Nkx6.1 controls migration and axon pathfinding of cranial branchio-motoneurons

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Summary

As many studies have focused on the mechanisms of motoneuron specification, little is known about the factors that control the subsequent development of postmitotic motoneurons. Previously, we showed that the transcription factor Nkx6.1 is required for the early specification of somatic motoneuron progenitors in the spinal cord. Our present analysis of hindbrain motoneuron development in Nkx6.1-deficient mouse embryos reveals that the early specification of branchio-motoneurons is independent of Nkx6.1 function, but that it is required for their subsequent development. In *Nkx6.1* mutant mice, we observed defects in the migration, as well as in the axon projections of branchio-motoneurons. A detailed analysis of the migratory defect in facial branchio-motoneurons reveals ectopic expression of the cell surface receptors *Ret* and *Unc5h3* in premigratory neurons, but no changes in the rhombomeric environment. Taken together, our findings demonstrate a requirement for Nkx6.1 in the development of postmitotic motoneurons, and suggest a cell-autonomous function in the control of branchio-motoneuron migration.

Key words: Nkx6.1, Nkx6.2, Hindbrain, Facial nucleus, Motoneuron, Neuronal migration, Neuronal differentiation, Mouse

Introduction

To generate the diversity of vertebrate central nervous system (CNS) neurons, neuronal progenitors must receive positional information along the anteroposterior (AP) and dorsoventral (DV) axes during development. The signals that confer such positional information induce the expression of specific complements of transcription factors, and determine the subsequent fate of the progenitors (Jessell, 2000; Lumsden and Krumlauf, 1996; Rijli et al., 1998). In the hindbrain, transcription factors of the Hox gene family have been identified as key regulators of cell identity along the AP axis (Rijli et al., 1998). Reflecting their role in AP specification, the anterior-most expression limits of different Hox genes adhere to morphologically distinct boundaries, which transiently subdivide the hindbrain into distinct lineage units, termed rhombomeres (r). Along the DV axis of the developing hindbrain, two discrete domains of progenitors give rise to three different classes of motoneurons, the branchio-motor (bm), visceromotor (vm) and somatic motor (sm) neurons. Both bm and vm neurons arise from a common ventral progenitor domain, defined by the expression of the homeodomain transcription factor Nkx2.2 (Briscoe et al., 1999). Downstream of Nkx2.2, the paired-like homeobox gene *Phox2b* is required for the differentiation of these progenitors into vm and bm neurons (Brunet and Pattyn, 2002; Pattyn et al., 2000). The sm neurons are generated dorsal to the vm/bm neurons from progenitors that express the homeodomain transcription factors Pax6, Olig2 and Nkx6.1 (Briscoe et al., 2000; Ericson et al., 1997; Novitch et al., 2001). Mutation of the *Nkx6.1* gene in mice leads to a mis-specification of sm neuron progenitors, resulting in the generation of interneurons instead of motoneurons (Sander et al., 2000a).

Although we are beginning to understand the genetic control of hindbrain motoneuron specification, little is known about the molecular mechanisms that control their subsequent development. A characteristic of bm neurons is their complex pattern of migration in the vertebrate hindbrain (Fritzsch, 1998). In mice, the most extensive migration is observed in bm neurons of the facial (VIIth) nerve, which are born in r4, and subsequently migrate within the mantle zone tangentially along the ventral midline, through r5, and into r6 (Altman and Bayer, 1982; Ashwell and Watson, 1983; Auclair et al., 1996; Fritzsch and Nichols, 1993). In r6, facial branchio-motor (fbm) neurons first migrate dorsolaterally and then radially to form the facial nucleus at the pial surface. However, not all fbm neurons initiate their migration simultaneously. The first neurons cross the r4/r5 boundary at embryonic day (E) 10.5, and reach their final destination in r6 at E12, while the last neurons only exit r4 at E12.5 and complete migration by E14. At E14, all fbm neurons are found in their final location in r6. In contrast to fbm neurons, bm neurons of the trigeminal (Vth) nerve remain within their rhombomere of origin, and translocate dorsolaterally to the Vth nerve exit point in the dorsal half of r2/r3 (Studer et al., 1996).

Given the extensive translocation of fbm neurons from r4 to r6, the mechanisms of bm neuron migration have been

predominantly explored in fbm neurons. Several observations suggest that environmental factors, as opposed to an underlying cell-intrinsic program, control the caudal migration of fbm neurons. In support of this view, fbm neurons in kreisler (*Mafb* – Mouse Genome Informatics) and *Krox20* (*Egr2* – Mouse Genome Informatics) mutant mice, which lack the entire r5, migrate out of r4, and continue with a dorsolateral and not a caudal migration within the anteriorly positioned r6 (Garel et al., 2000; Manzanares et al., 1999; McKay et al., 1997; Schneider-Maunoury et al., 1997; Seitanidou et al., 1997; Swiatek and Gridley, 1993). Further evidence for the role of environmental factors in the initiation of caudal migration has been provided by homotopic transplantation experiments between chick and mouse tissue (Studer, 2001). When chick r5 was replaced with mouse r5 or r6, chick fbm neurons, which normally lack a caudal migration, redirected their cell bodies toward the ectopic mouse tissue and followed a caudal migratory path, similar to mouse fbm neurons. Though these data suggest that r5-derived cues are required to initiate fbm neuron migration, it is largely unknown which cell-intrinsic factors enable fbm neurons to appropriately respond to such cues.

In this study, we provide evidence that Nkx6.1, which is expressed in postmitotic motoneurons, is required for bm neuron migration. In the absence of Nkx6.1, fbm neurons are born in normal numbers, but fail to initiate caudal migration. We show that their migratory defect coincides with an ectopic expression of the netrin receptor *Unc5h3*, as well as the GDNF receptor *Ret* in fbm neurons in r4, suggesting a cell-autonomous role for Nkx6.1 in the control of fbm neuron development.

Materials and methods

Mutant mice

The *Nkx6.1* null mutation was generated by gene targeting as previously described (Sander et al., 2000b). Midday of the day on which the vaginal plug was detected was considered as stage E0.5. For embryos at stages before E11.5 somite number was determined.

Immunohistochemistry and in situ hybridization

Embryos were fixed in 4% paraformaldehyde at 4°C overnight. Indirect immunofluorescence analyses were performed on cryosections as described previously (Briscoe et al., 2000). The following primary antibodies were used: rabbit anti-Nkx6.1 (Jensen et al., 1996); guinea pig anti-Nkx6.2 and mouse anti-Evx1 (Vallstedt et al., 2001); mouse anti-Islet1, mouse anti-2H3, mouse anti-Nkx2.2 and mouse anti-En1 (Developmental Studies Hybridoma Bank); and rabbit anti-Phox2b (Pattyn et al., 2000). Alexa Fluor-conjugated secondary antibodies (Molecular Probes) were used.

In situ hybridizations on whole-mount preparations and frozen sections were performed as described previously (Gradwohl et al., 1996; Wilkinson, 1992). The mouse neogenin (*Neo1* – Mouse Genome Informatics) riboprobe comprised base pairs (bp) 3369-4538 (GenBank Accession Number Y09535), the probe for mouse *Nkx6.2* bp 253-1244 (GenBank Accession L08074). The following other cDNA probes were used: peripherin (Escurat et al., 1990), *Hoxb1* (Murphy et al., 1989), *Epha4* (Gilardi-Hebenstreit et al., 1992), *Isl1* and *Isl2* (Osumi et al., 1997), *Dbx1* and *Dbx2* (Sander et al., 2000a), *Olig2* (Pabst et al., 2003), *Irx3* (Stolt et al., 2003), *Phox2b* and *Phox2a* (Pattyn et al., 1997), *Ret* (Pachnis et al., 1993), *Tag1* (*Cntn2* – Mouse Genome Informatics) (Garel et al., 2000), *Unc5h3* (Ackerman et al., 1997). For photography, hindbrains were dissected from surrounding tissue, flattened on microscope slides and mounted with 80% glycerol.

