stained using a *Ret* probe (*A, D, G*, and *J*), whereas a *Gfra1* probe (*B, E, H,* and *K*) stained more motoneurons than hybridization using a *Gfra2* probe (*C, F, I*, and *L*). *Arrows* in *B* and *C* indicate the same *Gfra1*- and *Gfra2*-positive column as in Figure 1, *C* and *D*. *Dashed lines* delineate motoneuron groups that are positive for only one α-receptor.

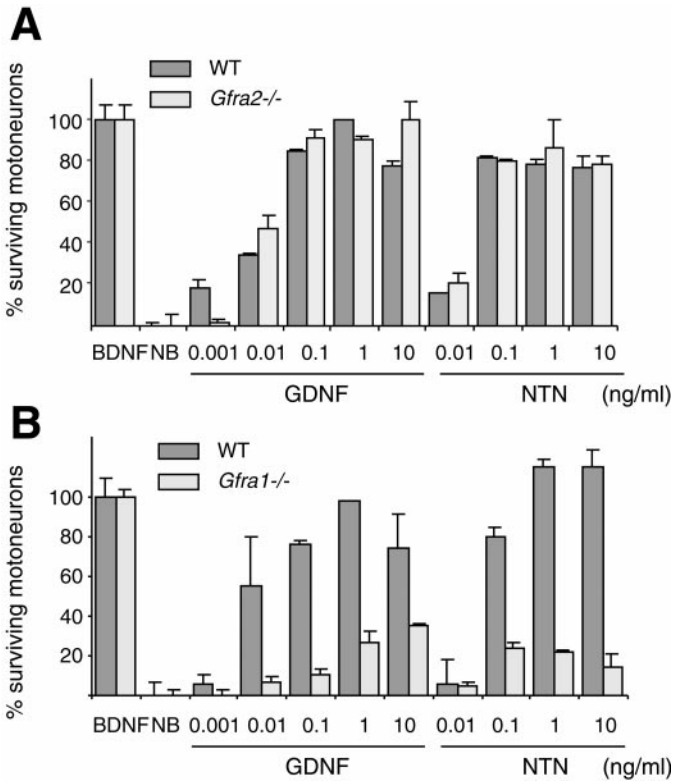

Figure 3. Survival of embryonic motoneurons from wild-type, *Gfra2*$^{-/-}$, and *Gfra1*$^{-/-}$ mice in the presence of GDNF and NTN. *A,* Survival of motoneurons from wild-type (*WT*) and *Gfra2*$^{-/-}$ E12.5 embryos in the presence of different concentrations of GDNF and NTN. *B,* Survival of motoneurons from wild-type (*WT*) and *Gfra1*$^{-/-}$ E13.5 embryos in the presence of different concentrations of GDNF and NTN. Survival values (mean ± SEM; $n = 3$) are normalized for survival in basal medium [Neurobasal (NB) defined as 0%] and expressed for each region as percentage of the number of motoneurons that developed in 1 ng/ml BDNF (defined as 100%).

of expression of *Gfra1* and *Gfra2* (Fig. 2*K,L*). In the mouse, *Gfra2* was expressed in fewer motoneurons than was *Gfra1* (Fig. 2, compare *K, L*). However, some groups of motoneurons were positive for *Gfra2* and negative for *Gfra1* (Fig. 2*K,L*). In conclusion, in the mouse spinal cord, most motoneurons express *Gfra1,* some of which are also positive for *Gfra2*, whereas few motoneurons express only *Gfra2*.

Differential responses of *Gfra1* or *Gfra2* null mutant motoneurons to GDNF and NTN

The distinct patterns of expression of *Gfra1* and *Gfra2* within the spinal cord raised the possibility that these receptor components might have distinct roles in motoneuron development. Because GDNF and NTN both have survival activity on motoneurons, we first examined the ability of mutant motoneurons lacking either GFRα1 or GFRα2 to respond to GDNF and NTN. GFRα2 is the favored receptor for NTN, and so we studied the NTN responsiveness *in vitro* of motoneurons purified from total spinal cords of E12.5 or E13.5 *Gfra*2 null mutant (*Gfra2*$^{-/-}$) mice. Surprisingly, in three independent experiments, motoneurons lacking *Gfra2* retained their responsiveness to NTN and GDNF at both low and high concentrations (Fig. 3*A*), suggesting that in motoneurons, survival responses are not mediated by GFRα2.

