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Complementary roles for Nkx6 and Nkx2 class proteins in the establishment of motoneuron identity in the hindbrain

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SUMMARY

The genetic program that underlies the generation of visceral motoneurons in the developing hindbrain remains poorly defined. We have examined the role of Nkx6 and Nkx2 class homeodomain proteins in this process, and provide evidence that these proteins mediate complementary roles in the specification of visceral motoneuron fate. The expression of Nkx2.2 in hindbrain progenitor cells is sufficient to mediate the activation of Phox2b, a homeodomain protein required for the generation of hindbrain visceral motoneurons. The redundant activities of Nkx6.1 and Nkx6.2, in turn, are dispensable for visceral motoneuron generation but are necessary to prevent these cells from adopting a parallel program of interneuron differentiation. The expression of Nkx6.1 and Nkx6.2 is further maintained in differentiating visceral motoneurons, and consistent with this the migration and axonal projection properties of visceral motoneurons are impaired in mice lacking Nkx6.1 and/or Nkx6.2 function. Our analysis provides insight also into the role of Nkx6 proteins in the generation of somatic motoneurons. Studies in the spinal cord have shown that Nkx6.1 and Nkx6.2 are required for the generation of somatic motoneurons, and that the loss of motoneurons at this level correlates with the extinguished expression of the motoneuron determinant Olig2. Unexpectedly, we find that the initial expression of Olig2 is left intact in the caudal hindbrain of *Nkx6.1/Nkx6.2* compound mutants, and despite this, all somatic motoneurons are missing. These data argue against models in which Nkx6 proteins and Olig2 operate in a linear pathway, and instead indicate a parallel requirement for these proteins in the progression of somatic motoneuron differentiation. Thus, both visceral- and somatic motoneuron differentiation appear to rely on the combined activity of cell intrinsic determinants, rather than on a single key determinant of neuronal cell fate.

Key words: CNS, Motoneuron, Hindbrain, Homeodomain protein

INTRODUCTION

The central control of movement and body homeostasis rely on two major classes of motoneurons generated at defined ventral positions in the spinal cord and brainstem during central nervous system (CNS) development. These are somatic motoneurons (sMNs) that innervate somite-derived skeletal muscles and visceral motoneurons (vMNs) that innervate either autonomic ganglia (general visceral) or branchial arch-derived muscles (special visceral). Extensive studies in the spinal cord have provided insight into the molecular pathway that controls sMN differentiation (Jessell, 2000; Shirasaki and Pfaff, 2002), whereas less is known about the genetic program that establish vMN identity in the hindbrain (Cordes, 2001).

All motoneurons in the developing CNS depend on Sonic hedgehog (Shh) signals for their generation (Ericson et al., 1996; Chiang et al., 1996). Shh is secreted by ventral midline cells of the notochord and floor plate and acts in a graded fashion, inducing distinct neuronal subtypes at different concentration thresholds (Jessell, 2000; Briscoe et al., 2001). A key activity of Shh in this process is to establish the patterned expression of a set of homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors, so that neural progenitor cells at different DV positions acquire distinct positional identities (Briscoe et al., 2000; Novitch et al., 2001). These transcription factors fall into two classes, class I and class II proteins, based on their regulation by Shh (Briscoe et al., 2000). The class I proteins are constitutively expressed by neural progenitors, and their expression is repressed by Shh. The class II proteins, in turn, depend on Shh signalling for their neural expression. Many of these proteins act directly as transcriptional repressors (Muhr et al., 2001; Novitch et al., 2001), and their repressor activities underlie selective cross-repressive interactions between class I and class II proteins necessary to establish and maintain boundaries between distinct ventral progenitor domains (Briscoe et al., 2000; Muhr et al., 2001; Vallstedt et al., 2001; Novitch et al., 2001). Once established, the expression profile of class I and class II

proteins appears also to control the fate of neurons by directing the activation of specific downstream determinants that establish the subtype identity of post-mitotic neurons (Briscoe et al., 2000; Muhr et al., 2001; Novitch et al., 2001; Pierani et al., 1999; Pierani et al., 2001; Zhou and Anderson, 2002). Subsequent to the period of neurogenesis, the patterned expression of these proteins has been shown to control the spatial generation of oligodendrocytes and astrocytes in the ventral neural tube (Zhou and Andersson, 2002; Lu et al., 2002).

Many of the basic features of cell patterning and neuronal differentiation have emerged from studies of somatic motoneuron differentiation in the spinal cord (Jessell, 2000; Shirasaki and Pfaff, 2002). sMNs are generated from a common ventral progenitor domain (referred to as the pMNs domain in this study) which spans the entire spinal cord and extends also into caudal levels of the hindbrain (Novitch et al., 2001; Arber et al., 1999). In the spinal cord, the pMNs domain is flanked ventrally by p3 progenitors that generate V3 neurons, and dorsally by p2 progenitors that give rise to V2 neurons (Ericson et al., 1997; Briscoe et al., 1999). Dorsal to the p2 domain, V1 and V0 neurons are generated from the p1 and p0 domains, respectively (Ericson et al., 1997; Pierani et al., 1999; Pierani et al., 2001). Within the pMNs domain, the HD proteins Pax6, Nkx6.1, Nkx6.2 and the pMNs domain specific bHLH protein Olig2 have been shown to promote the generation of sMNs (Ericson et al., 1997; Vallstedt et al., 2001; Novitch et al., 2001). In particular, the activities of Nkx6.1, Nkx6.2 and Olig2 are central to this process, and each of these proteins is sufficient to induce sMN differentiation at ectopic positions within the neural tube (Vallstedt et al., 2001; Novitch et al., 2001; Mizuguchi et al., 2001). Moreover, Nkx6.1 and Nkx6.2 (Nkx6 proteins) are partly redundant, and a virtual complete loss of sMNs is observed in *Nkx6.1/Nkx6.2* compound mutants (*Nkx6* mutants) (Vallstedt et al., 2001). A similar deficit of sMNs is also observed in mice lacking Olig2 function (Rowitch et al., 2002; Zhou and Andersson, 2002; Lu et al., 2002).

A remaining issue in sMN fate specification is the relative roles for Olig2 and Nkx6 proteins in this process. Both Olig2 and Nkx6 proteins function as repressors (Novitch et al., 2001; Muhr et al., 2001), and one role for these proteins is to prevent other repressor proteins from being expressed in the pMNs domain. Nkx6.1 and Nkx6.2 are necessary to constrain the expression of Dbx1 and Dbx2 to more dorsal p1 and/or p0 progenitor cells (Sander et al., 2000; Vallstedt et al., 2001), whereas Olig2 suppresses the p2 determinant Irx3 (Novitch et al., 2001; Zhou and Anderson, 2002). As Irx3 and Dbx proteins have been implicated in blocking sMN induction (Briscoe et al., 2000; Muhr et al., 2001; Vallstedt et al., 2001; Novitch et al., 2001), it is conceivable that the loss of sMNs in *Nkx6* and *Olig2* mutant mice primarily reflects the deregulated expression of these repressor proteins, or as yet unidentified repressor proteins, in the pMNs domain. However, Olig2 also has a crucial role in ensuring the progression of sMN differentiation by mediating the activation of the pro-neural bHLH protein Ngn2 in the pMNs domain (Novitch et al., 2001; Zhou and Anderson, 2002) and Nkx6 proteins have in turn been shown to be required for the expression of Olig2 in the spinal cord (Novitch et al., 2001). Thus, the loss of sMNs in *Nkx6* and *Olig2* mutants could also reveal a more general requirement for Nkx6 proteins to act upstream of Olig2 in the progression of sMN fate determination (Novitch et al., 2001; Zhou and Anderson, 2002).

The generation of vMN subtypes is primarily confined to the hindbrain and sacral and thoracic levels of the spinal cord (Jessell, 2000; Cordes, 2001). In the caudal hindbrain, sMNs and vMNs are generated at distinct DV positions, indicating that graded Shh signalling underlies the distinction between these MN subtypes at this level (Ericson et al., 1997). vMNs are generated in a position immediately ventral to sMNs and dorsal to the floor plate, from a progenitor domain that we term pMNv. Like the pMNs domain, cells in the pMNv domain express Nkx6.1 and Nkx6.2 (Sander et al., 2000; Pattyn et al., 2003). However, they also express Nkx2.2 and Nkx2.9 (Nkx2 proteins) (Ericson et al., 1997; Briscoe et al., 1999), but not the pMNs markers Pax6 or Olig2 (Ericson et al., 1997; Novitch et al., 2001). In addition to its expression in progenitors, Nkx6.1 has been shown also to be expressed in several vMN nuclei at advanced stages of brainstem development (Puelles et al., 2001). These patterns of expression imply that Nkx6 and Nkx2 class proteins contribute to the establishment of vMN identity, but genetic analyses have not yet uncovered a role for these proteins in this process (Briscoe et al., 1999; Sander et al., 2000; Pabst et al., 2003; Pattyn et al., 2003).