Retrograde labeling of cranial nerves

E11.5-E13.5 embryos were dissected and fixed in 4% paraformaldehyde. DiI and DiA (Molecular Probes) injections were performed as previously described (Fritzsch and Nichols, 1993). For retrograde labeling, DiI- or DiA-soaked filter strips were applied to the VIIth nerve lateral to the otocyst, to the mandibular branch of the Vth nerve near the angle of the jaw, and to the glossopharyngeal/vagal (IX/Xth) nerves near the jugular foramen, and allowed to diffuse for 2-5 days. Hindbrains were dissected, mounted on glass slides in glycerol, and images captured with a Biorad Radiance 2000 confocal system mounted onto a Nikon Eclipse 800 microscope. To visualize individual axons, the brains were gelatin embedded and vibratome sectioned at 100 μm before photography.

Results

Nkx6.1 is expressed in developing cranial motoneurons

To determine the pattern of *Nkx6.1* expression in the developing mouse hindbrain, we performed in situ hybridization using an antisense *Nkx6.1* probe on flat-mounted hindbrains. The first *Nkx6.1*-expressing cells appeared at E8.5 in the ventral neural tube immediately abutting the floor plate (Fig. 1A). At E10.5, when motoneurons differentiate, *Nkx6.1* was exclusively detected in two lateral stripes adjacent to the floor plate (Fig. 1B). Similar to spinal cord levels (Sander et al., 2000a), immunodetection of Nkx6.1 on coronal sections through the mouse hindbrain at E10.5 showed co-expression of Nkx6.1 with the vm/bm neuron progenitor marker Nkx2.2 (Fig. 1C), and with the motoneuron marker Isl1 (Fig. 1D). At E11.5, during bm neuron migration, *Nkx6.1* was detected in dorsolaterally migrating trigeminal bm neurons, as well as in caudally migrating fbm neurons (Fig. 1E). An additional lateral column of *Nkx6.1*-expressing cells, extending caudally from r4 to r8 at E11.5, corresponds from rostral to caudal to the inner ear efferent neurons, the vm neurons of the superior and inferior salivatory nuclei, and the bm neurons of the nucleus ambiguous (Fig. 1E). At E12.5, *Nkx6.1* was detected in the entire stream of migrating fbm neurons, from r4 into the dorsal half of r6 (Fig. 1F). To test if all classes of postmitotic motoneurons express *Nkx6.1*, we performed in situ hybridizations on transverse sections through the hindbrain at E12.5. *Nkx6.1* was expressed in all cranial motoneurons, including the oculomotor (Fig. 1G) and trochlear motor nuclei (Fig. 1H), the trigeminal bm neurons (Fig. 1I), the sm neurons of the abducens nerve (Fig. 1J), the facial bm neurons (Fig. 1J,K), as well as the bm neurons of the nucleus ambiguous, the vm neurons of the dorsal motor nucleus of the vagal nerve and the sm neurons of hypoglossal nerve (Fig. 1L). In addition, *Nkx6.1* was detected in the raphe nuclei (Fig. 1K,L), which contain serotonergic neurons that are generated subsequently to vm/bm neurons from the same progenitor domain (Briscoe et al., 1999). All these nuclei remained *Nkx6.1*-positive until the first postnatal week (data not shown), suggesting that Nkx6.1 may have a function in postmitotic motoneuron development.

Absence of somatic and reduced numbers of branchio-motoneurons in the hindbrain of late gestation *Nkx6.1* mutant embryos

To study the role of Nkx6.1 in hindbrain motoneuron development, we first analyzed late gestation *Nkx6.1* mutant

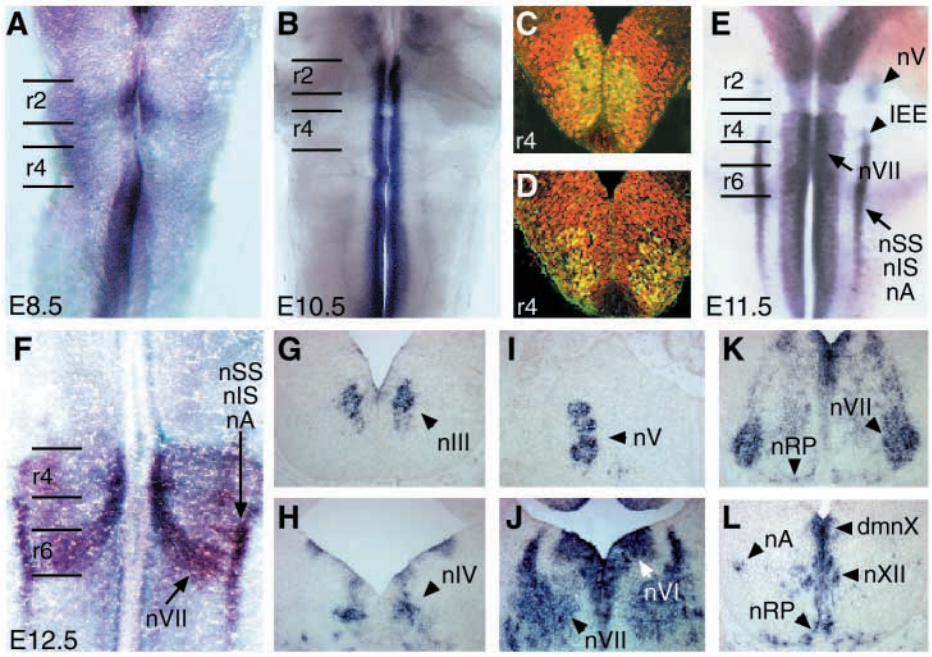

Fig. 1. *Nkx6.1* expression in the developing mouse hindbrain. In situ hybridization with a *Nkx6.1* probe on flat-mounted hindbrains at different embryonic stages (A,B,E,F) and on coronal hindbrain sections at E12.5 (G-L). Rhombomeric positions are indicated (r2 to r6). At E8.5 (A), *Nkx6.1* is expressed in ventral progenitor cells. At E10.5 (B), *Nkx6.1* expression spans the entire length of the ventral hindbrain. At E10.5, immunofluorescence detection of Nkx6.1 (red in C,D) with Nkx2.2 (green in C) or Isl1 (green in D) shows co-expression of Nkx6.1 with both Nkx2.2 and Isl1 (yellow appearing cells in C,D). At E11.5 (E), *Nkx6.1* expression is detected in the trigeminal (nV), and in migrating facial branchio-motor (fbm) neurons (nVII) from r4 to r6, as well as in a lateral column representing the motor nuclei of the inner ear efferents (IEE), the superior (nSS) and inferior (nIS) salivatory nucleus and nucleus ambiguous (nA). At E12.5 (F), *Nkx6.1* marks the entire stream of migrating fbm neurons from r4 into the dorsal half of r6, and is also detected in the oculomotor (nIII) (G), trochlear (nIV) (H), trigeminal (nV) (I), abducens (nVI) (J), facial nuclei (nVII) (K,J), the nucleus ambiguous (nA) (L), the dorsal motor nucleus of the vagal nerve (dmnX) (L) and the hypoglossal nucleus (nXII) (L). Nkx6.1 is also detected in the raphe nuclei (nRP) (K,L).

embryos for the presence of cranial motor nuclei. Using in situ hybridization for peripherin, which marks peripherally projecting neurons, the sm nuclei of the abducens and hypoglossal nerve were undetectable in *Nkx6.1* mutants (arrows in Fig. 2A-D). In contrast to sm nuclei, bm and vm nuclei were present in *Nkx6.1* mutants at E18.5. However, upon closer examination, the trigeminal (Fig. 2E,F) and facial bm nuclei (Fig. 2G,H), as well as the bm neurons of the nucleus ambiguous (Fig. 2I,J) showed a size reduction compared with wild-type embryos. In addition to its reduced size, we noticed that in *Nkx6.1* mutants the fbm nucleus was not found in its typical location close to the pial surface (Fig. 2G,H). Moreover, an analysis of histological sections through the entire hindbrain revealed that the fbm nucleus was positioned further rostrally in *Nkx6.1* mutants (data not shown), raising the possibility that they have a defect in fbm neuron migration. In the hindbrain at E18.5, trigeminal bm neurons had formed a compact nucleus in wild-type embryos (Fig. 2E), but were found scattered in *Nkx6.1* mutants (Fig. 2F). The purely visceral dorsal motor nucleus of the vagal nerve was normal in size and did not have a scattered appearance (arrowhead in Fig. 2C,D). Together, these findings show that cranial vm and bm nuclei are present in *Nkx6.1* mutants and suggest that Nkx6.1 could be involved in ensuring their proper migration.

