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GDNF Acts through PEA3 to Regulate Cell Body Positioning and Muscle Innervation of Specific Motor Neuron Pools

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Summary

Target innervation by specific neuronal populations involves still incompletely understood interactions between central and peripheral factors. We show that glial cell line-derived neurotrophic factor (GDNF), initially characterized for its role as a survival factor, is present early in the plexus of the developing forelimb and later in two muscles: the cutaneus maximus and latissimus dorsi. In the absence of GDNF signaling, motor neurons that normally innervate these muscles are mispositioned within the spinal cord and muscle invasion by their axons is dramatically reduced. The ETS transcription factor PEA3 is normally expressed by these motor neurons and fails to be induced in most of them in GDNF signaling mutants. Thus, GDNF acts as a peripheral signal to induce PEA3 expression in specific motor neuron pools thereby regulating both cell body position and muscle innervation.

Introduction

As an essential part of the development of coordinated muscle contraction, intrinsic and extrinsic signals control several sequential steps in the differentiation and growth of motor neurons. One of the first manifestations of this highly regulated process is apparent when, shortly after their generation, motor neurons become organized into discrete longitudinal columns along the anteroposterior axis of the ventral spinal cord. The median motor column (MMC), present at all segmental levels of the spinal cord, innervates axial and body wall muscles, whereas the lateral motor column (LMC), located only at limb levels, innervates limb muscles. The LMC is further divided into medial (LMCm) and lateral (LMCl) columns that send axons to the ventral and dorsal muscles of the limb, respectively (for review, see Jessell et al., 2000). These motor columns can be distinguished by the combinatorial expression of members of the LIM homeodomain proteins, referred to as the LIM code. Once motor neurons have settled into columns and send out axons toward their targets, motor neurons innervating specific muscles segregate into motor pools, spatially delimited groups of motor neurons located at specific rostrocaudal levels, reflecting a strong correlation between cell body positioning and axonal trajectory (Landmesser, 1978; Lin et al., 1998).

Within the neural tube, several transcription factors involved in controlling motor neuron diversity have been identified (for review, see Eisen, 1999; Jessell et al., 2000). For example, *Lim1* controls the trajectory of LMCl axons (Kania et al., 2000) and the forkhead-related protein TWH, as well as certain *Hox-c* and *Hox-d* genes control the development of LMC motor neurons (Carpenter et al., 1997; Dou et al., 1997; Tiret et al., 1998). Inductive signals also play an important role at several stages of this process. At early stages, SHH secreted by the axial mesoderm has a critical role in determining the identity of ventral progenitor cells and the fate of postmitotic neurons. Later on, motor neuron diversification along the rostrocaudal axis of the spinal cord depends on signals from the paraxial mesoderm (for review, see Eisen, 1999; Ensini et al., 1998; Jessell et al., 2000). Lastly, by producing retinoids, postmitotic LMCm motor neurons themselves signal to specify the fate of the later-born LMCl motor neurons (Sockanathan and Jessell, 1998).

Some aspects of motor neuron diversity may be acquired very early on before motor neurons reach their final position. For example, Lim1 is expressed by migrating LMCl motor neurons (Kania et al., 2000; Sockanathan and Jessell, 1998) and distinct electrical activity patterns (Milner and Landmesser, 1999), and fast or slow phenotypes (Milner et al., 1998) are already programmed before motor neurons reach their targets. The role of the periphery in the acquisition of motor neuron pool identity remains elusive. The onset of expression of transcription factors of the ETS family (PEA3 and ER81) by individual motor pools coincides with the arrival of motor axons at the base of the limb (Lin et al., 1998). In chick, early removal of the limb prevents the onset of *Pea3* and *Er81* expression by motor neurons, indicating that limb-derived signals are required for *Ets* gene expression by motor pools (Lin et al., 1998). However, the identity of these signals remains unknown.

Shortly after motor neurons make contact with their target muscles, about 50% are lost. During this period of programmed cell death, motor neurons are dependent on neurotrophic factors for their survival (for review, see Oppenheim, 1996; deLapeyrière and Henderson, 1997). Among the most potent trophic factors that can

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support survival of motor neurons is glial cell line-derived neurotrophic factor (GDNF) (Henderson et al., 1994). GDNF acts through a receptor complex involving the tyrosine kinase receptor RET and a glycosyl-phosphatidylinositol (GPI)-linked ligand binding subunit, GFRα1. Correspondingly, phenotypes of mutant mice lacking GDNF, RET, or GFRα1 are very similar: mutant mice fail to develop enteric neurons and kidneys and die perinatally (for review, see Airaksinen et al., 1999). *Ret* is expressed by nearly all motor neurons during the period of programmed cell death of motor neurons (Pachnis et al., 1993), and *Gfra1* is expressed by subsets of motor neurons (Garcès et al., 2000; Golden et al., 1999; Treanor et al., 1996), suggesting an important role for GDNF in motor neuron development. This is confirmed by a reduction of 25% in motor neuron numbers at birth of both *Gdnf* and *Gfra1* knockout mice (Cacalano et al., 1998; Moore et al., 1996; Sanchez et al., 1996). Strikingly, motor neurons affected in the mutants are not evenly distributed but are grouped at specific levels within the spinal cord, most noticeably at limb-innervating levels (Garcès et al., 2000; Oppenheim et al., 2000). These findings provided the first demonstration of a requirement of specific subsets of motor neurons for a given neurotrophic factor, but left open the question of motor neuron fate in the absence of GDNF. Although GDNF was initially characterized for its ability to prevent cell death of subsets of neurons during development, evidence has recently accumulated that it can also regulate proliferation and differentiation (Taraviras et al., 1999). This left open the possibility that in *Gdnf*$^{-/-}$ and *Gfra1*$^{-/-}$ mutant spinal cords, motor neurons might not be lost through cell death but may be affected at earlier developmental stages.

The restriction of the *Gdnf* and *Gfra1* mutant phenotype to a limited number of spatially delimited groups of motor neurons was also unexplained. Although GFRα1 expression is itself restricted to certain subsets, many GFRα1-expressing motor neurons are apparently unaffected in the mutants, suggesting that the specificity of the mutant phenotype has other determinants. Here and in the companion paper by Livet et al. (2002; [this issue of *Neuron*]), we show that both central and peripheral influences interact to determine the restricted requirement for GDNF. First, GDNF synthesized in the periphery induces PEA3 expression in motor neurons but can only act on certain prespecified subsets. In the absence of GDNF signaling, the majority of these motor neurons fail to express PEA3 and as a consequence settle at abnormal positions in the spinal cord and fail to innervate normally their target muscle. Second, the peripheral expression of GDNF itself is spatially restricted to the trajectory of specific populations of growing motor neurons, from their arrival in the brachial plexus to innervation of their target muscles. Thus, GDNF is a major peripheral signal required for induction of PEA3, which in turn defines the central and peripheral phenotype of specific motor pools.

Results

Dynamic and Specific Expression Patterns of *Gdnf* in the Developing Neuromuscular System

To understand the subset-specific nature of the *Gdnf*$^{-/-}$ and *Gfra1*$^{-/-}$ phenotypes (Garcès et al., 2000; Oppenheim et al., 2000), we first localized sites of production of GDNF in the developing neuromuscular system by analyzing the expression pattern of a GDNF/β-Gal fusion protein in heterozygous *Gdnf*$^{+/nlslacZ}$ embryos (Moore et al., 1996). GDNF expression was first detected around the 28 somite stage (corresponding to embryonic day [E]10) in a remarkably discrete region at the base of the developing forelimb bud (Figure 1A). By E11.5 (Figure 1B), GDNF expression had increased in intensity and expanded into the axillary region. At E12.5, high levels of GDNF/β-Gal were detected in the cutaneus maximus (CM) and latissimus dorsi (LD) muscles, as shown on whole embryos and on dissected muscles (Figures 1C, 1E, and 1F). These two GDNF^{+} muscles are inserted at the proximal humerus and together form the axillary arch (Baulac and Meininger, 1981). The CM constitutes a thin muscle layer under the skin spanning the entire thoracic and abdominal trunk (Theriault and Diamond, 1988); the LD is located underneath. Remarkably, GDNF expression was absent from other muscles in forelimb and hindlimb (Figures 1C and 1D).

In order to more precisely characterize the GDNF^{+} territories, we next performed an in situ hybridization analysis of serial transverse sections throughout the cervico-thoracic region. We used probes for *Gdnf* and *nlslacZ* in combination with probes for the early muscle cell markers *Pax3*, *myogenin*, and *MyoD*, and *Sox10*, a marker of early Schwann cells. *Gdnf* expression, assessed by *Gdnf* or *nlslacZ* antisense probes in *Gdnf*$^{+/-}$ embryos, perfectly matched the pattern previously revealed by X-Gal staining. At the 37 somite stage, *Gdnf*$^{+}$ cells found at the base of the limb did not express *Pax3*, *MyoD*, or *Sox10* (data not shown), in agreement with Wright and Snider (1996), and are likely of mesenchymal origin.