We have examined the role of Nkx6 and Nkx2 class proteins in the generation of MNs in the hindbrain. We provide evidence that Nkx6 proteins and Nkx2.2 mediate distinct and complementary activities at initial stages of vMN fate specification. Although Nkx2.2 appears to act upstream of the vMN determinant Phox2b (Pattyn et al., 2000; Dubreuil et al., 2000; Dubreuil et al., 2002) in the vMN differentiation pathway, Nkx6 proteins are necessary to ensure the molecular integrity of differentiating vMNs, by preventing these cells from initiating a parallel program of V0 neuron differentiation. Both Nkx6.1 and Nkx6.2 continue to be expressed in most differentiating vMNs, and consistent with this the migration and axonal projections of vMNs are severely affected in *Nkx6* mutant mice. We also find that the initial expression of Olig2 in the pMNs domain is unaffected in the hindbrain of *Nkx6* mutants. This is in contrast to the spinal cord where the expression of Olig2 depends on Nkx6 proteins. Despite the persistence of Olig2 expression in the hindbrain all sMNs are missing, indicating a parallel requirement for Nkx6 and Olig2 proteins in sMN fate determination. Together, these data provide insight into genetic pathways that control the generation of these distinct classes of MNs in the hindbrain, and also important loss-of-function support for the idea (Briscoe et al., 2000) that the combinatorial activities of class I and class II proteins are central in the specification of ventral neuronal subtypes.

MATERIALS AND METHODS

Mouse mutants

The generation and genotyping of *Nkx6.1* and *Nkx6.2* mutant mice have been reported previously (Sander et al., 2000; Vallstedt et al., 2001).

Chick in ovo electroporation

Full-length *Nkx2.2* and *Nkx6.1* inserted into a RCASBP(B) retroviral vectors (Briscoe et al., 2000) were electroporated into the hindbrain in HH stage 10 or 11 chick embryos (Briscoe et al., 2000).

After 36-48 hours, embryos were fixed and processed for immunohistochemistry and in situ hybridization histochemistry.

Immunohistochemistry and in situ hybridization histochemistry

Immunohistochemical localization of proteins was performed as described (Briscoe et al., 2000). Antibodies used were as follows: mouse (m), rabbit (r) and guinea pig (gp) Isl1/2, gp Nkx2.9, gp Irx3 (Briscoe et al., 2000), gp Nkx6.2, m anti-Evx1/2, r Dbx1, m Hb9 (Vallstedt et al., 2001), m Gata3 (Santa Cruz Biotechnology), m and r Nkx2.2, r Chx10, m Pax6 (Ericson et al., 1997), r Pax6 (Covance), r Phox2b (Pattyn et al., 2000), r Nkx6.1 (Briscoe et al., 1999), r β-gal (Cappel), gp Olig2 (Novitch et al., 2001). In situ hybridization histochemistry on sections or as wholemounts were performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993; Wilkinson, 1992) using mouse *Isl1*, *Dbx2*, *Nkx6.1*, *Nkx6.2*, peripherin, *Sox10*, *Pdgfra* probes and chick *Phoxb2* and *Shh* probes. Whole-mount X-gal staining was carried out as described elsewhere (Mombaerts et al., 1996).

RESULTS

Spatial generation of vMNs and sMNs in the hindbrain

vMNs and sMNs can be defined based on their selective expression of molecular markers. While both classes of neurons express the generic MN marker Isl1 (Fig. 1B) (Ericson et al., 1992), vMNs selectively express Phox2b, a HD protein required for the generation of all vMNs in the hindbrain (Fig. 1B,C) (Pattyn et al., 1997; Pattyn et al., 2000) and sMNs selectively express the HD protein Hb9 (Fig. 1D,N) (Arber et al., 1999; Thaler et al., 1999). In the caudal hindbrain, vMNs are generated from the pMNv domain that expresses Nkx2.2, Nkx2.9, Nkx6.1, Nkx6.2 and Phox2b (Fig. 1B,C,H-K) (data not shown) (Briscoe et al., 1999; Pattyn et al., 2000; Pattyn et al., 2003). sMNs, in turn, are generated dorsal to vMNs and ventral to V2 neurons, from progenitors that express Nkx6.1, Nkx6.2, Pax6 and Olig2 but not Nkx2.2 or the p2 progenitor marker Irx3 (Fig. 1D,F,H,J,L) (Briscoe et al., 2000; Vallstedt et al., 2001; Novitch et al., 2001). The generation of sMNs and vMNs differ also along the AP axis of the hindbrain. sMNs are confined to caudal axial levels [rhombomeres (r) 5, 7-8] (Arber et al., 1999), whereas vMNs are produced along the entire AP extent of the hindbrain (r2-8) (Lumsden and Krumlauf, 1996; Pattyn et al., 2000). The absence of sMNs in the anterior hindbrain prompted us to examine the patterning of neurons along the DV axis at this level. In r2-r4 at E10.5, no expression of Olig2 or Hb9 could be detected (Fig. 1E), and like Pax6 (Ericson et al., 1997), the p2 progenitor marker Irx3 extended down to the dorsal boundary of Nkx2.2 expression (Fig. 1G,M). These data indicated that V2 neurons might be generated immediately dorsal to vMNs in the anterior hindbrain. In support of this, no spatial gap could be detected between Chx10$^+$ V2 neurons and pre-migratory Phox2b$^+$/Isl1$^+$ vMNs at this level of the hindbrain (Fig. 1C,N-Q).

vMNs express interneuron characteristics in *Nkx6*-compound mutants

The role for Nkx6.1 and Nkx6.2 in the generation of sMNs in the spinal cord has been characterized, whereas their role in the differentiation of cranial MNs is less well understood (Briscoe

