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Nkx6-1 controls the identity and fate of red nucleus and oculomotor neurons in the mouse midbrain

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Little is known about the cues controlling the generation of motoneuron populations in the mammalian ventral midbrain. We show that Otx2 provides the crucial anterior-posterior positional information for the generation of red nucleus neurons in the murine midbrain. Moreover, the homeodomain transcription factor Nkx6-1 controls the proper development of the red nucleus and of the oculomotor and trochlear nucleus neurons. Nkx6-1 is expressed in ventral midbrain progenitors and acts as a fate determinant of the Brn3a$^+$ (also known as Pou4f1) red nucleus neurons. These progenitors are partially dorsalized in the absence of *Nkx6-1*, and a fraction of their postmitotic offspring adopts an alternative cell fate, as revealed by the activation of *Dbx1* and *Otx2* in these cells. Nkx6-1 is also expressed in postmitotic Isl1$^+$ oculomotor and trochlear neurons. Similar to hindbrain visceral (branchio-) motoneurons, Nkx6-1 controls the proper migration and axon outgrowth of these neurons by regulating the expression of at least three axon guidance/neuronal migration molecules. Based on these findings, we provide additional evidence that the developmental mechanism of the oculomotor and trochlear neurons exhibits more similarity with that of special visceral motoneurons than with that controlling the generation of somatic motoneurons located in the murine caudal hindbrain and spinal cord.

KEY WORDS: Red nucleus, Oculomotor/trochlear nucleus, Nkx6-1, Nkx6-2, Otx2, Dbx1, Mouse

INTRODUCTION

During mouse development, distinct neuronal populations organized in discrete nuclei arise in the ventral midbrain (VM). The best characterized are the substantia nigra and ventral tegmental area dopaminergic (mDA) neurons due to their clinical relevance, but less is known about the cues controlling the development of other nuclei in the VM or rostral hindbrain. These include the oculomotor nucleus (OM), the trochlear nucleus (TN) and the red nucleus (RN). The OM and TN give rise to the third (nIII) and fourth (nIV) cranial nerve, respectively. The nIII innervates the ipsilateral extraocular muscles and ciliary ganglion, thereby controlling most eye movements, eye accommodation and pupil contraction, and the nIV regulates the movements of the contralateral superior oblique muscle of the eye. The RN is located in close vicinity to the OM and mDA area (Evinger, 1988) and contains both excitatory glutamate- and inhibitory γ-aminobutyric acid (GABA)-synthesizing neurons, which project to the cerebellum, brainstem and spinal cord (Keifer and Houk, 1994). Together with the corticospinal tract, the rubrospinal tract plays a fundamental role in the control of limb movements (Kennedy, 1990).

Whereas little is known about the molecular mechanism and factors controlling the specification of the RN, similar cues for the motoneuron (MN) and interneuron (IN) populations arising in the vertebrate hindbrain and spinal cord appear to act during the development of the OM and TN (Eisen, 1999; Jessell, 2000; Jurata et al., 2000). In particular, the POU homeodomain (HD) transcription factor (TF) Pou4f1 (also known as Brn3a) is expressed in postmitotic RN neurons and is required for their survival (Agarwala and Ragsdale, 2002; Fedtsova and Turner, 1995; Turner et al., 1994; McEvilly et al., 1996; Xiang et al., 1996). A similar RN phenotype was reported in mouse mutants lacking the homeobox gene *Emx2* (Agarwala and Ragsdale, 2002). The LIM HD TF islet1 (Isl1), a generic MN marker, is expressed in the OM/TN (Agarwala and Ragsdale, 2002; Ericson et al., 1992) and, although Isl1 is necessary for survival of spinal MNs (Pfaff et al., 1996), its function in OM/TN development remains unknown. The paired-like homeobox gene *Phox2a* is expressed in OM/TN progenitors and, together with its paralog *Phox2b*, in their postmitotic offspring (Pattyn et al., 1997). The OM and TN are lost in *Phox2a*$^{-/-}$ but not in *Phox2b*$^{-/-}$ mice, indicating that Phox2a is a crucial fate determinant in OM/TN precursors (Coppola et al., 2005; Pattyn et al., 2000; Pattyn et al., 1997).