At E12.5, *Gdnf*$^{+}$ cells were present in the *myogenin*- and *MyoD*-labeled CM and LD muscles but absent from other muscles (Figures 1I–1K). Moreover, *Gdnf*$^{+}$ cells were not detected in *Sox10*-stained nerve branches (arrowhead in Figure 1L). In an attempt to establish the identity of GDNF^{+} cells in muscle, we analyzed by electron microscopy the en bloc X-Gal-stained upper thoracic region containing the CM and LD muscles. Electron-dense X-Gal precipitates were found in the nuclear cistern of morphologically poorly differentiated cells (Figures 1G and 1H). The β-Gal^{+} cells were occasionally mitotic and often located in immediate vicinity of cells harboring myofilaments, indicative of early myoblasts (Figure 1H). In contrast, cells that surrounded bundles of axons, presumably primitive Schwann cells, were never labeled (data not shown). In conclusion, these analyses revealed a highly dynamic and spatially restricted expression of GDNF: it is first expressed in the mesenchyme and then in undifferentiated cells within two hypaxial muscles.

Innervation of the Cutaneus Maximus and Latissimus Dorsi Muscles Is Specifically Affected in *Gdnf* and *Gfra1* Mutant Mice

To understand the precise relationship of the GDNF expression pattern to that of growing nerves, we used whole-mount neurofilament (NF) staining and combined X-Gal/NF staining of heterozygous *Gdnf*$^{+/-}$ embryos (10–25 embryos per stage). At E10.5, cervical motor ax-

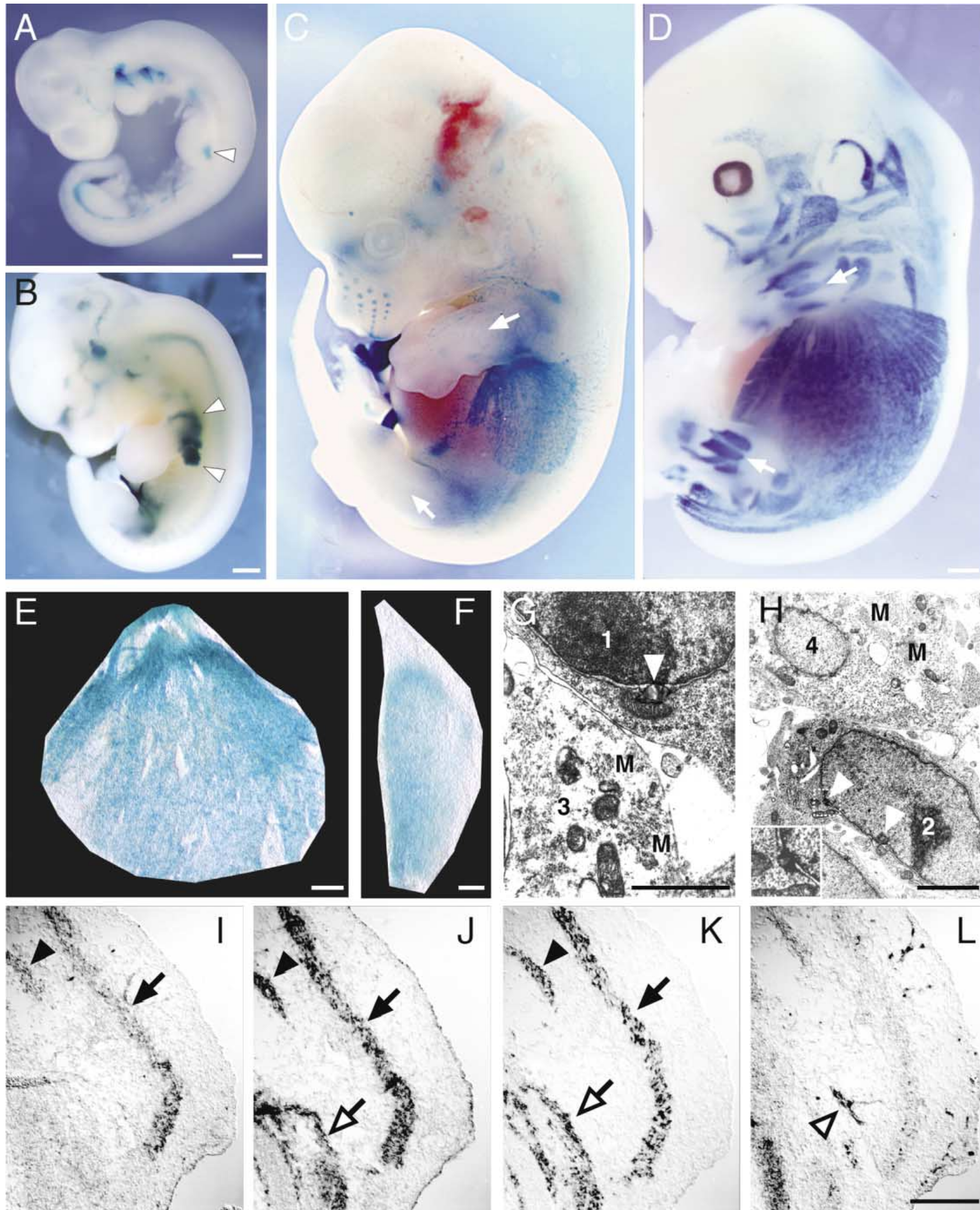

Figure 1. In the Developing Neuromuscular System, GDNF Expression Becomes Restricted to Specific Muscles

(A–F) X-Gal staining of eviscerated *Gdnf* heterozygous mice at E10 (30 somites) demonstrated localized production of GDNF/β-Gal in the proximal part of the forelimb bud (arrowhead in [A]). At E11.5, GDNF expression extended caudally to two developing hypaxial muscles (arrowheads in [B]); signal was also apparent in branchial arches and the floorplate/notochord. At stage E12.5, these muscles became located in the lateral and dorsal thoracic region (C). By dissection, these GDNF^{+} muscles were identified as the CM (E) and LD (F). Remarkably, GDNF/β-Gal expression was absent from other forelimb and hindlimb muscles (arrows in [C] and [D]) visualized in a transgenic mouse line (D) in which reporter gene expression is controlled by a myosin light chain promoter.

(G and H) Electron microscopy of the CM muscle from X-Gal-stained E12.5 *Gdnf*$^{+/-}$ mice revealed the presence of electron-dense X-Gal precipitates (arrowheads and inset in [H]) in the nuclear cistern of morphologically undifferentiated cells (1, 2). These GDNF/β-Gal expressing cells were often found in close association with myoblasts (3, 4) containing myofilaments (M). Inset in (H) is a 3.4× magnification of the dashed square.

(I–L) Transverse sections through the upper thoracic region of E12.5 *Gdnf*$^{+/-}$ embryos were stained by in situ hybridization with probes for *Gdnf* (I), *MyoD* (J), *myogenin* (K), or *Sox10* (L). *Gdnf* expression overlapped with *MyoD* and *myogenin* expression in the CM (filled arrow) and LD (filled arrowhead) muscles but was not detected in other muscles located in a more anterior position (open arrow). *Gdnf* was not expressed by *Sox10*$^{+}$ Schwann cells found in a nerve branch entering the CM muscle (open arrowhead).

Scale bars: 500 μm in (A)–(D), 250 μm in (E), 100 μm in (F), 0.5 μm in (G), 1 μm in (H), and 200 μm in (I)–(L).