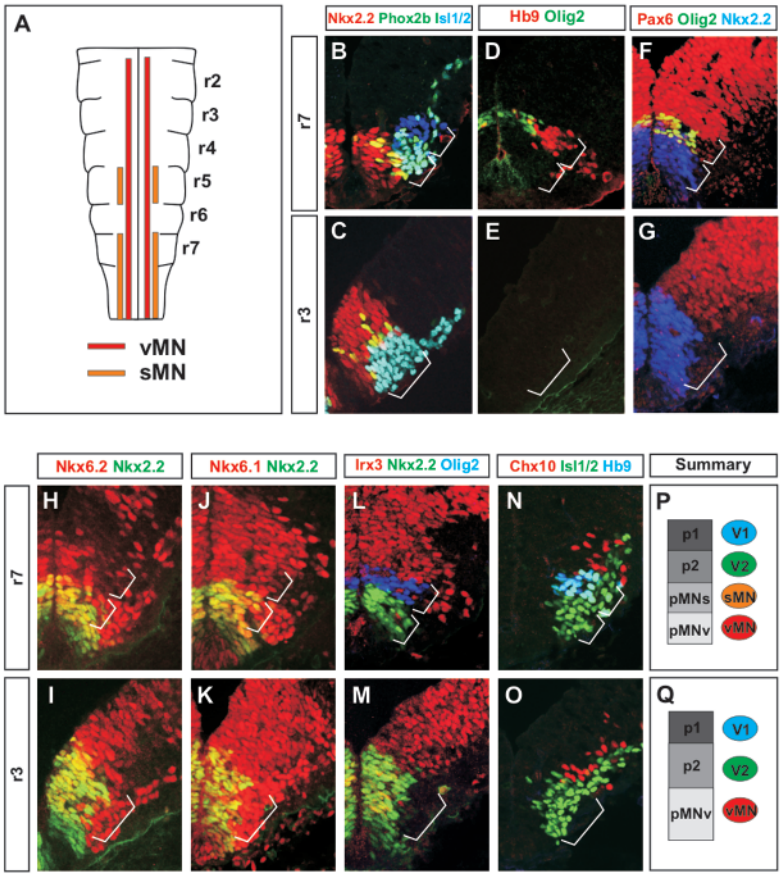

Fig. 1. Spatial generation of somatic- and visceral motoneurons in the hindbrain. (A) Schematic drawing of the embryonic hindbrain showing the distribution of visceral motoneurons (vMNs) (red) and somatic motoneurons (sMNs) (orange) along the AP axis. vMNs are generated in rhombomeres (r) 2-8 while sMNs are generated in r5 and r7-8 (Lumsden and Krumlauf, 1996; Cordes et al., 2001). (B-O) Transverse sections through r7 and r3 of wild-type (wt) embryos at E10.5. In r7, sMNs express Isl1/2 (B) and Hb9 (D), and are generated from the pMNs domain that express Olig2 (D,F) Nkx6.1 (J), Nkx6.2 (H), Pax6 (F) but not Nkx2.2 or Irx3 (B,L). In r3 and r7, vMNs express Isl1 and Phox2b (B,C) and are generated from the pMNv domain that expresses Nkx2.2 (B,C), Nkx6.1 (J,K) and Nkx6.2 (H,I) but not Olig2 (D,E), Pax6 (F,G) or Irx3 (L,M). sMNs are not generated in r3 and no expression of Olig2 or Hb9 is detected (E). In r3, the expression of Pax6 (G) and Irx3 (M) extends ventrally to the dorsal boundary of Nkx2.2 expression. Chx10$^+$ V2 neurons are detected immediately dorsal to pre-migratory Isl1$^+$ vMNs in r3 (O). In r7, Chx10$^+$ V2 neurons are detected dorsal to Is1$^+$/Hb9$^+$ sMNs (N). Ventral brackets in B,D,F,H,J,L,N indicates the pMNv domain and the dorsal bracket the pMNs domain. Brackets in C,E,G,I,K,M,O indicate pMNv domain and pre-migratory vMNs. (P,Q) Summary of ventral progenitor domains and neural subtypes generated in r7 (P) and r3 (Q).

et al., 2000; Sander et al., 2000; Vallstedt et al., 2001; Pattyn et al., 2003). To investigate in more detail the role of these proteins in the hindbrain, we first examined the initial generation of vMNs in *Nkx6.1* and *Nkx6.2* compound mutant mice (*Nkx6* mutants) (Vallstedt et al., 2001; Pattyn et al., 2003). In the hindbrain of *Nkx6* mutants at E10.5, Nkx2.2 was expressed and essentially normal numbers of neurons that expressed Phox2b and Isl1 could be detected (Fig. 2A-F,I-J). Analysis of *lacZ* expressed under the control of the *Nkx6.2* locus (Vallstedt et al., 2001) also revealed that the initial dorsal projections of vMN axons were similar in *Nkx6* mutants and in *Nkx6.2*$^{+/tlz}$ controls (Fig. 2C,D). In contrast to sMNs, V2 and V1 interneurons, which are missing or greatly reduced in number in *Nkx6* mutants (Fig. 2I,J; data not shown; see below) (Vallstedt et al., 2001), these data show that Nkx6 proteins are not required for the initial establishment of vMN identity. We noted, however, that the ventral expansion of *Dbx2* and Dbx1 expression observed in *Nkx6* mutants (Vallstedt et al., 2001) encroached also into the Nkx2.2^{+} pMNv domain (Fig. 2E-H). Moreover, most Isl1^{+}/Phox2b^{+} neurons generated caudal to r3 in *Nkx6* mutants had also initiated expression of the V0 neuronal determinant Evx1 (Moran-Rivard et al., 2001) at E10.5, a situation not observed in controls or in *Nkx6.1* and *Nkx6.2* single mutant mice (Fig. 2M-Q). Albeit dispensable for their generation, these data show that Nkx6.1 and Nkx6.2 function in a redundant manner to prevent differentiating vMNs from initiating a parallel program of V0 neuronal differentiation.

Nkx2.2 but not Nkx6.1 is sufficient to induce expression of the vMN determinant Phox2b

The generation of vMNs in *Nkx6* mutants raised the issue as to which factors might be directly involved in mediating the activation of downstream determinants of vMN fate, notably Phox2b (Pattyn et al., 2000; Dubreuil et al., 2000). Nkx2.2 and Nkx2.9 are candidates to be involved in this process (Briscoe et al., 1999), as these proteins are co-expressed in vMN progenitors (Pattyn et al., 2003) and are still expressed in the hindbrain of *Nkx6* mutants (Fig. 2E,F) (Pattyn et al., 2003). The generation of vMNs, however, is left largely unaffected in both *Nkx2.2* (Briscoe et al., 1999) and *Nkx2.9* mutants (Pabst et al., 2003) but it is possible that this may reflect functional redundancy between these proteins (Briscoe et al., 1999; Briscoe et al., 2000; Pabst et al., 2003). We therefore examined if forced expression of Nkx2.2 or Nkx6.1 was sufficient to induce expression of Phox2b at dorsal positions of the chick hindbrain. Stage 10 chick embryos were electroporated in ovo with RCAS-Nkx2.2 or RCAS-Nkx6.1 vectors (Briscoe et al., 2000) and after 36-48 hours of incubation embryos were harvested and analysed. Widespread expression of Nkx2.2 in the caudal hindbrain resulted in activation of *Phox2b* and Isl1 expression along the entire DV extent of the hindbrain, albeit the induction of *Phox2b* appeared to be more prominent in the ventral hindbrain (Fig. 3A-C). By contrast, *Phox2b* was not induced in response to Nkx6.1 (Fig. 3E,F). Consistent with the established role for Nkx6.1 in the specification of sMN fate (Briscoe et al., 2000), Nkx6.1 was sufficient to induce expression of the generic MN marker Isl1 (Fig. 3G) (see Briscoe et al., 2000). Forced expression of Nkx2.2 or Nkx6.1 had no effect on the expression of *Shh* (Fig. 3D,H). These data show that Nkx2.2 but not Nkx6.1 is sufficient to induce the vMN determinant Phox2b in the hindbrain, and provide evidence that Nkx6 and Nkx2 class proteins mediate separate and complementary activities at initial stages of vMN generation.

