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Met Signaling Is Required for Recruitment of Motor Neurons to PEA3-Positive Motor Pools

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Summary

Motor neurons in the spinal cord are grouped into motor pools, each of which innervates a single muscle. The ETS transcription factor PEA3 is a marker of a few such motor pools. Here, we show that *pea3* is first induced by GDNF in a caudal subset of the motor neurons that will constitute the *pea3*$^+$ population. Expansion of the *pea3* domain subsequently occurs by recruitment of neurons from more anterior segments. Signaling by Met, the HGF receptor, is required for the rostral expansion of the *pea3* domain, while the onset of *pea3* expression is independent of *met* function. *met* expression is observed in pioneer neurons but does not precede that of *pea3* in recruited neurons. We provide genetic evidence for a non-cell-autonomous function of *met* during the recruitment process. We propose the presence of a relay mechanism allowing cells induced by peripheral signals to recruit more anterior neurons to adopt the same motor pool-related phenotype.

Introduction

Patterning of the vertebrate CNS involves the generation of distinct classes of neurons, differing in neurotransmitter phenotype, cell body position and form, and axonal trajectory. These differences reflect the complexity of neuronal function, which can vary on a cell-to-cell basis, but also needs to be coordinated at the level of neuronal populations. Developmental signals must therefore trigger not only differentiation but also to a certain extent homogenization of neuronal fates.

Somatic motor neurons are a good example of both functional commonality, since all are involved in the contraction of skeletal muscle, and functional diversity, given the variety in the nature and position of their target muscles (Jessell, 2000). They are grouped in columns occupying stereotyped positions in the spinal cord that correlate with the position of their target muscles (Jessell, 2000; Landmesser, 1978). Motor neurons in the lateral motor columns (LMCs) selectively innervate limb muscles, while the medial motor columns (MMCs) contain motor neurons innervating axial and body wall muscles. Thus, the variety of muscle locations in the periphery requires that axons of each motor pool select a distinct trajectory. The anatomical segregation of motor columns correlates with the expression of distinct combinations of transcription factors of the LIM or forkhead families (Dou et al., 1997; Lin et al., 1998; Tsuchida et al., 1994). This combinatorial expression of transcription factors is induced by signals from both central and peripheral sources and controls the expression of many effectors of a given motor neuron subtype identity (reviewed in Jessell, 2000).

Within each motor column, the motor neurons innervating a given muscle are grouped within the spinal cord to form an anatomically distinct cluster, called a "motor pool" (Landmesser, 1978). For innervation of target muscles to occur in a precise manner, motor neurons of each pool should have molecular and functional properties that distinguish them from other pools, and they should have other common properties that lead them to behave as a group (Lance-Jones and Landmesser, 1980, 1981). Common properties potentially involved at different stages are electrical activity (Milner and Landmesser, 1999), electrical coupling by gap junctions (Personius et al., 2001), and the expression of receptors for axon guidance molecules (Shirasaki and Pfaff, 2002) or for specific neurotrophic factors (Garces et al., 2000; Oppenheim et al., 2000). Even more strikingly, transcription factors of the ETS family, such as PEA3, ER81, and TEL, are expressed by all motor neurons within certain individual motor pools in the spinal cord (Lin et al., 1998), and each motor pool expresses a characteristic combinatorial pattern of type II cadherins (Price et al., 2002).

pea3 is not only a marker for pools of motor neurons; it is also tightly regulated by peripheral interactions and required for normal innervation of the target. Thus, limb-derived signals were shown to be necessary for expression of *pea3* in embryonic chicken motor neurons (Lin et al., 1998). Recent reexamination of *gdnf* and *gfra1* knockout mice showed that the neurotrophic factor GDNF is one of the peripheral factors involved (Haase et al., 2002). Brachial motor neurons expressing PEA3 contribute axons to two nerves exiting the brachial plexus, the thoracodorsalis and medial anterior thoracic nerves, which innervate the *latissimus dorsi* (LD) and the *cutaneous maximus* (CM) muscles, respectively (Livet et al., 2002). In the absence of PEA3, resulting from inactivation of either *gdnf* or *pea3* itself, growth of these two nerves into the muscles was severely affected. The corresponding motor neurons did not die but were mispositioned within the spinal cord.

Two potential downstream targets of PEA3, *cadh-*

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erin-8 and *semaphorin3E*, were identified in these studies (Livet et al., 2002), and other cadherins have been shown to be induced by ETS genes (Livet et al., 2002; Price et al., 2002). It is likely that cadherins play a role in motor pool development downstream of GDNF and PEA3, since their combinatorial expression can direct the segregation and positioning of motor pools in the spinal cord. Similarly, the axonal guidance receptor EphA4, which is crucial for establishing the dorsal trajectory of lateral LMC axons in the limb, is a target of the transcription factor LIM-1 (Helmbacher et al., 2000; Kania and Jessell, 2003; Kania et al., 2000). Nevertheless, it remains unclear whether the nerve phenotype of *gdnf*$^{-/-}$ and *pea3*$^{-/-}$ embryos results directly from a loss of PEA3 function or whether it is a secondary consequence of mispositioning (Haase et al., 2002; Livet et al., 2002).

The peripheral nerve phenotypes of *gdnf* and *pea3* knockouts are strikingly similar to the previously described phenotype of mutants of the HGF receptor, the Met tyrosine kinase (Maina et al., 1997; Maina et al., 2001). Met is expressed in subpopulations of brachial and lumbar motor neurons, and HGF, which is produced by limb mesenchyme, was previously shown to promote axonal growth and survival of subpopulations of motor neurons in vitro (Ebens et al., 1996; Yamamoto et al., 1997). This suggested that the HGF/Met system too might be involved in development of the *pea3*-expressing neurons but left open the question of whether the actions of HGF might be on early differentiation, like GDNF, or on later processes, such as target innervation or survival.

Here, therefore, we have examined the function of *met* in the *pea3*-expressing population. To our knowledge, we describe a hitherto unknown process of motor neuron recruitment that is required to attain the final number of PEA3-expressing neurons and show that this recruitment is impaired in *met*-deficient embryos. We show that after induction of *pea3* in an initial pool of motor neurons by other limb-derived factors, such as GDNF, HGF promotes the recruitment of other motor neurons to the *pea3* pool, in a non-cell-autonomous manner. There is thus a remarkably complex interplay of peripheral and central factors in the establishment of a given motor unit.

Results

Met Is Required for Expression of *pea3* in Brachial Motor Neurons

The resemblance of the peripheral nerve phenotype of *pea3*$^{-/-}$ and *gdnf*$^{-/-}$ mutants to that of *met*$^{d/d}$ embryos, in which mutation of the cytoplasmic multifunctional docking site impairs signaling by the Met receptor (Maina et al., 1996), led us to investigate the PEA3^{+} motor neuron populations in *met*$^{d/d}$ mice. Inspection of *pea3* expression by in situ hybridization (ISH) in E11.5 spinal cords showed a significant reduction of the domain of *pea3* expression (Figures 1A and 1B). Quantification of PEA3^{+} nuclei on serial sections of spinal cords of E11.5 (49 somites) wild-type and *met*$^{d/d}$ embryos after immunocytochemisty with anti-PEA3 antibody showed that the PEA3^{+} pool was overall reduced by 40% (Figure 2H).