Nkx6.1 inactivation affects the migration and axonal pathfinding of branchio-motoneurons

To study the migratory pattern of bm neurons in more detail, we performed retrograde labeling with the fluorescent tracer DiI. As axon outgrowth precedes the migration of hindbrain motoneurons, migrating neurons can be retrogradely traced from their peripheral nerves. Application of DiI to the VIIth nerve labeled two populations of facial motoneurons, the vm neurons of the superior salivatory nucleus in r5, as well as the fbm neurons (Fig. 3A). At E12.5, DiI marked the entire migratory stream of fbm neurons from r4 to r6 in wild-type embryos. By contrast, the vast majority of fbm neurons in *Nkx6.1* mutants were located in r4, and only few were labeled in the rostral third of r5 (Fig. 3B). In r5, some fbm neurons appeared to have initiated a dorsolateral migration that is usually seen in fbm neurons in r6. However, most of the fbm neurons were positioned close to the floor plate in both r4 and r5. To exclude the possibility that neurons had actually migrated into r5/r6, but only failed to send their axons through the facial nerve, we also performed in situ hybridization with the bm/vm neuron marker *Phox2b*. Similar to the results from the DiI labeling experiments, fbm neurons in *Nkx6.1* mutants were exclusively detected in r4 and rostral r5 (Fig. 6C,D). At E13.5, virtually all fbm neurons were located in their final position in r6 in wild-type embryos, but were still positioned in r4 and rostral r5 in *Nkx6.1* mutants (data not shown).

We next studied the migratory pattern of trigeminal bm neurons by applying DiI to the mandibular branch of the Vth nerve in r2. In wild-type mice, trigeminal bm neurons migrate from their point of origin in ventral r2 and r3 dorsolaterally along the path of their axon. At E12.5, they have settled close to the exit point of the Vth nerve (Fig. 3C). In *Nkx6.1* mutants, trigeminal bm neurons appeared to migrate within a secondary nerve process, leading to the retrograde labeling of a loop-shaped structure (Fig. 3D). Moreover, the neurons only completed approximately two-thirds of their entire migratory path from the midline to the Vth nerve exit point, and failed to cluster, as seen in wild-type embryos. These data show that Nkx6.1 is required for normal migration of trigeminal and facial neurons in the hindbrain.

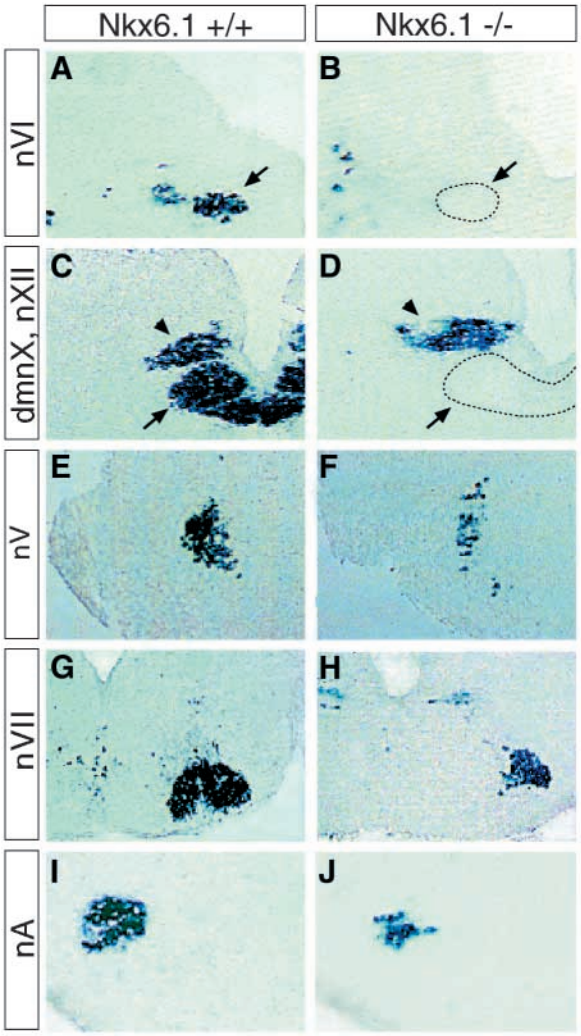

Fig. 2. Absence of somatic motor nuclei and reduced sizes of branchiomotor nuclei in *Nkx6.1* mutant embryos at E18.5. In situ hybridization with a peripherin probe on coronal sections through the hindbrain of wild type (left column) and *Nkx6.1* mutant (right column) embryos. In *Nkx6.1* mutant embryos, the somatic motor nuclei of the abducens (nVI) (arrow in A,B) and hypoglossal (nXII) (arrow in C,D) nerve are absent. The branchiomotor nuclei of the trigeminal (nV) (E,F) and facial (nVII) (G,H) nerve and the nucleus ambiguous (nA) (I,J) are present in *Nkx6.1* mutants, but reduced in size. By contrast, the dorsal motor nucleus of the vagal nerve (dmnX) (arrowhead in C,D) appears normal in size.

The retrograde DiI labeling experiments not only revealed defects in the migratory behavior of bm neurons, but also ectopic axon projections in the hindbrain of *Nkx6.1* mutants. When DiI was applied to the VIIth nerve in *Nkx6.1* mutants, scattered cell bodies were retrogradely labeled in r2/r3, as well as in r6/r7 (inset in Fig. 3B; Fig. 3E,F). Such ectopically projecting neurons were not observed in wild-type embryos (Fig. 3A; data not shown). Double labeling through the VIIth nerve in conjunction with the Vth (data not shown) or IX/Xth nerve (Fig. 3G,H) showed colocalization of these ectopically projecting neurons with the trigeminal bm neurons or the bm neurons of the nucleus ambiguous (arrows in Fig. 3H), respectively. Together, these data suggest that a subset of motor axons in r2/r3 and r6/r7 fail to recognize their proper exit points, and leave the hindbrain with the VIIth nerve in r4 (Fig. 3K).

To test if motor axons in *Nkx6.1* mutant embryos appropriately project their axons into the first, second and third branchial arches, respectively, we visualized the trajectories of these axons with an anti-neurofilament antibody on whole-mount embryos at E10.5. Analysis of the nerve branching patterns revealed accurate projections of the cranial nerves to the respective branchial arches in *Nkx6.1* mutant embryos (Fig. 3I,J), indicating that Nkx6.1 is not required for the pathfinding of peripheral motor axons.

Hindbrain segment identity is unaffected by *Nkx6.1* inactivation

Because the mechanisms which underlie bm neuron migration have been most extensively studied in fbm neurons, we focused in our subsequent analyses mainly on fbm neurons. Given the implication of environmental factors in the control of fbm neuron migration (Garel et al., 2000; Studer, 2001), a possible mechanism by which Nkx6.1 might regulate their migration is by altering the environment through which the neurons migrate. We therefore tested if the segmentation and patterning of the r4/r5 region was properly established in *Nkx6.1* mutants. We used *Hoxb1* as a marker for the r4 territory and *Epha4* for the r3 and r5 territories. The expression of *Hoxb1* (Fig. 4A,B) and *EphA4* (Fig. 4C,D), as well as the relative size of the rhombomeres, appeared normal in *Nkx6.1* mutants, suggesting that the molecular patterning of the r4/r5 region does not depend on Nkx6.1.

Normal differentiation of viscero- and branchio-motoneurons in the absence of Nkx6.1

As an alternative mechanism to an alteration of the environment, incorrect specification of fbm neurons or a delay in their differentiation could account for the aberrant migration of fbm neurons in *Nkx6.1* mutant mice. To explore this hypothesis, we studied the differentiation of cranial motoneurons at the early stages of motoneuron formation in the embryo. At E10.5, expression of the pan-motoneuron marker *Isl1* was similar in wild-type and *Nkx6.1* mutant embryos throughout most of the hindbrain (Fig. 5A,B), indicating that hindbrain motoneurons are generated independently of Nkx6.1 function. Given the reduced size of the facial nucleus at E18.5 (Fig. 2G,H), but the normal appearing *Isl1* signal in r4 at E10.5, we quantified the number of fbm neurons by counting Isl1-positive nuclei in immunohistochemistry at different stages of development (Fig. 5C). At E10.5, the number of Isl1-positive nuclei in r4 was similar in wild-type and *Nkx6.1* mutant embryos, suggesting that fbm neurons form in normal numbers in the absence of Nkx6.1. However, at E12.5, when a large number of fbm neurons have already migrated into their final position in wild-type embryos, we detected a significant reduction in the total number of fbm neurons in *Nkx6.1* mutants. A similar loss of fbm neurons has been reported in other mutants with defects in fbm neuron migration (Studer et al., 1996).