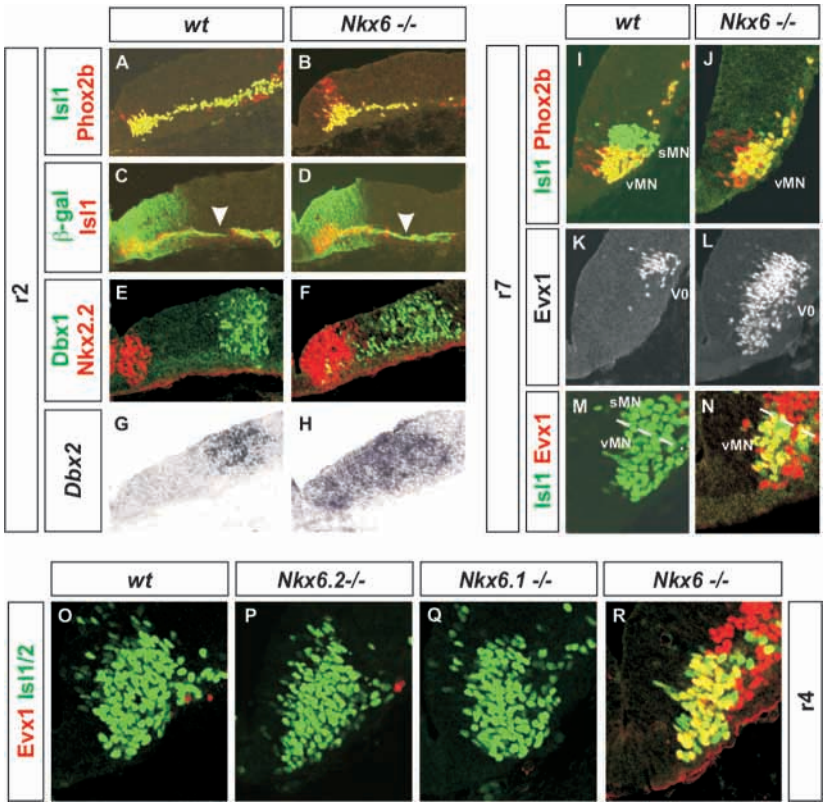

Fig. 2. Nkx6 proteins are required to suppress interneuron characteristics in vMNs.
(A-N) Transverse sections at r2 (A-H) and r7 levels (I-N) in wt and *Nkx6* mutants at E10.5. The number of cells that co-express Phox2b and Isl1 in r2 and r7 in *Nkx6* mutants (B,J) is similar to controls (A,I). The initial dorsal projections of vMN axons (arrowhead) are similar in *Nkx6.2*$^{+/tlz}$ controls and in *Nkx6* mutants at E10.5, as revealed by *lacZ* expression in Isl1^{+} neurons (C,D). sMNs, which express Isl1 but not Phox2b and are generated dorsal to vMNs in r7 (I), are extinguished in *Nkx6* mutants (J). The expression of the progenitor HD proteins *Dbx2* (G,H) and Dbx1 (E,F) expands ventrally into the Nkx2.2^{+} domain in *Nkx6* mutants. The generation of Evx1^{+} V0 interneurons is ventrally extended in *Nkx6* mutants (K,L), and the generation of these neurons occur at the expense of V1 and V2 interneurons and sMNs (I-L) (data not shown) (Vallstedt et al., 2001). Most Isl1^{+} vMNs generated in *Nkx6* mutants also express Evx1 at E10.5 (M,N). The expression of Evx1 in motoneurons appeared transient and no Isl1^{+}/Phox2b^{+} cells detected at E11.5 expressed Evx1 (data not shown). (O-R) Transverse sections through r4 of wt (O), *Nkx6.2*$^{tlz/tlz}$ (P), *Nkx6.1*$^{-/-}$ (Q) and *Nkx6* mutant (R) embryos. In *Nkx6* mutants, most vMNs expressed Evx1 (R). No expression of Evx1 could be detected in vMNs of wt embryos (O) or in *Nkx6.2*$^{tlz/tlz}$ (P) and *Nkx6.1*$^{-/-}$ (Q) single mutants at this level.

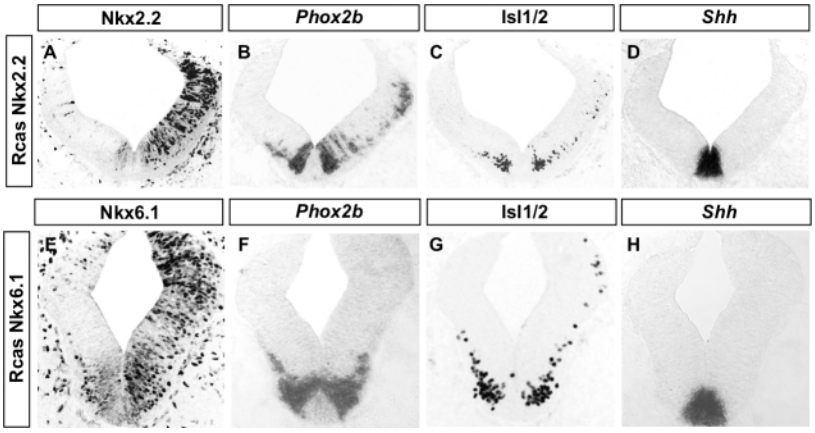

Fig. 3. Induction of the vMN determinant Phox2b by Nkx2.2. (A-D) Consecutive transverse sections through a stage 22 chick hindbrain electroporated with RCAS-Nkx2.2 construct. Forced expression of Nkx2.2 (A) induces expression of the vMN markers *Phox2b* (B) and Isl1/2 at ectopic dorsal positions (C). The expression of *Shh* was unaffected (D). No ectopic Isl1/2$^+$ neurons induced in response to Nkx2.2 co-expressed the V0 neuron determinant Evx1 (data not shown). (E-H) Consecutive sections through stage 22 chick hindbrain electroporated with RCAS-Nkx6.1 construct. Misexpression of Nkx6.1 (E) induces ectopic expression of Isl1/2 (G) but not *Phox2b* (F) or *Shh* (H).

Impaired migration and axonal projections of vMNs in *Nkx6* mutants

In addition to their expression in progenitor cells, Nkx6.1 and Nkx6.2 are also co-expressed in most hindbrain vMNs over the period that these cells differentiate, migrate and extend axons towards peripheral targets (Fig. 4C-F and data not shown). In r4-derived facial branchial (fb) MNs and GATA3$^+$ inner ear efferent (iee) (Karis et al., 2001) neurons, however, only the expression of Nkx6.1 persisted and Nkx6.2 was downregulated soon after their generation, and prior to the caudal migration (Studer et al., 1996) of fbMNs into r6 (Fig. 4G,H; data not shown). The prolonged expression of Nkx6.2 and/or Nkx6.1 in vMNs prompted us to examine later aspects of vMN differentiation in mice lacking *Nkx6* function. All vMNs, independent of subtype or origin along the AP axis, revealed impaired migratory and axonal projection properties in *Nkx6* mutants. In normal conditions, most r2-derived (trigeminal) MNs have completed their dorsal migration at E12, and settled close to the point at which MN axons exit the neural tube (Fig. 4J). In *Nkx6* mutants, vMNs in r2 were still dispersed along the migratory route at E12 and cells eventually settled at around E13.5 in an aberrant ventral position (Fig. 4L,S). Other vMNs subtypes that migrate in a strict ventral-to-dorsal fashion, such as vMNs of the glossopharyngeal and vagal nerve in the caudal hindbrain, revealed a similar migratory phenotype (data not shown). Moreover, the characteristic caudal migration of r4-derived fbMNs also failed to occur in *Nkx6* mutants; instead of initiating a caudal migration from r4 through r5 and into r6 (Studer et al., 1996), these MNs migrated in a strictly ventral-to-dorsal fashion within r4 (Fig. 4P). A similar, albeit less dramatic, migratory defect of fbMNs was observed also in *Nkx6.1* single mutants (Fig. 4O). In these mice, fbMNs failed to reach r6 and instead migrated dorsally in either r4 or r5. Importantly, as Evx1 is not expressed in r4-derived MNs in *Nkx6.1* single mutants (Fig. 2Q), these data show that the impaired migration of vMNs cannot simply reflect an early requirement of Nkx6 proteins to suppress the V0 determinant Evx1 in vMNs.

In addition, the axonal projection patterns of vMNs were severely affected by the loss of Nkx6.1 and Nkx6.2 function. Hindbrain vMNs initially extend their axons dorsally to leave the neural tube from dorsal exit points in even numbered rhombomeres, and project towards their peripheral targets (Cordes, 2001). Analysis of *tau-lacZ* expression (Vallstedt et al., 2001) revealed that many different aspects in the projection pattern of motor axons were impaired in *Nkx6* mutants. At caudal hindbrain levels, the majority of vagal and glossopharyngeal motor axons failed to recognise their exit points and instead, upon reaching a dorsal position of the neural tube, axons turned and projected caudally or rostrally within the CNS (Fig. 5A,B). These caudal groups of vMNs were eventually eliminated in *Nkx6* mutants, as indicated by the absence of *lacZ*$^+$ axonal projections in the vagal and glossopharyngeal nerves at E13.5 (Fig. 5C-F) and the complete lack of cells expressing peripherin in the caudal hindbrain at E16.5 (Fig. 5M,N). vMNs at more anterior hindbrain levels appeared less dramatically affected, and a trigeminal and facial nucleus were still detected in *Nkx6* mutants at E16.5. However, the total number of MNs in each nucleus was significantly reduced (Fig. 5G-L) and, consistent with the migratory defects, the trigeminal nuclei were displaced ventrally and the facial nuclei occupied an aberrant anterior position in *Nkx6* mutants at this stage (Fig. 5G-L). The fact that a trigeminal and facial nucleus could be detected as late as E16.5 in *Nkx6* mutants indicated that at least a subset of vMNs in these nuclei project axons out of the CNS and receive the necessary trophic support provided by peripheral targets (deLapeyriere and Henderson, 1997). In direct support for this, analysis of *lacZ* expression revealed that motor axons had projected into the trigeminal and facial nerve in *Nkx6* mutants at E13.5, although many axons at this stage followed aberrant peripheral routes (Fig. 5C-F). Together, these data reveal that Nkx6.1 and Nkx6.2 are required for both the migration and axonal navigation of vMNs, thus being consistent with a cell-autonomous requirement for Nkx6 proteins in differentiating vMNs.