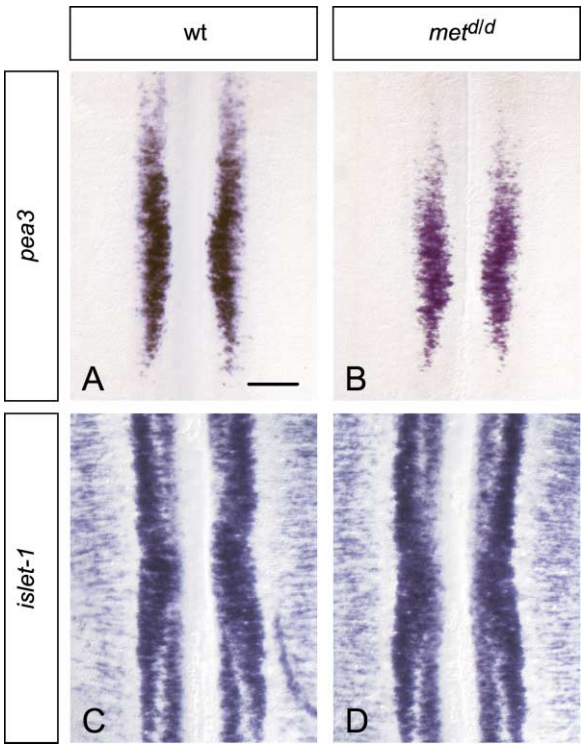

Figure 1. Met Signaling Is Required for Expression of PEA3 in a Subset of Brachial Motor Neurons

(A and B) Analysis by ISH of E11.5 (49 somites) flat-mounted spinal cords using a *pea3* probe, showing that the *pea3* expression domain was shortened in *met*$^{d/d}$ spinal cords. The midline and ventral structures are in the center, while dorsal structures are on either side of the pictures. Scale bar, 150 μm.

(C and D) *Islet-1* ISH on whole-mount wild-type (C) and *met*$^{d/d}$ (D) E11.5 (49 somites) spinal cords, showing similar levels of *islet-1* expression in the C5-C8 region.

To determine whether the reduction of the *pea3*$^{+}$ domain reflected a loss of neurons or a failure to express PEA3, we looked for effects on *islet-1*, which is also expressed by this population (Figure 2D; Livet et al., 2002). Using whole-mount ISH, no difference in *islet-1*$^{+}$ motor pools was observed between wild-type and *met*$^{d/d}$ embryos at E11.5 (49 somites; Figures 1C and 1D). Consistent with this, quantification of the number of Isl1/2^{+} motor neurons on E11.5 spinal cord sections corresponding to the brachial *pea3* domain (C5 to C8, see later) showed no difference between wild-type and *met*$^{d/d}$ embryos (Figure 2G). Accordingly, at the same stage, there was no increase in the number of TUNEL-positive cells in the C5-C8 region of *met*$^{d/d}$ compared to wild-type spinal cords (data not shown). These results indicate that the reduction of *pea3* expression domain in the absence of Met signaling is not caused by the death of motor neurons but rather suggest that a subset of neurons of the *pea3* domain fail to turn on PEA3 expression.

To determine the specificity of this motor neuron phenotype in *met*$^{d/d}$ mutants, we examined the expression of other motor neuron-specific markers at E12.5. At this stage, the reduction in *pea3* expression was even more pronounced (Supplemental Figures S1A and S1B avail-

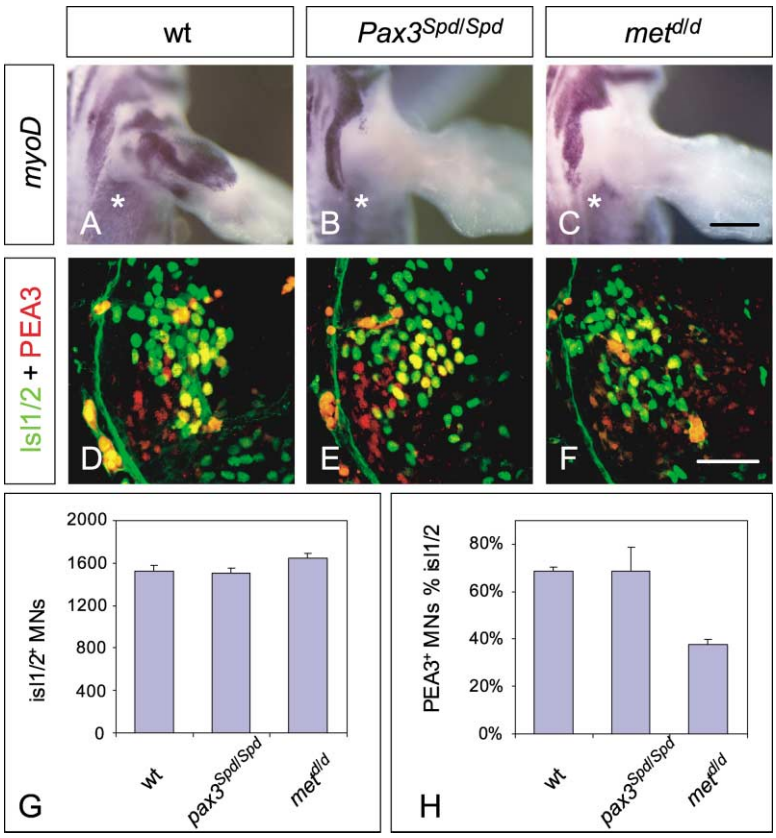

Figure 2. The Establishment of *pea3* Expression Is Independent of Muscles

(A–C) Whole-mount *MyoD* ISH showing the forelimb musculature of E12.5 wild-type (A), *pax3*$^{Spd/Spd}$ (B), and *met*$^{d/d}$ (C) embryos. Limbs of *pax3*$^{Spd/Spd}$ and *met*$^{d/d}$ embryos are similarly devoid of all migratory muscles. White asterisk indicates the cutaneus maximus (CM) muscle, present in (A) and absent in both mutants (B and C). Scale bar, 0.6 mm (A–C).

(D–F) Double immunofluorescence of wild-type (D), *pax3*$^{Spd/Spd}$ (E), and *met*$^{d/d}$ (F) E11.5 (49 somites) spinal cords with PEA3 (red) and Isl1/2 (green) antibodies. While the number of Isl1/2 motor neurons is unchanged in *pax3*$^{Spd/Spd}$ and *met*$^{d/d}$ spinal cords, the number of PEA3^{+} motor neurons is reduced in *met*$^{d/d}$ but not in *pax3*$^{Spd/Spd}$ spinal cords. Scale bar, 40 μm (D–F).

(G–H) Isl1/2+ and PEA3+ motor neurons were quantified by immunohistochemistry on serial sections. While the number of Isl1/2 motor neurons in the C5-C8 region of wild-type, *pax3*$^{Spd/Spd}$, and *met*$^{d/d}$ spinal cords is not significantly different (G), there is an overall 40% reduction of the number of PEA3^{+} neurons in *met*$^{d/d}$ (t test, $p < 0.00004$) but not in *pax3*$^{Spd/Spd}$ spinal cords (significantly different from *met*$^{d/d}$; t test, $p < 0.00002$). For each stage and genotype, three spinal cords were serially sectioned, stained with Isl1/2 and PEA3 antibodies, and the number of stained cells was counted and pooled to reconstitute the PEA3 population. Left and right sides were considered separately. Error bars, SEM.

able online at http://www.neuron.org/cgi/content/full/39/5/767/DC1), but no changes could be detected in the expression of either the transcription factor *scip*, which labels a neighboring subpopulation of LMC motor neurons (Supplemental Figures S1C and S1D) or of *Ret* in *pea3*-negative columns of LMC motor neurons (Supplemental Figures S1E and S1F). Thus, the *met*$^{d/d}$ phenotype is specific to the *pea3*-expressing motor neurons and does not affect other LMC motor pools.

The Establishment of *pea3* Expression Is Independent of Muscle

Our results showed that *met* is required for normal PEA3 expression by motor neurons. However, the *met* mutation also impairs myoblast precursor migration at limb levels, leading in *met*$^{d/d}$ embryos to a complete absence of all migratory muscles, including the two target muscles of *pea3*$^{+}$ neurons (the LD and CM muscles) (Maina et al., 1996; C. Ponzetto, personal communication). At E11.5, when we already observed defects in PEA3 expression, myoblasts are still migrating from the somites to their final destination (Figure 1). Although this made it unlikely that changes in PEA3 resulted from defects in contact with target muscle, we needed to rule out the possibility that they might be secondary to the absence of migrating myoblasts.