The induction of fate-determining TFs by extrinsic and intrinsic signals along the dorsoventral (D/V) and anterior-posterior (A/P) axis of the neural tube is a crucial process that is likely to precede the differentiation of RN and OM/TN neurons in the VM/rostral hindbrain (Agarwala and Ragsdale, 2002; Fedtsova and Turner, 2001). Relevant studies support a general model in which the glycoprotein sonic hedgehog (Shh), secreted from the ventral midline of the neural tube (the floor plate, FP), is essential for D/V patterning by controlling the expression of different sets of HD TFs

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(Dessaud et al., 2008; Jessell, 2000). Among these, the HD TFs Nkx6-1 and Nkx6-2 are transcriptional repressors induced by Shh signaling in the ventral hindbrain and spinal cord (Briscoe et al., 2000; Cheesman et al., 2004; Muhr et al., 2001; Qiu et al., 1998), and are required for the specification of hindbrain and spinal somatic MNs (sMNs) and INs (Broihier et al., 2004; Hutchinson et al., 2007; Sander et al., 2000a; Vallstedt et al., 2001), as well as for proper muscle nerve formation and innervation patterns (De Marco Garcia and Jessell, 2008). Moreover, Nkx6-1 and Nkx6-2 are also required for the proper migration and axon pathfinding of hindbrain visceral (branchio-) MNs (vMNs), but not for their initial specification (Müller et al., 2003; Pattyn et al., 2003). Ablation of Nkx6 function leads to the derepression of the Shh-repressed HD TFs developing brain homeobox 1 and 2 (Dbx1 and Dbx2) in the ventral spinal cord, and to a fate-switch of MNs and ventral (V2) INs into more dorsal (V0) INs (Sander et al., 2000a; Vallstedt et al., 2001). Notably, most of these studies have been focussed on spinal cord and hindbrain, thus leaving largely unknown the role of Nkx6-1 and Nkx6-2 in the development of VM neuronal populations (Cai et al., 2001; Puelles et al., 2004; Puelles et al., 2001).

The mammalian HD protein Otx2 controls fore- and mid-brain patterning (Acampora et al., 2005). Conditional inactivation of *Otx2* in the mid-/hind-brain region of the mouse results in a complete loss of the RN and hypoplasia of the OM (Puelles et al., 2004). Here we studied the functional involvement of Otx2 and Nkx6-1 in specification of identity and fate of RN and OM/TN neuronal populations. We show that Otx2 is necessary and sufficient for the maintenance and ectopic induction of Nkx6-1 in the VM/rostral hindbrain of mutant mice. Moreover, Nkx6-1 is required for the generation of Pou4f1$^+$ RN neurons and for the proper migration and axon outgrowth of OM/TN neurons, thus revealing intriguing similarities between the midbrain phenotype of *Nkx6-1*$^{-/-}$ mice and the sMN and vMN defects previously described in the same mutants. These findings suggest that functional aspects of the mechanism controlling hindbrain and spinal cord MN development have been conserved and recruited to a more rostral position in the mouse brain.

MATERIALS AND METHODS

Mutant mice

Generation and genotyping of *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$, *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$; *Nkx2-2*$^{-/-}$, *Nkx2-2*$^{-/-}$, *En1*$^{+/Wnt1}$, *Nkx6-1*$^{-/-}$, *Nkx6-2*$^{tlz/tlz}$ and *Nkx6-1*$^{-/-}$; *Nkx6-2*$^{tlz/tlz}$ mice has previously been described (Panhuysen et al., 2004; Prakash et al., 2006; Puelles et al., 2004; Sander et al., 2000b; Sussel et al., 1998; Vallstedt et al., 2001). Animal treatment was conducted under federal guidelines for the use and care of laboratory animals and was approved by the HMGU Institutional Animal Care and Use Committee.

Radioactive in situ hybridization (ISH) and immunohistochemistry

Mouse embryo paraffin sections (8 μm) or cryosections (16 μm) were processed for radioactive ISH or for immunohistochemistry (Fischer et al., 2007; Prakash et al., 2006). Details on riboprobes, antibodies and image acquisition are available upon request. The whole-mount neurofilament staining procedure was performed as described in Huber et al. (Huber et al., 2005).

BrdU treatments

BrdU treatments were performed as described in Omodei et al. and Puelles et al. (Omodei et al., 2008; Puelles et al., 2006).