In contrast to r4, the *Isl1* signal in r5 appeared to be reduced in *Nkx6.1* mutants. In r5, a large percentage of *Isl1*-positive motoneurons constitute the sm neurons of the abducens nucleus, while a smaller percentage represents the vm neurons

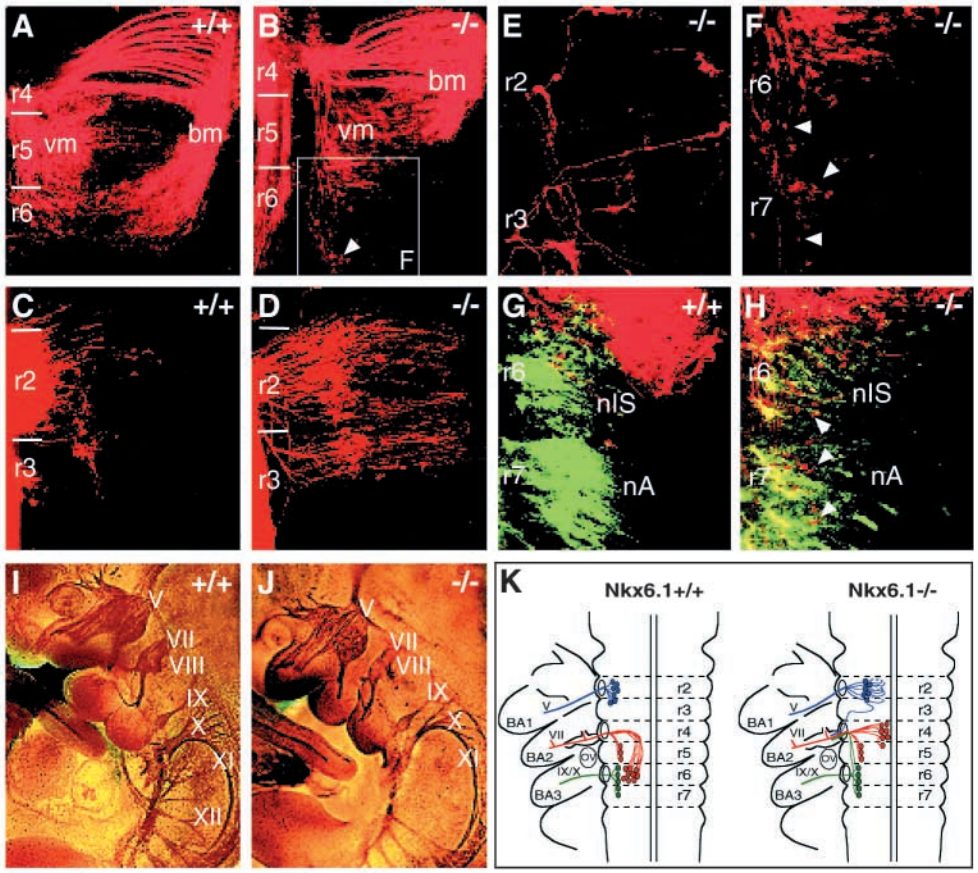

Fig. 3. Defects in branchio-motoneuron migration and axonal pathfinding in the hindbrain of *Nkx6.1* mutant embryos. Application of DiI to the facial (A,B,E,F,G,H) and trigeminal nerve (C,D) in wild-type (A,C,G) and *Nkx6.1* mutant embryos (B,D,E,F,H) at E12.5. While facial branchiomotor (bm) neurons are backlabeled along their entire migratory stream from r4 into dorsal r6 in wild-type embryos (A), most facial bm neurons are clustered close to the ventral midline in r4 and few in the rostral third of r5 in *Nkx6.1* mutants (B). No difference in the location of facial visceromotor (vm) neurons is observed between wild-type and *Nkx6.1* mutant embryos (A,B). In wild-type embryos, trigeminal motoneurons are found at the trigeminal nerve exit point (C), but scattered between the floor plate and the exit point in *Nkx6.1* mutants (D). Note the backlabeling of an additional nerve process in *Nkx6.1* mutants (D). DiI application to the facial nerve labeled neurons in r2/r3 (E) and r6/r7 (arrowheads in B,F) in *Nkx6.1* mutants, but not in wild-type embryos. Simultaneous application of DiI (red) to the facial and DiA (green) to the glossopharyngeal/vagal nerve shows that neurons, which project through the facial nerve in *Nkx6.1* mutants (arrowheads in H), are localized within the territory of the inferior salivatory nucleus (nIS) and nucleus ambiguous (nA). (I,J) Lateral view of E10.5 embryos stained with 2H3 anti-neurofilament antibody. The branching pattern of the cranial nerves does not differ between wild-type (I) and *Nkx6.1* mutant embryos (J). (K) Schematic summary of the differences in the position and axonal projections of hindbrain motoneurons between wild-type and *Nkx6.1* mutant embryos. Trigeminal (V), facial (VII), vestibuloacoustic (VIII), glossopharyngeal (IX), vagal (X), spinal accessory (XI) and hypoglossal (XII) nerves; branchial arch (BA); otic vesicle (OV).

of the superior salivatory nucleus. To test if the reduced *Isl1* signal in r5 results from a defect in sm neuron differentiation, we examined the expression of the sm neuron marker *Isl2*. At E10.5, *Isl2*-positive motoneurons were completely absent from the *Nkx6.1* mutant hindbrain (Fig. 5E), showing an absolute requirement of Nkx6.1 for sm neuron differentiation at hindbrain levels. These data confirm our findings at spinal cord levels that demonstrated incorrect specification of sm neuron progenitors in the absence of Nkx6.1 (Sander et al., 2000a). In spinal cord sm neuron progenitors, Nkx6.1 is required for expression of Olig2 and similar to the phenotype observed in *Nkx6.1* mutants, *Olig2* mutant embryos also display an absence of sm neurons (Novitch et al., 2001). We therefore tested if a similar requirement of Nkx6.1 for *Olig2* expression exists at hindbrain levels. Surprisingly, in the caudal hindbrain *Olig2* expression was maintained in the absence of Nkx6.1 (Fig. 5F,G), suggesting that Nkx6.1 and Olig2 function in a parallel rather than linear pathway in sm neuron differentiation. Consistent with the maintenance of *Olig2* expression, the domain of *Irx3*, which is repressed by Olig2 (Lu et al., 2002; Zhou and Anderson, 2002), was not changed in *Nkx6.1* mutants (Fig. 5H,I).

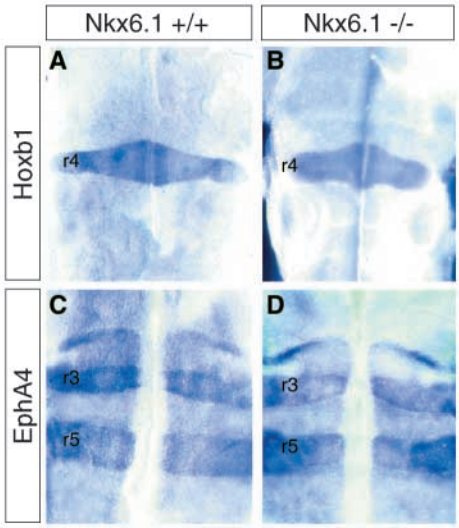

Fig. 4. Rhombomere identity is not affected by the absence of Nkx6.1 activity. In situ hybridization on flat-mounted hindbrains at E10.5. The expression pattern of *Hoxb1* (A,B), and *Epha4* (C,D) is similar in wild-type (A,C) and *Nkx6.1* mutant embryos (B,D).