We therefore analyzed *pea3* expression in mutants that affect myoblast precursor migration without altering *met* functions in the spinal cord. The *Splotch* and *Splotch-delayed* spontaneous mutations of the *pax3* gene were previously shown to lead as well to a complete absence of migratory muscles, including the LD and CM muscles (Bober et al., 1994; Epstein et al., 1991; Franz et al., 1993). ISH using a *myoD* probe on E12.5 embryos showed that the muscle phenotype in *pax3*$^{Sp/Sp}$ or *pax3*$^{Spd/Spd}$ embryos was as severe as in *met*$^{d/d}$ embryos (Figures 2A–2C). Spinal cords of E11.5 (49 somites) *pax3*$^{Spd/Spd}$ and *met*$^{d/d}$ embryos were serially sectioned and analyzed by immunocytochemistry using anti-PEA3 and anti-Isl1/2 antibodies (Figures 2D–2H). Despite their identical limb muscle defect, we found that, in contrast to the 40% reduction observed in *met*$^{d/d}$ embryos, *pax3*$^{Spd/Spd}$ embryos showed no significant reduction in the number of PEA3^{+} motor neurons (Figure 2H). However, at later stages, as expected, the absence of target muscle led to massive cell death of all LMC motor neurons in both mutants (Supplemental Figure S1G–S1L, and data not shown), further reinforcing the specificity of the early muscle-independent role of *met*.

Biphasic Development of PEA3 Expression in Brachial Spinal Cord

Our results indicated that *met* was required for a subset of motor neurons of the *pea3* domain to express PEA3. However, we recently showed that GDNF was the limb-derived factor that induced *pea3* (Haase et al., 2002). To better characterize their respective roles, we examined the normal spatial development of the *pea3* expression domain at brachial levels in mouse embryos using the *pea3*^{NLZ} allele, which faithfully drives lacZ expression at normal sites of *pea3* expression (Livet et al., 2002). On flat-mounted spinal cord preparations of *pea3*$^{NLZ/+}$

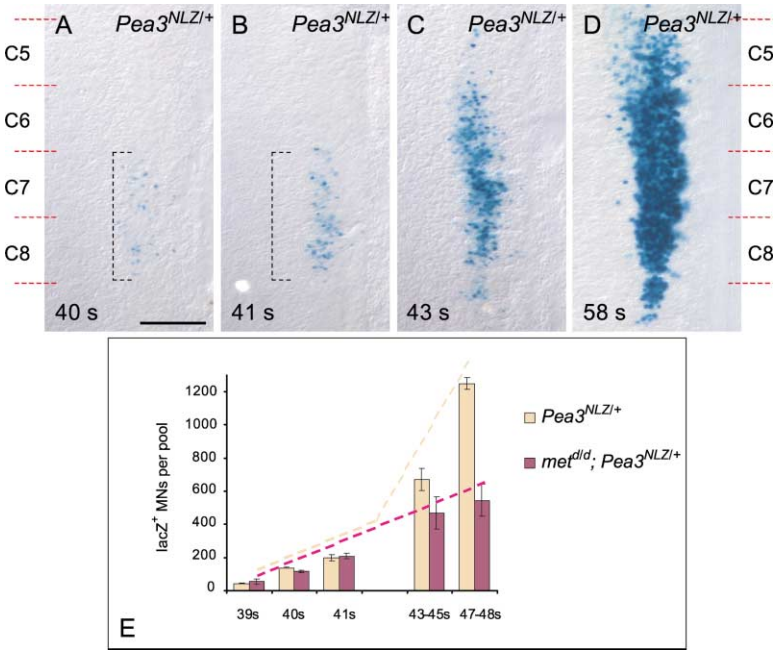

Figure 3. Met Signaling Is Required for Normal Expansion of PEA3 Expression

(A–D) The spatial evolution of *pea3-lacZ* expression in *pea3*$^{NLZ/+}$ embryos was analyzed on flat-mounted spinal cord preparations. Pictures are centered on the *pea3* pool on the left side of the spinal cord. The midline is on the right, and dorsal structure is on the left. The position of the C5-C8 spinal segments is indicated. Before 43 somites, *lacZ*$^{+}$ neurons are confined to the C7-C8 segment. Note the anterior spreading of *pea3-lacZ* expression starting from 43 somites. Scale bar, 150 μm.

(E) Evolution of the number of β-galactosidase-positive motor neurons per *pea3* motor pool in *pea3*$^{NLZ/+}$ and *met*$^{d/d}$; *pea3*$^{NLZ/+}$ spinal cords between E10.5 (39 somites) and E11.5 (49 somites). For each stage and genotype, at least three spinal cords were stained for β-galactosidase activity, serially sectioned, and the number of stained cells was counted and pooled to reconstitute left and right *pea3* pools. No significant difference in the number of β-gal^{+} motor neurons was detected between *pea3*$^{NLZ/+}$ and *met*$^{d/d}$; *pea3*$^{NLZ/+}$ embryos before 43 somites. At 43/45 somites and later, *met*$^{d/d}$; *pea3*$^{NLZ/+}$ spinal cords contained significantly fewer *pea3-lacZ*-positive neurons compared to *pea3*$^{NLZ/+}$ spinal cords. Dashed lines indicate gross evolution of β-gal^{+} neuron numbers in both genotypes. While in *pea3*$^{NLZ/+}$ spinal cords the rate of increase switches from a slow to a rapid phase, *met*$^{d/d}$; *pea3*$^{NLZ/+}$ embryos maintain the initial slow rate of increase. t test: 43–45 somites, $p < 0.05$; 47–48 somites, $p < 0.00002$.

embryos, *pea3* expression was first detected at E10.5 (38 somites), as reported by Haase et al. (2002), in a few motor neurons in a restricted domain spanning the C7-C8 spinal segments (Figure 3A). Up to the 42 somite stage, the number of β-gal^{+} cells increased, but the *pea3*$^{+}$ domain remained restricted to C7-C8 (Figure 3B). In contrast, from the 43 somite stage onward, addition of β-gal^{+} cells was observed in more anterior positions, resulting in a progressive anterior spreading of *pea3* expression (Figure 3C). The *pea3*$^{+}$ column reached its maximal extent at E12.5 (58 somites), when it spanned the C5-C8 spinal segments (Figure 3D). The increase in number of *pea3*$^{+}$ neurons also resulted in a densification of the *pea3* pool at the C7-C8 level. Over the same period, the total number of PEA3^{+} neurons was counted on serial sections (Figure 3E). Numbers increased slowly up to the 42 somite stage and then increased more steeply up to 48 somites. There are thus two phases in the evolution of *pea3* expression. After initial demarcation of a restricted pool of *pea3*$^{+}$ cells in C7-C8, additional neurons turn on *pea3* both at the same level and in more anterior segments, resulting in the rostral spreading and overall expansion of *pea3* expression.

met Is Required for Spreading of *pea3* Expression but Not for Its Onset

We next compared the number of *pea3*-expressing motor neurons in *pea3*$^{NLZ/+}$ and *pea3*$^{NLZ/+}$; *met*$^{d/d}$ spinal cords over the same period. Using stage-matched embryos, we found that the first phase of *pea3* expression in C7-C8 was normal in *met*$^{d/d}$ embryos (Figure 3E), demonstrating that initial induction of *pea3* expression is independent of *met* function. However, from the 43/45 somite stage onward, the increase in the number of β-gal^{+} motor neurons in *met*$^{d/d}$; *pea3*$^{NLZ/+}$ embryos failed to switch from its linear rate to the exponential rate observed in wild-type spinal cords (Figure 3E), resulting in a 40% reduction at 48 somites. The slow rate of increase in the number of β-gal^{+} neurons in *met*$^{d/d}$ embryos, suggests that this phenotype reflects a lack of induction rather than lack of stabilization of *pea3* expression.