Differential regulation of Olig2 expression by Nkx6 proteins in the hindbrain

We next turned our attention to sMNs generated dorsal to vMNs at caudal levels of the hindbrain. Nkx6.1 and Nkx6.2 are required for the generation of sMNs (Sander et al., 2000; Vallstedt et al., 2001), but the precise role for these proteins in the specification of sMN fate is unclear; Nkx6.1 and Nkx6.2 mediate their neural patterning activities by functioning as transcriptional repressors (Muhr et al., 2000), indicating that they promote sMN generation by suppressing the expression of other repressors, such as Dbx1 and Dbx2, in sMN progenitors (Briscoe et al., 2000; Sander et al., 2000; Vallstedt

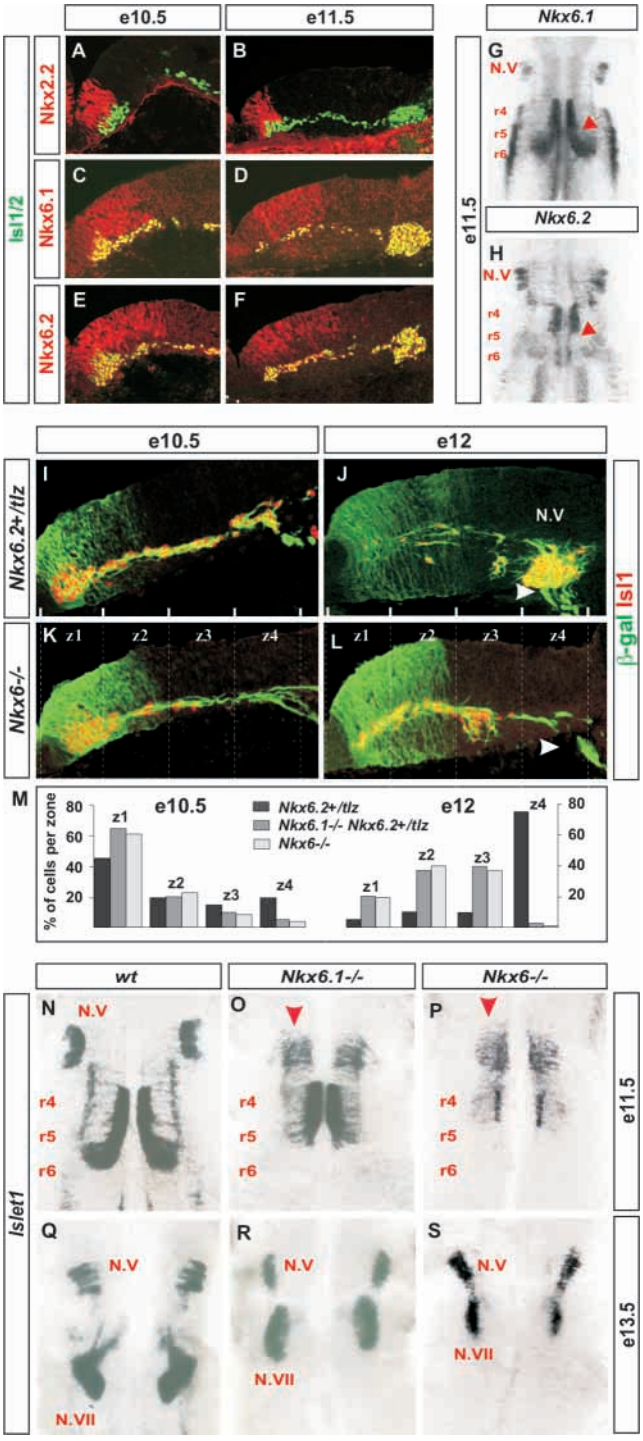

Fig. 4. The migration of vMNs is impaired in *Nkx6.1* and *Nkx6* mutants. (A-F) Transverse sections through r2 of wild-type embryos at E10.5 and E11.5. The expression of Nkx6.1 (C,D) and Nkx6.2 (E,F) but not Nkx2.2 (A,B) persists in Isl1^{+} trigeminal vMNs as they migrate from the pMNv domain towards a more dorsal position of the hindbrain. (G,H) Dorsal view of flat-mounted hindbrain at E11.5. Caudally migrating fbMNs originate in r4 and migrate caudally through r5 (arrowhead) into r6 where they turn dorsally and settle in a dorsal position (Studer et al., 1996). Nkx6.1 is expressed in caudally migrating fbMNs (G), whereas Nkx6.2 only is detected only in pre-migratory fbMNs in r4 (H). Both *Nkx6.1* (G) and *Nkx6.2* (H) are expressed in trigeminal vMNs (indicated as N.V). The color reaction in G,H was underdeveloped to reveal the expression of *Nkx6.1* and *Nkx6.2* in post-mitotic neurons over the expression of these genes in ventral progenitor cells.
(I-M) Transverse sections through r2 at E10.5 (I,K) and E12 (J,L) of *Nkx6.2*$^{+/tlz}$ controls (I,J) and in *Nkx6* mutants (K,L) showing impaired dorsal migration of trigeminal MNs in *Nkx6* mutants. The position of trigeminal MNs was determined by Isl1/2 expression and their axonal projections was visualized by the expression of *lacZ*. In *Nkx6* mutants at E10.5 (K), Isl1/2^{+} cells are detected closer to the ventral midline compared with *Nkx6.2*$^{+/tlz}$ controls (I). At E12, most trigeminal MNs have reached their final position close to the trigeminal nerve exit point in *Nkx6.2*$^{+/tlz}$ controls (J), whereas many cells are positioned along the migratory pathway in *Nkx6* mutants at this stage (L). Note that the trigeminal nerve exit point appears unaffected *Nkx6* mutants (arrowhead in J,L), but that cells settle in an aberrant ventral position in *Nkx6* mutants compared with controls (J,L). (M) Quantification of vMN migratory defects in r2 of *Nkx6.1* and *Nkx6* mutant mice. The position of migrating Isl1^{+} cells along their migratory route was assessed by arbitrarily dividing r2 into four equivalent zones (indicated as z1-z4 in K,L) between the site of generation and the trigeminal nerve exit point (arrowhead in J,L). The percentage of Isl1^{+} cells located in each zone at E10.5 and E12 in wild-type controls, *Nkx6.1* single mutants and *Nkx6* mutants is indicated (M). No migratory defects were observed in *Nkx6.2* single mutant mice (data not shown). (N-S) Dorsal view of flat-mounted hindbrains showing *Isl1* expression in wild-type (N,Q), *Nkx6.1* mutants (O,R) and *Nkx6* mutants (P,S) at E11.5 and E13.5. In wild-type embryos at E11.5, *Isl*$^{+}$ fbMNs generated in r4 have initiated their caudal migration through r5 into r6 (N), where they accumulate and settle in a lateral position at E13.5 (Q, indicated as N.VII). In *Nkx6.1* mutants, fbMNs fail to migrate into r6 and instead migrate to occupy positions in r4 or r5 (O,R). In *Nkx6* mutants, all fbMNs fail to initiate a caudal migration, and cells instead migrate strictly dorsally within r4 (P,S). In wild-type embryos at E11.5, *Isl1*$^{+}$ trigeminal MNs (indicated as N.V) in r2 and r3 have migrated away from the ventral midline towards their final settling position (N,Q). As indicated above in I-M, the migration of *Isl1*$^{+}$ trigeminal MNs occur at a slow pace in *Nkx6.1* mutants (arrowhead in O) and *Nkx6* mutants (arrowhead in P).

et al., 2001). However, Nkx6.1 and Nkx6.2 are also necessary for the expression of Olig2 in the spinal cord (Novitch et al., 2001). It is possible, therefore, that the loss of sMNs in *Nkx6* mutants (Vallstedt et al., 2001) primarily reflects the loss of Olig2 expression in these mice (Novitch et al., 2001; Zhou and Andersson, 2002). In the caudal hindbrain of *Nkx6* mutants, the expression of Dbx2 is derepressed in the sMN progenitor domain (Fig. 6G,H) in a fashion similar to that previously reported at the spinal cord level (Vallstedt et al., 2001). Unexpectedly, however, we found that the expression of Olig2 appeared unaffected in the caudal hindbrain at E10 (Fig. 6A-B,E-F). Even more strikingly, in ventral positions of the anterior hindbrain (r2-r4), where Olig2 is not normally expressed over the period that MNs are generated, the loss of Nkx6.1 and Nkx6.2 function resulted in ectopic activation of Olig2 expression (Fig. 6K,L). In the caudal hindbrain, the expression of Olig2 in *Nkx6* mutants was progressively lost and could no longer be detected at E11.5 (Fig. 6I,J). By contrast, the domain of ectopic Olig2 expression in the anterior hindbrain persisted over this period (data not shown). These

data reveal an unanticipated differential regulation of Olig2 expression by Nkx6 proteins along the AP axis of the neural tube, and while Nkx6.1 and Nkx6.2 are necessary for Olig2 expression in the spinal cord (Novitch et al., 2001), these same proteins are required to suppress the expression of Olig2 in the anterior hindbrain. The mechanism by which Nkx6 proteins suppresses the expression of Olig2 in the anterior hindbrain is unclear, but does not seem to involve overall changes in AP-identity and Hox-gene expression in the hindbrain of *Nkx6* mutants (Pattyn et al., 2003) (data not shown).