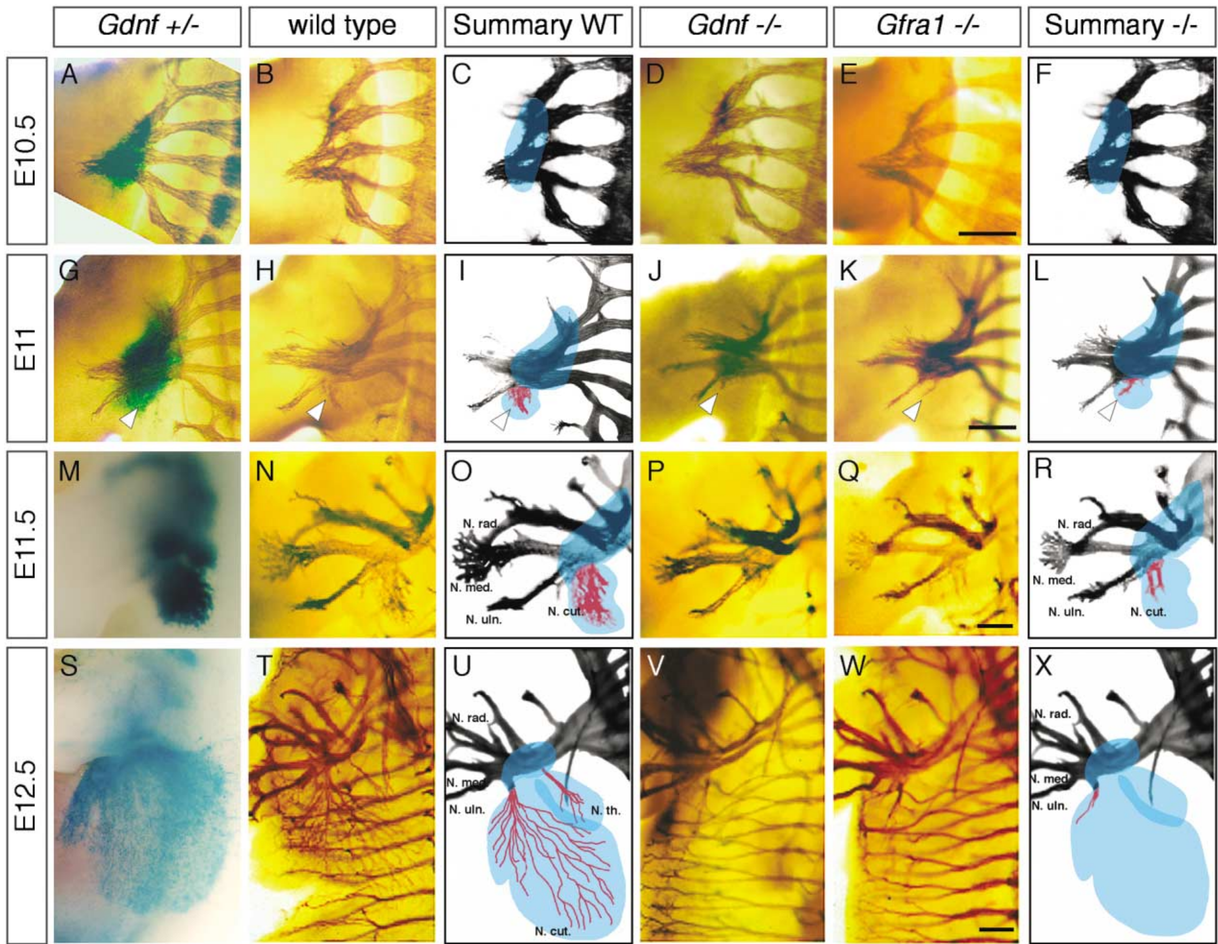

Figure 2. The Development of Brachial Motor Nerves Is Perturbed in *Gdnf* and *Gfra1* Mutant Embryos

The formation of brachial nerves was analyzed at stages E10.5 (36 somites, [A–F]), E11 (43 somites, [G–L]), E11.5 (M–R), and E12.5 (S–X) by NF staining in wild-type (B, H, N, and T), $Gdnf^{-/-}$ (D, J, P, and V), and $Gfra1^{-/-}$ mice (E, K, Q, and W). The territory of GDNF expression was analyzed in $Gdnf^{+/-}$ mice of the corresponding stages by X-Gal (M and S) or by combined X-Gal/NF staining (A and G). Summary diagrams with brachial nerves and $GDNF^{+}$ territories are shown for wild-type (C, I, O, and U) and mutant (F, L, R, and X) embryos. Nerves affected in the mutants are indicated in red.

At stage E10.5, C5–C8 spinal nerves had access to GDNF at their axon tips (A) and showed a similar growth pattern in wild-type and mutant mice (B–F). At E11, nerve fibers to the developing CM muscle normally exited the plexus in a sharp turn (arrowheads in [G]–[I]) but were arrested in their growth or misrouted in *Gdnf* and *Gfra1* mutant mice (arrowheads in [J] and [K]). In wild-type E11.5 embryos, the nerve to the CM (N. cut.) gave rise to numerous branches (N) that continued to grow in a caudal direction (T) in the $GDNF^{+}$ CM muscle (S and U). At stages E11.5 and E12.5, these branches were either absent or reduced to few nerve fiber bundles in both *Gdnf* (P and V) and *Gfra1* mutants (Q and W). The thoracodorsalis nerve (N. th. in [U]), which normally innervates the LD muscle, was also affected in *Gdnf* and *Gfra1* mutants (V and W). In contrast, segmental skin-innervating nerves, which provide the sensory input to CM motor neurons, seemed normal in the mutants (deliberately omitted for clarity in diagrams [U] and [X]). The growth and branching of three major forelimb nerves, i.e., N. medianus (N. med.), N. ulnaris (N. uln.) and N. radialis (N. rad.), was also normal in both mutants.

Scale bars: 200 μm.

ons have exited the spinal cord through ventral roots C5–C8 and converged to begin to form the brachial plexus (Figure 2B). At this stage, GDNF expression was confined to a small region around the plexus where the C5–C8 nerve fibers intermingled (Figures 2A and 2C). By E11, individual nerves from the plexus began to defasciculate from the brachial plexus (Figure 2H) and grow into the forelimb and neck and caudally into the axillary region (Figures 2H and 2I). At the same stage, the zone of GDNF expression expanded specifically in the direction taken by the caudal nerve branches (Figures 2G and 2I). From E11.5–E12.5, GDNF expression was not only detected around the brachial plexus, but also extended into the CM and LD muscles (Figures 2M, 2O, 2S, 2U, and Figure 1) around the rapidly branching corresponding nerves (Figures 2N and 2T) (Greene, 1963).

The strong correlation between restricted GDNF expression and the growth of the motor nerves innervating the CM and LD muscles led us to analyze the role of GDNF signaling in this process. No differences in the overall pattern of nerve growth could be seen between wild-type and $Gdnf^{-/-}$ or $Gfra1^{-/-}$ mutant embryos at E10.5 (Figures 2A–2F). However, already by E11, caudal

nerve branches of the plexus in *Gdnf*$^{-/-}$ mice displayed abnormalities: they were atrophied and occasionally misrouted (Figure 2J). Similar defects were observed in *Gfra1*$^{-/-}$ mice (Figure 2K). At E11.5 and E12.5, the nerve growth deficits became even more obvious (Figures 2P–2R and 2V–2X): in all *Gdnf* and *Gfra1* mutants analyzed ($n > 15$), the nerves to the CM and LD were missing or reduced to a small number of fibers. This did not reflect a disorganization of the muscle since *Gdnf*$^{-/-}$ embryos displayed the same β-Gal expression pattern as heterozygotes (Figure 2S, and data not shown), indicating that the differentiation and migration of GDNF^{+} cells was not perturbed. Moreover, the phenotype was specific for the CM and LD nerves since: (1) intercostal nerves innervating the same region (visible as horizontal stripes in Figures 2T, 2V, and 2W) were unchanged, and (2) the medianus, ulnaris, and radialis nerves that innervate the forelimb displayed indistinguishable progression and caliber at E11.5–E12.5 (Figures 2P–2R and 2V–2X) and innervated their target muscles normally (data not shown).

To examine whether the innervation defects in the CM and LD persisted throughout later stages of development, we also analyzed dissected individual muscles at E17.5, shortly before death of the mutants around postnatal day (P)0 (Moore et al., 1996). NF/synaptophysin staining of normal CM muscles ($n = 14$) showed that motor nerve branches entered this muscle at the axillar level, extended caudally in parallel with the muscle fibers and arborized into numerous presynaptic terminals distributed over the entire muscle (Figure 3A and data not shown). In CM muscles ($n = 20$) from *Gdnf*$^{-/-}$ embryos, the number and caudal progression of motor nerves was severely reduced (Figure 3B). In LD muscles from *Gdnf*$^{-/-}$ embryos ($n = 7$), the innervation of the distal synaptic zones was severely affected: only one out of normally three to four distal branches had formed and this branch was reduced in caliber and length (Figures 3C and 3D). Very similar changes were observed in CM and LD muscles from *Gfra1*$^{-/-}$ embryos (data not shown). As at the earlier stages, these deficits did not affect nerves to neighboring muscles, such as the pectoralis (data not shown). In conclusion, axonal growth and arborization of two specific motor nerves depend on a GDNF signal present in the immediate environment of their axons and in their target muscles.

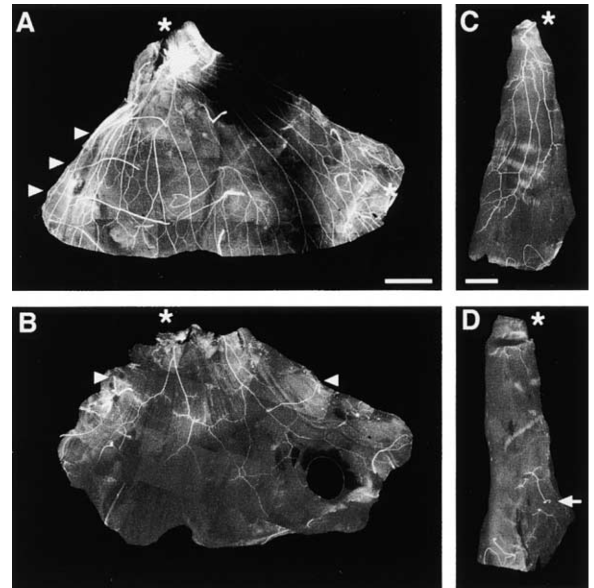

Figure 3. The Cutaneus Maximus and Latissimus Dorsi Muscles Are Not Properly Innervated in *Gdnf* Mutant Mice

Whole-mount NF/synaptophysin labeling of CM (A and B) and LD (C and D) muscles in E17.5 wild-type (A and C) and *Gdnf*$^{-/-}$ (B and D) mice.