We examined the precise anteroposterior location of the remaining *pea3* neurons on serial sections of spinal cords of E11.5 embryos (49 somites) using unambiguous morphological landmarks (see Experimental Procedures). PEA3^{+} nuclei were counted in wild-type and *met*$^{d/d}$ embryos after immunocytochemistry with anti-PEA3 antibody. Strikingly, although the PEA3^{+} pool was overall reduced by 40% (see Figure 2H), numbers at C7 and C8 were less strongly affected (Figure 4). In contrast, the anterior segments C5 and C6 showed a massive reduction in the number of PEA3-positive motor neurons (Figure 4). This observation is consistent with our initial observation by ISH of the shortening of the domain of *pea3* expression along its anteroposterior extent in E11.5 *met*$^{d/d}$ spinal cords (Figures 1A and 1B). In wild-type embryos, spreading also occurs at the C7 and C8 levels but only at later stages, leading to the thickening of the *pea3* domain in these two segments at E12.5. Observation of *pea3* ISH at E12.5 revealed that spreading at C7-C8 levels was also compromised in *met*$^{d/d}$ spinal cords (Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/39/5/767/DC1).

Initial activation of *pea3* expression in a pioneer subpopulation in C7-C8 is thus independent of *met*, consistent with the idea that GDNF is the limb-derived factor that initially induces *pea3* (Haase et al., 2002). Subsequently, a second group of neurons acquires *pea3* expression in a *met*-dependent manner during the second (exponential) phase of expansion.

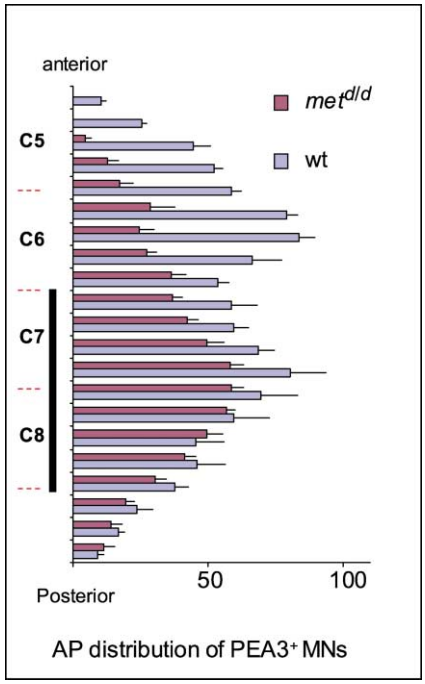

Figure 4. Loss of PEA3 Expression in *met*$^{d/d}$ Spinal Cords Is Confined to Neurons from C5-C6

The number of *pea3*$^{+}$ neurons was quantified by immunohistochemistry on serial sections along the anteroposterior axis of the brachial spinal cord, in three embryos of each genotype, left and right sides being considered separately. DRGs C7 and C8 are indicated as a black bar along the positional axis (vertical). The more anterior the sections, the greater the reduction in the number of *pea3*$^{+}$ neurons. t test: at anterior C7 levels, $p < 0.05$; at anterior C6 and C5 levels, $p < 0.0001$. Error bars, SEM.

HGF Cooperates with GDNF to Induce *pea3* in Spinal Cord Explants

Our results suggested that HGF might either have the capacity to induce *pea3* expression in brachial motor neurons or to cooperate with GDNF to shape the *pea3* domain. To test whether HGF has such an inductive effect, we used in vitro culture of fragments of spinal cords containing the prospective *pea3* region and isolated from *pea3*$^{NLZ/+}$ embryos at stages (30–37 somites) preceding the onset of *pea3* expression. We compared the effects of growing the explants alone or in the presence of GDNF or HGF, for a period of 30–48 hr. *pea3* expression was not detected in motor neurons in explants grown alone (Figure 5A). After 48 hr, irrespective of the treatment, a very low level of *LacZ* expression was occasionally detected in a subset of dorsal neurons, always recognizable by their position. These dorsal neurons were disregarded in our quantification. GDNF treatment induced *pea3* expression (visualized by X-gal staining) in numerous motor neurons, mostly concentrated in a region of the explant corresponding to the segments C7-C8 ($n = 8$, Figure 5C), consistent with its effects after 24 hr (Haase et al., 2002). In contrast, HGF alone failed to induce *pea3* ($n = 16$, Figures 5B and 5E). To test for cooperation between HGF and GDNF, the left and right sides of each spinal cord explant were cultured separately, with GDNF alone or both HGF and GDNF. We found that addition of HGF with GDNF induced 2-fold more *pea3*$^{+}$ motor neurons compared to GDNF alone ($n = 7$, Figures 5D and 5E). In most explants, this increase corresponded to a thickening of the group of *pea3*$^{+}$ neurons in the prospective C7-C8 region. In some cases, a clear rostral spreading in more anterior segments of the explant (C5-C6) was also observed (Figure 5D).

met Expression in the Caudal *pea3* Domain Is Induced by GDNF and PEA3

Whereas HGF had no *pea3*-inducing activity on its own, it efficiently enhanced the response to GDNF. This suggested that GDNF enables some neurons to respond to HGF. We therefore asked whether *met* was coexpressed with *pea3* and whether this expression was altered in *gdnf*$^{-/-}$ mutants by performing double ISH for *met* and *pea3* on whole-mount spinal cords from wild-type and *gdnf*$^{-/-}$ E11.5 embryos (49 somites). In wild-type spinal cords, *met* was coexpressed with *pea3* in C7-C8 neurons (Figure 5F) but not in the anterior part of the *pea3* domain (see later). In *gdnf*$^{-/-}$ mutants, in the prospective domain of *pea3*/*met* coexpression, the number of *met*-expressing neurons was severely reduced (Figure 5G), indicating that GDNF is required for *met* expression in these neurons. Sites of *met* expression outside the *pea3* domain were all unaffected in *gdnf*$^{-/-}$ spinal cords (note in particular *met* expression in motor neurons just anterior to the prospective *pea3* domain [arrow in Figures 5F–5H]). Other effects of GDNF on these motor neurons are mediated through PEA3. Examination of *pea3*$^{-/-}$ spinal cords at E11.5 showed that *met* expression in the presumptive area of *pea3*/*met* coexpression was also severely reduced, while all other sites of *met* expression appeared again normal (Figure 5H), confirming that PEA3 is also required for *met* expression and reinforcing the idea that *pea3* mediates the induction of *met* by GDNF, thus allowing HGF to act on these cells.

Met Is Not Expressed in Recruited Neurons

To better understand how GDNF and HGF might control the establishment of *pea3* expression domain, we analyzed in detail the spatial evolution of *met* and *pea3* expression during the spreading period, by double ISH on whole-mount spinal cords. At 41 somites, *met* was clearly detected in MMC motor neurons but not in the *pea3*$^{+}$ domain (Figure 6A, arrow), and most of the LMC in the C5-C8 spinal segments was largely devoid of *met* expression. At the 44 somite stage, a few neurons in the posterior-most part of the *pea3*$^{+}$ domain (at the level of C8) started to express *met* (Figure 6B). The number of neurons coexpressing *met* and *pea3* rapidly increased between the 47 and 52 somite stages, resulting in an anterior progression of the anterior limit of *met* expression within the *pea3*$^{+}$ domain (Figures 6C–6E). However, although *met* expression progresses anteriorly from the 44 to 52 somite stages, nearly all anterior-most *pea3*$^{+}$ neurons in C5-C6 are devoid of *met* expression over this period (Figures 6B and 6C). Moreover, delimitation of the prospective domain of *pea3* expression (black dotted line in Figures 6A–6D, defined by *pea3* expression at E12.5, see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/39/5/767/DC1) showed

