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Integrity of Developing Spinal Motor Columns Is Regulated by Neural Crest Derivatives at Motor Exit Points

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Summary

Spinal motor neurons must extend their axons into the periphery through motor exit points (MEPs), but their cell bodies remain within spinal motor columns. It is not known how this partitioning is established in development. We show here that motor neuron somata are confined to the CNS by interactions with a neural crest subpopulation, boundary cap (BC) cells that prefigure the sites of spinal MEPs. Elimination of BC cells by surgical or targeted genetic ablation does not perturb motor axon outgrowth but results in motor neuron somata migrating out of the spinal cord by translocating along their axons. Heterologous neural crest grafts in crest-ablated embryos stop motor neuron emigration. Thus, before the formation of a mature transitional zone at the MEP, BC cells maintain a cell-tight boundary that allows motor axons to cross but blocks neuron migration.

Introduction

The existence of boundaries between distinct compartments is a fundamental aspect of nervous system organization, and its most overt manifestation in vertebrates is the segregation of the central nervous system (CNS) from the peripheral nervous system (PNS). This division is exemplified in the spinal cord at specialized spinal nerve root transitional zones located both at the motor exit point (MEP), where motor axons leave the cord in ventral roots, and at the dorsal root entry zone (DREZ), where the afferents of primary sensory dorsal root ganglion (DRG) neurons enter the spinal cord in the dorsal roots. At these sites, the mature CNS:PNS interface is characterized by the cellular apposition of astrocytes and Schwann cells, glial cells derived embryologically from the neural tube and neural crest, respectively (Golding and Cohen, 1997). In development, the earliest manifestation of these interfaces is the appearance at the neural tube surface of clusters of boundary cap (BC) cells (Altman and Bayer, 1984). These were shown to be neural crest derivatives that originate in the ventral migratory stream and arrest at prospective hindbrain and spinal nerve root entry/exit points (Golding and Cohen, 1997; Niederlander and Lumsden, 1996). The only known marker of BC cells in the mouse embryo is the *Krox20* gene, which encodes a zinc finger transcription factor shown to control hindbrain segmentation and PNS myelination (Schneider-Maunoury et al., 1993; Topilko et al., 1994; Wilkinson et al., 1989). Its inactivation did not result in any obvious BC cell phenotype in the spinal cord, preventing investigation of their possible function during development (Schneider-Maunoury et al., 1993; Topilko et al., 1994). In the chick embryo, BC cells have been shown to express high levels of *Cad-7* in addition to *Krox20* (Nakagawa and Takeichi, 1995; Niederlander and Lumsden, 1996).

Axons initially enter and exit the spinal cord in complex, multistage processes that are poorly understood. The entry/exit sites must be specified for axon targeting, and the respective contributions of neural crest BC cells outside the spinal cord and of neuroepithelial cells within is unclear (Golding and Cohen, 1997; Niederlander and Lumsden, 1996). The idea that neural crest derivatives such as Schwann cell precursors may guide spinal motor axon outgrowth in the periphery has received the most attention. The evidence suggests that rather than neural crest cells guiding axons, glial migration in the embryonic PNS is itself probably guided by axons (Bhattacharyya et al., 1994; Carpenter and Hollyday, 1992; Gilmour et al., 2002).

Little is known about the possible role of neural crest cells in earlier patterning events, however, in particular the initial specification of MEPs and the establishment of boundaries between emerging CNS and PNS compartments. As there is no obvious barrier (e.g., a continuous basal lamina) at developing interfaces to impede cell migration (Golding and Cohen, 1997), a previously overlooked requirement at the early MEP and DREZ is for a mechanism that allows axons to cross freely between CNS and PNS compartments but blocks cell mixing between them.

In this study, we investigated the role of neural crest BC cells in the organization of an early CNS:PNS interface, the spinal MEP, using three progressively refined experimental models: (1) surgical neural crest ablation in chick embryos; (2) *Splotch* (*Pax3* mutant) mouse embryos where crest cells fail to delaminate in the trunk region of the neural tube (Auerbach, 1954; Henderson et al., 1997; Serbedzija and McMahon, 1997); and (3) selective, targeted ablation of BC cells in the mouse embryo using a knockin of the gene encoding the diphtheria toxin A subunit in the *Krox20* locus. We find that in all three ablation scenarios, the absence of BC cells at MEPs has little effect on the patterning of motor axon outgrowth but results in motor neuron cell bodies migrating into the periphery. Moreover, grafting heterolo-

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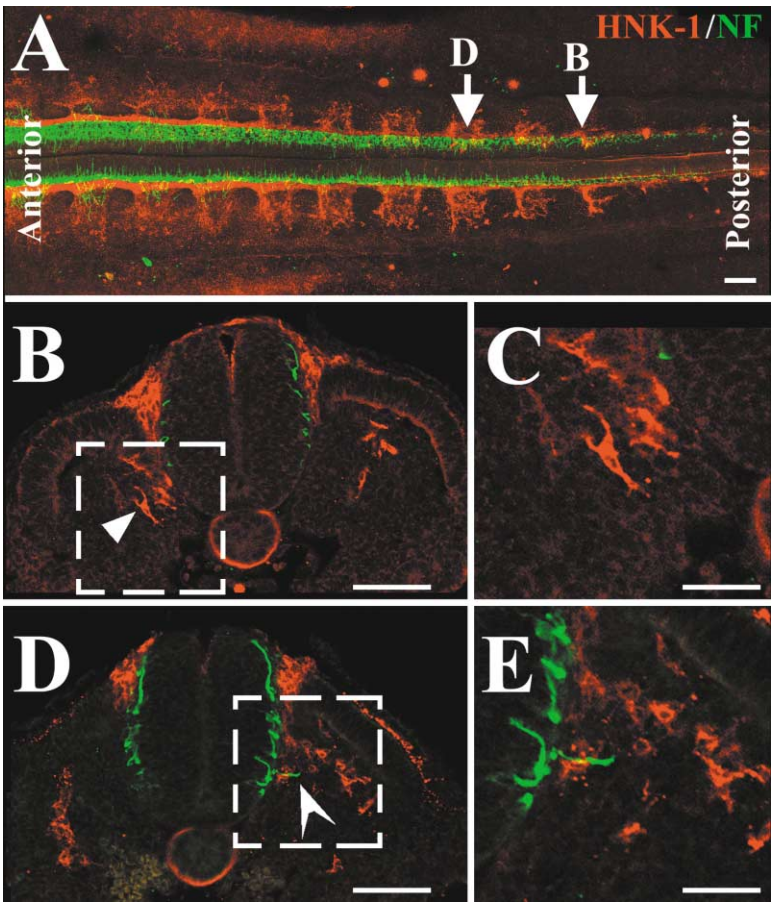

Figure 1. Migrating Neural Crest Cells Prefigure Spinal Cord MEPs

(A) Dorsal view of a confocal microscopic image, caudal to right, of an E2 (HH stage 17) chick embryo trunk double-immunostained for the neural crest cell marker HNK-1 (red) and neurofilament heavy chain (NF; green). The first motor axons are seen anterior to the front of migrating neural crest.

(B) Transverse sections of the embryo shown in (A) (white arrow B) at the level of the second posteriormost somite containing HNK-1-positive cells. Neural crest cells are seen delaminating from the dorsal neural tube, accumulating at the dorsal staging area, and migrating ventrally alongside the neural tube. The leading (ventral) front of the neural crest is adjacent to a prospective MEP (plain arrowheads), but no motor axons are visible.

(C) Higher power view of the region delineated by the dashed box in (B).

(D) Transverse section of (A) (white arrow D) taken two somites rostral to those in (B) and (C). The first motor axons that emerge from the neural tube and enter the sclerotome (notched arrowheads) are surrounded medially by neural crest cells.

(E) High power view of region delineated by the dashed box in (D). All images are confocal views; (A) is a montage of several flattened z series. Bars in (A), (B), and (D): 50 μm; in (C) and (E): 25 μm.

gous (quail) neural crest cells into dorsal neural tube-ablated chick embryos prevented motor neuron emigration. This identifies an unanticipated function for neural crest derivatives, maintaining the integrity of spinal motor columns by the imposition of a cell-impermeable boundary at the immature MEP.

Results

Identified Neural Crest Derivatives Prefigure Spinal MEPs

To locate the front of the early migrating neural crest stream relative to prospective MEPs in the spinal cord (Loring and Erickson, 1987; Rickmann et al., 1985; Serbedzija et al., 1989), we used confocal microscopy analysis on whole-mount antibody-labeled embryos. We combined either HNK-1 or anti-Cad-7 (Nakagawa and Takeichi, 1995) antibodies, which label neural crest derivatives, with an anti-neurofilament-H chain antibody (NF) to label motor axons (Figure 1A). In sections taken through the most caudal regions of embryos displaying HNK-1/Cad-7-positive neural crest cells, many labeled cells were located at the pial surface of the neural tube. Some of these were present as small clusters in ventral positions, in the vicinity of prospective MEPs (Figures 1B and 1C), two to three somites posterior to the position where NF-positive axons first emerged from the ventral neural tube (Figures 1D and 1E). Since the embryo develops in an antero-posterior gradient, this suggests that at any given position along this axis, neural crest cells are present at MEPs some hours before motor axons exit the neural tube. The position and antigenic phenotype of these cells corresponded to neural crest derivatives designated boundary cap (BC) cells in earlier studies of mouse (Schneider-Maunoury et al., 1993; Wilkinson et al., 1989), rat (Golding and Cohen, 1997), and avian (Niederlander and Lumsden, 1996) neural tube development.