Unbiased stereology and cell countings

Pou4f1$^+$ and Th$^+$ cells were evaluated by the optical fractionator method and phospho-Histone H3$^+$ cells were counted using the Stereo Investigator 5.05.4 software (MBF Bioscience, Williston, VT, USA) on serial coronal midbrain cryosections or paraffin sections from E18.5 or E11.0 embryos. Averaged cell numbers for each genotype were subjected to a Student's *t*-test for estimation of statistical significance. Counting of Nkx6-1$^+$, Nkx6-2$^+$, Pou4f1$^+$ and Isl1$^+$ single- and double-labeled cells was performed on *z*-stack confocal image series (1 μm intervals) recorded from VM sections of E12.5 embryos.

Bioinformatics prediction of Nkx6-1 target genes

All gene symbols, promoter and transcript identifiers in Table S1 (in the supplementary material) were derived from the promoter sequence retrieval database ElDorado (Genomatix, Munich, Germany). A genome-wide scan for conserved orthologous promoters with Nkx6-1 binding sites (BSs) was performed with the Gene2Promoter program (Genomatix). The selection of putative Nkx6-1 target genes was based on the *Nkx6-1*$^{-/-}$ midbrain phenotype and on their implication in neural development and/or axon guidance. Promoter sequences from up to seven different mammalian species were aligned with the DiAlign TF program (Cartharius et al., 2005) in the Genomatix software suite GEMS Launcher to evaluate overall promoter similarity and to identify conserved Nkx6-1 BSs. The promoter regions were defined as ~900 bp upstream, including the proximal region, and 100 bp downstream of the transcription start site (TSS). Position weight matrices were used according to Matrix Family Library Version 7.0 (October 2007) for promoter analyses. BSs were considered as 'conserved BSs' only if the promoter sequences for all given orthologs could be aligned in the region of the Nkx6-1 BS with the help of the DiAlign TF program (using default settings).

RESULTS

Midbrain basal plate progenitors and postmitotic OM/TN neurons express Nkx6-1

To establish the function of Nkx6 TFs in the development of VM progenitors and postmitotic neurons, we first performed a detailed expression analysis for Nkx6-1 and Nkx6-2 and a related TF, Nkx2-2, in the embryonic mouse midbrain at mid-gestational stages. From the earliest stage analyzed (E9.0), Nkx6-1 is expressed in the midbrain basal plate (BP) initially overlapping with the adjacent Nkx6-2$^+$ territory in the ventrolateral midbrain, but becoming mutually exclusive at E9.5 (Fig. 1A,B). The medial FP expresses Nkx6-1 at E9.0 but not at later stages (Fig. 1A,B). At E11.0, an intermediate zone of weaker Nkx6-1 expression is apparent between the Nkx6-1$^+$ ventricular/subventricular zone (VZ/SVZ) and mantle zone (MZ) (Fig. 1D). This subdivision into an Nkx6-1$^+$ domain within the VZ/SVZ and one within the MZ is clearly discernible at E12.5 (Fig. 1I). Although the Nkx6-1$^+$ and Nkx6-2$^+$ domains appear to be mutually exclusive, some cells expressing Nkx6-2 are detected within the Nkx6-1$^+$ MZ from E10.5 onwards (Fig. 1C,D). At E12.5, ~63% of the Nkx6-1$^+$ cells in the BP MZ coexpress Nkx6-2. Nkx6-1 is also expressed within the dorsal Nkx6-2$^+$ domain in the VZ/SVZ of the lateral midbrain at this stage (Fig. 1I). Expression of Nkx2-2 overlaps with the Nkx6-2$^+$ domain in the lateral midbrain and initially (at E9.0-9.5) extends further ventrally into the BP (Fig. 1E,F). From E10.5 onwards, Nkx2-2 expression is confined to a narrow transverse stripe coinciding with the ventrolateral sulcus of the midbrain (His, 1904), and overlapping with the Nkx6-2$^+$ domain (Fig. 1G,H,M). In contrast to Nkx6-1 and Nkx6-2, Nkx2-2 expression extends throughout the neuroepithelium including the VZ/SVZ and MZ at E12.5 (Fig. 1M). At this stage, Nkx2-2 expression also expands ventrally into the Nkx6-1$^+$ VZ/SVZ (Fig. 1I,M).