(A) In wild-type mice, motor nerve branches enter the CM at the axillar level (asterisk) and run in parallel with the muscle fibers. Some sensory nerve branches (arrowheads in [A] and [B]) emanating from intercostal nerves can also be observed while others have been stripped off during the dissection procedure.

(B) In *Gdnf*$^{-/-}$ mice, motor branches are severely reduced in number and often do not extend beyond the most rostral part of the CM.

(C) In the normal LD, the thoracodorsalis nerve (asterisk) enters proximally, gives rise to short branches to the proximal synaptic zone and divides into three to four long branches supplying distal synaptic zones.

(D) In mutant LD muscles, long distal nerve branches are either absent or severely reduced in caliber and length. In rare cases, residual distal motor innervation is provided by ectopic nerves entering the muscle at a caudal level (arrow).

Scale bars: 1 mm.

Pea3^{+} Motor Pools Are Specifically Affected in *Gdnf* and *Gfra1* Mutants

The specific alterations detected in the projection pattern of motor axons raised the question of the fate of the corresponding motor pools. In chicken (Lin et al., 1998) and mouse (Arber et al., 2000), the differential expression of *Er81* and *Pea3* (two members of the ETS class of transcription factors) defines motor pools in the lateral motor column (LMC). We therefore analyzed the expression of these *Ets* transcription factors, using in situ hybridization on whole-mount E12.5 cervical spinal cords. In normal embryos, strongly *Pea3* positive motor pools were detected in segments C5 to C8 (Figure 4A). In striking contrast, the number of the *Pea3*$^{+}$ motor neurons was dramatically reduced in both *Gdnf*$^{-/-}$ and *Gfra1*$^{-/-}$ mutants (Figures 4C and 4D). By analyzing the position and number of PEA3-immunoreactive motor neurons in serial sections from C5 to C8 *Gdnf*$^{-/-}$ spinal cords (Figures 4E–4J), we showed that the number of PEA3^{+} motor neurons was drastically reduced in segments C6 and rostral C7 (Figure 4E) where the cell bodies of the majority of the LD motor neurons reside (Baulac and Meininger, 1981). We also observed a reduced number of PEA3^{+} motor neurons at a more ventromedial position in segments C7/C8, which is normally occupied by PEA3^{+} motor neurons belonging to the CM pool (Baulac and Meininger, 1981; Livet et al. 2002). *Gdnf* heterozygous mice displayed a phenotype that was intermediate between mutant and wild-type embryos (Figure 4B). The total number of PEA3-immunoreactive motor neurons in segments C5 to C8 was reduced by 89.7% ± 4% (mean ± SEM, $n = 4$) in homozygous *Gdnf*$^{-/-}$ embryos and by 49.7% ± 5% in heterozygous *Gdnf*$^{+/-}$ embryos (Figures 4E and 4J). The few remaining PEA3^{+} motor neurons in the *Gdnf*$^{-/-}$ and *Gfra1*$^{-/-}$ mutants were found in the most medial portion of the LMCm (Figures 4H and 4I). There was not a generalized requirement

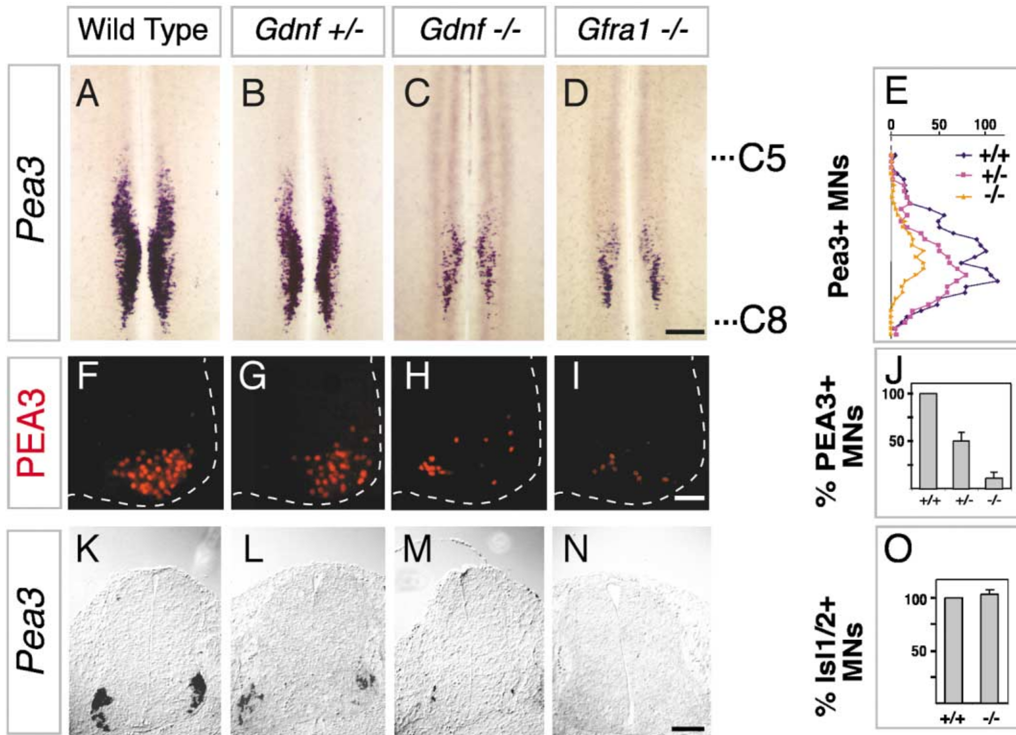

Figure 4. PEA3 Expression Is Dramatically Reduced in *Gdnf* and *Gfra1* Mutant Spinal Cords

(A–D) Cervical spinal cords from E12.5 wild-type (A), *Gdnf*$^{+/-}$ (B), *Gdnf*$^{-/-}$ (C), or *Gfra1*$^{-/-}$ (D) mice were analyzed by whole-mount in situ hybridization with *Pea3*. Whole mounts are presented in an "open-book" configuration in which the midline and ventral structures are central, and dorsal structures appear to the left and right.

(E–J) PEA3 expression was detected at E12.5 by immunofluorescence on serial transverse sections comprising segments C5–C8. (E) Number of PEA3$^+$ motor neurons per section plotted as a function of rostrocaudal position, showing two major PEA3$^+$ motor neuron groups in wild-type embryos. In *Gdnf*$^{-/-}$ mice, PEA3 expression was reduced along the entire rostrocaudal axis. In (F)–(I), ventral half-spinal cords. At the C7 level, PEA3$^+$ motor neurons in *Gdnf*$^{+/-}$ mice (G), although less numerous, occupied the same region as in wild-type embryos (F), whereas in *Gdnf*$^{-/-}$ (H) and G*fra1*$^{-/-}$ (I) mice the few remaining PEA3$^+$ motor neurons were concentrated in a medial position. (J) Total number of PEA3$^+$ motor neurons in segments C5–C8 in *Gdnf*$^{+/-}$, *Gdnf*$^{-/-}$ and wild-type mice.

(K–N) In situ hybridization analysis of E10.75 cervical spinal cord sections from wild-type (K), *Gdnf*$^{+/-}$ (L), *Gdnf*$^{-/-}$ (M), and G*fra1*$^{-/-}$ (N) mice demonstrated that *Pea3* expression was already missing in early stage mutant motor neurons.

(O) The total number of Isl1/2$^+$ motor neurons in the C5–C8 ventral horns did not differ between wild-type and *Gdnf*$^{-/-}$ mice at E12.5. Graph represents counts from one typical experiment out of four performed (E); histograms represent mean ± SEM of four embryos per genotype (J and O).

Scale bars: 250 μm in (A)–(D), 50 μm in (F)–(I), and 100 μm in (K)–(N).

for GDNF for ETS gene expression: *Er81*$^+$ motor pools, which were normally confined to upper cervical levels, appeared unaltered in both *Gdnf*$^{-/-}$ and *Gfra1*$^{-/-}$ mice (data not shown).