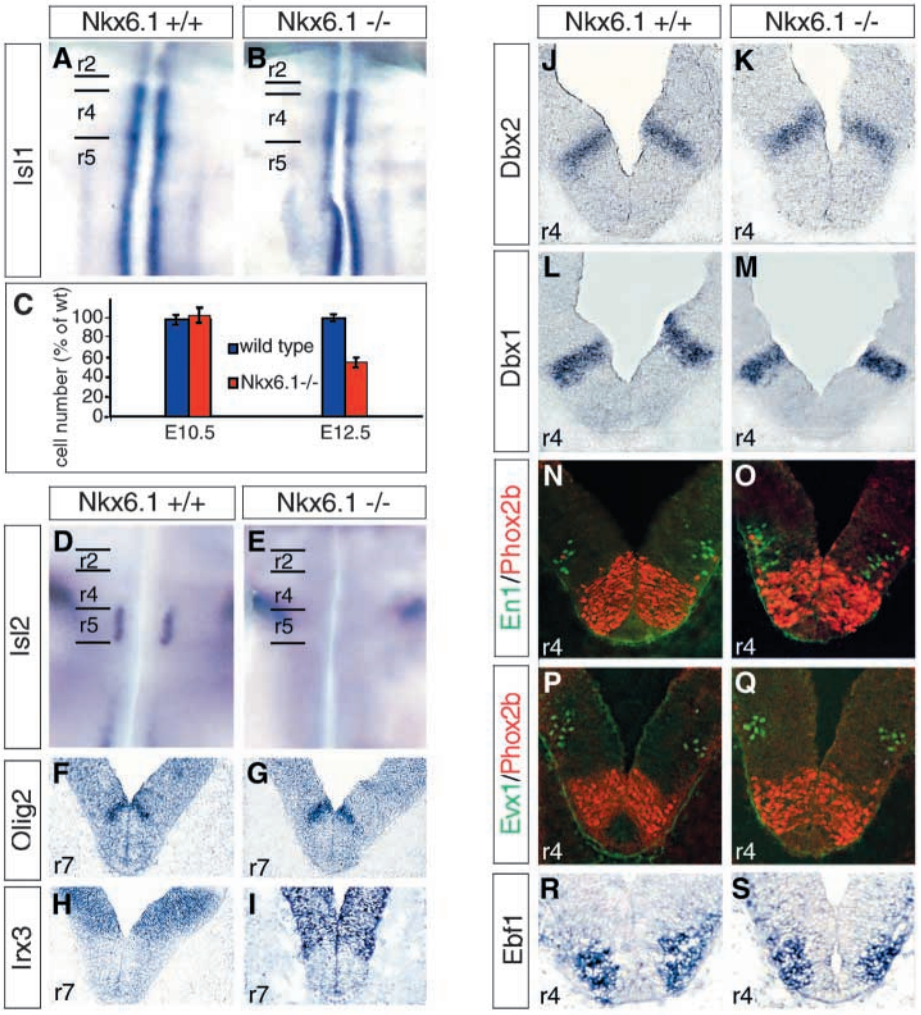

Fig. 5. Specification of facial branchio-motoneurons is not affected by *Nkx6.1* inactivation. In situ hybridization with *Isl1* (A,B) and *Isl2* (D,E) on flat-mounted hindbrains of E10.5 wild-type (A,D) and *Nkx6.1* mutant (B,E) embryos. (B) *Nkx6.1* mutant embryos generate *Isl1*-positive cells throughout the hindbrain. (C) Quantification of Isl1-positive facial branchio-motoneurons at r4 levels of wild-type and *Nkx6.1* mutant embryos at E10.5 and E12.5. Using immunohistochemistry with an anti-Isl1 antibody on coronal hindbrain sections, Isl1-positive nuclei on 12 representative sections for each genotype and age were counted. Values are shown as % of wild type, mean±s.d. (D,E) *Isl2* marks somatic motoneurons and the otic ganglion. *Isl2*-positive motoneurons are not detected in hindbrains of *Nkx6.1* mutants (E). (F-S) In situ hybridization with *Olig2* (F,G), *Irx3* (H,I), *Dbx2* (J,K), *Dbx1* (L,M) and *Ebf1* (R,S) and co-immunofluorescence detection of En1 (N,O) or Evx1 (P,Q) together with Phox2b (N-Q) on sections through r4 of wild-type (F,H,J,L,N,P,R) and *Nkx6.1* mutant embryos (G,I,K,M,O,Q,S) at E10.5. These markers are similarly expressed in wild-type and in *Nkx6.1* mutant embryos.

We next investigated whether fbm neurons in r4 were correctly specified as bm neurons. Between E9.5 and E11.5, when fbm neurons differentiate in r4, wild-type and *Nkx6.1* mutant embryos showed no difference in the expression of the bm neuron markers Nkx2.2, Isl1, *Phox2a* and Phox2b (data not shown, Fig. 5C,N,O). As we have previously observed an expansion of V1 interneurons into the motoneuron domain in the spinal cord of *Nkx6.1* mutants (Sander et al., 2000a), we also tested if fbm neurons or their progenitors carry characteristics of V1 or V0 interneurons. In contrast to spinal cord, we did not detected a ventral expansion of the V1 or V0 progenitor markers *Dbx2* or *Dbx1*, respectively (Fig. 5J-M). Likewise, fbm neurons did not misexpress the V1 interneuron marker En1 or the V0 interneuron marker Evx1 (Fig. 5P,Q).

Given that mice deficient for the transcription factor *Ebf1* also have a defect in fbm neuron migration (Garel et al., 2000), we also tested if fbm neurons in *Nkx6.1* mutants have normal *Ebf1* expression. The expression of *Ebf1* in fbm neurons was not affected by the *Nkx6.1* mutation (Fig. 5R,S). Likewise, *Nkx6.1* was normally expressed in fbm neurons in *Ebf1* mutant embryos (data not shown). Collectively, these data suggest that r4 progenitors generate normal numbers of correctly specified fbm neurons in the absence of Nkx6.1.

Migrating facial branchio-motoneurons show aberrant expression of guidance receptors in *Nkx6.1* mutant embryos

Based on these results, it seemed unlikely that defects in the early specification of bm neurons or in the establishment of a correct rhombomeric environment account for the aberrant migration of fbm neurons. We therefore examined the possibility that Nkx6.1 regulates the expression of cell surface molecules, and might thereby influence the ability of fbm neurons to interpret guidance cues in their environment. First, to visualize fbm neurons at different stages of their migration, we performed whole-mount in situ hybridization with the vm/bm neuron marker *Phox2b*. Though most fbm neurons were still located in r4 at E10.75, a few had progressed into the rostral third of r5 in both wild-type and *Nkx6.1* mutant embryos (arrowhead in Fig. 6A,B), suggesting that early during their migration a few fbm neurons cross the r4/r5 boundary in the absence of Nkx6.1. At E12.5, fbm neurons in wild-type embryos were detected along their entire migratory path from r4 to r6, but in *Nkx6.1* mutants were clustered in ventral r4 (Fig. 6C,D). In *Nkx6.1* mutants, the distribution of fbm neurons at E12.5 was almost indistinguishable from their pattern at E10.75 (Fig. 6B,D), indicating that most fbm neurons have remained at their point of origin in r4.