To identify the GFRα receptor essential for the GDNF and NTN survival responses, we next studied knock-out mice deficient for GFRα1 (*Gfra1*$^{-/-}$). In three independent experiments, motoneurons from E13.5 *Gfra1*$^{-/-}$ mutants retained normal responsiveness to BDNF but no longer responded to low concentrations of GDNF (≤0.1 ng/ml) (Fig. 3*B*), whereas the same concentration of GDNF (0.1 ng/ml) prevented the death of 75 ± 2.6% of motoneurons purified from wild-type littermates. More surprisingly, *Gfra1*$^{-/-}$ motoneurons were also less responsive to NTN. Only 24 ± 3% of the *Gfra1* null mutant motoneurons were rescued by 1 ng/ml NTN, and this figure did not increase with higher concentrations of NTN, although in the same experiment 1 ng/ml NTN saved 80 ± 2% of wild-type motoneurons (Fig. 3*B*). At higher concentrations (1 and 10 ng/ml) GDNF rescued 27 ± 3.5% and 35 ± 1% of mutant motoneurons, respectively, compared to 98 ± 0.1% and 74 ± 17% of wild-type motoneurons. We presumed that *Gfra1*$^{-/-}$ motoneurons kept alive by GDNF and NTN expressed GFRα2. Our results identify GFRα1 as the principal survival co-receptor for GDNF and NTN.

Loss of specific subpopulations of motoneurons in the *Gfra1* knock-out mice

Our *in vitro* results showing that GFRα2 was not necessary to induce motoneuron survival in the presence of GDNF or NTN corroborated the absence of gross abnormalities observed in the