The reduced PEA3 expression in specific motor pools before programmed cell death raised the question of whether these motor neurons do not express PEA3 or are lost. To follow motor neuron generation and/or survival, we first counted the number of motor neurons expressing the transcription factors Isl1/2 at E12.5 (Tsuchida et al., 1994). In normal embryos, all PEA3$^+$ motor neurons expressed Isl1/2, and their number represented at least 25% of the total number of Isl1/2$^+$ motor neurons in the ventral horn of segments C5–C8 (data not shown). The total number of Isl1/2$^+$ cells in these segments differed by less than 1% ± 3% (mean ± SEM; n = 4) between wild-type and *Gdnf* mutant embryos (Figure 4O), making it unlikely that motor neuron generation or death was altered in the mutants at this stage. Accordingly, the number of apoptotic cells, detected by TUNEL staining at the position of the PEA3 motor pools, was not increased in mutant spinal cords at any stage from E9 to E12 (data not shown).

Specific Motor Neuron Populations Are Mispositioned in *Gdnf* and *Gfra1* Mutant Mice

Since motor neurons that normally express PEA3 were present in normal numbers, we studied their fate in mutant spinal cords. As a first step, we determined the stage at which PEA3 deficits became apparent. At E10.75, when the first innervation deficits were observed, *Pea3* expression in motor neurons was already missing in *Gfra1*$^{-/-}$ and *Gdnf*$^{-/-}$ embryos and was severely reduced in heterozygous *Gdnf*$^{+/-}$ embryos (Figures 4K–4N). Thus, in the absence of GDNF signaling, PEA3 is not induced in the majority of motor neurons.

With the aim of localizing motor neurons that failed to express PEA3 in the mutants, we analyzed the expression profile of genes normally coexpressed by PEA3$^+$ motor neurons such as *Isl1* or *Hb9* (Arber et al., 2000).

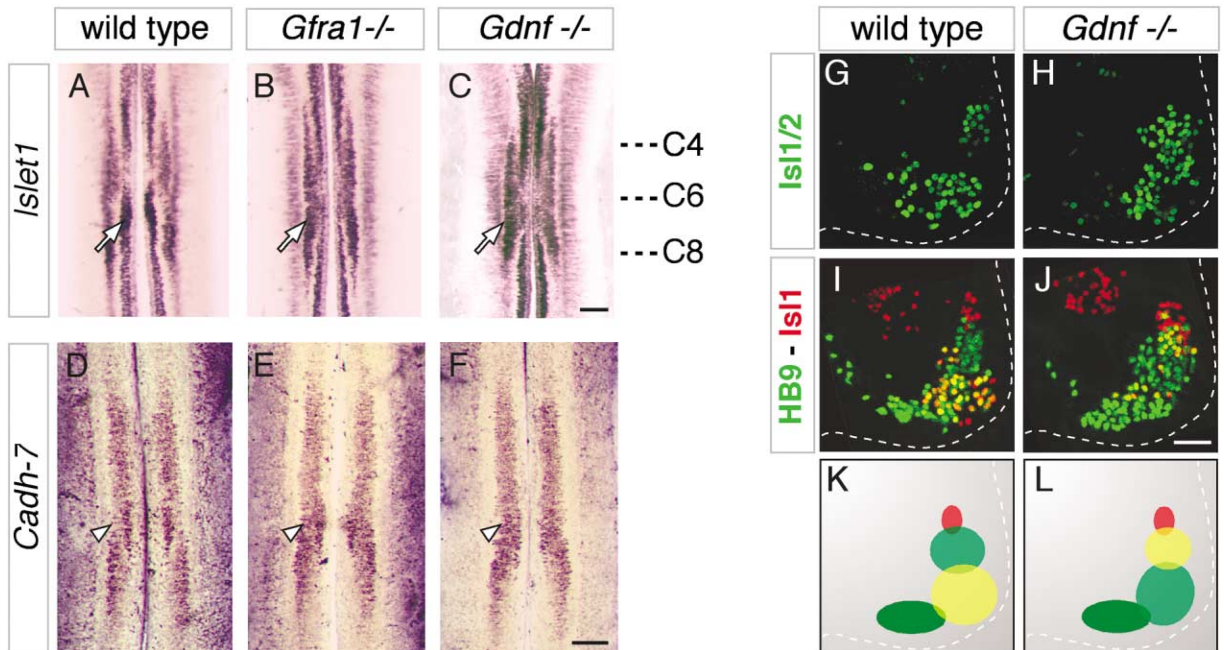

Figure 5. Absence of GDNF Signaling Causes Mispositioning of Cervical Motor Neurons

(A–F) *Isl1* and *Cadherin-7* in situ hybridization in E12.5 spinal cords from wild-type (A and D), *Gfra1*$^{-/-}$ (B and E), and *Gdnf*$^{-/-}$ (C and F). In mutant *Gdnf* and *Gfra1* spinal cords, *Isl1* expression was much reduced at positions normally corresponding to PEA3^{+} motor neuron pools (arrow, compare with Figure 4). Conversely, at C5–C7 levels, a population of *Isl1*$^{+}$ neurons (arrow) was detected in a more dorsolateral position in both mutants. At the same rostrocaudal levels, *Cadherin-7*$^{+}$ neurons in the mutants were not only found in their normal position, but also in a dorsolaterally expanded territory (arrowheads in [D]–[F]).

(G–J) E12.5 cervical spinal cord sections from wild-type (G and I) and *Gdnf*$^{-/-}$ (H and J) mice. In comparison with wild-type embryos (G), the number of Isl1/2^{+} motor neurons in *Gdnf*$^{-/-}$ mice (H) was reduced medially and increased dorsolaterally. Similarly, Isl1/HB9 double-positive motor neurons (in yellow) were apparently shifted from a more medioventral position in the wild-type (I) to a more dorsolateral position in *Gdnf*$^{-/-}$ mice (J).

(K–L) Model illustrating the mispositioning of motor neurons in the mutants as an apparent “changing of places” between misspecified, presumptive PEA3^{+} neurons of the lateral part of the LMCm (in yellow) and neurons of the dorsal part of the LMCl (in green).

Scale bars: 250 μm in (A)–(F) and 50 μm in (G)–(J).

Gdnf and *Gfra1* mutant spinal cords, when analyzed by whole-mount in situ hybridization (Figures 5A–5C), displayed a reduction in the density of *Isl1*-expressing cells in the medial LMC, which was paralleled by an increase in *Isl1*-expressing cells in a more dorsolateral region normally occupied by the lateral LMC. Immunohistochemical analysis of sections further confirmed that motor neuron populations positive for Isl1/2 (Figures 5G and 5H) or double positive for Isl1/HB9 (Figures 5I and 5J) were increased in number at a position corresponding normally to the lateral LMC. These changes were most pronounced in segments C6–C7, which contained the highest numbers of PEA3^{+} motor neurons in wild-type embryos and which were most affected in the mutants.

To analyze the consequences of these changes on cell populations normally adjacent to PEA3^{+} motor neurons, we analyzed the expression profile of *Cadherin-7*, which labels a small subset of motor neurons positioned dorsal to PEA3^{+} motor neurons (Livet et al., 2002). Interestingly, in both *Gdnf*$^{-/-}$and *Gfra1*$^{-/-}$ mice, *Cadherin-7*$^{+}$ cells were found in a broader dorsolateral territory than in wild-type spinal cords (Figures 5D–5F). There is thus a subtle disorganization of the mutant spinal cord with disappearance of PEA3^{+}/Isl1^{+}/HB9^{+} motor neurons from their normal position and the appearance of abnormally elevated numbers of Isl1^{+}/HB9^{+}/*Cadherin-7*$^{+}$ neurons in a more dorsolateral position (Figures 5K and 5L). Thus, in addition to its role in target innervation, peripheral GDNF signaling is required for the correct positioning of cervical motor neuron pools.

GDNF Is an Early Limb-Derived PEA3 Regulator

By early limb ablation, Lin et al. (1998) showed that peripheral factors from the developing limb are required for *Ets* gene induction in chicken motor neurons. The identity of these factors, however, has remained enigmatic. The critical time period for peripheral upregulation of *Ets* expression in the chicken forelimb is around HH stages 18 (Lin et al., 1998), approximately equivalent to mouse E10.25. We therefore tested the hypothesis that GDNF might represent a limb-derived factor for *Pea3* gene induction. We considered that there are several prerequisites for GDNF to play this role. (1) GDNF should be expressed at the right time, i.e., before or concomitantly with the onset of *Pea3* expression in motor neurons. (2) GDNF should be accessible to the axons of presumptive PEA3 motor neurons. (3) These motor neurons should express the GDNF receptors GFRα1 and RET. (4) GDNF must be able to induce PEA3 in vitro.