Neural Crest Ablation Does Not Affect the Emergence of Spinal Motor Axons or Their Extension in the Periphery

Since neural crest BC cells appeared to prefigure motor axons at exit points, we next tested the extent to which they controlled axon outgrowth, by unilateral surgical ablations of the dorsal neural tube. These were carried out in thoracic regions of E2 chick embryos, by removing the dorsal half of the neural tube at presomitic levels, before the onset of neural crest migration. The manipulated embryos were then allowed to develop in ovo for up to 5 days. The extent of crest ablation and its effects were examined at various times post-operation by dual immunostaining of adjacent transverse cryostat sections with antibodies to either HNK-1 or Cad-7 combined with anti-NF antibody. On the unoperated side, neural crest derivatives, including DRG neurons, satellite cells, root entry and exit point BC cells, and Schwann cell precursors, were all normally positioned, but these were

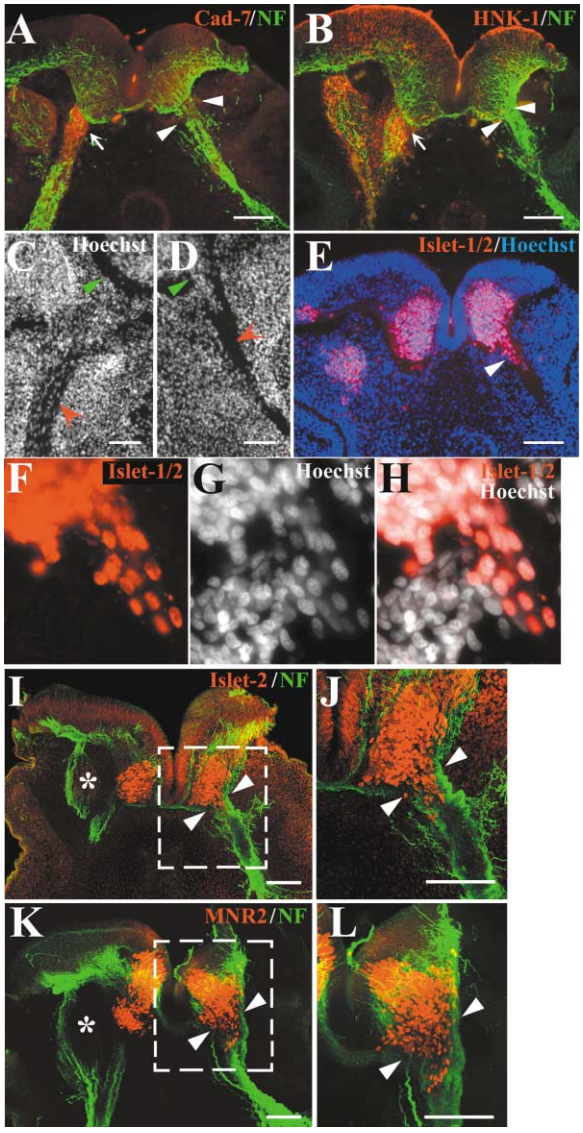

Figure 2. Absence of Neural Crest Cells at the MEP Does Not Affect Motor Axon Outgrowth but Leads to Motor Neuron Emigration into the Periphery

(A–H) Transverse sections of an E4.5 (HH stage 23) embryo 49 hr after ablation of the dorsal half of the right side of the neural tube. On the unoperated side (left), neural crest cells and their derivatives are clearly detectable by dual immunostaining for Cad-7 (red in [A]) or with HNK-1 (red in [B]) combined with neurofilament heavy chain (NF, green). Note that compared with the large numbers of neural crest derivatives in the vicinity of MEPs on the unoperated side (white arrows in [A] and [B]), none are evident on the ablated side (white arrowheads in [A] and [B]). In the absence of neural crest cells (right), motor axons exit the neural tube appropriately, extend in the ventral root, and branch to form a dorsal ramus in a similar manner to motor axons on the unoperated side.

(C–D) Hoechst nuclear staining of the ventral portion of the spinal cord reveals that, on the unoperated side of the embryo (C), the nuclei of Schwann cell precursors are distributed in typical, longitudinal arrays along the ventral root (red notched arrowhead), while the marginal zone (prospective white matter, green arrowhead) is devoid of nuclei. In contrast, on the operated side (D), the ventral root is free of cell nuclei (red notched arrowhead), but a stream of cell nuclei is evident in the marginal zone (green arrowhead) forming a bridge to the spinal ventral horn gray matter.

entirely absent from the operated side (Figures 2A and 2B). The only neural crest derivatives that were occasionally seen on the operated side were scattered clusters of neurons beneath the notochord, in positions equivalent to sympathetic chain ganglia on the unoperated side. Since the neural tube remained open postoperation, these probably derived from small numbers of neural crest cells that rerouted from the unoperated side and had migrated ventrally around the notochord (data not shown). The absence of neural crest derivatives on the operated side was also evidenced by the acellular nature of ventral roots (Figure 2D), compared to the unoperated side where the nuclei of Schwann cell precursors were distributed in characteristic longitudinal arrays along nerve roots (Figures 2C and 2D, red notched arrow). Despite this, the pattern and extent of motor axon outgrowth was remarkably similar on both operated and control sides, with respect to the ventral root projection and distal branching patterns (Figures 2A and 2B), including emergence of a dorsal ramus at the appropriate position. Neural crest derivatives are thus not essential for motor axons to locate exit points in the ventral spinal cord.

Neural Crest Ablation Leads to Motor Neuron Emigration from the Spinal Cord

In addition to the acellular ventral roots, a consistent feature revealed by nuclear staining of ablated embryos was the presence on the operated side of a wedge of large cells, forming a cellular bridge extending from the spinal ventral horn to adjacent marginal zone. In some cases, this extended into proximal motor nerve roots

(E) Section from the same embryo, immunostained with Islet-1/2 (red) and counterstained with Hoechst (blue). There is no noticeable difference in the distribution of Islet-1/2-positive motor neurons within the ventral neural tube on the operated and unoperated sides. In contrast, all the ectopic cells streaming out of the neural tube, in the ventral marginal zone and up to 50 μm along the ventral root on the operated side, are Islet-1/2 positive (white arrowhead).

(F–H) High-power view of the stream of nuclei within the ventral root shown in (E) (arrowhead). The nuclei of all ectopic motor neurons are immunolabeled with Islet-1/2 (red, [F and H]), and they correspond to all cells within the stream, as revealed with Hoechst nuclear stain (gray, [G and H]). Nuclei stained with Hoechst alone identify cells outside the nerve root.

(I and J) Transverse sections of an E5 (HH stage 26) embryo, 68 hr after ablation, dual immunolabeled with antibodies to the motor neuron-specific marker Islet-2 (red) and to neurofilament (green). The specificity of Islet-2 antibody for motor neurons is confirmed by the absence of labeling in the DRG (star). Many spinal motor neurons in the ventral horn are strongly Islet-2 positive, and a subset of these have escaped the confines of the spinal cord and extended into the ventral root. ([J] is a high power view of area defined by dashed box in [I].)

(K and L) Transverse sections of an E5 (HH stage 26) embryo, 65 hr after ablation, dual immunolabeled with antibodies to the motor neuron-specific marker MNR2 (red) and neurofilament (green). As with Islet-2 (I and J), MNR2 antibody does not label the DRG (star) but labels a different subpopulation of motor neurons, some of which also exit the spinal cord on the operated side. ([L] is a high power view of area defined by dashed box in [K].) The white arrowheads in (I)–(K) demarcate the position of MEPs. Images in (I)–(L) are all flattened images of confocal microscopic z series of 50 μm thick vibratome sections of whole-mount, double immunolabeled embryos. Bars in (A)–(E): 100 μm; (I)–(L): 150 μm.

(compare Figures 2C and 2D). Since the stream of migrating cells appeared to originate exclusively from ventral horn gray matter in the spinal cord (Figure 2D), this suggested that the absence of neural crest BC cells at ventral exit points resulted in the large-scale emigration of cells normally confined to the spinal cord.