The detection of Olig2 expression in the caudal hindbrain at E10 in *Nkx6* mutants prompted us to examine the loss of sMNs at this level (Sander et al., 2000; Vallstedt et al., 2001) in more detail. Pax6 is required for the generation of sMNs in the hindbrain (Ericson et al., 1997), whereas Nkx2.2 and Irx3 have been implicated to suppress sMN generation (Briscoe et al., 1999; Briscoe et al., 2000). At E10 in *Nkx6* mutants, the majority of Olig2$^+$ cells in the caudal hindbrain expressed Pax6 but not Nkx2.2 or Irx3 (Fig. 6C-F). Thus, the early loss of Hb9$^+$ sMNs does not appear to reflect a loss of Pax6 expression, or a deregulated expression of Nkx2.2 or Irx3 proteins in sMN progenitors. At later stages in *Nkx6* mutants, however, the expression of Nkx2.2 had begun to encroach dorsally into the sMN progenitor domain, and by E10.5 many cells which co-expressed Nkx2.2 and Pax6 could be detected (data not shown). This expansion of Nkx2.2 expression coincided with the progressive loss of Olig2 expression observed in the caudal hindbrain (Fig. 6I,J; data not shown). As Nkx2.2 is a known repressor of Olig2 expression (Novitch et al., 2001), it is possible that the progressive loss of Olig2 expression at this level in *Nkx6* mutants reflects a requirement for Nkx6 proteins to constrain the expression of Nkx2.2 to vMN progenitors.

In the anterior hindbrain, the ectopic expression of Olig2 in *Nkx6* mutants was not accompanied by any generation of Hb9$^+$ sMNs (Fig. 6K,L). In addition, in contrast to the caudal hindbrain, most ectopic Olig2$^+$ cells co-expressed Nkx2.2 and Pax6 already by E10 (Fig. 6M,N and data not shown). A dorsal expansion of Nkx2.2 expression, and the co-expression of Nkx2.2 and Olig2 in ventral progenitor cells, has previously been linked to the specification of oligodendrocyte precursors (OLPs) in the spinal cord (Zhou et al., 2001). We therefore considered if Olig2$^+$/Nkx2.2$^+$ progenitors in the anterior hindbrain resulted in a premature generation of OLPs. However, no expression of *Sox10* or *Pdgfra*, two early markers of OLP differentiation (Hall et al., 1996; Kuhlbrodt et al., 1998), could be detected in the anterior hindbrain between E10-E11.5 either in controls or *Nkx6* mutants (Fig. 6O-R; data not shown). At caudal hindbrain levels we instead observed a loss of *Sox10* and *Pdgfra* expression in *Nkx6* mutants compared to controls (data not shown) (M. Qui, personal communication). This observation is consistent with the progressive loss of Olig2 expression observed at this level (Fig. 6I), indicating that Nkx6 proteins are required for the

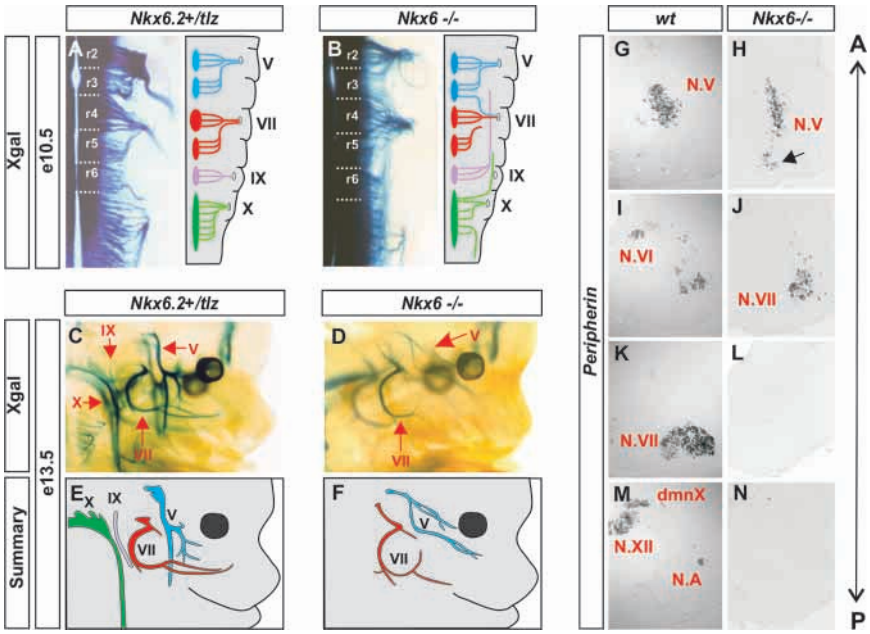

Fig. 5. Abnormal projection patterns of vMN axons in *Nkx6* mutant mice. (A,B) Dorsal view of the hindbrain of *Nkx6.2*$^{+/tlz}$ controls (A) and *Nkx6* mutants (B) showing *lacZ* expression (detected by X-gal staining) at E10.5. On the right side of each micrograph in A,B, a schematic summary of axonal projections of vMN subtypes is included; blue indicates trigeminal MNs (V) generated in r2-3, red indicates r4-5 derived vMNs projecting in the facial nerve (VII), pink: indicates r6-derived vMNs projecting into the glossopharyngeal nerve (IX) and green indicates vMNs projecting into the vagal nerve (X). Open circles indicate the axonal exit point of vMN subtypes. In *Nkx6* mutants, most vMNs generated in the caudal hindbrain fail to project out of the neural tube through their normal exit points in r6 and r7. Instead, axons turn and extend either in caudal or rostral directions within the neural tube (A,B; data not shown). Axonal projections of vMNs into the VIIth and Vth nerve is less affected and most axons converge at their respective exit point. In contrast to controls, few axons in *Nkx6* mutants have at this stage exited the neural tube (A,B). A significant number of r3-derived trigeminal MNs at E10.5, arbitrarily project caudally towards the exit point in r4, rather than their normal exit point in r2. (C-F) Lateral view of E13.5 embryos showing *lacZ* expression in vMN axons in *Nkx6.2*$^{+/tlz}$ controls (C) and *Nkx6* mutants (D). In *Nkx6.2*$^{+/tlz}$ embryos, the normal pattern of peripheral projections of the trigeminal (V), facial (VII), glossopharyngeal (IX) and vagal (X) nerves is detected. In *Nkx6* mutants (D), *lacZ*$^+$ projections of vMNs into the trigeminal (V), and facial (VII) nerves are severely impaired, although the overall shape of the facial nerve resembled that of control embryos. At more caudal levels of *Nkx6* mutants, no *lacZ*$^+$ axonal projections into the vagal (X) and glossopharyngeal nerves (IX) are detected. Schematic summary of axonal projections in control (*Nkx6.2*$^{+/tlz}$) and *Nkx6* mutants at E13.5 (E,F). (G,H) Transverse sections through the brainstem of wild type (G,I,K,M) and *Nkx6* mutants (H,J,L,N) at E16.5, hybridized with a peripherin probe to visualize MN nuclei. Sections are shown in an anterior (upwards) to posterior (downwards) direction. In *Nkx6* mutants, the trigeminal (N.V; H) and facial (N.VII; J) nuclei are reduced in size compared with controls (G,K) and the facial nucleus is displaced rostrally (J, compare with wild type in K). The nuclei of the vagal nerve, nucleus ambiguus (N.A) and the dorsal motor nucleus (dmnX), are absent in *Nkx6* mutants (N, compare with wild type in M). In addition, sMNs of the abducens (N.VI; I) and the hypoglossal (N.XII; M) nuclei are missing in *Nkx6* mutants (J,N).