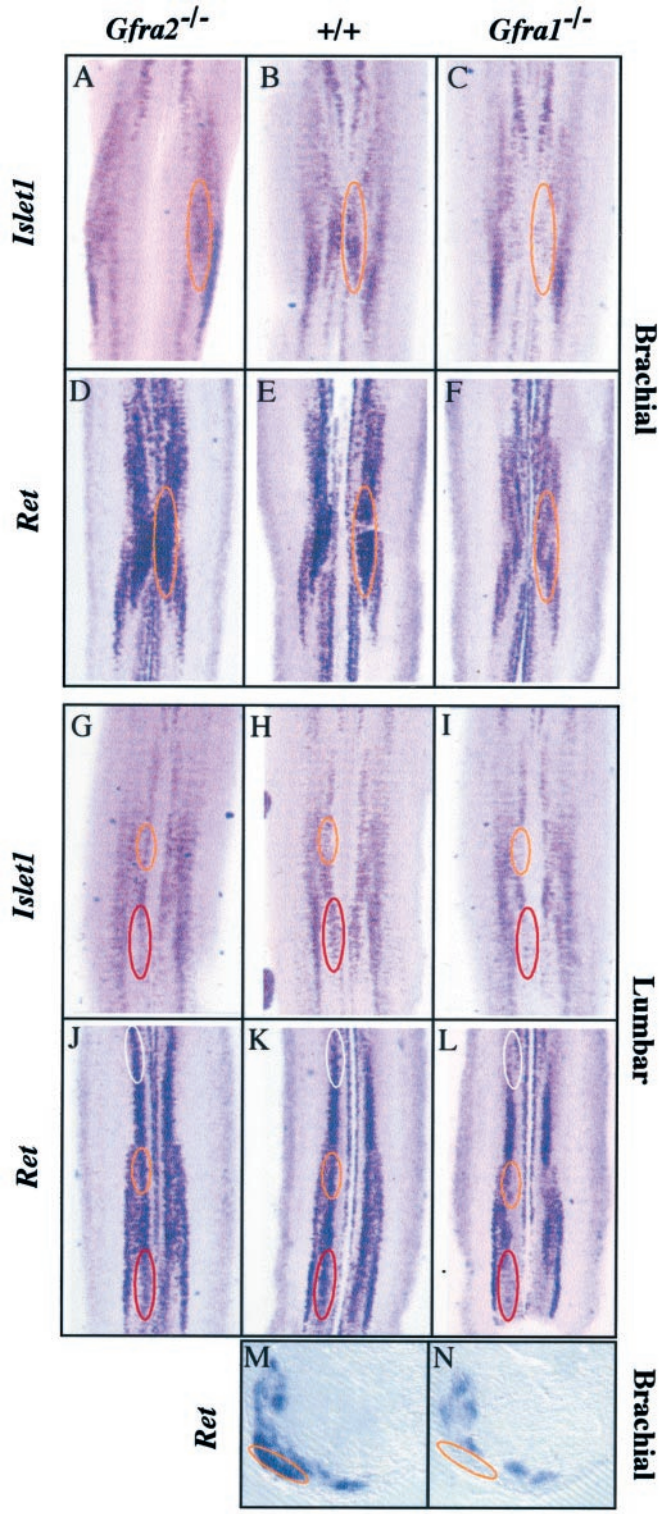

Figure 4. Loss of motoneurons in *Gfra1*$^{-/-}$ spinal cords. Whole-mount ISH was performed using probes to *Islet1* (*A–C* and *G–I*) or *Ret* (*D–F* and *J–L*) on E15.5 spinal cords from wild-type (*B, E, H,* and *K*), *Gfra1*$^{-/-}$ (*C, F, I,* and *L*), or *Gfra2*$^{-/-}$ (*A, D, G,* and *J*) mice. Analysis of brachial (*A–F*) and lumbar (*G–L*) regions shows that several groups of motoneuron are missing in the *Gfra1*$^{-/-}$ mutant mice (*orange, red*, and *white lines*). At the brachial level the *Ret* staining apparent within the *orange lines* corresponds to *Islet1*-negative motoneurons present on a different focal plane within the whole-mount views. The negative column is localized in the brachial swelling that corresponds to LMC; this has been confirmed by transverse sections of whole-mount ISH that clearly show that the lateral motor column circled *orange* in the wild-type (*M*) is missing in the *Gfra1*$^{-/-}$ mutant mice (*N*). Only partial loss (*red lines*) is observed in the *Gfra2*$^{-/-}$ lumbar spinal cords. Note that in *A*, the most lateral part of the object represents the lateral limit of the ventral horn. Each panel is representative of at least four spinal cords.

Gfra2$^{-/-}$spinal cords (Rossi et al., 1999). Likewise, the absolute requirement for GFRα1 in the survival activity of GDNF on a subpopulation of motoneurons in culture was in accordance with the loss of motoneurons observed in the spinal cord of mutant mice lacking *Gfra1* (Cacalano et al., 1998). Thus, we sought to characterize motoneuron subpopulations potentially lost in *Gfra1*$^{-/-}$ and *Gfra2*$^{-/-}$ mutant mice. Spinal cords were dissected from wild-type, *Gfra1*$^{-/-}$, and *Gfra2*$^{-/-}$ embryos at E15.5, the end of the programmed cell death (PCD) period in mouse motoneurons (Yamamoto and Henderson, 1999). To get an overall view of the motoneuron organization in these mice, we first used ISH on whole-mount spinal cord using a probe to the motoneuron marker *Islet1*. At the brachial level (Fig. 4*A–C*), comparison of mutant and wild-type spinal cord staining clearly showed that a group of motoneurons was present in the wild-type and the *Gfra2*$^{-/-}$ samples (Fig. 4*A,B*) but was missing in the *Gfra1*$^{-/-}$ mutant (Fig. 4*C*). This group of motoneurons belonged to the lateral motor column (LMC) and could be easily distinguished in whole-mount spinal cords by its characteristic position in the brachial swelling of the neural tube. It corresponds to the column shown to be strongly positive for *Gfra1* expression but negative for *Gfra2* (Fig. 1*C,D, white lines*). This was further confirmed using probes to *ChAT* (data not shown) and to *Ret*: wild-type and *Gfra2*$^{-/-}$ spinal cords showed similar labeling patterns (Fig. 4*D,E*), but the same column was missing in *Gfra1*$^{-/-}$ spinal cords (Fig. 4*F, orange lines*).

In the lumbar region, *Islet1* and *Ret* staining revealed the possible loss of three groups of motoneurons in the *Gfra1*$^{-/-}$ mutant (Fig. 4*I,L*) compared to their wild-type counterparts (Fig. 4*H,K*). In the *Gfra2*$^{-/-}$ mutant we observed a small decrease in the expression of *Islet1* and *Ret* in only one of these groups of motoneuron (Fig. 4*G,J, red lines*).