To test this possibility, sections of spinal cord tissue from neural crest-ablated embryos were immunostained with an antibody that recognized both Islet-1 and Islet-2, members of the Islet subgroup of LIM homeodomain proteins expressed in motor neurons (Ericson et al., 1992; Tsuchida et al., 1994). Strikingly, the nuclei of 100% of the ectopically migrating cells found on the operated side were Islet-1/2 positive (Figures 2E, 2F, and 2H), indicating that the absence of neural crest BC cells selectively allows spinal motor neurons to migrate into the periphery. While most ectopic neurons were located within the ventral marginal zone of the spinal cord and in proximal ventral roots, some cells were detected as far as 300 μm along distal nerve roots, beyond the branch point of the dorsal ramus. Although the extraspinal Islet-1/2-positive cells appeared to derive exclusively from spinal motor columns, it remained a possibility that some were displaced DRG or sympathetic neurons, since these are also Islet-1-positive. Therefore, sections from crest-ablated embryos were immunolabeled with antibodies to alternative, motor neuron-specific markers, Islet-2 and MNR2, which label only subsets of motor neurons (Tanabe et al., 1998; Tsuchida et al., 1994) (Figures 2I–2L). As observed in Islet-1/2-immunolabeled embryo sections, many of the extraspinal cells were Islet-2 (Figures 2I and 2J) and MNR2 positive (Figures 2K and 2L), confirming their motor neuron identity.

We analyzed more than 200 dorsal ablated chick embryos, all of which displayed ectopic motor neurons. The amount and extent of emigration varied as a function of several factors, including embryo age, the rostrocaudal position of the operation site, the duration of the experiment, and the detection method used. Generally, ectopic motor neurons were first detected at 24 hr postoperation (E3), and their incidence increased with survival time. Emigration was consistently most pronounced at the wingbud level; at this level in one section from a representative embryo that had developed 68 hr post ablation, 134 motor neurons (34% of the Islet-1/2-positive motor pool) were ectopically located outside the spinal cord gray matter. Motor neurons at the front of this ectopic migratory stream extended a distance of ~300 μm from the MEP along the ventral root. In caudalmost positions of the ablation, corresponding to mid-thoracic regions of the same embryo, in contrast, a representative section contained only four ectopic motor neurons (1.3% of the corresponding motor pool), and none had migrated further distally than the spinal cord marginal zone. Furthermore, in regions where ablation was incomplete and some neural crest had survived, ectopic motor neurons were never observed.

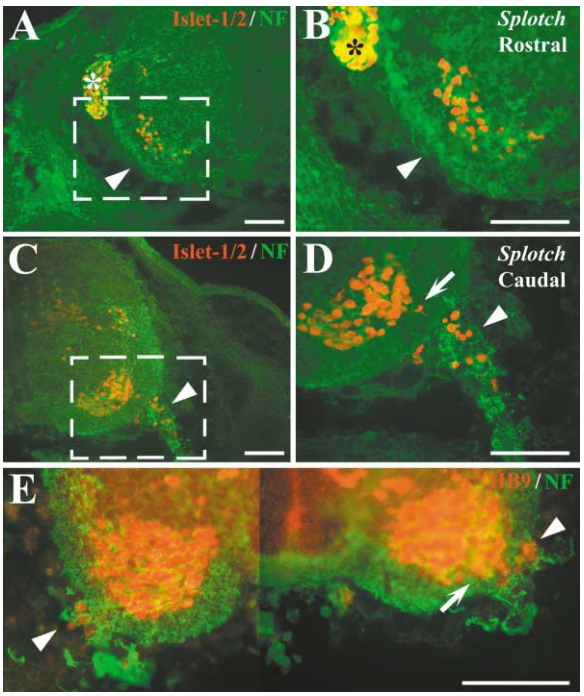

Figure 3. Motor Neurons Emigrate from the Trunk Spinal Cord in *Splotch* Mutant Embryos

(A–D) Transverse sections of the thoracic region of an E13.5 *Splotch* embryo dual immunostained with antibodies to Islet-1/2 (red, [A–D]) and neurofilament (green). The position of both Islet-1/2-positive motor neurons within the spinal motor column and dorsal interneurons is unaffected in the mutant. In rostral parts of the embryo (A and B), where neural crest derivatives are present as evidenced by DRGs (star), Islet-1/2-positive motor neurons are exclusively confined within the spinal cord gray matter (arrowheads). In contrast, in more caudal regions (C and D), the lack of neural crest derivatives evidenced by the complete absence of DRGs results in Islet-1/2-positive cells emigrating from the spinal cord gray matter into the marginal zone (arrow in [D]) and the ventral root (arrowheads in [C] and [D]). (D) shows a high power view of boxed area designated in (C).

(E) Transverse sections of a neural crest-free region of an E12.5 Splotch embryo dual immunostained with the motor neuron specific antibody HB9 (red) and anti-neurofilament antibody (green). Ectopic motor neurons are detected both within the marginal zone (arrow) and in the ventral roots (arrowheads). Bars (A–E): 100 μm.

Motor Neurons Emigrate from the Spinal Cord in *Splotch*/*Pax3* Mutant Embryos

While the simplest interpretation of the above results is that motor neurons escape the confines of the spinal cord because of the absence of neural crest cells at the MEP, it is possible that some side effect of surgical manipulation was partly responsible. We therefore chose an alternative, genetic approach to test the function of neural crest derivatives at MEPs. In *Splotch* (*Pax3*) mouse mutants, neural crest emigration fails to take place in lower thoracic and lumbar regions of the spinal cord (Auerbach, 1954; Franz, 1990; Henderson et al., 1997; Serbedzija and McMahon, 1997). In rostral regions of mutant spinal cord, in which neural crest migration proceeds to some extent, as evidenced by the presence of small numbers of crest derivatives including DRGs, no ectopic motor neurons were observed (Figures 3A and 3B). In contrast, in thoracic regions of homozygous mutant embryos at E11.5 and E13.5 (data not shown) that were completely devoid of neural crest derivatives (Figures 3C and 3D; note absence of DRG in Figure 3C),

numerous Islet-1/2-positive motor neurons were located ectopically both in the marginal zone and along ventral nerve roots (Figures 3C and 3D), and their motor neuron identity was confirmed using HB9 antibody, a marker specific for motor neurons (Tanabe et al., 1998) (Figure 3E). Thus, the absence of neural crest derivatives at MEPs arising from the *Splotch* mutation has identical consequences to surgical crest ablation, resulting in motor neuron somata escaping the confines of spinal motor columns.

Although less substantial in its extent, the distribution and location of ectopic motor neurons were more consistent in the *Splotch* mutant than in crest-ablated chick embryos. In five embryos examined aged between E11.5 and E13.5, ectopic motor neurons were first seen at the forelimb level, corresponding to the rostrocaudal level devoid of neural crest cells, as judged by the absence of DRGs. Down to the lower abdominal regions, an average of 20 ($\pm$8) ectopic Islet-1/2-positive motor neurons/section were found outside the spinal cord gray matter. The front of migrating neurons extended within the ventral root up to 100 μm from the MEP (Figures 3C and 3D). In more caudal regions, spinal cord development was impaired by the mutation, and many fewer neurons emigrated from the ventral gray matter (data not shown).

Heterologous Neural Crest Grafts Abolish Extraspinal Motor Neuron Migration in Crest-Ablated Embryos

Each of the above models of neural crest depletion could have undesirable side effects that may disrupt spinal cord development, such as perturbed dorsoventral patterning or impeded development of the paraxial mesoderm (Mansouri et al., 2001; Schubert et al., 2001), since both processes are dependent on signals emanating from the dorsal neural tube (Basler et al., 1993; Briscoe et al., 1999; Stern et al., 1995). However, if the phenomenon of spinal motor neuron emigration is a direct consequence of the removal of neural crest cells, the phenotype should be rescued by grafting exogenous neural crest into these sites. We therefore implanted re-aggregates of purified quail neural crest cells (McGonnell and Graham, 2002) into the neural tube of E2 chick embryos immediately after unilateral dorsal neural tube ablation and allowed the embryos to develop for 3 days. Grafted quail neural crest cells, detected by the quail-specific marker QCPN, were located primarily on the ablated side of the embryo along the pathways of crest migration in the host trunk, within both the dorsolateral and the ventromedial streams. They also showed a remarkable propensity for homing to the vicinity of MEPs/ventral nerve roots on the ablated side (Figure 4A). In all the segments where the presence of quail crest derivatives at these sites could be confirmed by QCPN and Cad-7 immunostaining (Figures 4A and 4B), motor neurons remained confined within spinal motor columns (Figure 4C). Notably, the presence of very few quail crest cells at host root exit points appeared sufficient to prevent the emigration of motor neurons.