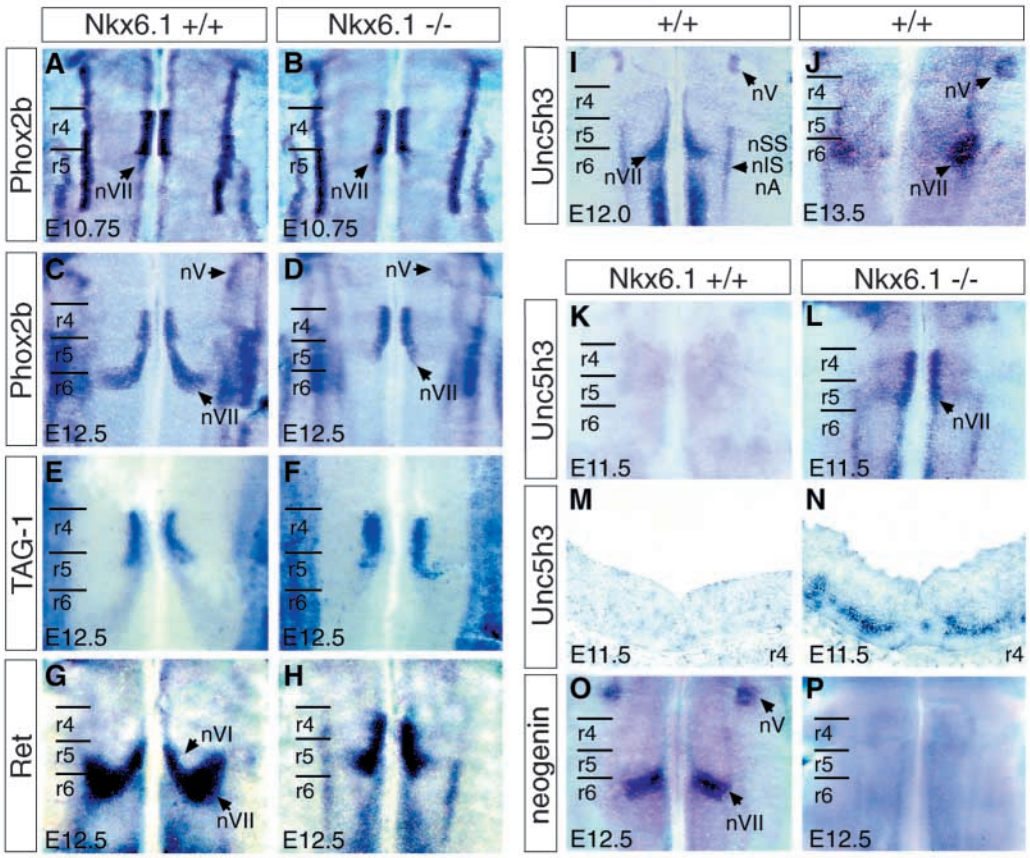

Fig. 6. Ectopic expression of cell-surface receptors in facial branchio-motoneurons of *Nkx6.1* mutants. Whole-mount in situ hybridization with the indicated probes on flat-mounted hindbrains from wild-type (left columns) and *Nkx6.1* mutant (right columns, except J) embryos at the indicated ages. *Phox2b* marks facial branchio-motor (fbm) neurons (nVII, A-D). At E10.75, few fbm neurons have migrated into r5 in wild type (arrowhead in A) and *Nkx6.1* mutants (arrowhead in B). At E12.5, some fbm neurons have completed migration into r6 in wild-type embryos (C), but no neurons are found caudal to upper r5 in *Nkx6.1* mutants (D). *Tag1* is normally expressed in fbm neurons in r4 in both wild-type (E) and *Nkx6.1* mutant embryos (F). *Ret*, which is normally restricted to migrating fbm neurons in r5 and r6 (G), is ectopically expressed in r4 in *Nkx6.1* mutants (H). In wild-type embryos, *Unc5h3* is first detected at E12.0 (I) in facial and trigeminal (nV) motoneurons, and motoneurons of the superior (nSS) and inferior salivatory nucleus (nIS), as well as the nucleus ambiguous (nA). The expression of *Unc5h3* in these neurons is maintained at E13.5 (J). Neogenin is expressed in dorsolaterally migrating facial and trigeminal motoneurons (O). In *Nkx6.1* mutants, fbm neurons ectopically express *Unc5h3* (L) and do not express neogenin (P). In situ hybridization for Unc5h3 on coronal sections through r4 verifies that the ectopic expression is specific to fbm neurons (N). Abducens nucleus, nVI.

Previous work has shown that fbm neurons regulate the expression of cell-surface receptors in a rhombomere-specific fashion (Garel et al., 2000). In r4, they strongly express the cell adhesion molecule *Tag1*, but become *Tag1*-negative in r5 and r6 (Fig. 6E) (Garel et al., 2000). By contrast, the GDNF receptor subunit *Ret* is not detected in fbm neurons in r4, but expressed in caudally and dorsolaterally migrating fbm neurons in r5 and r6 (Fig. 6G) (Garel et al., 2000). To study if Nkx6.1 activity is required for the rhombomere-specific expression of these cell surface molecules, we tested *Tag1* and *Ret* expression in *Nkx6.1* mutants. Although *Tag1* was normally expressed in *Nkx6.1* mutants (Fig. 6F), fbm neurons showed an ectopic expression of *Ret* in r4 at E11.5 and E12.5 (Fig. 6H, data not shown). Notably, at E10.5, at the onset of fbm neuron migration, fbm neurons were *Ret* negative in both wild-type and *Nkx6.1* mutant embryos (data not shown).

Recent studies have suggested that facial motoneurons in culture are responsive to the diffusible guidance molecule netrin 1 (Varela-Echavarria et al., 1997). Netrin 1 has been implicated in both neuronal migration and axon guidance in vertebrates, and can either act as a chemoattractant or chemorepulsive signal (Bloch-Gallego et al., 1999; Causeret et al., 2002; Finger et al., 2002; Przyborski et al., 1998). Through the interaction with the transmembrane receptor DCC and its homologue neogenin netrin 1 mediates chemoattraction, while interaction of netrin 1 with the *Caenorhabditis elegans* UNC5-related receptors mediates a repulsive response (Ackerman et al., 1997; Hong et al., 1999; Leonardo et al., 1997; Meyerhardt et al., 1997).

To explore a possible role for netrin signaling in fbm neuron migration, we studied the expression of the three mammalian UNC5 homologues (UNC5H) UNC5H1, UNC5H2 and UNC5H3, as well as DCC and neogenin, in mouse embryos during fbm neuron migration. Although *Unc5h1* and *Unc5h2* were not detected in migrating fbm neurons, fbm neurons expressed *Unc5h3*. In wild-type embryos, *Unc5h3* was not expressed before E12.0 (Fig. 6K). At E12.0, faint *Unc5h3* expression was detected in r4, and a strong signal was seen in fbm neurons in r5 and r6 (Fig. 6I). At E13.5, when their migration is almost complete, *Unc5h3* marked fbm neurons in r6 (Fig. 6J). *Unc5h3* was also detected in dorolaterally migrating trigeminal motoneurons (Fig. 6I,J). Among the DCC homologues, we detected only neogenin in cranial motoneurons, which as *Unc5h3* was localized in fbm neurons migrating away from the midline in r6 at E12.5 (Fig. 6O). Colocalization of *Unc5h3* and neogenin was also observed in dorsolaterally migrating trigeminal motoneurons (Fig. 6I,J,O).

To test if Nkx6.1 is required for the coordinated expression of netrin receptors in fbm neurons, we studied *Unc5h3* and neogenin expression in *Nkx6.1* mutant

hindbrains. Although no *Unc5h3* expression was detected in wild-type embryos at E11.5 (Fig. 6K,M), strong expression of *Unc5h3* was found in fbm neurons in *Nkx6.1* mutants (Fig. 6L,N). This ectopic expression in r4 fbm neurons was maintained at E12.5 (data not shown). We observed that the onset of ectopic *Unc5h3* expression coincided with the onset of the migratory defect. At E10.5, when early-born fbm neurons migrate into r5 in *Nkx6.1* mutant embryos (Fig. 6B), no ectopic expression of *Unc5h3* was observed (data not shown). However, at E11.5, when caudal migration has stopped in *Nkx6.1* mutants, they expressed *Unc5h3* ectopically (Fig. 6L,M). Fbm neurons in *Nkx6.1* mutants did not express neogenin (Fig. 6P). These findings demonstrate that Nkx6.1 controls the cell surface characteristics of migratory fbm neurons, and reveal a temporal link between the ectopic expression of cell surface receptors in fbm neurons and their migratory defect.

Nkx6.1 and Nkx6.2 are co-expressed in r4

Given that early migratory fbm neurons progress into the r5 territory in *Nkx6.1* mutants, we considered that other factors with similar function as Nkx6.1 might be present in r4. A close relative to Nkx6.1, Nkx6.2, has previously been shown to have similar activity as Nkx6.1 in promoting the generation of motoneurons in the spinal cord (Vallstedt et al., 2001). Based upon this finding, we examined if Nkx6.2 is expressed during fbm neuron development. At E10.5, we detected Nkx6.2-positive cells in a broad ventral domain in r4. Within this domain, the ventral and dorsal limit of expression of Nkx6.2 coincided with the limits of Nkx2.2 expression, and virtually all Nkx2.2-positive cells co-expressed Nkx6.2 (Fig. 7A). The most laterally located Nkx6.2-positive cells produced Isl1 (Fig. 7B). As Nkx6.1 is expressed in a similar domain as Nkx6.2 (Fig. 1C,D), we next tested if these two factors are co-expressed in r4. At E10.5, essentially all Nkx6.2-positive cells co-expressed Nkx6.1, but the domain of Nkx6.1-positive cells extended beyond the dorsal limit of Nkx6.2 expression (Fig. 7C). *Nkx6.2* expression was also detected in trigeminal bm neurons in r2, and was maintained in these neurons during their dorsolateral migration in r2/r3 (data not shown; Fig. 7E,G). Although *Nkx6.1* was detected in the entire migratory stream of fbm neurons from r4 to r6 between E11.5 and E12.5 (Fig. 1E,F), *Nkx6.2* was confined to fbm neurons in r4, and absent from fbm neurons in r5 and r6 (Fig. 7E,G). Notably, the level of *Nkx6.2* expression in r4 fbm neurons decreased markedly after E11, and Nkx6.2 was not detected in postmigratory fbm neurons (Fig. 7G). This suggests that compensation by Nkx6.2 for Nkx6.1 might only be effective during the early stages of fbm neuron development. Consistent with such early compensatory function of Nkx6.2, *Nkx6.1/Nkx6.2* double mutant embryos show a complete lack of caudal migration into r5 (Pattyn et al., 2003).