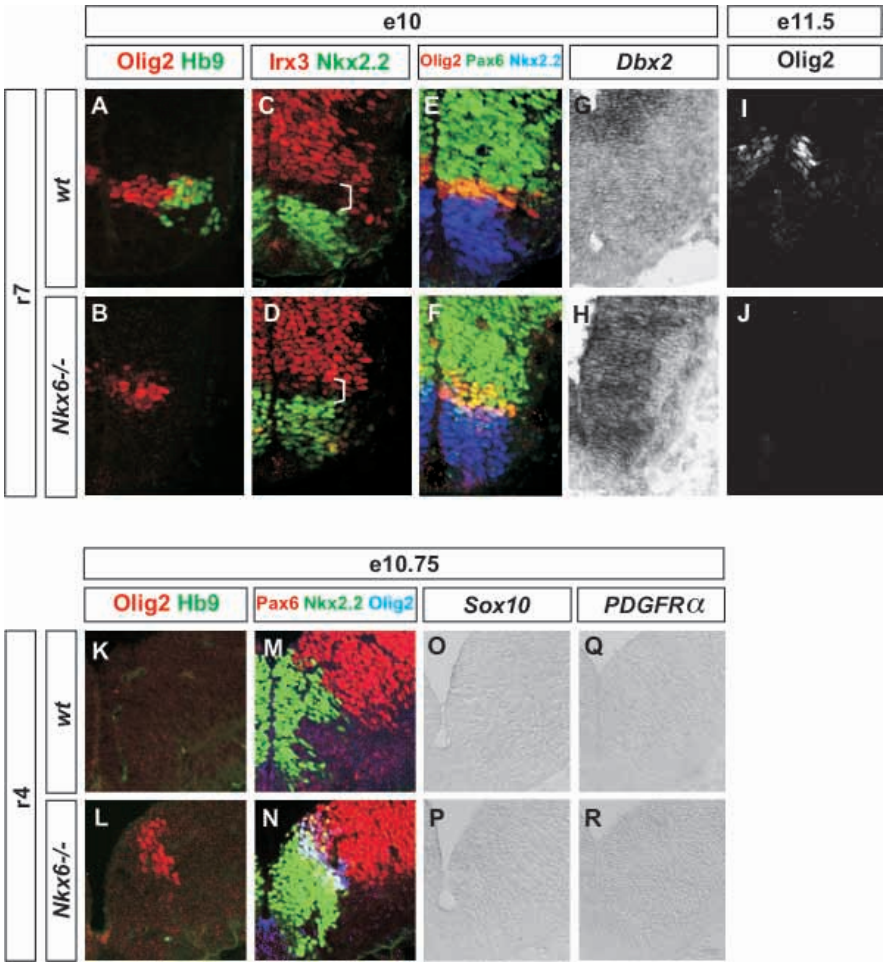

Fig. 6. Olig2 is expressed but sMNs are missing in the hindbrain of *Nkx6* mutants. (A-R) Transverse sections through r7 or r4 of wild type and *Nkx6* mutant embryos at E10 (A-H), E11.5 (I-J) or E10.75 (K-R). (A-H) In r7, the expression of Olig2 is similar in controls and *Nkx6* mutants at E10 (A,B). $Hb9^{+}$ sMNs are detected in controls (A) but not *Nkx6* mutants (B). The pattern of Nkx2.2, Pax6 and Irx3 is similar in controls and *Nkx6* mutants in r7 at E10 (C-F). The expression of Dbx2 is expanded ventrally and ectopically expressed in the pMNs domain (G,H). By E11.5, the expression of Olig2 is extinguished at the level of r7 in *Nkx6* mutants (I,J). (K-R) In r4 of wild-type embryos at E10.75, sMNs are not generated and no expression of Olig2 or Hb9 can be detected (K). In *Nkx6* mutants, Olig2 is expressed ectopically in r4, but no $Hb9^{+}$ neurons are detected (L). Most ectopic $Olig2^{+}$ cells in r4 of *Nkx6* mutants co-express Pax6 and Nkx2.2 at E10.75 (M,N). No expression of the oligodendrocyte precursor cell markers *Sox10* or *Pdgfra* is detected either in controls (O,Q) or in *Nkx6* mutants (P,R) at E10.75.

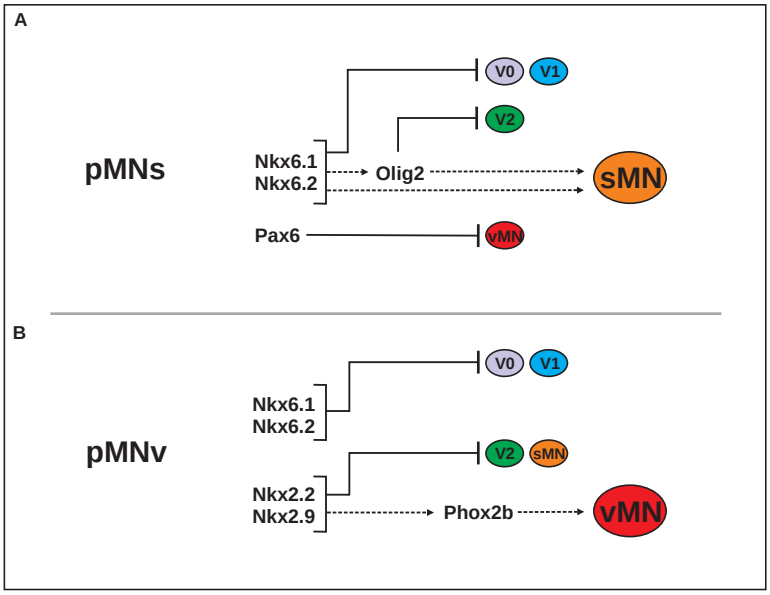

Fig. 7. Model for the role of Nkx proteins in somatic- and visceral MN generation. (A) sMN generation: The combinatorial expression of Nkx6 proteins (Nkx6.1 and Nkx6.2), Olig2 and Pax6 act to suppress cells in the pMNs domain from undertaking other ventral differentiation programs; Nkx6.1 and Nkx6.2 suppress V0 and V1 fate (Briscoe et al., 2000; Vallstedt et al., 2001), Olig2 suppresses V2 fate (Novitch et al., 2001; Zhou and Anderson, 2002) and Pax6 blocks vMN fate (Ericson et al., 1997). In addition to its role in suppressing V2 fate, Olig2 also promote cells to progress along the sMN differentiation pathway (Novitch et al., 2001; Zhou and Andersson). In part, Nkx6.1 and Nkx6.2 promote sMN generation by acting upstream of Olig2 (Novitch et al., 2001) (this study), but Nkx6 proteins also act in parallel with Olig2 in the progression of sMN fate determination. (B) vMN generation: The combinatorial activity of Nkx6 and Nkx2 class proteins in the pMNv domain suppress more dorsal sMN and interneuron differentiation programs. Nkx6.1 and Nkx6.2 suppress V0 and V1 fate (Vallstedt et al., 2001) (this study), while Nkx2.2 and Nkx2.9 suppress sMN and V2 neuronal fate (Briscoe et al., 1999; Briscoe et al., 2000; Pabst et al., 2003). In the pMNv domain, Nkx6 proteins are dispensable for the progression of vMN differentiation, and Nkx2 class proteins mediate the activation of the vMN determinant Phox2b (Pattyn et al., 2000). Once induced, Phox2b and Nkx2 proteins may also cooperate in subsequent steps of vMN fate determination (Dubreuil et al., 2002). For further details, see text.

generation, or correct temporal specification, of OLPs in the caudal hindbrain.

DISCUSSION

Complementary roles for Nkx2 and Nkx6 class proteins in vMN fate specification

In this study, we provide evidence that Nkx6 and Nkx2 class proteins mediate complementary activities in the specification of vMN fate. Nkx6.1 and Nkx6.2 are not required for the generation of vMNs, and instead these proteins ensure that differentiating vMNs do not initiate a parallel V0 differentiation program. The role for Nkx6 proteins to suppress Dbx gene expression and the V0 determinant Evx1 (Moran-Rivard et al., 2001) in vMNs appears to be analogous to their role in more dorsal sMN progenitors (Vallstedt et al., 2001). However, while progenitors in the sMN domain in *Nkx6* mutants give rise to V0 neurons at the expense of sMNs (Vallstedt et al., 2001), vMNs are generated in close to normal numbers but with a mixed vMN/V0-neuronal identity. This difference can most easily be explained by the fact that Nkx6 proteins are necessary, either directly or indirectly, for the activation of downstream determinants of sMN fate (see below), whereas the initiation of Phox2b expression in the vMN pathway is mediated by a separate activity. Nkx2.2 and Nkx2.9 are expressed in the hindbrain of *Nkx6* mutants (Pattyn et al., 2003), and we show that Nkx2.2 is sufficient to induce Phox2b expression in the hindbrain. These data indicate that Nkx2.2 acts upstream of Phox2b in the vMN pathway. Consistent with this idea, the expression of Nkx2.2 is unaffected in *Phox2b* mutants despite the fact that all vMNs are missing in these mice (Pattyn et al., 2000). Nkx2.2, however, is not required for the generation of vMNs (Briscoe et al., 1999), but this may reflect functional redundancy between Nkx2.2 and Nkx2.9 (Briscoe et al., 1999; Pabst et al., 2003). The fact that Nkx2.2 and Nkx2.9 are induced independently of each other in the hindbrain (Briscoe et al., 1999; Pabst et al., 2003) and that the generation of vMNs is also left largely unaffected in Nkx2.9 mutants (Pabst et al., 2003), supports this idea. Recent data, however, has shown that Nkx2.9 cannot compensate for the loss of Nkx2.2 function by rescuing the generation of serotonergic (S) neurons in the hindbrain (Pattyn et al., 2003), despite the fact that Nkx2.9, in *Nkx2.2* mutants, is expressed in the S neuron progenitor domain. These data indicate differences in the intrinsic properties of Nkx2.2 and Nkx2.9. Thus, although data may favour redundancy between these proteins in vMN fate specification, this idea needs to be established by future analyses of *Nkx2.2* and *Nkx2.9* compound mutant mice.