Genetic Targeted Ablation of BC Cells in the Mouse Embryo

Since neither of the strategies we adopted for neural crest ablation specifically targeted BC cells, it could be

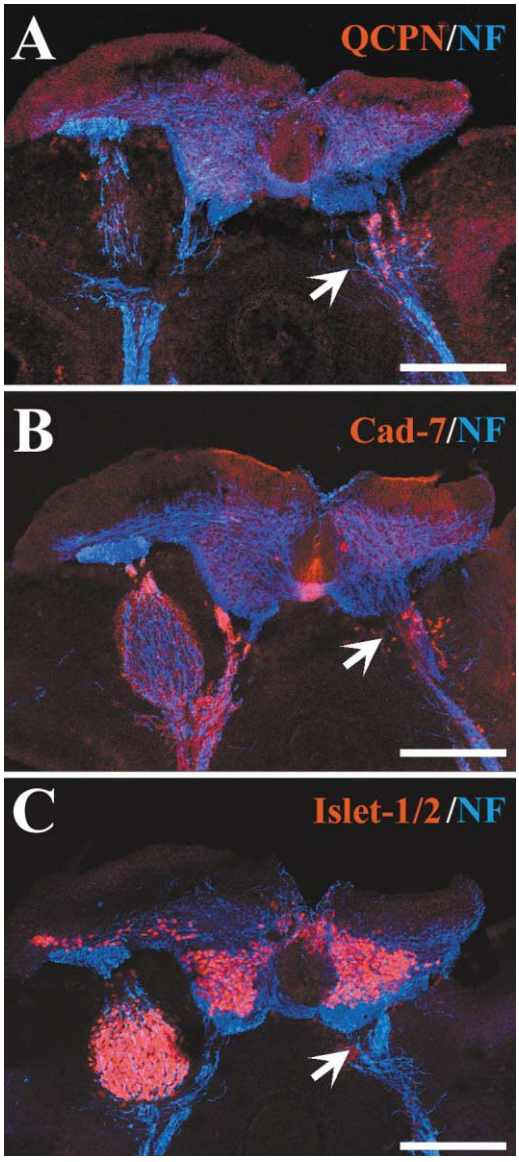

Figure 4. Heterotopic Neural Crest Cell Grafting Prevents the Extraspinal Migration of Motor Neurons following Dorsal Neural Tube Ablation

(A–C) Adjacent transverse sections of an E5 (HH stage 26) embryo, 69 hr after unilateral dorsal neural tube ablation, followed immediately throughout the zone of ablation by the grafting of pellets of purified quail neural crest cells into the lumen of the neural tube. (A) On the operated side, most grafted neural crest cells, identified with the quail-specific antibody marker QCPN (red), appear to home to the vicinity of the host motor exit point and proximal ventral root (arrowhead in [A]–[C], host motor axons visualized by anti-neurofilament antibody labeling [NF, blue]). (B) As seen with host neural crest cells on the unoperated side, grafted quail cells (arrow) express a high level of the neural crest marker Cad-7 (red). (C) In the presence of quail neural crest cells, despite the ablation, all Islet-1/2-expressing motor neurons remain confined within the anterior horn of the host spinal cord on the operated side. Neither the marginal zone nor ventral roots on the ablated side, visualized with anti-neurofilament antibody (NF, blue), contain ectopic neurons, and the pattern of motor axon outgrowth resembles that seen on the unoperated side (left). Bar: 200 μm.

argued that the resulting phenotype was a more widespread consequence of removing all neural crest derivatives. To test whether spinal motor neuron emigration in the *Splotch* mutant and crest-ablated chick embryos was due to the specific absence of ventral root BC cells, we turned to a more selective ablation strategy in the mouse embryo. The expression of the *Krox20* gene is initially restricted to BC cells in early spinal cord development (Schneider-Maunoury et al., 1993; Wilkinson et al., 1989), and this allowed us to target them for elimination. To achieve this, we produced a *Krox20*$^{GFP(DT)}$ allele in which the gene encoding the diphtheria toxin A subunit (DT) has been inserted at the *Krox20* locus by homologous recombination in ES cells (Figures 5A and 5B). A genomic region surrounding the *Krox-20* initiation codon was replaced by the GFP coding sequence, flanked by two *loxP* sites, and immediately followed by the DT gene (Figure 5A). The expression of the toxin, which is normally prevented by the presence of the GFP gene, was expected to be turned on upon Cre-mediated GFP excision, leading to the generation of a *Krox20*^{DT} allele (see Experimental Procedures and Figure 5A). *Krox20*$^{GFP(DT)/+}$ embryos expressed GFP in BC cells associated with ventral and dorsal roots, recapitulating the endogenous *Krox20* expression pattern (Figure 5D, arrowheads) (Schneider-Maunoury et al., 1993). GFP was observed in *Krox20*$^{GFP(DT)/+}$ embryo MEP BC cells from E9.75 onward (data not shown), at about the time when motor axons first emerge from the spinal cord.

Because *Krox20* is the only known marker of BC cells in the mouse embryo, their ablation upon DT expression had to be assessed by the absence of *Krox20* expression in peripheral nerve roots. Therefore, we made use of another *Krox20* allele carrying a knockin of the *E. coli lacZ* gene, *Krox20*^{lacZ}. Embryos carrying this allele specifically express *lacZ* in BC cells (Schneider-Maunoury et al., 1993). *Krox20*$^{lacZ/DT}$ embryos were generated by introducing the *Krox20*^{lacZ} and *Krox20*$^{GFP(DT)}$ alleles together with a transgene expressing the Cre-recombinase in the germ line (PGK-Cre^{m}; Lallemand et al., 1998). Analysis performed at E11. 5 indicated that *Krox20*$^{lacZ/DT}$ embryos were devoid of β-galactosidase reporter activity at the level of sensory and motor nerve roots, whereas β-galactosidase-positive BC cells were normally detected in *Krox20*$^{lacZ/GFP(DT)}$ littermates (Figure 5E). This strongly suggests that excision of the GFP cassette and subsequent expression of the toxin resulted in the complete elimination of BC cells in *Krox20*$^{lacZ/DT}$ embryos. As a further indication of the efficiency of the toxin system, we verified that its activation in *Krox20*$^{DT/+}$ embryos resulted in the ablation of rhombomeres 3 and 5, sites of *Krox20* expression in the hindbrain (data not shown).

Ablation of BC Cells Is Sufficient to Allow Emigration of Spinal Motor Neurons

Using the *Krox20*^{DT} allele, we next tested whether the targeted elimination of MEP BC cells would result in the emigration of motor neurons from the spinal cord. *Krox20*$^{DT/+}$ and wild-type E11.5 embryo sections were dual immunolabeled with the axonal marker β-III-tubulin (TuJ1) and motor neuron markers Islet-1/2 and HB9 (Figure 6). This showed that, in common with surgically ablated and *Splotch* mutant embryos, and in contrast to control embryos, many Islet-1/2-positive cells were located ectopically in the marginal zone or within proximal ventral roots in *Krox20*$^{DT/+}$ embryos (Figures 6A and 6B, arrow and arrowhead, respectively). Since Islet-1/2 also labels sensory neurons in the DRG (Figures 6E–6G, star), immunostaining with the anti-HB9 antibody, which exclusively labels a subset of motor neurons, confirmed the identity of the extraspinal neurons in *Krox20*$^{DT/+}$ E11.5 embryos (Tanabe et al., 1998). Many HB9-positive cells were again found associated with the ventral root (Figures 6E, 6F, 6I, and 6J). In a total of six mutant embryos examined, extraspinal motor neurons were found at every rostrocaudal level of the spinal cord at E11.5 and were still present at E12.5, but only in caudalmost regions (data not shown). At its greatest extent in a typical E11.5 mutant embryo, 21 extraspinal motor neurons were identified in one section, 15 of which had extended into the ventral root. However, no ectopic HB9-positive motor neurons were detected more distal than 200 μm from the MEP, suggesting that they either remain in the vicinity of the spinal cord or migrate further, displacement from the CNS environment leading to loss of motor neuron-specific markers and/or cell death.

Do Ventral Root Cells Migrate into the Spinal Cord in the Absence of MEP BC Cells?

Emigration of motor neurons outside the spinal cord raised the possibility that, in the absence of BC cells at the MEP, PNS cells may cross the CNS:PNS boundary in the reverse direction. To investigate this issue we stained E11.5 embryo sections with an antibody directed against the neuregulin receptor ErbB3, which labels neural crest-derived Schwann cell precursors (Meyer and Birchmeier, 1995; Meyer et al., 1997), cells normally confined exclusively to the PNS. We found that ErbB3-positive cells were present within the distal part of ventral roots and in peripheral nerves in both wild-type and mutant embryos (Figures 6M and 6N, arrows). No ErbB3-positive cells were detected within the spinal cord of *Krox20*$^{DT/+}$ embryos, suggesting that elimination of BC cells is not sufficient to allow inward migration of PNS cells into the spinal cord. In addition, immunolabeled cells were undetectable in the proximal part of the ventral root in *Krox20*$^{DT/+}$, unlike in wild-type embryos (Figures 6N and 6M, arrowheads), suggesting that this part of the ventral root remains devoid of Schwann cell precursors as a consequence of BC cell ablation.

In conclusion, these data identify BC cells as the neural crest derivatives specifically responsible for maintaining the integrity of spinal motor columns. In contrast, they may not be involved in regulating the migratory behavior of Schwann cells at the MEP.