Based upon the observation that Nkx6.1 can repress Nkx6.2 in spinal motoneurons (Vallstedt et al., 2001), we tested if fbm neurons ectopically activate Nkx6.2 expression in the absence of Nkx6.1. In contrast to spinal cord, fbm neurons showed a similar pattern and level of Nkx6.2 expression in wild-type and *Nkx6.1* mutant embryos between E10.5 and E12.5 (Fig. 7C-H). Thus, unlike in spinal cord motoneuron progenitors, Nkx6.1 is not responsible for the downregulation of Nkx6.2 in fbm neurons.

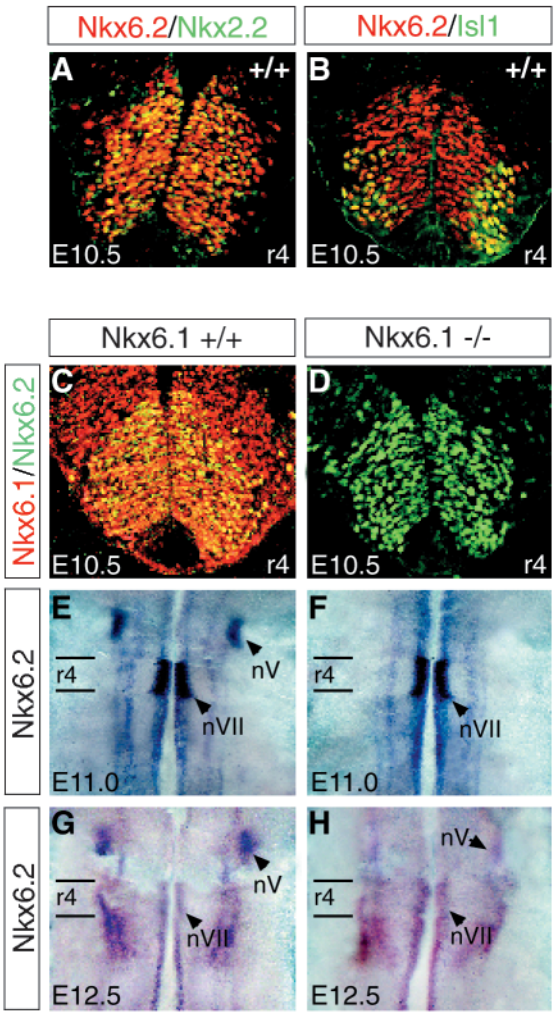

Fig. 7. Facial branchio-motoneurons in r4 co-express Nkx6.1 and Nkx6.2. Immunofluorescence detection of Nkx6.2 (red in A,B and green in C,D) with Nkx2.2 (green in A), Isl1 (green in B), Nkx6.1 (red in C,D) on sections through r4 in wild-type (A-C) and *Nkx6.1* mutant (D) embryos at E10.5. Nkx6.2 is co-expressed with Nkx2.2 (yellow appearing cells in A), Isl1 (yellow appearing cells in B) and Nkx6.1 (yellow appearing cells in C). The number of Nkx6.2-expressing cells and the level of Nkx6.2 expression are not changed in *Nkx6.1* mutants (D). (E-H) Whole-mount in situ hybridization with *Nkx6.2* on flat-mounted hindbrains from E11.0 (E,F) and E12.5 (G,H) wild-type (E,G) and *Nkx6.1* mutant embryos (F,H). At E11.0, *Nkx6.2* is strongly expressed in facial branchio-motor (fbm, nVII) neurons in r4, but not in migrating fbm neurons in r5 or r6 (E). At E12.5, expression of *Nkx6.2* in fbm neurons is markedly downregulated (G). Trigeminal branchio-motoneurons (nV) express *Nkx6.2* at E11.0 (E) and E12.5 (G). *Nkx6.2* expression in fbm neurons does not differ between wild-type and *Nkx6.1* mutant embryos (E-H). However, in *Nkx6.1* mutants, *Nkx6.2* does not mark the trigeminal branchio-motoneurons at E11.0 (F), and is only faintly detected in these neurons at E12.5 (H). The signal may be decreased in *Nkx6.1* mutants, because trigeminal neurons are not clustered as a compactly as in wild-type embryos.

Discussion

In this study, we investigated the role of the transcription factor Nkx6.1 in the development of hindbrain motoneurons. Our results show that Nkx6.1 is dispensable for the early

specification of vm and bm neurons, but is required for their subsequent development. Despite proper establishment of rhombomeric segment identities in the absence of Nkx6.1, subsets of cranial motor axons fail to recognize their correct exit points. In addition, trigeminal and facial bm neurons do not migrate into their appropriate positions in the hindbrain. A detailed analysis of fbm neurons in *Nkx6.1* mutant mice shows that these neurons ectopically express the guidance receptors *Ret* and *Unc5h3* before initiating migration. Our data support a model in which Nkx6.1 has a cell-autonomous function in the control of bm neuron development.

Nkx6.1 function in branchio-motoneuron development

Correct selection of a migratory path requires a constant crosstalk between the migrating neuron and the environment through which the cell body translocates (Hatten, 2002; Nadarajah and Parnavelas, 2002). The aberrant migration of trigeminal and facial bm neurons in *Nkx6.1* mutants could therefore either result from changes in the surrounding environment, or from a cell-intrinsic defect in the neurons themselves. As the mechanisms that underlie bm neuron migration have been most extensively studied in fbm neurons, we performed a detailed analysis of the defects in only these neurons. As illustrated by normal expression of *Hoxb1* and *Epha4* in the r4 and r5 territories, we did not find any alterations in the expression of markers that specify the regional identity of the territory through which the neurons migrate. Although these data argue against a role of extrinsic factors and favor a cell-autonomous function for Nkx6.1 in the control of fbm neuron migration, we cannot exclude that lack of Nkx6.1 in either the adjacent progenitors or in other cell types leads to subtle changes in the environment that were not detected by our markers.

As a cell-intrinsic mechanism, it is conceivable that defects in the early specification of bm neurons or in the timing of their differentiation could result in aberrant neuronal migration. Our analysis of fbm neuron development argues against such an early function of Nkx6.1, as we found no alterations in the timing of motoneuron generation, and normal expression of the bm neuron markers Nkx2.2, Isl1, Phox2b, *Phox2a* and *Hoxb1*. Moreover, fbm neurons did not co-express markers of more dorsally located interneurons, excluding that motoneurons have a mixed identity. Instead, it appears that Nkx6.1 has a cell-autonomous function in modulating the expression of cell-surface receptors in postmitotic fbm neurons. This hypothesis is supported by our finding, that fbm neurons in *Nkx6.1* mutants ectopically express the guidance receptors *Unc5h3* and *Ret* in r4 (Fig. 8B). As fbm neurons in *Nkx6.1* mutants expressed *Unc5h3* before any expression was detected in wild-type embryos, our data suggest that the aberrant *Unc5h3* expression in r4 fbm neurons is not merely a result of their inability to migrate, but indeed indicates ectopic activation. However, it also needs to be considered that the misexpression of guidance receptors is a consequence and not the cause of the migratory defect.