Our combined analysis of peripheral innervation and GDNF expression (Figures 1 and 2) had already demon-

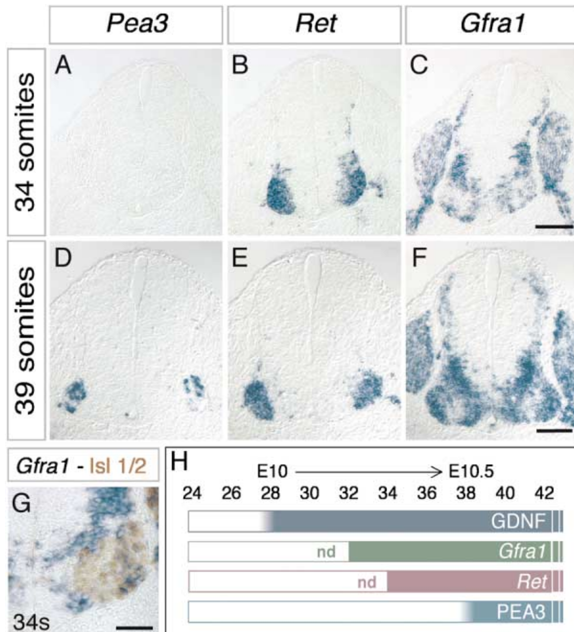

Figure 6. Cervical Motor Neurons Express *Gfra1* and *Ret* before *Pea3*

(A–F) In situ hybridization from wild-type embryos of the 34 somite stage. *Pea3* expression was undetectable in cervical spinal cord (A), whereas expression of *Ret* (B) and *Gfra1* (C) was readily detected. At the 39 somite stages, *Pea3* expression was found in motor neurons (D) coexpressing *Ret* (E) and *Gfra1* (F). Note the presence of motor neurons positive for *Gfra1* but negative for *Pea3*.

(G) Double in situ hybridization and immunostaining of a ventral half-spinal cord at the 34 somite stage showing *Gfra1* expression (in blue) in Isl1/2$^+$ motor neurons (in brown).

(H) The onset of *Gdnf, Gfra1*, or *Ret* expression in the 28 to 34 somite period preceded that of *Pea3* at 38 somites. Abbreviations: n.d., not determined.

Scale bars: 100 μm in (A)–(F) and 50 μm in (G).

strated that GDNF was produced in the proximal forelimb from the 28 somite stage onward and that the C5 to C8 motor nerve fibers gained access to this GDNF source as early as at the 36 somite stage. To precisely determine the onset of *Pea3* expression in cervical motor neurons, we analyzed normal embryos by in situ hybridization and *Pea3*$^{+/nlslacZ}$ embryos by X-Gal staining at different time points (17 to 42 somite stages). Using either method, *Pea3* expression could not be detected before the 37 somite stage (Figures 6A and 6H) but was clearly present from the 38/39 somite stage onward (Figure 6D). Thus, the onset of GDNF expression at the 28 somite stage preceded that of *Pea3* (38 somites) by about 12 hr. We therefore analyzed expression of the two components of the GDNF receptor complex, *Gfra1* and *Ret,* by in situ hybridization. Within the spinal cord, *Ret* expression could be observed as early as at the 34 somite stage in nearly all motor neurons (Figure 6B) from all rostrocaudal levels (data not shown). *Gfra1* expression was apparent at $\sim$32 somites in a subset of motor neurons located along the entire cervical spinal cord (Figures 6C and 6G). At later stages ($\sim$39 somites), cervical *Pea3*$^+$ motor neurons were shown to coexpress *Gfra1* and *Ret* (Figures 6D–6F).

To assess whether GDNF could induce *Pea3* expression, we isolated segments of the neural tube from cervical or thoracic levels of heterozygous *Pea3*$^{+/nlslacZ}$ embryos at 30–36 somite stages (Figure 7A). At the time of isolation, no *Pea3* expression could be detected in neural tube (Figure 6A). The explants were then grown in vitro for 24 hr in the presence or absence of GDNF. In cervical explants cultured in the absence of GDNF, no X-Gal precipitates were observed (Figure 7B). In contrast, in the presence of GDNF, the level of PEA3 induction increased markedly over the 24 hr culture period in 15 out of 19 explants. At 24 hr, numerous cells expressing PEA3 were found in a restricted region spanning intermediate and lower cervical segments (Figure 7C). This pattern of PEA3 expression closely mimicked the pattern seen in vivo in the 40 somite stage embryos. In order to more precisely characterize the PEA3$^+$ cells in the explants, we stained transverse sections through cultured cervical explants with antibodies against β-Gal, Isl1, and HB9 (Figures 7F–7M). In triple-labeling experiments, PEA3 expression was detected only in HB9$^+$/Isl1$^+$ motor neurons and not in HB9$^+$/Isl1$^-$ motor neurons (Figure 7M). This pattern of PEA3 expression was again strikingly reminiscent of the in vivo situation (see Figure 5). In the absence of GDNF, proportions between HB9$^+$/Isl1$^+$ and HB9$^+$/Isl1$^-$ motor neurons number were apparently normal (Figures 7F–7I), indicating that the effect of GDNF is to induce PEA3 expression and not to promote motor neuron survival.

In contrast, motor neurons from upper cervical levels, although they strongly expressed *Ret* and *Gfra1*, did not upregulate PEA3 in response to GDNF (Figure 7C, and data not shown). The spatial restriction of the PEA3 response was further confirmed by the observation that thoracic neural tube explants grown for 24 hr in the presence of GDNF never stained positive for X-Gal (Figures 7D and 7E). Thus, even among *Ret*$^+$ motor neurons, only a predetermined subset is able to respond to GDNF by upregulating the ETS transcription factor PEA3. In conclusion, GDNF is an early limb-derived PEA3 regulator.

Discussion

Interactions between neurons and their target tissues have long been known to be important in regulating neuronal survival during the cell death period that follows target contact. However, there are growing indications that such interactions may also be relevant at earlier stages. For example, neurotrophic factors can act to enhance axonal outgrowth (Ebens et al., 1996; Tucker et al., 2001), and peripheral factors are required for correct differentiation of spinal motor pools (Lin et al., 1998). Here and in the companion paper by Livet et al. (2002), we describe a system of interplay between intrinsic and extrinsic factors that determines central positioning and axonal arborization of specific motor pools (summarized in Figure 8). Motor neurons intrinsically programmed to project toward the limb grow toward an intermediate target that represents a highly localized source of the neurotrophic factor GDNF. Only in response to peripheral GDNF do motor neurons express the ETS transcription factor PEA3. In the absence of GDNF signaling,

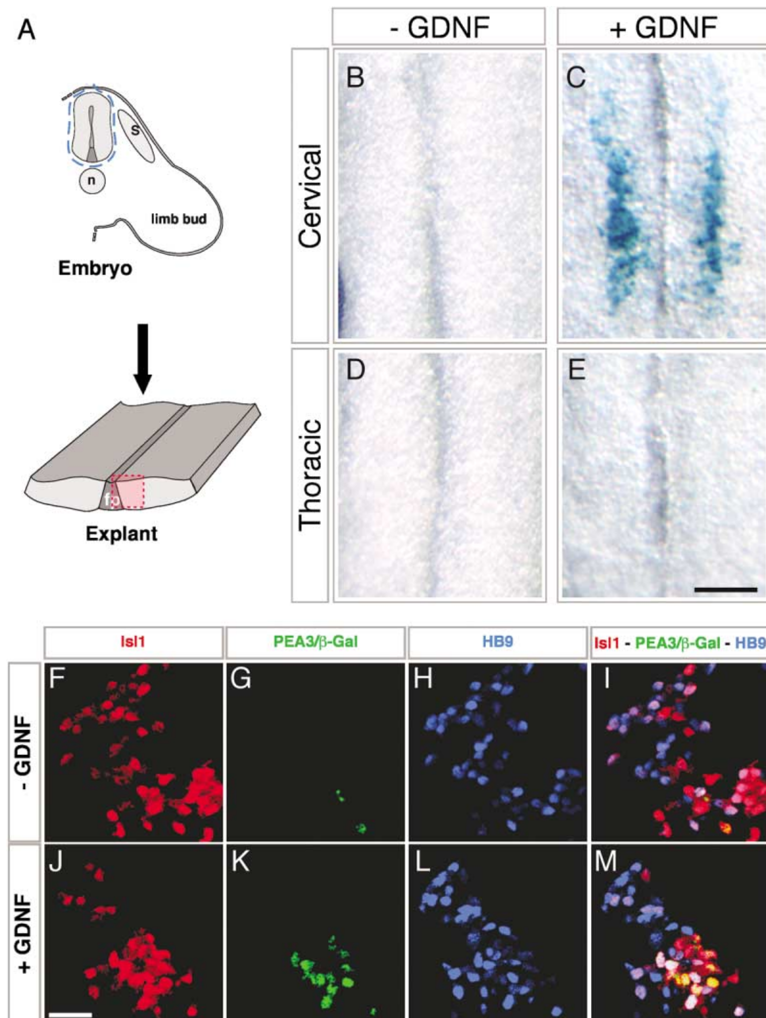

Figure 7. GDNF Is Able to Induce PEA3 Expression in Explant Cultures from Cervical Neural Tubes

(A) Schematic representation of neural tube dissection: neural tube explants were isolated from the 30–36 somite stage *Pea3*$^{+/nlslacZ}$ heterozygous embryos and flattened after being opened dorsally.