Extraspinal Motor Neurons Exit the Spinal Cord by Translocating along Their Axons

The implication of the above findings is that motor neurons may need to be actively constrained within spinal motor columns by continuous signaling from MEP BC cells to prevent them otherwise following their axons into the periphery. To understand more about the underlying mechanisms, we analyzed the mode of emigration in ablated chick embryos in greater detail. Thus, the ventral neural tube of E2 chick embryos was electropor-

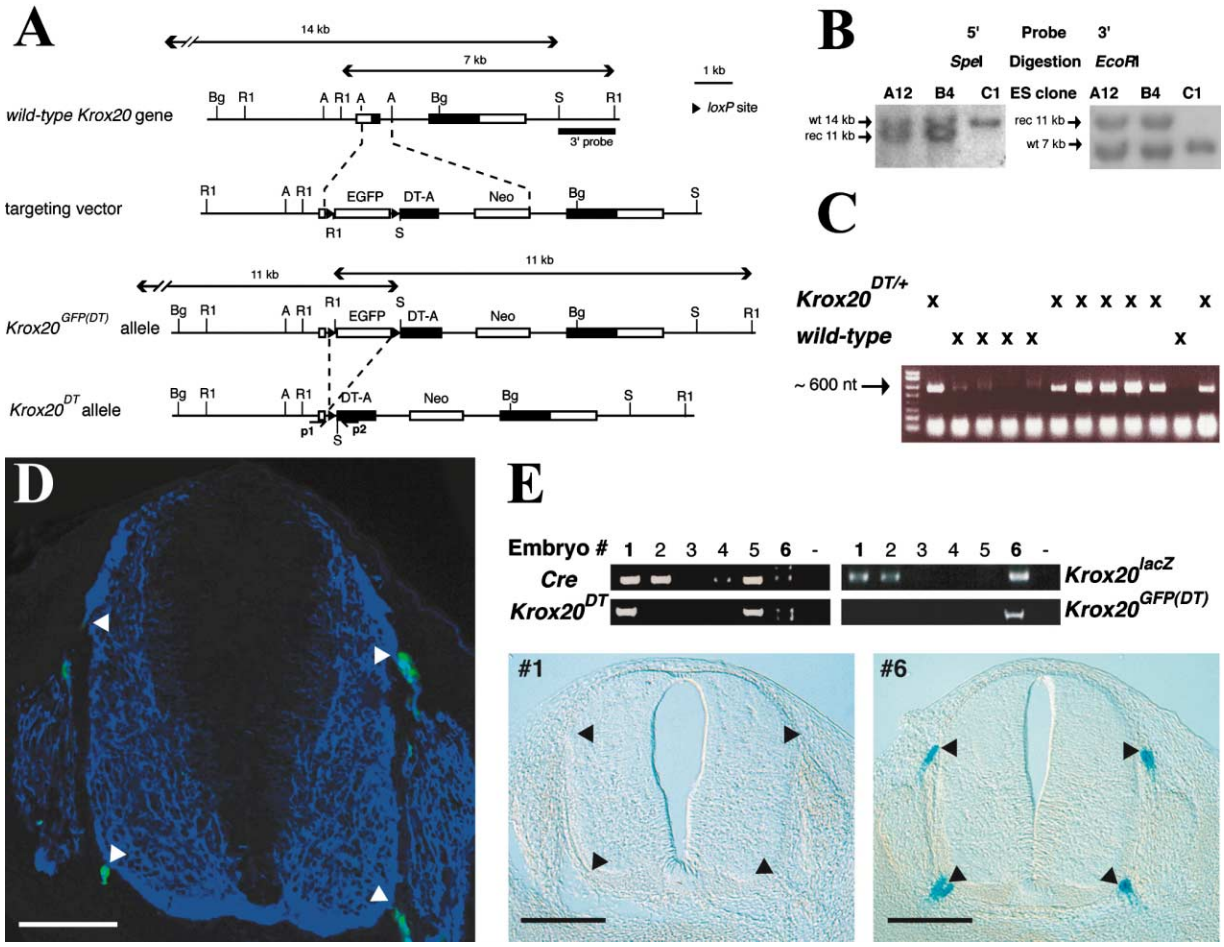

Figure 5. Conditional Genetic Ablation of *Krox20*-Expressing BC Cells

(A) Schematic representation of the wild-type *Krox20* gene, the targeting vector, and the unrecombined *Krox20*$^{GFP(DT)}$ and recombined *Krox20*^{DT} alleles. In the targeting vector, an AvrII-AvrII fragment containing the *Krox20* gene initiation codon was replaced by an *EGFP/DT-A* cassette followed by the *neoR* resistance gene. In the *Krox20*$^{GFP(DT)}$ allele, the *GFP* gene is flanked by two *loxP* sites (triangles) and prevents expression of the gene encodoing the diphtheria toxin A subunit (DT-A). Upon Cre-mediated recombination, the GFP coding sequence is excised, and the DT-A gene can be expressed from the *Krox20* locus. Bg, BglII; R1, EcoRI; A, AvrII, S, SpeI.

(B) Southern blot of genomic DNA from different ES clones, digested with SpeI and EcoRI, and hybridized with 5′ and 3′ probes (shown in [A]), respectively. Two independent recombinant (A12 and B4) and one wild-type (C1) clones are shown.

(C) *Krox20*$^{DT/+}$ embryos were obtained by breeding *Krox20*$^{GFP(DT)/+}$ males with Cre-expressing females (see Experimental procedures) and genotyped by PCR using primers pr1 and pr2 (positioned as indicated in [A]). PCR analysis of one litter is shown as an example.

(D) GFP expression is restricted to BC cells (arrowheads) within proximal regions of both ventral and dorsal roots. Transverse section of an E11.5 *Krox20*$^{GFP(DT)/+}$ embryo immunostained with antibodies to GFP (green) and the axonal marker β-III-tubulin (blue).

(E) Targeted expression of diphtheria toxin leads to the complete elimination of BC cells. Transverse 60 μm thick sections of E11.5 embryos stained for β-galactosidase (lower panels) and genotyped by PCR for the presence of the *PGK-Cre*^{m} transgene and of the *Krox20*$^{GFP(DT)}$, *Krox20*^{DT}, and *Krox20*^{lacZ} alleles (top panels). Combination of the different PCR results indicates that the genotype of embryo #1 is *Krox20*$^{DT/lacZ}$, whereas the genotype of embryo #6 is *Krox20*$^{GFP(DT)/lacZ}$. No β-galactosidase activity was detected within nerves roots of embryo #1 (left panel, arrowheads), indicating that BC cells have been completely eliminated. In contrast, embryo #6 shows strong β-galactosidase expression in BC cells (right panel, arrowheads). Note that the faint smears present in gel lanes #6 correspond to nonspecific PCR products amplified with Cre and DT-A primers. Bars: 200 μm.

ated with a GFP reporter plasmid immediately prior to unilateral dorsal neural tube ablation. This procedure allows the cellular morphology of individual extraspinal motor neurons to be visualized in their entirety, since only a subset of motor neurons emigrates from the neural tube upon removal of BC cells, and of these, the GFP electroporation process labels only a small proportion. Surgically manipulated, GFP-electroporated embryos were immunolabeled as whole mounts using a combination of MNR2 and Islet-2 antibodies to specifically mark subpopulations of spinal motor neurons, and anti-NF antibody to label their processes (Figures 7A, 7B, and 7E). This combination of markers was employed here rather than Islet-1/2 antibody that labels all ectopic motor neurons (Figures 2F–2H), both to confirm the motor neuron identity of ectopic GFP-positive cell soma and to allow individual soma to be more readily assigned to their axon trajectories in the ventral root. Confocal microscopic analysis of sections of these embryos revealed that many ectopic, GFP-expressing motor neurons displayed a bipolar morphology, their spindle-shaped cell bodies projecting a trailing process in the