Together, the combined expression of these TFs defines three adjacent domains along the D/V axis of the midbrain: the ventral-most expressing only Nkx6-1 in the BP; the intermediate coexpressing Nkx6-2 and Nkx2-2; and the dorsal-most expressing Nkx6-2 in the lateral midbrain. It is noteworthy that Nkx6-1 expression also initiates in more dorsal progenitors at late mid-gestation. Next, we examined the identity of the Nkx6-1$^+$ and Nkx6-

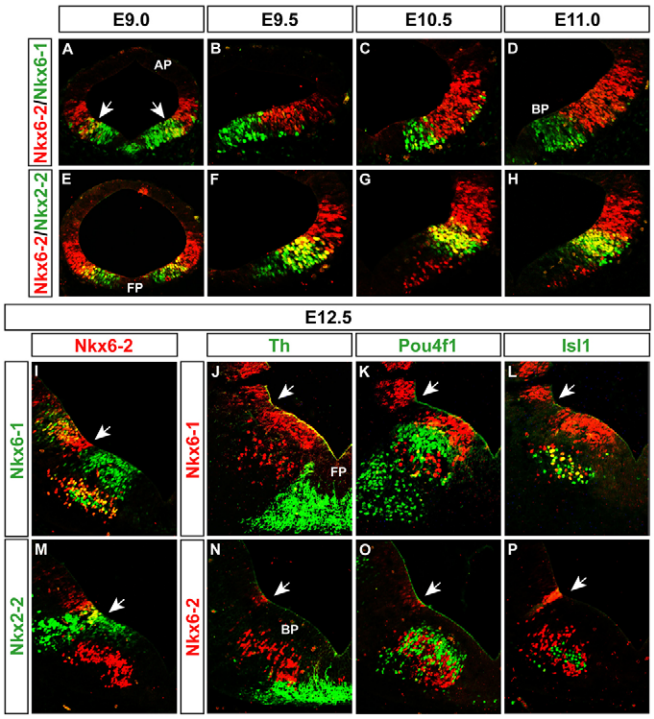

Fig. 1. Midbrain BP progenitors and postmitotic OM neurons express Nkx6-1. (**A-P**) Representative coronal midbrain sections of E9.0 (A,E), E9.5 (B,F), E10.5 (C,G), E11.0 (D,H) and E12.5 (I-P) WT mouse embryos. (A-D) Nkx6-1$^+$ cells are confined to the midbrain BP at E9.0-E11.0. Arrows in A indicate cells initially coexpressing Nkx6-1 and Nkx6-2. (E-H) Coexpression of Nkx2-2 and Nkx6-2 in the ventrolateral midbrain. (I-P) Nkx6-1 is expressed in two distinct progenitor domains ventral and dorsal to the ventrolateral midbrain sulcus (arrows) at E12.5. Some Nkx6-1$^+$ cells in the BP MZ coexpress Nkx6-2 (I). Dorsal Nkx6-2$^+$ progenitors partly overlap with Nkx2-2$^+$ cells (M). Th$^+$ mDA neurons arise from the Nkx6-1$^-$ and Nkx6-2$^-$ FP (J,N). Pou4f1$^+$ RN and Isl1$^+$ OM neurons arise from the Nkx6-1$^+$ midbrain BP (K,L,O,P). Note that at E12.5, most Pou4f1$^+$ neurons are Nkx6-1$^-$ and Nkx6-2$^-$ (K,O), and Isl1$^+$ OM neurons coexpress Nkx6-1 but not Nkx6-2 (L,P). AP, alar plate; BP, basal plate; FP, floor plate.

2$^+$ cells in the VM MZ by analyzing the coexpression of tyrosine hydroxylase (Th), Pou4f1 and Isl1, which label mDA, RN and OM neurons in the midbrain, respectively. Nkx6-1 or Nkx6-2 are not expressed in Th$^+$ mDA neurons arising from the FP (Fig. 1J,N) (Bonilla et al., 2008; Kittappa et al., 2007; Ono et al., 2007); some Pou4f1$^+$ RN neurons coexpress Nkx6-1 (~9%) or Nkx6-2 (~15%) (Fig. 1K,O); and a considerable number of Isl1$^+$ OM neurons (~69%) are Nkx6-1$^+$ but not Nkx6-2$^+$ at E12.5 (Fig. 1L,P). The E9.0-E12.5 rostral hindbrain displays a similar spatial pattern of Nkx6-1, Nkx6-2, Nkx2-2 and Isl1 expression, although Nkx6-2 expression within the ventral MZ is not detected at this brain level (not shown).