Because several groups of motoneuron are no longer detectable in the *Gfra1*$^{-/-}$ spinal cords but only a few groups of motoneuron are missing in the *Gfra2*$^{-/-}$ spinal cords, we further focused on the *Gfra1*$^{-/-}$ mutant mice. This possible motoneuron loss in this mutant was studied by ISH using markers of subpopulation of motoneurons. We used two probes that have been shown to be markers of chick LMC motoneurons localized in the limb-innervating regions: *EphA4* (Ohta et al., 1996) and *Raldh2* (Zhao et al., 1996; Sockanathan and Jessell, 1998). In both brachial and lumbar regions, the number of *EphA4*-expressing motoneurons was dramatically reduced in the *Gfra1*$^{-/-}$ mutant compared to the wild-type (Fig. 5*A–D*). Similar results were obtained after staining using the *Raldh2* probe. In the brachial region, the motoneurons stained by *Raldh2* that appear the most medial in this view (Fig. 5*E*) were absent in the *Gfra1*$^{-/-}$ mutant (Fig. 5*F*). In the lumbar region, a group of motoneurons localized in the rostral part (Fig. 5*G*) was lost in the *Gfra1*$^{-/-}$ mutant (Fig. 5*H*), whereas the level of expression of *Raldh2* decreased in a group of motoneurons that appeared more caudal (Fig. 5*G,H*).

Increased apoptosis of motoneurons lost in the *Gfra1*$^{-/-}$ mice

The absence of motoneurons in the *Gfra1*$^{-/-}$ mutant spinal cord suggested that they had died owing to the loss of GDNF or NTN

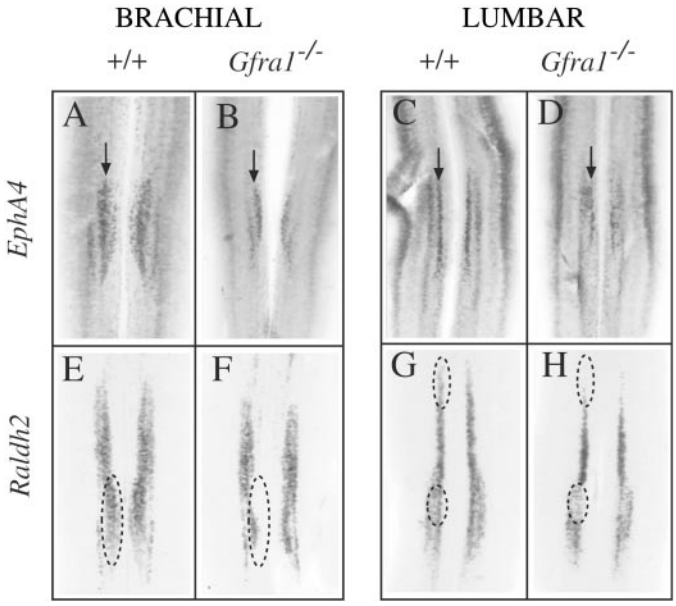

Figure 5. EphA4- and *Raldh2*- expressing motoneurons are lost in *Gfra1*$^{-/-}$ mutant mice. Whole-mount ISH was performed using probes to *EphA4* (*A–D*) or *Raldh2* (*E–H*) on E15.5 spinal cords from wild-type (*A, E, C,* and *G*) or *Gfra1*$^{-/-}$ (*B, F, D,* and *H*) mice. Analysis of brachial (*A, B* and *E, F*) and lumbar (*C, D* and *G, H*) regions shows that the number of motoneurons expressing *EphA4* decreased in the mutant as compared to the wild-type (*arrows*). *Raldh2* expression is greatly reduced in several groups of motoneuron in the *Gfra1*$^{-/-}$ mutant mice (rostral, *dashed lines*), and the staining is also decreased in a further group of motoneurons (caudal, *dashed lines*). Each panel is representative of at least four spinal cords.