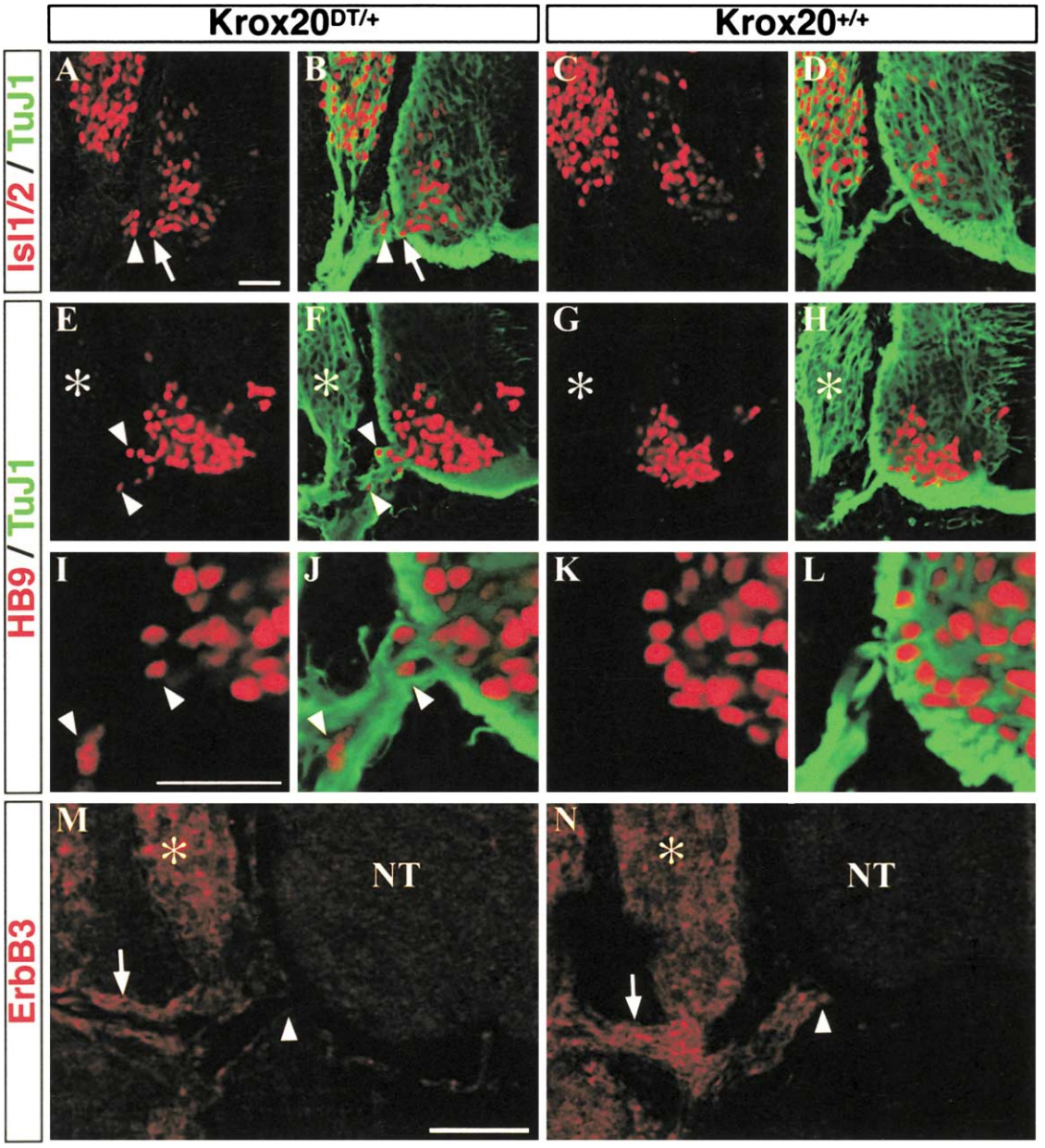

Figure 6. Genetic Ablation of BC Cells Results in Emigration of Motor Neurons out of the Spinal Cord

(A–D) Transverse sections of E11.5 *Krox20*$^{DT/+}$ (A and B) and wild-type (C and D) embryos immunostained with antibodies to the axonal marker β-III-tubulin (TuJ1, green) and to Islet-1/2 (red), which labels both spinal motor and DRG sensory neurons. In the mutant, ectopic Islet-1/2-positive cells are frequently found in the marginal zone and within the ventral root ([A and B], arrowheads). These cells are well separated from the DRG and are therefore likely to be motor neurons rather than displaced DRG neurons. Extraspinal Islet-1/2-positive neurons were never observed in wild-type embryos (C and D).

(E–L) Transverse sections of E11.5 *Krox20*$^{DT/+}$ (E, F, I, and J) and wild-type (G, H, K, and L) embryos immunostained with antibodies to β-III-tubulin (TuJ1, green) and the motor neuron-specific marker HB9 (red), which is not expressed in DRG neurons (stars in [E]–[H]). In *Krox20*$^{DT/+}$ mutant embryos, HB9-positive neurons were observed in the marginal zone and within the ventral root ([E, F, I, and J], arrowheads), but not in wild-type embryos (G, H, K, L). (I) and (J) are higher magnifications of separate sections.

(M and N) Transverse sections of E11.5 *Krox20*$^{DT/+}$ (M) and wild-type (N) embryos immunostained with an antibody against the Schwann cell precursor marker ErbB3 (red). Labeling in the DRG (stars) and within the distal part of the ventral roots and in peripheral nerves is seen in both mutant and wild-type embryos (arrows) but is absent from proximal parts of the ventral root (arrowheads) in *Krox20*$^{DT/+}$embryos. No ErbB3-positive cells were observed in the neural tube (NT) of *Krox20*$^{DT/+}$ embryos. Bars: (A–L), 50 μm; (M and N), 100 μm.

neural tube and extending a leading process into the ventral root (Figures 7C and 7D). We conclude, therefore, that in absence of BC cells, ectopic motor neuron cell bodies leave the spinal cord by translocating along their axons (Figures 7F and 7G).

Discussion

The boundaries between CNS and PNS compartments in the spinal cord are manifest in specialized transitional regions within spinal nerve roots at both the DREZ and

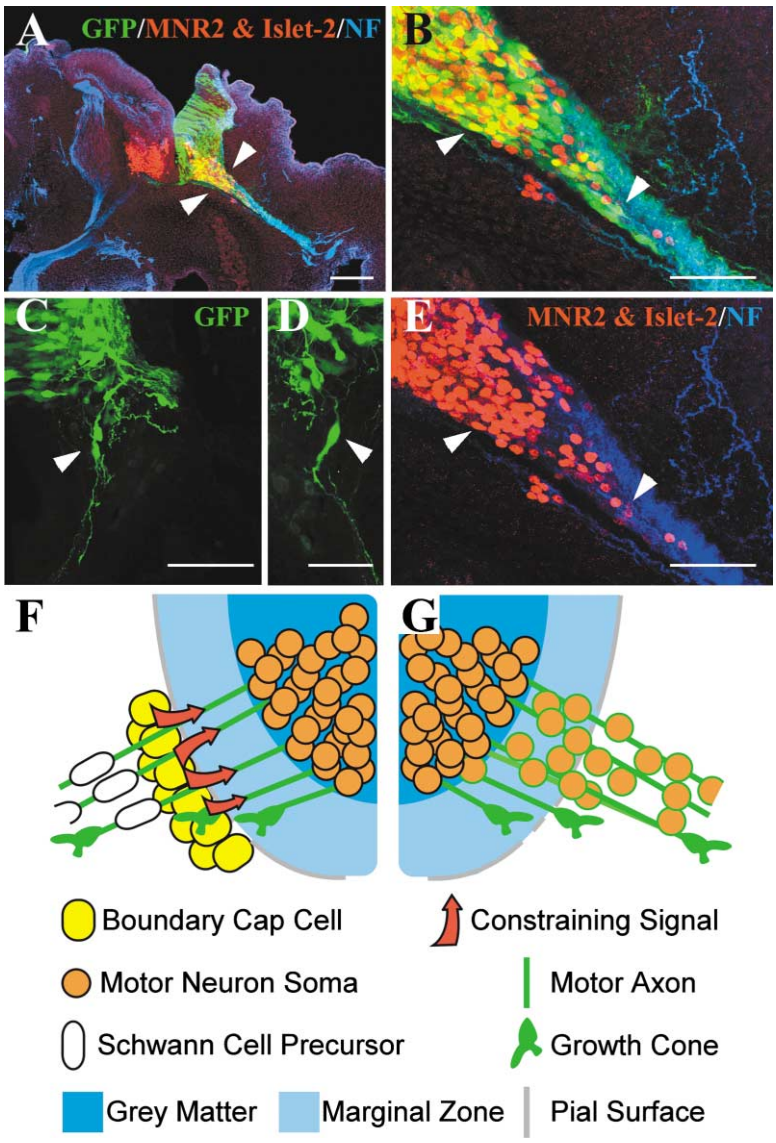

Figure 7. Extraspinal Motor Neuron Cell Bodies Can Migrate by Translocating along Their Axons but Are Normally Confined within the CNS by Motor Exit Point Neural Crest BC Cells

(A, B, and E) Transverse sections of an E5 (HH stage 26) embryo 71 hr after unilateral GFP reporter plasmid electroporation and dorsal neural tube ablation. This embryo has been immunostained with a mixture of antibodies to neurofilament (blue) and two specific motor neuron markers, Islet-2 and MNR2 (red). Numerous motor neurons exit the spinal cord on the operated side only (arrowheads); GFP-expressing (green, [A and B]) ectopic neurons in the ventral root have a distinct bipolar morphology (shown at higher power in [B], arrowheads). Comparison between (B) and (E) shows that most (~90%) of the GFP-positive neurons are also Islet-2/MNR2 positive.