We concluded that Pou4f1$^+$ RN and Isl1$^+$ OM neurons might derive from Nkx6-1$^+$ progenitors and designated the Nkx6-1$^+$ VZ/SVZ in the midbrain BP as 'ventral Nkx6-1$^+$ progenitor domain', in contrast to the Nkx6-1$^+$ VZ/SVZ in the lateral midbrain, which we designated as 'dorsal Nkx6-1$^+$ progenitor domain'. Moreover, our findings suggest that, with the exception of a few Pou4f1$^+$ RN neurons, Nkx6-2 labels a distinct and as yet unidentified cell population in the mouse VM.

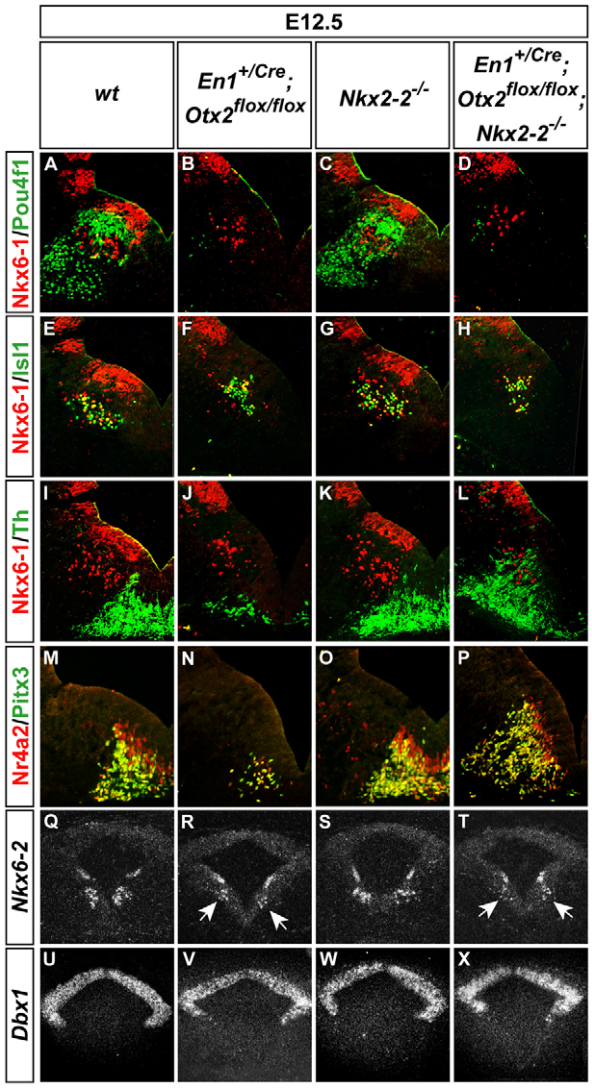

Fig. 2. Otx2 is required for maintenance of the VM Nkx6-1$^+$ progenitor domain. (**A-X**) Representative coronal midbrain sections of E12.5 WT embryos (A,E,I,M,Q,U), *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ (B,F,J,N,R,V), *Nkx2-2*$^{-/-}$ (C,G,K,O,S,W) and *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$; *Nkx2-2*$^{-/-}$ (D,H,L,P,T,X) mutant embryos. (B,D) Loss of ventral Nkx6-1 expression and of Pou4f1$^+$ RN neurons in *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ mice is not rescued in *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$; *Nkx2-2*$^{-/-}$ triple mutants. (F,H) There is a slight reduction of Isl1$^+$ OM neurons in both *Otx2* mutants. (J,L,N,P) Th/Pitx3$^+$ mDA neurons are lost in *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ but rescued in *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$; *Nkx2-2*$^{-/-}$ mutants. (C,G,K,O) Nkx6-1, Pou4f1, Isl1, Th and Pitx3 expression is unaffected in *Nkx2-2*$^{-/-}$ mice. (Q-X) There is a strong reduction of the ventral *Nkx6-2*$^+$ domain (arrows in R,T) but unaltered *Dbx1* expression in *Otx2* mutants.