signaling. At E13.5, the moment of the naturally occurring motoneuron cell death (Yamamoto and Henderson, 1999), TUNEL-positive cells were apparent in wild-type spinal cord (Fig. 6*A*). In *Gfra1*$^{-/-}$ spinal cords the number of TUNEL-positive cells increased dramatically in the lumbar region (Fig. 6*B*). At E14.5, wild-type PCD had decreased as compared to E13.5 (Fig. 6*A,C*) but in the mutant, TUNEL staining was still higher than in the wild-type (Fig. 6*C,D*). At E15.5, when normal motoneuron PCD is nearly complete (Yamamoto and Henderson, 1999), increased TUNEL staining was no longer apparent in the mutant (data not shown). We also did not observe any significant changes in the number of apoptotic cells in the brachial region from E12.5 to E15.5 (data not shown). To identify the cells dying by apoptosis, spinal cords were double-stained with TUNEL and antibodies to Islet1/2. Confocal analysis of these preparations showed that, in both wild-type and mutant spinal cords, most TUNEL-positive nuclei represent apoptotic motoneurons (Fig. 6*E–H*). Therefore, the loss of lumbar motoneurons observed in the *Gfra1* mutants could be attributed, at least in part, to an increase in cell death during the period of motoneuron PCD.

DISCUSSION

We have shown that subpopulations of spinal motoneuron can be distinguished by the combinatorial expression of the ligand-binding subunits GFRα1 and GFRα2, at the stage at which their programmed cell death is about to begin. Contrary to expectation, analysis of mutant mice in which *Gfra1* has been deleted clearly showed that GFRα1 is required for survival activity of both GDNF and NTN on most purified motoneurons. Accordingly, groups of motoneuron that strongly express *Gfra1* but not *Gfra2* identified by specific independent markers are missing in the *Gfra1*$^{-/-}$ mice. This loss, at least in part, reflects an increase in cell death during the period of naturally occurring PCD. These findings substantiate the hypothesis that there are different subpopulations of motoneuron with different trophic requirements and demonstrate that GFRα1 signaling plays a vital role in the development of specific motoneuron pools.

Distinct expression patterns of *Gfra1* and *Gfra2* in spinal motoneurons

As a first step to gain insight into the role of GDNF and NTN on motoneuron development, we studied the expression of their receptor complex components in normal mice. To get an overview of the spinal cord and to better distinguish the organization of motoneurons expressing these receptors, we used whole-mount