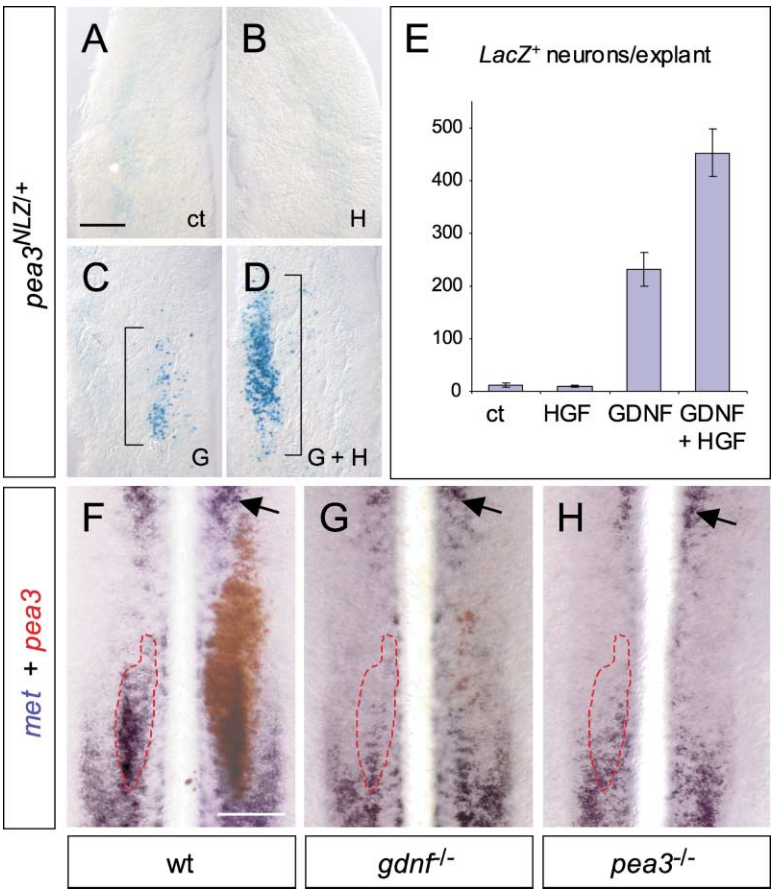

Figure 5. HGF/GDNF Cooperation to Induce *pea3*: GDNF Enables *pea3*$^+$ Neurons to Respond to HGF by Activating *met* Expression

(A–E) C4-T1 explants isolated from 30–37 somite stage *pea3*$^{NLZ/+}$ embryos were grown alone (A) or in the presence of HGF (B), GDNF (C), or GDNF + HGF (D). *pea3-lacZ* expression was detected by β-galactosidase staining. (E) These effects were quantified by counting the number of β-gal$^+$ motor neurons per explant. For each condition, at least seven explants were quantified. Contrary to GDNF (C), which efficiently induces *pea3* expression ($p < 0.00004$), HGF (B) does not have any *pea3*-inductive capacity. However, HGF enhances the inductive effect of GDNF (D) by 2-fold ($p < 0.0017$). Error bars, SEM. Scale bars, 150 μm in (A–D).

(F–H) Analysis of *met* (blue) and *pea3* (red) expression in the spinal cord of E11.5 (52 somites) wild-type (F), *gdnf*$^{-/-}$ (G), and *pea3*$^{-/-}$ (H) embryos. (F and G) Mirror images of one spinal cord side showing either *met* only (left side) or *met* and *pea3* (right side) probes. The extent of *met* expression in the wild-type *pea3* domain at this stage is indicated by a red dotted line. *met* expression in this domain is severely reduced in both *gdnf*$^{-/-}$ and *pea3*$^{-/-}$ embryos, while all other sites (black arrow) of expression remain unaffected. Scale bars, (F–H).

that, at any intermediate stage, most neurons that had not turned *pea3* on yet (white area in Figure 6E) were devoid of *met* expression. Thus most C5-C6 LMC neurons do not express detectable levels of *met* before they turn on *pea3*, and most of the newly added *pea3*$^+$ neurons are still devoid of *met* mRNA. Since the phenotype in *met*$^{d/d}$ mutants was confined to the *met*-negative C5-C6 segments, these data suggest a non-cell-autonomous requirement of *met* for spreading of *pea3* expression.

met Is Required Non-Cell-Autonomously for *pea3* Expression in C5-C6 Motor Neurons

We could not exclude the possibility that *met* might be expressed in most C5-C6 neurons at a level escaping detection by ISH, prior to the onset of *pea3* expression. To provide genetic evidence for the non-cell-autonomous actions of HGF/Met, we took advantage of the differential location of the two subgroups of *pea3* neurons. Our results suggested that *pea3* expression in more anterior neurons is induced by a signal produced by the posterior neurons, which themselves are instructed to do so by HGF. We therefore focused on induction of *pea3* expression in the C5 segment. Spinal cord segments corresponding to either C5 or C7-C8 were isolated from 30–37 somites embryos and recombined in collagen gel, by placing each C5 explant anterior to a C7-C8 explant of the same embryo side having the same DV and AP orientation.

We first tested whether the cooperation between HGF and GDNF could also be observed in C5, despite the physical interface (and sometimes distance) between the posterior and anterior explants. As in the case of explants containing the whole *pea3* region, explant combinations grown without factor or with HGF alone did not express significant levels of *pea3* (data not shown). In the presence of GDNF, the number of *pea3*$^+$ neurons in C5 explants was always extremely low compared to C7-C8 explants (Figure 7A), consistent with the idea that the GDNF-responsive neurons are essentially confined to the C7-C8 segments. In the presence of both GDNF and HGF, the number of *pea3*$^+$ neurons in C5 explants, although still low compared to posterior explants, was significantly increased with respect to cultures with GDNF alone ($n = 6$, Figures 7B and 7C). This increase was accompanied by a densification of the *pea3*$^+$ population in C7-C8 explants as previously observed. In most cases, induction of *pea3* in C5 had occurred in the motor neurons located in the zone of contact between the two explants, occasionally spreading up to the anterior part of the C5 motor column. These observations confirm that, while the neurons that activate *pea3* expression in response to GDNF are essentially located in C7-C8, recruitment of more C5 motor neurons to the *pea3* population is mainly achieved by addition of HGF.

To directly address the non-cell-autonomous requirement for *met*, we next asked whether *met* function was dispensable for expression of *pea3* in C5 motor neurons. This question was addressed by testing the effect of

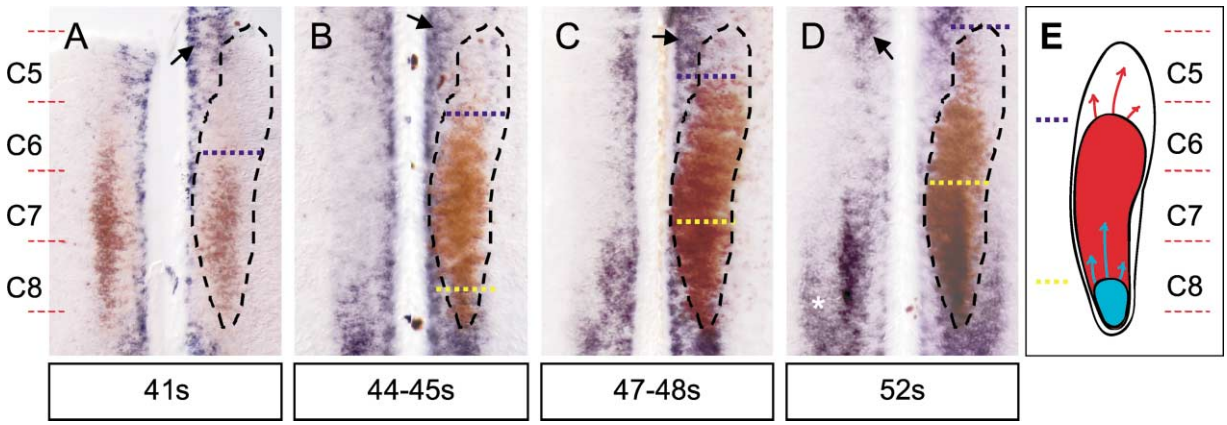

Figure 6. Met Is Not Expressed in Anterior Neurons of the *pea3* Domain

(A–D) Time course showing the onset of *met* expression in the *pea3*-expressing motor neurons, on flat-mounted spinal cords. Double ISH was performed using probes for *pea3* (red) and *met* (blue). (B–D) Mirror images of one spinal cord hybridized with either *met* probe only (left side) or *met* and *pea3* probes (right side). The position of the C5-C8 spinal segments is indicated. At 41 somites, *met* expression is not detected in the *pea3* territory, while it is already expressed in thoracic motor neurons (A). The maximal extent of the $pea3^{+}$ domain, as determined on E12.5 flat mounts, is indicated by a dotted line on the right side of each stage. While *pea3* expression progresses anteriorly (blue dotted line, [A–E]), at any given stage, neurons in this domain that do not express *pea3* yet do not express *met* either. Instead, *met* is first detected in the caudal part of the $pea3^{+}$ domain at 44 somites (B). The anterior limit of *met* expression in the caudal *pea3* (yellow dotted line, [B–E]) domain progressively shifts anteriorly (C and D). All other neurons expressing *met* in this area do not coexpress *pea3* at any stage (arrow in [A]–[E], white asterisk in [E]).