(B–E) Explants isolated from cervical (B and C) or thoracic (D and E) neural tubes were grown in the presence (C and E) or absence (B and D) of GDNF for 24 hr. PEA3^{+} cells (stained with X-Gal) were only detected in the presence of GDNF and were confined to a specific area within the cervical region.

(F–M) Transverse sections of the ventral region (red dashed line in [A]) of neural tube explants grown without (F–I) or with (J–M) GDNF and labeled with antibodies directed against Isl1 (in red), β-Gal (in green), and HB9 (in blue). In the presence of GDNF, motor neurons positive for PEA3 (β-Gal^{+}) are seen in the cervical region (K); these motor neurons are Isl1^{+}/HB9^{+} (J and L) and appear white in the merged image (M).

Scale bars: 250 μm in (B)–(E) and 25 μm (F)–(M).

PEA3 is not expressed; as a consequence, motor neurons settle in an abnormal position in the spinal cord and fail to project normally within their target area. Thus, peripheral factors control the transcriptional phenotype of specific motor neurons and thereby allow for correct cell body positioning and target innervation.

GDNF Is a Signal from the Limb Bud Regulating PEA3 Expression in Motor Neurons

The striking similarity of the mutant phenotypes for GDNF, GFRα1, and PEA3 provides strong genetic evidence that they are involved in the same pathway (Figure 8). First, we found no differences in the phenotypes of *Gdnf* and *Gfra1* cervical mutant spinal cords: GDNF signaling through GFRα1 is thus required for correct development of motor neurons of the LMC, in accordance with the requirement of GFRα1 for GDNF-mediated motor neuron survival in vitro (Garcès et al., 2000). Strikingly, embryos lacking *Pea3* (Livet et al., 2002) showed a similar disorganization of LMC motor neurons. The similarity is not only limited to the cell positioning defect, but also extends to the pattern of target innervation. The early death of *Gdnf* and *Gfra1* mutants (Moore et al., 1996; Cacalano et al., 1998) compared to *Pea3*$^{-/-}$ mice prevented us from comparing the postnatal phenotypes. However, deficits in early axon growth and in innervation of the LD and the CM muscles at different embryonic stages were closely similar in *Gdnf*, *Gfra1*, and *Pea3* mutants.

In the chick, limb-derived signals coordinately regulate the expression of ETS genes in motor neurons and sensory neurons as their axons approach the base of the limb (Lin et al., 1998). Since 90% of motor neurons in the *Gdnf*, *Ret* (data not shown), and *Gfra1* mutants never express *Pea3*, we further analyzed the possibility that GDNF might be the peripheral signal inducing *Pea3*. GDNF fits all the prerequisites expected for such a signal. First, GDNF synthesis in the forelimb bud starts as early as at the 28 somite stage before the onset of *Pea3* expression by motor neurons, which occurs at the 38 somite stage. Second, this source of GDNF is accessible to motor neurons that express the GDNF receptors before they initiate *Pea3* expression. Lastly, positive regulation of PEA3 by GDNF can be demonstrated in vitro. Altogether these results strongly suggest that GDNF is a prime candidate for being the limb-derived signal necessary for PEA3 induction.

GDNF induces PEA3 expression only in the region of

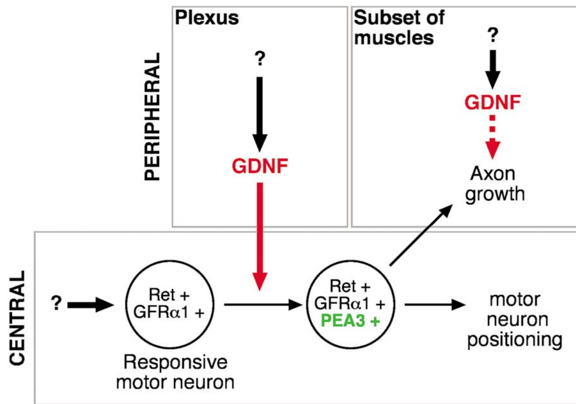

Figure 8. Model for GDNF Signaling through PEA3 in Specific Motor Neuron Pools

At early stages, peripheral GDNF produced by mesenchymal cells located around the brachial plexus induces PEA3 expression in a subset of cervical RET$^+$ GFRα1$^+$ motor neurons. This is a necessary step for controlling motor neuron positioning in the cord and axonal invasion of target muscles (Livet et al., 2002). Later on, GDNF expressed by cells within specific muscles might be required to maintain PEA3 expression and might directly contribute to axon growth and arborization. Question marks indicate as yet unidentified signals involved in predetermination of the subsets of motor neurons responsive to GDNF and in spatial restriction of GDNF expression in the periphery.

endogenous PEA3 expression, suggesting that at this time only subsets of motor neurons are predisposed to receive the signal for PEA3 induction. This is in agreement with chick neural tube reversal experiments, which demonstrate that the pattern of ETS gene expression by LMC motor neurons is specified between stages 13 and 15 (corresponding to about the 19–24 somite stages in the mouse) (Lin et al., 1998). Other regulatory elements must also be present to restrict this activity to the responding motor neurons either by repressing the effects of GDNF signaling in other motor neurons that express GDNF receptors or by functioning cooperatively with GDNF to induce *Pea3* only in appropriate motor neurons. Putative factors involved in the control of these processes are *Hox-c* genes whose expression is position dependent along the rostrocaudal axis of the spinal cord (Liu et al., 2001) and whose inactivation can lead to alterations in motor innervation of specific limb muscles (Tiret et al., 1998).

GDNF may not be the only signal involved in PEA3 regulation. In the absence of GDNF, *Pea3* was still expressed in many DRG neurons (data not shown). Moreover, about 10% of motor neurons still expressed PEA3 in *Gdnf* and *Gfra1* mutant spinal cords. These PEA3$^+$ motor neurons might be responsible, at least in part, for the small nerve branches observed in the mutant CM and LD muscles. Thus there are two classes of PEA3$^+$ motor neurons: the majority (90%) whose PEA3 expression is dependent on GDNF and the other 10% which express PEA3 independently of GDNF.

Restricted Expression of GDNF in the Environment of Growing Motor Axons

Expression of *Gdnf* before the period of motor neuron programmed cell death has previously been reported in the proximal part of the forelimb bud (Wright and Snider, 1996). Here, we visualized a tightly localized and dynamic expression pattern of GDNF. The factors responsible for the restricted expression of GDNF remain to be elucidated. Positive or negative regulator genes might be involved: Pax2 is necessary for expression of GDNF in the metanephric mesenchyme (Brophy et al., 2001) whereas the forkhead transcription factor Foxc1 is a negative regulator of *Gdnf* (Kume et al., 2000). GDNF expression started around the 28 somite stage (E10) in the forelimb bud shortly before the arrival of the C5–C8 motor nerves. At the 36–39 somite stages (E11), GDNF was expressed at high levels in the mesenchymal tissue in the plexus region; all motor axons of the brachial plexus should have access to this source of GDNF. What is the role of GDNF at this stage? A first possibility was that GDNF might be a chemoattractant for motor neurons growing toward the plexus. This is unlikely since at E11, a stage where axons reach the plexus, the initial route of axons into the plexus is not affected in *Gdnf* and *Gfra1* mutants. Since the limb plexus is known to serve as an intermediate target where motor neurons defasciculate before growing into the limb itself (Lance-Jones and Landmesser, 1981), a second possibility was that like commissural interneurons (Wang and Tessier-Lavigne, 1999), motor neurons depend on survival factors derived from intermediate targets and that GDNF is one of these factors. However, at E12.5, when many motor axons have already passed through the brachial plexus, neuronal numbers are not altered in *Gdnf* mutant spinal cords and cell death is not modified, making this hypothesis unlikely. Finally, since the plexus is also the region where individual motor neuron populations make their first target-related pathfinding decisions (for review, see Landmesser, 2001), it was possible that GDNF might be involved in control of target innervation. Our results suggest that GDNF may influence this process by inducing expression of genes such as *Pea3* and its downstream effectors that, in turn, control late steps in axon growth and arborization.

At stages E11–E12.5, individual nerves growing into the forelimb were no longer in contact with GDNF at their tips, whereas the nerves to the CM and the LD remained enveloped by GDNF. Since GDNF is required for *Pea3* expression (Figure 8), it is difficult to assess whether GDNF might have a PEA3-independent role in axon growth at these stages. However, there are several arguments in favor of GDNF not being only a *Pea3* inducer. First, GDNF expression at E11.5–E12.5 (i.e., after *Pea3* induction) is confined to the region around the growing nerves, which are affected in the mutants and thus might also be required for *Pea3* maintenance. Second, previous studies have indeed shown that in vitro GDNF can stimulate motor neuron neurite outgrowth (Zurn et al., 1996) and cause hyperinnervation of muscle targets in vivo (Nguyen et al., 1998), even in muscles that are not innervated by PEA3$^+$ motor neurons such as the sternomastoideus or the spinotrapezius (Keller-Peck et al., 2001; Livet, personal communication). This model is reminiscent of the association between regions of high neurotrophic factor expression and the positions of growing axons reported for NT-3 during peripheral nerve growth in the limb (Farinas et al., 1996). In addition, there are interesting parallels between our results and

those concerning the HGF/MET system. HGF has both growth-promoting and in vitro survival-promoting actions mainly on limb-innervating motor neurons that express higher levels of the MET receptor than non-limb-innervating thoracic motor neurons (Ebens et al., 1996; Yamamoto et al., 1997). Strikingly also, the same specific nerves as observed in this study are absent in two MET signaling mutant mice (Maina et al., 1997). It will be of great interest to determine how GDNF and HGF signaling act together or sequentially to control the innervation of these muscles.