(C and D) Transverse section of an E5 (HH stage 26) embryo 69 hr after unilateral GFP reporter plasmid electroporation and dorsal neural tube ablation. Because few extraspinal motor neurons express GFP, their spindle-shaped bipolar morphology (white arrowheads) and both their trailing processes in the spinal cord and long leading processes extending in the ventral root are clearly visualized. Bars (A): 150 μm; (B, C, and E), 50 μm; (D): 25 μm. All pictures are flattened confocal z series.

(F–G) Schematic diagram illustrating the role of motor exit point BC cells. (F) Under normal circumstances, clusters of BC cells (yellow) define the PNS side of the CNS:PNS interface at the motor exit point. Here, the basal lamina (gray line) of the neural tube is discontinuous, and motor axons (green lines) leave the neural tube. Motor neuron cell bodies (orange discs) are confined within the CNS by factors deployed by BC cells (red arrows), either by contact-mediated retrograde signaling along axons or diffusion toward motor neuron soma within the motor column (dark blue). (G) In the absence of neural crest BC cells, the integrity of the CNS:PNS interface at the MEP is compromised. The lack of BC cell constraining signals allows motor neuron cell soma to escape the confines of the lateral motor column by translocating along their axons into the marginal zone (light blue) and out into the periphery via the MEP.

MEP. These interfaces comprise a unique cellular apposition between two types of glia, astrocytes and Schwann cells, which arise embryologically from distinct sites, the neural tube and neural crest, respectively. Since these sites represent the points of entry and exit of axons connecting the developing spinal cord with the periphery, a selective gating mechanism must be in place at early interfaces that allows axons unhindered access but prohibits the mixing of cells between emerging CNS and PNS compartments.

The objective of the work described in this paper was to determine how interfaces between CNS and PNS compartments are first established at spinal MEPs, and how they function. Using a series of progressively refined ablation techniques in chick and mouse embryos, we have identified a particular subpopulation of neural crest derivatives, BC cells, that we propose perform a key gating function at immature MEPs, confining motor neurons within the spinal cord and thereby maintaining the integrity of spinal motor columns.

BC Cells Prefigure Spinal MEPs but Do Not Influence Motor Axon Exit or Outgrowth

Since there was some uncertainty about the relative timing of arrival of migrating neural crest cells at prospective MEPs and the initiation of motor axon outgrowth (Loring and Erickson, 1987; Rickmann et al., 1985; Serbedzija et al., 1989), it was first necessary to establish this with some precision. We showed by two-color whole-mount embryo immunolabeling and confocal microscopy analysis that, shortly before spinal motor axons emerge from the CNS, groups of neural crest cells begin to occupy the sites of prospective MEPs. By virtue of their position and antigenic phenotype, these correspond to BC cells, the earliest neural crest derivatives to prefigure the sites of nerve root entry and exit at the

surface of the embryonic avian hindbrain (Niederlander and Lumsden, 1996) and spinal cord (Golding and Cohen, 1997; Schneider-Maunoury et al., 1993). To analyze the function of BC cells at MEPs, the neural crest was ablated, either surgically in chick embryos or by exploiting the neural crest-free phenotype of the spinal cord in trunk regions of *Splotch* mutant mouse embryos. This had little effect on the pattern of motor axon outgrowth, demonstrating, first, that motor axons target ventral exit points either by a cell autonomous mechanism or are guided to them from within the spinal cord by signals from neuroepithelial cells (Niederlander and Lumsden, 1996). Second, motor axon pathfinding in the periphery seems to proceed independently of neural crest derivatives. This finding concurs with recent studies in zebrafish embryos (Gilmour et al., 2002), where growing axons were shown unambiguously to lead peripheral glia.

Neural Crest Ablation Leads to Motor Neuron Emigration through MEPs

The absence of neural crest cells at MEPs, however, had a dramatic consequence, leading in both model systems to the emigration of large numbers of identified spinal motor neurons into the periphery. The simplest interpretation of this finding is that neural crest BC cells comprise the PNS side of the CNS:PNS interface at immature MEPs, and in their absence, the integrity of the boundary is compromised, allowing motor neurons to escape the confines of spinal motor columns. This adds a novel dimension to our understanding of the epigenetic mechanisms that regulate the settling patterns of motor neurons in the embryonic spinal cord (Price et al., 2002; Sockanathan and Jessell, 1998), since it raises the novel possibility that neural crest cell-motor neuron interactions help maintain the integrity of early developing motor columns.

Interestingly, our findings also help resolve a long-standing controversy concerning the possible neural tube origins of avian trunk ventral root glia (Le Douarin et al., 1991), since prior surgical dorsal neural tube ablation was a common feature in all previous studies that assigned a Schwann cell-like (or "sheath" cell) identity to neural tube emigrants within ventral roots (Lunn et al., 1987; Rickmann et al., 1985; Weston, 1963). In the present work, however, we show that all the ectopic neural tube emigrants found in the periphery as a result of neural crest ablation were Islet-1/2-expressing neurons. It is also noteworthy that we have never observed ectopic neurons under normal circumstances. Our observations do not, therefore, correspond to the phenomenon of ventral root exiting cells described by Sohal and colleagues (Sohal et al., 1996, 1998a, 1998b), the existence of which has been called into question by recent work (Yaneza et al., 2002).

Grafts of Purified Quail Neural Crest Cells Rescue the Ectopic Motor Neuron Phenotype in Crest-Ablated Chick Embryos

The removal of neural crest by means of surgical ablation, or as a result of the *Splotch* mutation, could have had effects on dorsoventral patterning of the spinal cord and/or on somitic development. It was important, therefore, to establish that ectopic motor neurons arose as a direct consequence of the absence of neural crest from MEPs. We show here that the effect can be completely rescued in surgically ablated chick embryos by grafts of purified quail neural crest cell aggregates. Two further aspects of the rescue experiment deserve comment. First, it was apparent that the presence of very few exogenous crest derivatives at sites in the vicinity of the MEP was sufficient to prevent motor neuron emigration in ablated regions of the host spinal cord. This concurs with observations in operated chick embryos at the A-P extremities of dorsal neural tube ablated regions (data not shown) and in anterior thoracic regions of *Splotch* mutant embryos (Figures 3A and 3B). This suggests that signaling from neural crest cells, possibly by a retrograde mechanism mediated by axonal contact, is sufficient to constrain motor neurons within the CNS despite the continued absence of the dorsal half of the neural tube. Second, there was a clear preference of the grafted quail cells to migrate into host ventral roots, toward MEPs (see, e.g., Figure 4A), possibly by tracking along host motor axons or in response to a putative exit point chemoattractant.

Targeted Genetic Elimination of Preexisting MEP BC Cells Leads to Motor Neuron Emigration

Finally, in this work, by targeting BC cells for elimination in embryos carrying the *Krox20*^{DT} allele, we provide direct evidence that they are the neural crest subpopulation specifically responsible for gating the MEP. Thus, this subpopulation of neural crest derivatives is necessary and sufficient to maintain the integrity of the CNS:PNS boundary with respect to spinal motor neurons. However, elimination of MEP BC cells in *Krox20*$^{DT/+}$ embryos did not lead to the inward migration of PNS glial cells from the ventral root. This suggests that processes of radial neuroepithelial cells that comprise the CNS side of the immature MEP may also play a part in regulating cell mixing at this interface. The variation in the numbers of extraspinal motor neurons seen in the three models examined in this study probably arises because of differences in timing and of precision of neural crest removal. Thus, unlike in operated chick embryos and *Splotch* mutant mice, where neural crest derivatives are completely absent from the outset, most derivatives, including BC cells and Schwann cell precursors, form on schedule in the *Krox-20*^{DT} line, since the expression of DT-A is under control of *Krox-20*, which is expressed only after the BC cell clusters form and begin to differentiate. This may allow other cell autonomous and target-derived factors constraining motor neuron migration to exert their effects and partially compensate for the elimination of BC cell-derived signals. Alternatively, ectopic motor neurons once outside the CNS environment could die or more rapidly downregulate expression of specific markers, including Islet-1/2 and HB9 in older embryos.

We have yet to explore in detail the corresponding behavior of BC cells at the DREZ and the consequences of their targeted ablation for development of the DREZ and the subsequent ingrowth there of primary afferent projections from DRG neurons in *Krox-20*$^{DT/+}$ embryos. However, it is likely that BC cells at the DREZ interface

perform a similar function to those at the MEP. This idea is consistent with the finding that in *Sox-10* mutant mice, where gliogenic derivatives of the neural crest are largely eliminated, the DREZ is absent, and in its place, a syncytium of cells forms a bridge linking the DRG directly to the dorsal spinal cord (Britsch et al., 2001; Sonnenberg-Riethmacher et al., 2001).