(E) Scheme representing the evolution of *pea3* and *met* expression in the presumptive *pea3* domain during the recruitment period. The black line indicates the full extent of *pea3* expression at E12.5. Domains in blue and red represent neurons coexpressing *met* and *pea3* or expressing *pea3* only, respectively, at an intermediate step of the recruitment period. The white area represents the neurons that have not yet activated *pea3* expression but that will subsequently be recruited to do so.

HGF on C5 explants isolated from $met^{d/d}$ embryos and combined with wild-type C7-C8 explants in the presence of GDNF. An HGF-induced increase in the number of $pea3^{+}$ motor neurons was observed not only in wild-type C7-C8 explants but also in $met^{d/d}$ C5 explants, at the site of contact with the posterior explant (n = 6, Figures 7D–7F). Since the signaling response mediated by the Met^{d} receptor is severely affected (Maina et al., 1996; Ponzetto et al., 1994), the efficient induction of *pea3* in $met^{d/d}$ explants can only be attributed to a signal produced by the wild-type posterior explant. Indeed, when instead the posterior C7-C8 explants originated from $met^{d/d}$ embryos, addition of HGF with GDNF failed to induce recruitment, either in $met^{d/d}$ posterior explants or in anterior explants, irrespective of the genotype of the latter (n = 3, Figures 7G–7I).

We wished to exclude the possibility that apparent recruitment of $PEA3^{+}$ neurons in C5-C6 might reflect the migration of motor neurons that differentiated in C7/8. We cultured C5-$pea3^{NLZ/+}$ explants either alone or in combination with a posterior C7-C8-wt explant, in the presence of both GDNF and HGF, and monitored expression of the *pea3-lacZ* allele by β-galactosidase staining. We found that addition of C7-C8 explants to C5 resulted in a >5-fold increase in the number of $LacZ^{+}$ motor neurons in the C5 explants (n = 5, Figures 7J–7L). Since, in these explant combinations, activation of *pea3-lacZ* expression can only occur in $pea3^{NLZ/+}$ motor neurons, this result shows that caudorostral migration of motor neurons cannot account for the HGF-induced recruitment. These data further confirm that the ability of HGF to cooperate with GDNF and promote *pea3* expression in C5 motor neurons requires the presence of the posterior explant, strongly suggesting that HGF is acting on C7-C8 neurons and not on C5 motor neurons.

Discussion

Recent reports have shown that the pools of brachial motor neurons that innervate the CM and LD muscles are a particularly interesting model system for understanding the interplay between peripheral and central factors in the correct establishment of a functional neuromuscular unit. By studying the expression during development of one key determinant of their differentiation, the ETS transcription factor PEA3, we have brought to light a novel process of recruitment of motor neurons to the PEA3 pools. We show that this involves a non-cell-autonomous role of the HGF/Met signaling and propose that the development of these motor pools requires a new level of coordination between factors derived from the periphery and those derived from neighboring neurons within the spinal cord.

Recruitment of Neighboring Neurons as a Mechanism for Attaining the Final Size of the PEA3 Population

Our analysis of *pea3* expression in $met^{d/d}$ embryos revealed a significant shortening of the *pea3* expression domain. The lack of increase in TUNEL staining and the constant number of Islet-1-positive motor neurons in the corresponding region suggested that this reduction was not due to cell death but to a failure of some motor neurons to express *pea3*. Moreover, by using mice also lacking all limb muscles like *met* mutants, including the target muscles of *pea3* neurons, we excluded the possi-

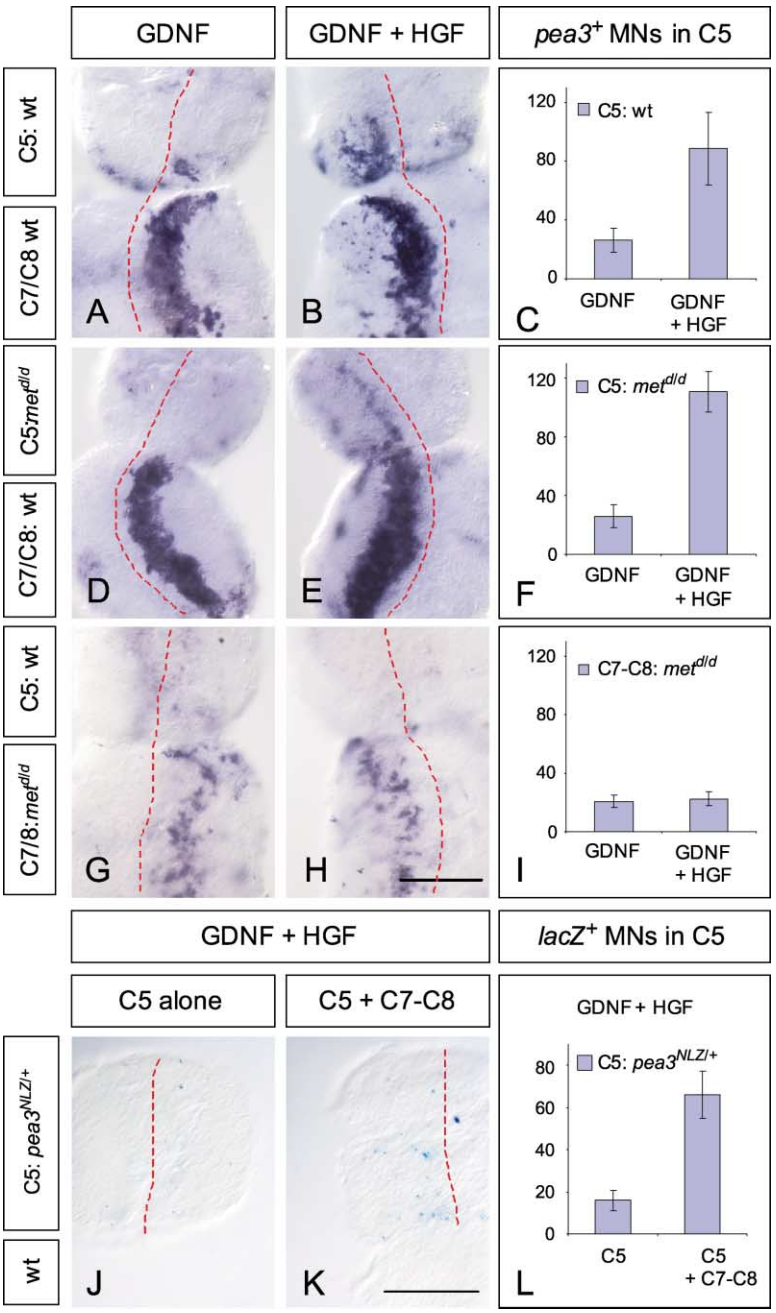

Figure 7. *met* Is Required Non-Cell-Autonomously to Promote *pea3* Expression in C5 Motor Neurons

(A–I) Explants from C5 brachial segments were isolated from wild-type (A–C and G–I) or $met^{d/d}$ (D–F) 30–37 somite embryos and recombined with C7-C8 explants from wild-type (A–F) or $met^{d/d}$ (G–I) embryos of similar stages. Left and right side explant combinations from the same embryo pair were treated with either GDNF (A, D, and G) or GDNF and HGF (B, E, and H) for 30 hr in culture. *pea3* expression was analyzed by ISH, and the number of $pea3^{+}$ neurons in C5 was quantified in at least six explants per condition (C and F) and in three explants per condition (I) to evaluate the inductive effect of HGF. In the presence of a wild-type C7-C8 explant, while GDNF has little effect on C5 motor neurons, addition of HGF to GDNF leads to efficient *pea3* induction in motor neurons of both wild-type (t test, $p = 0.0184$) and $met^{d/d}$ (t test, $p < 0.0002$) C5 explants. In contrast, when the C7-C8 explant derives from $met^{d/d}$ embryos, while induction of *pea3* by GDNF in C7-C8 explants is unaltered, no further enhancements of this effect by addition of HGF can be can be observed in C7-C8. Moreover, the lack of *met* signaling in the posterior explant abolishes HGF-induced recruitment in wild-type C5 explants (t test, $p = 0.405$). Scale bar, 150 μm.