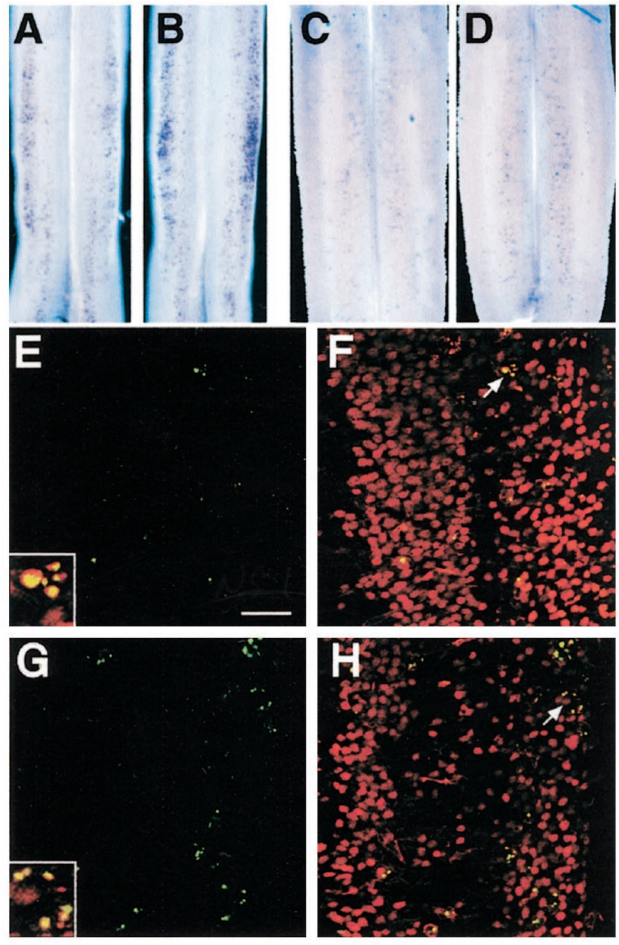

Figure 6. Increased apoptotic cell death of motoneurons in the *Gfra1*$^{-/-}$ mutant detected by whole-mount TUNEL labeling. *Top panels,* Lumbar spinal cords from wild-type (*A, C*) or *Gfra1*$^{-/-}$ (*B, D*) mice from stage E13.5 (*A, B*) or E14.5 (*C, D*). Note the increased intensity of staining in mutant spinal cords as compared to wild-type. Because in *A* and *B* spinal cords are not completely flattened, the most lateral part of the image in *A* and *B* represents the lateral limit of the ventral horn, but in *C* and *D* represents the dorsal limit of the spinal cord. *Middle* and *bottom panels,* Confocal micrographs of double-labeled lumbar spinal cord preparations from wild-type (*E, F*) or *Gfra1*$^{-/-}$ (*G, H*) E13.5 embryos stained by TUNEL (in *green*) and antibodies to Islet 1/2 (in *red*). TUNEL-positive nuclei are more numerous in the mutant (*G*) than in the wild type (*E*). Superposition of TUNEL and Islet1/2 fluorescence images (*F, H,* and *insets*) shows that nearly all TUNEL-positive nuclei represent apoptotic motoneurons. Scale bar (for *E–H*), 50 μm.

ISH on predissected spinal cord, which provides complete and detailed information on the receptor expression patterns. Within the mouse spinal cord, most motoneurons express *Gfra1,* and some are also positive for *Gfra2*, whereas few motoneurons express only *Gfra2*. Distinct patterns of expression of *Gfra1* and *Gfra2* have already been observed in the nervous system or in developing organs (Treanor et al., 1996; Baloh et al., 1997; Jing et al., 1997; Klein et al., 1997; Trupp et al., 1997; Widenfalk et al., 1997; Yu et al., 1998; Soler et al., 1999). However, our results highlight the heterogeneity within a single type of central neurons and strengthen parallels between motoneuron subpopulations and those already described in peripheral sensory ganglia (for review, see Snider and Wright, 1996; Davies, 1997).

During the period of motoneuron cell death, GDNF and NTN are expressed in the environment of motoneurons. GDNF is expressed by floor-plate, limb bud mesenchyme, and by Schwann cells and, at later stages, by some muscles (Henderson et al., 1994; Sanchez et al., 1996; Wright and Snider, 1996; Golden et al., 1999; our unpublished results). NTN has been found in limb bud and skeletal muscles (Golden et al., 1999). Because different subpopulations of motoneuron can be distinguished by the *Gfra* receptor they express, we assessed whether these different subpopulations might exhibit different responsiveness to GDNF or NTN, and we determined which receptors are involved in the survival response to which factor. To this end, we studied the responsiveness to GDNF and NTN of motoneurons purified from mutant mice in which *Gfra1* or *Gfra2* had been deleted.

GFRα2 is not essential for motoneuron survival

Surprisingly, motoneurons lacking *Gfra2* respond equally well in culture to GDNF and NTN. This result suggests that in motoneurons both GDNF and NTN signal for survival mainly through the complexes formed by RET and GFRα1 receptor. This is in accordance with the report that RET-GFRα1-expressing fibroblasts respond similarly to both GDNF and NTN (Baloh et al., 1997). One might predict that motoneurons that normally express *Gfra2* but not *Gfra1* should not respond to GDNF or NTN when cultured from *Gfra2*$^{-/-}$ embryos. However, their total number is probably too small to be detected in our survival assay, and *Gfra1* may be upregulated *in vitro* in the presence of GDNF or NTN. Alternatively, at the stage of motoneuron purification, motoneurons dependent on GFRα2 for their survival are already lost. This is unlikely because there are no gross defects in spinal motoneurons in these mutants (Rossi et al., 1999; our results). In addition, we cannot rule out the role of another GFRα family member, GFRα4, for example (Thompson et al., 1998).

The normal survival response of motoneurons lacking *Gfra2 in vitro* is consistent with the absence of gross motoneuron defects in *Gfra2*$^{-/-}$ or *Ntn*$^{-/-}$ mice (Airaksinen et al., 1999; Heuckeroth et al., 1999). However, the possibility of minor defects cannot be ruled out as demonstrated by the decrease in *Islet1* and *Ret* expression in some lumbar motoneurons of *Gfra2*$^{-/-}$ mice.