Ectopic Motor Neuron Somata Emigrate from the Spinal Cord by Translocating along Their Axons

GFP electroporation of ectopic motor neurons in crest-ablated embryos suggested migration into the periphery appeared to involve perikarya following their axons as they exited the spinal cord. This behavior may indicate the existence of a more general developmental mechanism whereby neuronal migration is tightly coupled to axon extension that, in the absence of constraining/repellent factors, leads neuron cell bodies to follow their axons (Figures 7F and 7G). Interestingly in one class of neurons, cerebellar granule cells, in which the behavior of precursors migrating from the rhombic lip into the cerebellar anlage has been characterized in vivo, perikarya were seen to translocate along their leading axons toward the external germinal layer (Gilthorpe et al., 2002; Wingate and Hatten, 1999). Our results also raise questions about how exit point BC cells normally signal to block inappropriate motor neuron migration. Is there reciprocal signaling between motor neurons and BC cells mediated by diffusible or contact mediated mechanisms? Are the differential responses of axonal growth cones and motor neuron cell bodies reflected in corresponding asymmetric patterns of expression of receptors for putative BC cell repellent molecules? The existence of such a mechanism could explain the observation of ectopic motor neurons in *HB9* mutant mice (Arber et al., 1999). Thus, the transcription factor HB9 could regulate the responsiveness of motor neurons to a putative repellent signal from BC cells, perhaps by controlling expression of receptors on their growth cones and/or soma.

Experimental Procedures

In Ovo Microsurgery

The neural crest was unilaterally ablated before neural crest delamination by removal of the dorsal half of the right side of the neural tube of HH stage 10–14 chick embryos, from presomitic levels up to the last three formed somites. The length of tissue removed was equivalent to four to eight somites. Briefly, the eggs were windowed, and the embryos were bathed in Tyrode's saline. A solution of 5% India ink (Pelikan Fount) in Tyrode's was injected below the blastodisc to help visualize the embryo. The neural tube was opened at the chosen site of surgery, and its dorsal half was separated from the paraxial mesoderm using a 15° microfeather scalpel (John Weiss). The neural tube was then cut longitudinally with a pulled glass needle, and the dorsal neural tube was removed. The embryos were left to develop in ovo at 38.5°C for 2–4 days. As expected, at the site of dorsal surgical ablation, the neural tube remained open. This procedure allows spinal cord development using the unoperated side as a control. There was no evidence of neural crest cells migrating from medial positions of the unoperated part of the embryo into ablated regions.

Neural Crest Culture

E2 quail neural tubes were dissected from presomitic levels up to eight somites. These were separated from notochord, paraxial mesoderm, and epidermis with a solution of Dispase plus DNase (Sigma). The neural tube explants were then cultured for 24 hr in 10% sheep serum in F12 with Glutamax (GIBCO) on a substratum of fibronectin (Sigma) in precoated plastic tissue culture dishes to allow for the migration of neural crest cells from the explant. The neural tube component of the explants was excised from the culture dish with a tungsten needle and discarded. The neural crest cells in the outgrowth zone ware harvested by a short incubation in trypsin, and the recovered cells were resuspended in 10% sheep serum in L15. They were then allowed to aggregate into pellets in 2 ml volumes of 10% sheep serum in L15 plated in a nontissue culture plastic dish for 1 hr at 37°C in a CO_2 incubator. Clusters of quail crests were inserted, using a tungsten needle, in lumen of the neural tube of E2 chick embryos within the zone of neural tube ablation immediately after surgery. The embryos were then left to develop at 38.5°C for 48 hr.

GFP Plasmid Electroporation

Prior to dorsal neural tube ablation, small amounts pCA-β-EGFPm5 (gift of Dr Jon Gilthorpe) in Fast Green FCF were injected in the lumen of the neural tube, at presomitic level, using a picospritzer (WPI). The injected DNA was then unilaterally pulse-electroporated in the neural tube of chick embryos (Itasaki et al., 1999).

Antibodies Used for Immunolabeling

Mouse monoclonal anti-Islet-1/2 (39. 5D5), anti-Islet-2 (51. 4H9), and anti-MNR2 (81. 5C10) were all developed by Dr Thomas Jessell. QCPN was developed by Drs. Carlson and Carlson. All these antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. Rabbit anti-Islet-1/2 and anti-HB9 were a gift from Dr. Thomas Jessell. Mouse monoclonal anti-Cad-7 was a gift from Dr. S. Nakagawa. Rabbit anti-laminin (L9393) was obtained from Sigma; mouse monoclonal HNK-1 from Serotec; rabbit anti-neurofilament (NF1991) from Chemicon; mouse monoclonal anti-β-III-tubulin (TuJ1) from BabCo; rabbit anti-ErbB3 from Santa Cruz; rabbit anti-GFP from Molecular Probes.

Dual Immunostaining for Whole-Mount Embryo and Section Confocal Microscopy Analysis

E2.5 chick embryos were fixed in methanol-DMSO (4:1) for 24 hr at 4°C, rehydrated through decreasing methanol series in PBS/0.05% Tween 20 (PTW), and blocked overnight in 1% milk/0.1% Triton/PBS (blocking solution). Antibodies were then mixed at the appropriate dilution in blocking solution and left to incubate overnight at 4°C. The embryos were then washed extensively in PTW and incubated overnight at 4°C with fluorescent secondary antibodies (Molecular Probes) at the appropriate concentration in blocking solution. The embryos were then washed in PTW and cleared in increasing concentrations of glycerol/PTW before mounting for whole-mount confocal microscopy analysis. The same embryos were mounted in 30% gelatine/PBS and sectioned (50 μm thickness) on a vibratome (Leica). Mouse embryo sections were cut on a cryostat (16 μm) and blocked 1 hr at room temperature in PTW containing 10% serum. Primary antibodies were applied in the same solution overnight at 4°C. Secondary antibodies (Jackson ImmunoResearch) were incubated in PTW for 2 hr at room temperature. Sections were mounted in Vectashield (Vector).

Immunostaining of Embryos Post-Surgery

Whole-mount immunostaining of operated chick embryos was as described above, with the following modification: embryos were gutted and fixed for 2 hr in ice-cold PFA. They were then washed in ice-cold PBS for 2 hr and blocked overnight in blocking solution.

For section antibody staining, the fixed embryos were cryoprotected in 30% sucrose/PBS overnight, frozen in OCT, and cryosectioned (Bright cryostat; 20 μm) in a sequential series through the operated region. The sections were preincubated in 1% milk/0.1% Triton/PBS for 1 hr, washed extensively in PBS, and incubated overnight at 4°C with primary antibodies at the appropriate dilution in 1× MEM/1% BSA (antibody diluent). Sections were washed extensively in PBS and incubated with secondary antibody (Molecular

Probes) in antibody diluent for 1 hr at room temperature. They were then washed in PBS and counterstained with Hoechst nuclear dye (bisbenzimide, 4 μM in PBS, Sigma) before mounting.

Generation of *Krox20$^{GFP(DT)}$* Mutant Mice

A 10 kb genomic fragment derived from the *Krox20* locus in which an AvrII-AvrII fragment containing the initiation codon had been replaced by a *neo^R* selection cassette (a gift from O. Voiculescu) was used as the starting construct. A 3 kb cassette containing a modified form of the EGFP coding sequence (a gift from H. Le Mouellic) flanked by two *loxP* sites and followed by the diphtheria toxin A gene was inserted 5′ to the *neo^R* gene in the targeting vector (Figure 5A). ES cells (CK35 line, a gift from C. Kress and C. Babinet) were maintained, transfected, and selected as described previously (Schneider-Maunoury et al., 1993). 300 G418-resistant colonies were screened for homologous recombination Southern blotting (Figure 5B) using a 0.7 kb EcoRI-EcoRI fragment as a 5′ probe and a SpeI-EcoRI fragment as a 3′ probe, respectively (Figures 5A and 5B). Chimeric founder mice were produced by C57BL/6J blastocysts injection: 1 out of 8 positive clones injected transmitted the targeted allele. *Krox20$^{GFP(DT)/+}$* heterozygous animals were obtained upon crossing the chimeras with C57Bl6/DBA2 mice and were identified by PCR analysis and/or detection of GFP fluorescence in hair follicles. *Krox20$^{DT/+}$* embryos were generated by crossing *Krox20$^{GFP(DT)/+}$* males with *PGK-Cre^m* females (a gift from Y. Lallemand; Lallemand et al., 1998) that express the Cre-recombinase in the germ line. In the resulting litters, complete recombination was observed in all the embryos, and the *Krox20$^{GFP(DT)}$* allele was no longer detectable. *Krox20$^{DT/+}$* embryos were identified by PCR using primers pr1 (5′, in the *Krox20* coding sequence) and pr2 (3′, in the DT-A coding sequence), as indicated in Figure 5C. *Krox20$^{DT/lacZ}$* and *Krox20$^{GFP(DT)/lacZ}$* embryos were generated by crossing *Krox20$^{GFP(DT)/+}$* females with *Krox20$^{lacZ/+}$;PGK-Cre^m* heterozygous males. Recombination of the *Krox20$^{GFP(DT)}$* allele was only observed in embryos carrying the *PGK-Cre^m* transgene.

X-Gal staining was performed as described (Schneider-Maunoury et al., 1993).

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