The persistent expression of Nkx2.2 and Nkx2.9 in *Nkx6* mutants also provides a logic as to why vMNs express V0 neuron characteristics, but not traits typical of other ventrally generated neurons. Previous studies have shown that Nkx2.2 suppresses the generation of sMNs in the spinal cord (Briscoe et al., 1999; Briscoe et al., 2000), most likely due to its role in repressing the expression of Olig2 (Novitch et al., 2001). Nkx2.2 and/or Nkx2.9 are also strong candidates to suppress V2 neuronal fate, as expression of the V2 determinant Irx3 only extends ventrally to the dorsal boundary of Nkx2.2 expression in both *Nkx6* and *Olig2* mutant mice (this study) (Zhou and Anderson, 2002; Lu et al., 2002). Thus, while high levels of Nkx6 activity act to suppress V0 and V1 neuronal fate (Vallstedt et al., 2001), Nkx2 class proteins instead appear to block programs of sMN and V2 neuron differentiation. Considering that Nkx2 class proteins act upstream of Phox2b, the combined activities of Nkx6 and Nkx2 class proteins appear sufficient therefore to account for the initial steps of vMN fate specification.

Sequential roles for Nkx6 proteins in vMN differentiation

The expression of Nkx6.1 and/or Nkx6.2 is maintained in differentiating vMNs, indicating that these proteins are involved in sequential steps of vMN differentiation. In support of this, we find that both the migration and axonal projection properties of vMNs in the hindbrain are affected by the loss of Nkx6.1 and Nkx6.2 function; the dorsal migration of vMN subtypes occurs at a slow pace, and r4-derived fb MNs fail to migrate caudally into r6 and instead migrate strictly dorsally within r4. In addition, the overall navigation of vMN axons, both within the CNS and in the periphery, is perturbed. The altered properties of differentiating vMNs are consistent with a cell-autonomous role for Nkx6 proteins in post-mitotic differentiating vMNs, but as Nkx6.1 and Nkx6.2 are also broadly expressed in ventral progenitor cells, we cannot exclude that migratory and axonal pathfinding defects also involve changes in the environment that vMNs encounter as they differentiate. In addition, the early role for Nkx6 proteins in vMN specification makes it difficult to definitively link the requirement for Nkx6.1 and Nkx6.2 to postmitotic neurons. Our analysis of *Nkx6.1* single mutants, however, show that the impaired migration of vMNs cannot only be a secondary consequence of their early role to suppress Evx1 expression in vMNs, because Nkx6.2 alone is sufficient to suppress Evx1 in r4-derived fbMNs, but not to compensate for the loss of Nkx6.1 by fully restoring the subsequent migration of these neurons. We have noticed that the expression of cadherin 8 (Garel et al., 2000; Korematsu and Redies, 1997) is not initiated in differentiating fb MNs in *Nkx6* mutants (data not shown), and Muller and colleagues have found that the expression profile of netrin receptors are altered in vMNs in *Nkx6.1* single mutants (M. Sander, personal communication). These data provide additional, albeit indirect, support for a cell-autonomous role for Nkx6 proteins in vMNs, and raise the possibility that the deregulated expression of these proteins may contribute to the impaired differentiation of vMNs observed in *Nkx6.1* and *Nkx6* mutant mice.

A parallel requirement for Nkx6 and Olig2 proteins in sMN fate determination

Our current analysis provides new insight also into the role of Nkx6 and Olig proteins in the generation of sMNs. Olig2 has previously been shown to have a dual role in sMN fate determination; it suppresses the expression of Irx3 in sMN progenitors, and also promotes cell-cycle exit and neuronal differentiation by derepression of the pro-neural bHLH protein Ngn2 in the sMN progenitor domain (Novitch et al., 2001; Mizuguchi et al., 2001; Zhou and Anderson, 2002; Lu et al., 2002). Nkx6 proteins are required for the expression of Olig2 in the spinal cord (Novitch et al., 2001), and there is a similar deficit of sMNs in *Nkx6* mutants, *Olig2* mutants and *Olig1/2*

compound mutants (Vallstedt et al., 2001; Zhou and Anderson, 2002; Lu et al., 2002). Because forced expression of Nkx6.1 in the chick spinal cord results in the ectopic activation of Olig2 expression (Novitch et al., 2001) and the expression of Nkx6.1 is left unaffected in *Olig* mutants (Zhou and Anderson, 2002), a model in which Olig2 acts downstream of Nkx6 proteins in the sMN pathway has been proposed (Novitch et al., 2001; Zhou and Anderson, 2002). In contrast to spinal cord levels, we find that the initial phase of Olig2 expression is unaffected in the caudal hindbrain in *Nkx6* mutants, and neither the expression of Irx3 nor Nkx2.2 have encroached into the sMN progenitor domain at this stage. Despite this, all sMNs are missing. These data reveal a requirement for Nkx6.1 and Nkx6.2 in sMN fate specification that is unrelated to their role in promoting Olig2 gene expression, and further indicate that Olig2, in the absence of Nkx6 protein function, is not sufficient to specify sMN fate in the hindbrain. These findings seem to exclude the possibility that Nkx6 and Olig proteins operate in a strict linear pathway. As both Nkx6 and Olig proteins mediate their inductive activities by acting as repressors (Muhr et al., 2001; Novitch et al., 2001), it appears more likely that these proteins act in parallel to exclude different sets of repressor proteins from the sMN progenitor domain (Muhr et al., 2001; Novitch et al., 2001). If expressed in sMN progenitors in either *Nkx6* or in *Olig* mutant mice, such Olig2 of Nkx6 regulated repressor proteins would be predicted to act independently of each other to block sMN generation at a step downstream of Olig2. This idea gains support by the fact that forced expression of Irx3 within the sMN progenitor domain, is sufficient to inhibit sMN generation (Briscoe et al., 2000).

In previous analyses, the genetic ablation of individual class I or class II proteins has typically resulted in a transformation of progenitor domain identity, followed by a predictable switch in neuronal subtype identity (Ericson et al., 1997; Vallstedt et al., 2001; Pierani et al., 2001; Novitch et al., 2001; Zhou and Anderson, 2002). Although these data highlight a central role for class I and class II proteins in the establishment of progenitor domains, the early transformation of progenitor domain identity has precluded attempts to evaluate the relevance of the combinatorial expression of these proteins in neuronal fate determination. It remained possible, for example, that the only role for Pax6 (Ericson et al., 1997; Novitch et al., 2001), Nkx6.1 and Nkx6.2 in the pMNs domain (Vallstedt et al., 2001; Novitch et al., 2001) was to ensure the expression of Olig2, which in turn directed all downstream events necessary for the establishment of sMN identity (Novitch et al., 2001; Zhou and Anderson, 2002). In our current hindbrain analysis, we provide evidence for a parallel requirement of Nkx6 and Olig2 proteins in sMN fate specification, and furthermore that Nkx6 and Nkx2 class proteins mediate complementary activities in the specification of vMN fate. Importantly, as Nkx6.1 and Nkx6.2 are not required for the initial establishment of either the pMNs or the pMNv progenitor domain, these findings suggest that the combinatorial activities of class I and/or class II protein expression in distinct progenitor domains (Briscoe et al., 2000) also is necessary for the rigid specification of ventral neuronal subtypes.

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