(J–L) The inductive effect of HGF requires the presence of C7-C8 explants. Left and right side C5 explants from $pea3^{nlz/+}$ embryos were cultured either alone (J) or in combination with C7-C8 explants isolated from wild-type embryos (K), both in the presence of GDNF and HGF. The number of $lacZ^{+}$ neurons in C5 explants was quantified in five explants per condition (L). Addition of C7-C8 explants contributes to a 5-fold induction of *pea3-lacZ* expression in C5 motor neurons (t test, $p = 0.0016$). Expression of *lacZ* in induced neurons shows that HGF-induced recruitment does not result from migration of $pea3^{+}$ motor neurons from the wild-type posterior explants. Scale bar, 150 μm.

bility that HGF might indirectly affect *pea3* expression by acting on migrating myoblasts. While GDNF had previously been shown to be a limb-derived factor responsible for *pea3* expression in brachial motor neurons (Haase et al., 2002), these results show that a second factor, HGF, also participates in the establishment of the *pea3* expression domain. However, unlike GDNF, HGF failed to promote *pea3* expression in naive spinal cord explants. Instead, it efficiently enhanced the inductive effect of GDNF. These results show that, rather than acting independently on two different fractions of the *pea3* domain, HGF and GDNF act on the same subset of motor neurons and cooperate to induce the recruitment of additional motor neurons to the *pea3* population.

Our results show that, within the population of neurons that express PEA3, two principal groups can be distinguished, which differ both by their rostrocaudal position and by the signals that are responsible for the induction of *pea3* in each of them (Figure 8). The first group contains motor neurons situated at C7 and C8 levels in which *pea3* expression is independent of *met* functions. These neurons, which express PEA3 from early stages, express high levels of the GDNF receptor subunit GFRα1 (Garces et al., 2000), can be induced to express *pea3* by addition of GDNF to naive explants (Haase et al., 2002), and fail to do so in embryos lacking GDNF. *met* expression in these neurons is abolished in both *gdnf* and *pea3* mutants, consistent with the idea that GDNF induces *met* in a PEA3-dependent manner.

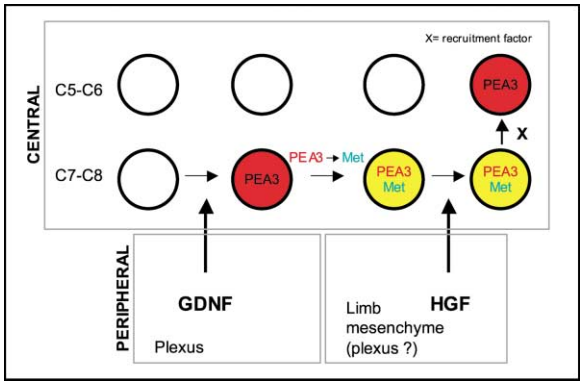

Figure 8. Summary of Motor Neuron Phenotypes in *met*$^{d/d}$ Mutants and Model for the Induction and Spreading of the *pea3* Domain

Model for induction of *pea3* expression in motor pool. Limb mesenchyme-derived GDNF induces *pea3* expression (red) in a predetermined group of C7-C8 motor neurons expressing Ret and GFRα1. *met* expression is activated downstream of PEA3 (yellow). *met*-expressing cells exposed to limb-derived HGF, recruit neighboring motor neurons (mostly from C5-C6) by inducing them to express *pea3*. Such a non-cell-autonomous function of *met* is likely to be achieved though transcriptional activation of an effector protein (secreted molecule X). The induction by GDNF is an ongoing process but is represented as acting once for simplicity. Moreover, some of the newly induced *pea3*-expressing motor neurons in turn express *met* and become competent as well to recruit other neurons.

A second group of motor neurons only expresses PEA3 at later stages, during a hitherto undescribed process of recruitment leading to the spatial expansion of the *pea3* domain, which progressively spreads rostrally, while the density of PEA3$^+$ cells at all levels increases. *met* is required for recruitment, but throughout the period of *pea3* anterior spreading, most neurons in the prospective *pea3* area in the C5-C6 segments (including both the recently recruited *pea3*$^+$ neurons and the neurons that are still to be recruited) do not express detectable levels of *met* mRNA, suggesting a non-cell-autonomous role for Met in this process. Genetic evidence for this came from an explant recombination assay, combining C5 and C7-C8 explants from different genetic backgrounds and examining *pea3* expression in C5 explants in these various contexts. We were able to demonstrate that *pea3* expansion requires *met* in C7-C8 neurons but not in recruited neurons. These results confirm that HGF acts on the same neurons in which GDNF has induced *pea3* and *met* expression and that recruitment is a non-cell-autonomous consequence of *met* signaling.

Collectively, these results show that production of a recruitment signal by the C7-C8 neurons requires proper specification induced in these cells by GDNF, enabling them to respond to a second instructive signal from the limb mesenchyme, HGF (Ebens et al., 1996; Yamamoto et al., 1997). One prediction of our model is that different limb-derived signals are required at early and late stages for full induction of *pea3* expression. This is in striking agreement with early limb ablations in chicken embryos (stage 18), which led to complete absence of ETS factor (PEA3 and ER81) expression (Lin et al., 1998). In contrast, when ablations were performed slightly later (stage 20), PEA3 expression was detected in a spatially restricted domain, corresponding to a subset of the motor pools in which it is normally expressed (Lin et al., 1998). Consequently, a brief exposure to early limb-derived signals is only sufficient for the onset and persistence of ETS gene expression in a subset of motor neurons: full expression requires later signals as well. In the light of our results, it is tempting to speculate that for the PEA3$^+$ population, the early factor in these experiments was GDNF and the late factor HGF. The similar effect of early and late hindlimb ablations on expression of another ETS transcription factor, ER81, suggests that the bifactorial mechanism we describe might not be unique to the PEA3 population.

Parallels within the Developing Nervous System

The model suggested by our data (Figure 8) has some striking parallels at earlier stages of neural tube development. Motor neurons differentiate early toward motor column-specific phenotypes, reflecting the area within the embryo to which their axons will project. Acquisition of columnar identity requires interplay between peripheral signals, such as those from the paraxial mesoderm (Ensini et al., 1998; Matise and Lance-Jones, 1996), and signals from neighboring motor neurons within the spinal cord, such as retinoic acid (Sockanathan and Jessell, 1998). Our results here suggest that a similar situation might prevail for the determination of pool-specific identity. Thus, in addition to the role of peripheral GDNF, there is an essential role of signaling from neighboring motor neurons for expansion of the PEA3 population and for normal invasion of target muscles.

In a second example, a strikingly similar case of recruitment of unspecified cells to expand a given identity was shown to contribute to hindbrain segmentation. In the vertebrate hindbrain, which is divided into compartments (rhombomeres) alternating between odd- and even-numbered identities, the transcription factor Krox-20 plays a key role in the acquisition of cellular identity (Seitanidou et al., 1997). While the hindbrain neuroepithelium initially acquires an even-numbered character, expression of *Krox-20* is induced in two stripes of cells, most likely by signals from the environment. Subsequently, *Krox-20* is sufficient not only to promote odd-number characteristics but also to mediate the recruitment of other cells in order to expand odd-numbered rhombomeres (Giudicelli et al., 2001). In a remarkable parallel, this recruitment involves a non-cell-autonomous ability of Krox-20 to induce its own expression in neighboring cells.