GFRα1 is required for development of distinct subpopulations of motoneuron

Most of the motoneurons purified from mice lacking *Gfra1* did not respond to GDNF or NTN *in vitro*. Thus, in the majority of motoneurons isolated by our purification procedure, GFRα1 is absolutely required for the survival activity of either GDNF or NTN. NTN and GDNF rescued ~25% of the *Gfra1*$^{-/-}$ motoneurons, so this subpopulation of motoneurons might express GFRα2. Altogether these results suggest a lack of α-receptor/ligand specificity in purified motoneurons in contrast to the *in vivo* specific α-receptor/ligand interaction observed in DRG neurons and in other neurons (Leitner et al., 1999; for review, see Airaksinen et al., 1999). In addition, the low percentage of motoneurons expressing *Gfra2* observed by *in situ* hybridization could be in accordance with the low percentage (~ 25%) of *Gfra1*$^{-/-}$ motoneurons saved by NTN or GDNF.

Our results with motoneurons differ from those obtained using dopaminergic and nodose neurons isolated from *Gfra1*$^{-/-}$ mice. E12 *Gfra1*$^{-/-}$ nodose neurons do not respond to GDNF even at high concentrations, even though these neurons express *Gfra2* at E15 and E18 (M. S. Airaksinen, unpublished observations). It will be important to determine whether *Gfra2* is expressed by *Gfra1*$^{-/-}$ nodose neurons at E12. *Gfra1*$^{-/-}$ dopaminergic neurons do not respond to either GDNF or NTN whatever the concentration (Cacalano et al., 1998), in accordance with the lack of expression of *Gfra2* in these neurons (Horger et al., 1998). Motoneurons are thus the only neurons described, which, in the absence of GFRα1, retain a partial responsiveness to both GDNF and NTN.

In vivo, mice lacking *Gdnf* or *Gfra1* show a significant loss of lumbar motoneurons (Moore et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Oppenheim et al., 2000) in accordance with our results showing that *Gfra1* is required for GDNF survival effects. Nevertheless, ~75% of the motoneurons survive at birth in contrast to the ~75% of the motoneurons, which die *in vitro* in the presence of GDNF alone. Several hypotheses might be put forward to explain this observation: (1) motoneurons still alive in *Gfra1*$^{-/-}$ mice express *Gfra2* and are saved by NTN. However, the fact that only 25% of purified motoneurons from the *Gfra1*$^{-/-}$ mice were saved by NTN does not argue for this hypothesis; (2) although in wild-type mice <75% of motoneurons express *Gfra2*, *in vivo* some GFRα2 molecules can act in "trans" in knock-out mice (Trupp et al., 1997; Yu et al., 1998); (3) *in vivo* neurotrophic factors from other families may also compensate.

To characterize the motoneurons lost in the mutant we performed whole-mount ISH using several markers. This study revealed that groups of motoneuron expressing several markers such as *ChAT*, *Islet1*, *Ret*, *EphA4,* and *Raldh2* are lacking in the mutant. Although we cannot completely exclude the possibility that the lack of these markers in the mutant is attributable to their downregulation in the absence of GDNF signaling, this is unlikely because this implicates all these markers in the same GDNF-dependent pathway. The lack of some subpopulations of motoneuron in the mutant might be attributable to an increase in apoptotic death, as suggested by the increased levels of TUNEL labeling in lumbar regions of E13.5 and E14.5 mutant spinal cords. Our double-staining experiments identifying dying cells as motoneurons are in accordance with the increased number of pyknotic motoneurons observed by Oppenheim et al. (2000) during the PCD in *Gdnf*$^{-/-}$ mice. This observation indicates that the loss of motoneurons in *Gfra1*$^{-/-}$ spinal cord takes place during the period of PCD. Altogether these observations implicate GDNF signaling through GFRα1 as a major actor in survival of subpopulations of motoneuron, especially in lumbar spinal cord. The absence of increased TUNEL staining in the brachial regions of mutant spinal cords where motoneurons are clearly missing raises an additional hypothesis that GDNF signaling is a critical factor for earlier motoneuron development processes. Further studies are needed to explore this possibility.

Conclusion

We have shown that *Gfra1* and *Gfra2* exhibit complex expression patterns in mouse spinal cord. *In vitro* and *in vivo* GFRα1 is necessary for the survival of subpopulations of motoneuron as demonstrated using purified *Gfra1*$^{-/-}$ motoneurons and by observation of increased cell death during the period of PCD in the *Gfra1*$^{-/-}$ mice. The absence of distinct groups of motoneuron expressing several markers in the *Gfra1*$^{-/-}$ mice demonstrates for the first time that signaling through a neurotrophic factor receptor is absolutely required for the development of specific subpopulations of motoneuron.

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