Lack of GDNF Leads to Localized Misorganization of Motor Pools in the Spinal Cord

In the absence of GDNF, there is no reduction in overall motor neuron numbers over the period we have studied and no increase in TUNEL$^+$ cells in the corresponding motor pools. However, there is a disorganization of the cervical spinal cord that strongly affects PEA3$^+$ motor neurons belonging to the LMCm. In this region, fewer Isl1$^+$ motor neurons than normal are observed in positions normally corresponding to the LMCm, whereas higher numbers are observed in a more dorsolateral position. The changes in the organization of the cervical spinal cord in the mutants correspond phenotypically to a mispositioning of motor neuron cell bodies affecting both the lateral and the medial part of the LMC, meaning that not only PEA3$^+$ motor neurons are affected. Given the fact that LMCm motor neurons can regulate the fate of LMCl motor neurons (Sockanathan and Jessell, 1998), two hypotheses might be put forward to explain this aspect of the *Gdnf* knockout phenotype. (1) LMCm motor neurons remain in their normal position, do not express *Pea3* and *Isl1*, and fail to induce the LMCl phenotype (extinction of *Isl1*) in late-born neurons. (2) In the absence of PEA3, LMCm motor neurons settle in an abnormal dorsal position in the spinal cord where they keep their molecular LMCm identity, and this leads secondarily to mispositioning of LMCl neurons. While both hypotheses fit with our observations, we favor the latter hypothesis (Figures 5K and 5L) since in *Pea3* mutants (Livet et al., 2002), β-Gal$^+$ motor neurons are dorsally mispositioned at E12.5, and a late stage of pool segregation within LMCm and LMCl motor neurons does not occur. Since Type II cadherins, which are deregulated in *Pea3*$^{-/-}$ mutants (Livet et al., 2002), control motor pool segregation in the developing chick spinal cord (Price et al., 2002), the mispositioning of motor pools observed in *Gdnf*$^{-/-}$, and *Gfra1*$^{-/-}$ mutant spinal cords might result from a deregulation of Type II cadherin expression downstream of *Pea3*.

In summary (Figure 8), GDNF, initially discovered through its role as a survival factor at later stages, is synthesized from early stages in the brachial plexus around the converging spinal nerves and induces PEA3 expression in a restricted prespecified subset of motor neurons. In the absence of GDNF signaling, these motor neurons do not differentiate properly, their cell bodies are mispositioned within the spinal cord, and the invasion and branching of motor axons within their target muscles is perturbed. At all stages analyzed, these changes are very similar to those observed by Livet et al. (2002) in *Pea3* mutant mice. We have therefore defined a molecular pathway by which GDNF acts through PEA3 to control the central positioning of motor neuron cell bodies as well as the growth and arborization of their axons.

Experimental Procedures

Animals and Genotype Analysis

Heterozygous *Gfra1*$^{+/-}$ (Cacalano et al., 1998) and *Gdnf*$^{+/-}$ (Moore et al., 1996) mice, generously provided by A. Rosenthal (Genentech, Inc., South San Francisco), were backcrossed to C57Bl/6 or CD1 mice, respectively, for at least six generations and mated to obtain homozygous or heterozygous embryos; wild-type embryos from the same litters were used as controls. The day of the positive vaginal plug was recorded as E0.5. Genotype analysis of *Gfra1* mutant embryos was performed by PCR as described (Garcès et al., 2000); *Gdnf*$^{+/-}$, *Gdnf*$^{-/-}$, and wild-type embryos were distinguished by PCR using primers in *Gdnf* (5′-ATTTTATTCAAGCCACCATT and 5′-TGCCTCTGCCTCCGCCATCT) or in *nlslacZ* (5′-CGCATCGTAACCGTGCATCTGCCAGTTTGA). Heterozygous *Pea3*$^{+/nlslacZ}$ embryos were genotyped as described by Livet et al. (2002). Transgenic *MLC 3F nlslacZ* mice (Kelly et al., 1995) were used as homozygotes.

Immunohistochemistry

Cryostat sections were processed for immunohistochemistry studies as described (Tsuchida et al., 1994). Primary antibodies were used at the following dilutions: rabbit anti-Isl1/2 serum, 1:2500 (Tsuchida et al., 1994); mouse monoclonal anti-Isl1/2, 1:100 and 1:500 (4D5, 2D6, Developmental Hybridoma bank); guinea pig anti-Isl1, 1:1000; rabbit anti-PEA3, 1:500 and rabbit anti-HB9, 1:1600 (Arber et al., 1999); and mouse anti-β-Gal, 1:1000 (Promega). Alexa-488-, CY3- and CY5-coupled secondary antibodies were obtained from Jackson Laboratory. Images were collected on a Zeiss Axiophot microscope, a Zeiss LSM 410, or a Leica confocal microscope and superimposed in Adobe Photoshop to identify double- and triple-labeled cells.

In Situ Hybridization

In situ hybridization was performed on 16 or 20 μm thick frozen sections or in whole-mount spinal cords as described previously (Garcès et al., 2001). The *Gfra1* and *Ret* clones were previously described (Garcès et al., 2000). Double in situ hybridization/immunohistochemistry was carried out as described (Carroll et al., 2001). Plasmid clones for rat *Isl1* and mouse *Raldh2*, *Pea3*, and *Cadh7* were kindly provided by T.M. Jessell. The rat *Gdnf* clone was from P. Ernfors, the mouse *Pax3* clone was from P. Charnay, the mouse *MyoD* and *myogenin* clones were from M. Buckingham, the *nlslacZ* clone was from P. Carroll, and the mouse *Sox10* clone was from R. Wegner.

Analysis for β-Galactosidase Activity

X-Gal staining of whole-mount embryos was performed according to standard techniques. After staining, individual muscles were dissected out and photographed. The cervico-thoracic region was taken out, fixed for 3 hr in 2.5% glutaraldehyde and for 2 hr in 1% osmium tetroxide, and embedded in epoxy resin (Embed 812, Electron Microscopy Science, Ft. Washington, PA). Electron microscopy of thin sections was performed as described (Sorensen et al., 1998).

Motor Neuron Cell Counts

In E12.5 embryos, cervical dorsal root ganglions were identified, and the fourth cervical spinal cord segment was labeled by injection of Coomassie blue (Sigma) before embedding and cutting of 10 μm cryosections. Isl1/2 and PEA3 immunoreactive motor neurons were counted on both sides of the spinal cord on every third section; no correction was made for split nuclei. The same number of sections was counted for wild-type, *Gdnf*$^{+/-}$ and *Gdnf*$^{-/-}$ spinal cords throughout the cervical segments C5–C8 where the PEA3$^+$ motor neurons are normally located.

Innervation Analysis

Whole-mount NF staining was performed as described (Maina et al., 1997). For combined X-Gal/NF staining, embryos were fixed in 4% PFA/PBS for 1 hr at 4°C, X-Gal stained, washed in PBS, postfixed for 30 min, and then processed for whole-mount NF staining. For NF/synaptophysin labeling of nerve-muscle preparations, E17.5 embryos were fixed in 4% PFA/PBS, and the CM and LD were dissected out together with their nerves. After postfixation, the preparations were incubated overnight with anti-NF-145 kDa (Chemicon Ab1987, 1:300) and anti-synaptophysin (Boehringer, clone SY-38, 1:200) antibodies and then incubated with secondary antibodies.

Culture of Neural Tube Explants

Neural tube explants were dissected at cervical and thoracic levels from the 30–36 somite stage embryos and cultured in a collagen matrix as described (Yamada et al., 1993) in Neurobasal medium (Life Technologies) supplemented with B27 supplement (Life Technologies), 0.5 mM L-glutamine, 10% fetal calf serum, 4 mM glucose, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Recombinant NT3 (10 ng/ml, R&D) was added in every culture as neurotrophic support. Explants were cultured for up to 24 hr with or without recombinant GDNF (10 ng/ml, R&D) and then removed from the collagen matrix, fixed for 30 min at 4°C in 4% PFA/PBS, and X-Gal-stained or prepared for cryosectioning and immunohistochemistry. Nineteen neural tube explants at both levels were analyzed in the presence of GDNF and 16 in the absence of GDNF.

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