HGF Induces Expression of a Recruitment Factor

One important question is the identity of the recruitment factor, whose expression must be restricted to neurons coexpressing *met* and *pea3* and controlled in these cells by GDNF, PEA3, and HGF. Moreover, given constant two-segment distance between the anteriormost *met*/*pea3* cells and the next anterior cells in which *pea3* should be induced, the signal is likely to be a diffusible molecule. These criteria eliminate all the candidates we have tested. Among those, Cadherin 8 and Semaphorin 3E, as well as the Eph receptor EphA4 (Garces et al., 2000; Helmbacher et al., 2000) were expressed normally in the population of *pea3*$^+$ neurons remaining in *met*$^{d/d}$

spinal cords (Supplemental Figure S4 at http://www.neuron.org/cgi/content/full/39/5/767/DC1). Another candidate factor was retinoic acid, which was previously shown to mediate the cell fate change induced by LMCm neurons in later-born LMCl neurons (Sockanathan and Jessell, 1998). However, expression of RALDH2 is not restricted to neurons coexpressing *met* and *pea3*, and normal levels of RALDH2 expression were observed in $met^{d/d}$ spinal cords (data not shown). Finally, two factors known to induce PEA3 expression in other systems, FGF8 and Wnt-1 (Howe et al., 2001; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001), were not detected in ventral spinal cord (data not shown).

Conclusion

Although mature motor pools may each contain motor neurons with different functional modalities, their coordinated development requires that they express common properties as well. Previously reported examples include the early characteristic bursts of spontaneous activity as they grow toward their targets (Milner and Landmesser, 1999) and the later coupling of motor neurons within a given pool by gap junctions (Personius et al., 2001). We have focused here on one molecular characteristic that is also of key functional importance: the expression of the ETS factor PEA3. Together with the recent reports by Livet et al. (2002) and Haase et al. (2002), our results demonstrate that there are multiple levels of control of motor pool differentiation with time, each of which is vital for successful innervation of target muscles.

Experimental Procedures

Animals and Genotype Analysis

The generation of the signaling-deficient allele of *met* used in this study (met^d) has been previously described (Maina et al., 1996). Genotype analysis by PCR was performed as described. Mice carrying the $pea3^{NLZ}$ allele were genotyped as described by Livet et al. (2002). *Splotch* and *Splotch-delayed* mutant mice were obtained from the Jackson Laboratories and maintained on a C57Bl/6 background. *gdnf* mutants were maintained and genotyped as described (Haase et al., 2002).

In Situ Hybridization

Spinal cords were dissected in cold PBS and fixed in 4% PFA. Whole spinal cord in situ hybridization was performed as described (Garces et al., 2000), with digoxigenin-labeled RNA probes for *met* (Yamamoto et al., 1997), *pea3*, and *Cadherin8* (obtained from T. Jessell), *islet-1* (from F. Sedel), *ret* (from A. Rosenthal), *scip* (from P. Carroll), *sema3E* (from J. Livet), *MyoD* (from C. Ponzetto), *EphA4* (from P. Charnay). For double ISH, one of the probes was labeled with Fluorescein-UTP. Digoxigenin and Fluorescein were detected sequentially with alkaline phosphatase-conjugated antibodies (Roche 1/2000). NBT/BCIP staining (blue) was always performed first, and the antibody was stripped with 0.1 M Glycine (pH 2.2). Spinal cords were then incubated with the second antibody, washed, and stained with INT/BCIP (orange/red).

Immunohistochemistry and Quantification of Motor Neuron

Spinal cords were dissected from E11.5 embryos (48–50 somites), previously fixed for 2 hr in 4% PFA. All DRGs except C7 and C8 were removed. Fragments of spinal cords including the C4-T2 segments were frozen in PBS, 20% sucrose, 7.5% gelatin, and transverse sectioned (14 μm) on a cryostat. Antibodies used were anti-PEA3 (Arber et al., 1999; 1/1000), anti-Isl1/2 (39-4D5, 1/100; 40-2D6, 1/500, obtained from the developmental studies hybridoma bank [DSHB]).

The quantitative analysis was performed by counting the number of PEA3 and $Isl1/2^+$ nuclei on one out of every four sections, throughout the entire length of the $PEA3^+$ region. Although Isl1/2 is expressed at all levels of the spinal cord, $Isl1/2^+$ motor neurons were counted on an identical number of sections corresponding to the wild-type extent of PEA3 expression. $Isl1/2^+$ interneurons were not included in this analysis.

For the analysis of the anteroposterior distribution of $PEA3^+$ motor neurons, we first dissected the spinal cord together with all DRGs, allowing unambiguous identification of every spinal segment. All DRGs except C7 and C8 were removed from the preparation. The C7 and C8 DRGs were used as internal landmarks to determine the precise anteroposterior level of each section. This allowed us to assign to every section its AP coordinates and determine an average number of $PEA3^+$ motor neurons for every AP level. For each genotype, the value was calculated from three different embryos, counting left and right sides separately.

β-Galactosidase Staining

X-gal staining was performed according to standard techniques on dissected spinal cords or whole embryos. For *pea3-lacZ* motor neuron counts, after staining for β-galactosidase activity, embryos were postfixed in 4% PFA. The spinal cord was then dissected from cervical segment C5 to thoracic segment T2, where *pea3*-positive neurons are located, and embedded in gelatin/Albumin. Fifteen micrometer vibratome serial cross-sections were collected for the quantification of β-gal-positive nuclei. Throughout the entire length of the $lacZ^+$ region, neurons of every spinal cord section were counted in at least three different embryos of each stage and genotype. Each side was considered separately. Analysis of the AP distribution of $\beta\text{-gal}^+$ neurons was performed as described for the immunocytochemistry on four control and four mutant embryos (49 somites) and gave results similar to those obtained by in situ hybridization (data not shown).

Cultures of Spinal Cord Explants

Spinal cord explant cultures were isolated from E10.5 mouse embryos (30–36 somites), embedded in collagen matrices as described (Haase et al., 2002), and cultured in L-15 medium (invitrogen) supplemented with 2% horse serum, 3.6 mg/ml glucose, progesterone (6.25 ng/ml), modified N-2 supplement, sodium bicarbonate (25 mM), penicillin, and streptomycine, in the presence of combinations of GDNF or HGF (both 10 ng/ml, R&D). Recombinant NT3 (10 ng/ml, R&D) was added in every culture as neurotrophic support. Since the amount of $pea3^+$ neuron can vary with the stage of the embryos or because of imprecise AP identification during the dissection, comparison between different factor combinations was performed in every case, by culturing the left and right sides of a same spinal cord fragment separately.

The global cooperation between HGF and GDNF (Figure 5) was tested on explants corresponding to spinal segments C4 to T1 of pea3NLZ/+ embryos. After 30–48 hr culture, explants were dissected out of the collagen matrices and stained for β-galactosidase activity. Quantification was performed by counting the number of X-gal^+ nuclei on explants cultured for 30 hr (seven to ten explants per condition).

For explant recombination assays (Figure 7), spinal cord segments corresponding to either C5 or C7-C8 were dissected and recombined in collagen gel, by placing each C5 explant anterior to a C7-C8 explant of the same embryo side, thus having the same DV and AP orientation. Again, combinations corresponding to left and right spinal cord sides were cultured separately, in the presence of GDNF alone or both HGF and GDNF. When combining explants from different genotypes, comparison was performed on left and right side explant combinations from a same set of $met^{d/d}$ and wild-type embryos. After 30 hr of culture, explant combinations were taken out of the collagen, and *pea3* expression was analyzed by ISH or β-gal staining.

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