It has been suggested that axon guidance and tangential neuronal migration require similar guidance molecules to transduce the guidance signal (Alcantara et al., 2000; Yee et al., 1999). Our analysis of migration and axon pathfinding in *Nkx6.1* mutant hindbrains indicates that Nkx6.1 controls both

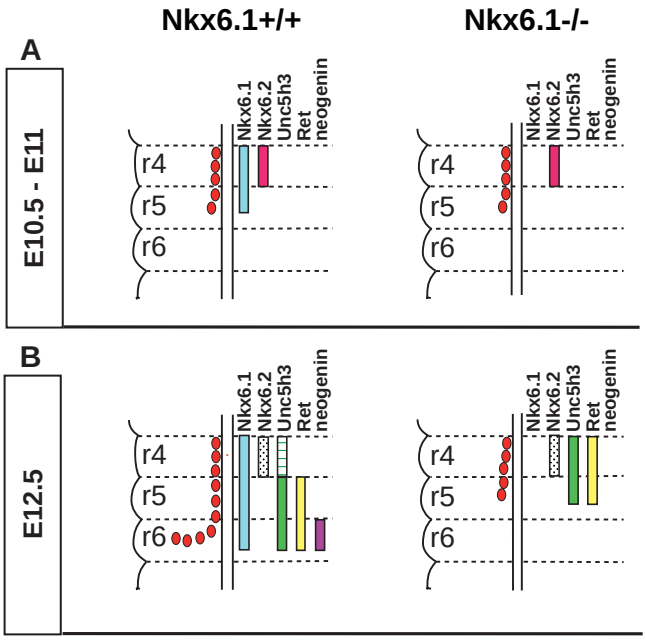

Fig. 8. Summary of the molecular changes in facial branchio-motoneurons of *Nkx6.1* mutant embryos. (A) Between E10.5 and E11 pre-migratory facial branchio-motor (fbm) neurons in r4 express both Nkx6.1 and Nkx6.2. Early migratory populations of fbm neurons progress into rostral r5 in both wild-type and *Nkx6.1* mutant embryos, and do not express the cell-surface receptors *Unc5h3*, *Ret* and neogenin. (B) Although Nkx6.2 is strongly expressed at the early stages of fbm neuron migration, later populations of pre-migratory fbm neurons at E12.5 express only low levels of Nkx6.2. At E12.5, some fbm neurons have already migrated from r4 into r6, and *Unc5h3*, *Ret* and neogenin are expressed in these neurons in a rhombomere-specific pattern. In *Nkx6.1* mutants, fbm neurons do not migrate caudally, and show ectopic expression of *Unc5h3* and *Ret* in r4.

aspects of neuronal navigation. Given that axon outgrowth precedes the migration of hindbrain bm neurons, it is important to note that both processes are not necessarily coupled, and may involve different factors. Indeed, a recent study in zebrafish showed that the Hox gene co-factor *lazarus/pbx4* controls bm axon pathfinding and migration through distinct mechanisms (Cooper et al., 2003). Our finding that only a small number of motor axons show ectopic projections in *Nkx6.1* mutants, while the majority appears to recognize their correct exit points, suggests that other factors may compensate for Nkx6.1 in axon pathfinding. A good candidate for such redundant function is the Nkx6.1 homolog Nkx6.2, which we found to be co-expressed with Nkx6.1 in newly differentiated trigeminal and facial bm neurons (Fig. 7E; Fig. 8A). The finding that *Nkx6.1*/*Nkx6.2* double mutants show more severe axon pathfinding defects than *Nkx6.1* single mutants directly confirms that the two factors have such redundant function (Pattyn et al., 2003).

The role of guidance molecules in facial branchio-motoneuron migration

The appropriate navigation of fbm neurons through the different substrates along their migratory path from r4 to r6 most likely requires a position-dependent modification of their

cell surface characteristics. This view is supported by the observation that fbm neurons regulate the expression of *Tag1*, *Ret* and *Cad8* in a rhombomere-specific fashion (Garel et al., 2000). There is substantial evidence that these cell surface molecules play a role in neuronal migration in the CNS (Enomoto et al., 2001; Enomoto et al., 2000; Kyriakopoulou et al., 2002), but their direct involvement in fbm neuron migration remains to be demonstrated.

Our present analysis shows that migrating fbm neurons also express the netrin receptors *Unc5h3* and neogenin in a rhombomere-specific pattern. UNC5 netrin receptors have been shown to interact with DCC-type receptors, transforming attraction as a response to netrin 1 mediated by DCC into a repulsive function (Hong et al., 1999). Further functional analyses in *Drosophila* revealed that expression of *Unc5* alone results in short-range repulsion, while co-expression of *Unc5* with the *DCC* homolog *frazzled* mediates long-range repulsion (Keleman and Dickson, 2001). Our finding that dorsolaterally migrating fbm neurons in r6, as well as dorsolaterally migrating trigeminal motoneurons, co-express the attractant netrin receptor neogenin and the repulsive netrin receptor *Unc5h3*, raises the possibility that floor plate derived netrin 1 could be involved in driving these neurons away from the midline. Consistent with this view, fbm neurons, which express *Unc5h3*, but fail to express neogenin in *Nkx6.1* mutants, remain close to the ventral midline and do not complete a dorsolateral migration.

Control of facial branchio-motoneuron migration by different transcription factors

Previous genetic studies in mice have shown that Nkx6.2 partially compensates for Nkx6.1 in the development of spinal cord motoneurons (Vallstedt et al., 2001). Our finding that fbm neurons co-express Nkx6.1 and Nkx6.2 in r4, but only maintain the expression of *Nkx6.1* after crossing the r4/r5 boundary, raises the possibility that Nkx6.2 might compensate for Nkx6.1 function in r4. As *Nkx6.2* is expressed at significant levels only until E11 and downregulated thereafter, this hypothesis is consistent with our observation that some fbm neurons progress into r5 at the onset of their migration. Direct genetic evidence for a compensatory function of Nkx6.2 is provided by the observation that fbm neurons in *Nkx6.1*/*Nkx6.2* double mutant embryos show a complete lack of caudal migration (Pattyn et al., 2003). Strikingly, the downregulation of *Nkx6.2* in fbm neurons after E11 temporally coincides with the onset of ectopic *Ret* and *Unc5h3* expression in r4 (Fig. 8), suggesting the possibility that in early development Nkx6.2 alone may be sufficient to prevent the expression of these receptors, and may thereby maintain responsiveness of fbm neurons to r5-derived cues.

Three other transcription factors that have been implicated in the control of fbm neuron migration are Hoxb1 (Goddard et al., 1996; Studer et al., 1996) and the Gata factors, Gata2 and Gata3 (Nardelli et al., 1999; Pata et al., 1999). In r4 progenitor cells, *Hoxb1*, *Gata2* and *Gata3* function in a regulatory cascade, in which Hoxb1 is required to activate *Gata2*, and Gata2 in turn to activate *Gata3*. We did not find any indication that Nkx6.1 functions directly up- or downstream of this regulatory cascade, as we observed no alterations in the expression of *Hoxb1*, *Gata2* and *Gata3* in *Nkx6.1* mutants (data not shown). Likewise, *Nkx6.1* shows a normal pattern and level of expression in *Gata3* mutant embryos (I. Pata and A. Karis, unpublished).

A late migratory defect of fbm neurons has been observed in mice, which are mutant for the transcription factor *Ebf1* (Garel et al., 2000). In *Ebf1* mutant mice, a subset of fbm neurons fails to migrate into r6 and undergo premature dorsolateral migration in r5. Similar to Nkx6.1, Ebf1 is expressed in migrating fbm neurons, and the migratory defect in *Ebf1* mutants is also associated with the premature expression of *Ret* in fbm neurons in r4. These findings suggest that both transcription factors might regulate similar targets. However, it appears that Ebf1 and Nkx6.1 do not function in a regulatory cascade, as *Ebf1* expression was not affected in *Nkx6.1* mutant embryos, and vice versa (data not shown).

In summary, our study demonstrates a role for Nkx6.1 in the development of postmitotic motoneurons. Although Nkx6.1 is required for the correct specification of sm neuron progenitors, we show that the early specification and generation of vm/bm neurons is independent of Nkx6.1 function. Our data support a model in which Nkx6.1 functions cell-autonomously in postmitotic bm neurons to ensure their correct migration in the hindbrain. Given the ectopic expression of guidance receptors in pre-migratory fbm neurons of *Nkx6.1* mutants, it will be interesting to further explore the role of cell-surface receptors in fbm neuron migration.

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