Otx2 is required for maintenance of the VM Nkx6-1$^+$ progenitor domain

We next investigated which signals apart from Shh control the expression of Nkx6 TFs in the murine VM. As reported before, inactivation of *Otx2* in the mid-/hind-brain region, including the VM, of *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ mutants, which were generated by crossing mice carrying a floxed *Otx2* allele (Puelles et al., 2003) with *En1*$^{+/Cre}$ knock-in mice (Kimmel et al., 2000), resulted in the loss of the ventral but not dorsal Nkx6-1$^+$ progenitor domain at E12.5 (Fig. 2B,F,J) (Puelles et al., 2004). Furthermore, Pou4f1$^+$ RN neurons were

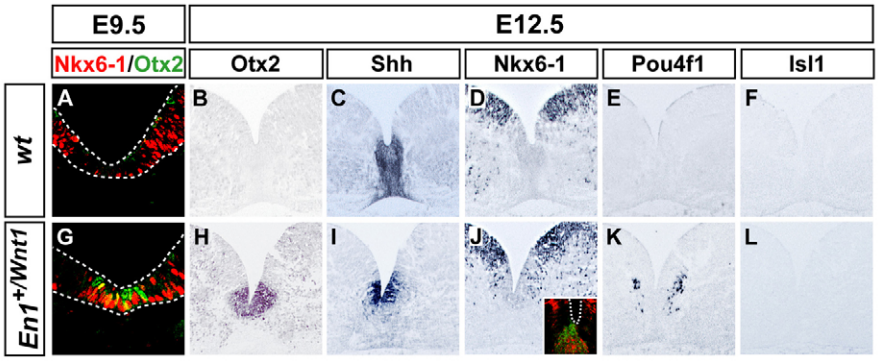

Fig. 3. Otx2 is sufficient for the ectopic induction of Nkx6-1 and Pou4f1$^+$ RN neurons. (**A-L**) Representative coronal sections from the rostral hindbrain of E9.5 (A,G) and E12.5 (B-F,H-L) WT (A-F) and *En1$^{+/Wnt1}$* (G-L) embryos. (A,G) At E9.5, ectopic expression of Otx2 induces Nkx6-1 ectopically in the FP of the *En1$^{+/Wnt1}$* rostral hindbrain. (D,J) Ectopic Nkx6-1$^+$ cells are located lateral to the Otx2$^+$ domain (inset in J; Nkx6-1 in red, Otx2 in green) in the *En1$^{+/Wnt1}$* rostral hindbrain FP at E12.5. (E,K) Ectopic Pou4f1$^+$ cells arise from the Otx2$^+$/Shh$^+$/Nkx6-1$^+$ mutant FP. (F,L) Isl1$^+$ cells are not detected at this caudal level of the WT and mutant brain. Plane of sectioning in B-F and H-L is more horizontal than in the inset in J.

completely lost and Th/Pitx3$^+$ mDA neurons were strongly reduced, whereas Isl1$^+$ OM neurons were slightly decreased but otherwise unaffected, and some of them still coexpressed Nkx6-1 in these mutants (Fig. 2A,B,E,F,I,J,M,N) (Puelles et al., 2004). We reasoned that the loss of the ventral Nkx6-1$^+$ progenitor domain in *En1$^{+/Cre}$*; *Otx2$^{flox/flox}$* mutants might be due to either a repression of Nkx6-1 by the ventrally expanded Nkx2-2 (Puelles et al., 2004), or a requirement (direct or indirect) of Otx2 for the maintenance of the ventral Nkx6-1 expression domain. To discriminate between these two possibilities, we crossed the *En1$^{+/Cre}$*; *Otx2$^{flox/flox}$* mice with *Nkx2-2$^{-/-}$* mice (Sussel et al., 1998) to generate compound *En1$^{+/Cre}$*; *Otx2$^{flox/flox}$*; *Nkx2-2$^{-/-}$* triple mutants (Prakash et al., 2006). As previously reported, the mesencephalic Nkx6-1 expression domains and the three analyzed neuronal populations were unaffected in *Nkx2-2$^{-/-}$* embryos at E12.5 (Fig. 2C,G,K,O) (Briscoe et al., 1999). Removal of *Nkx2-2* in *En1$^{+/Cre}$*; *Otx2$^{flox/flox}$* mutants resulted in a rescue of Th/Pitx3$^+$ mDA neurons but not of Pou4f1$^+$ RN neurons at E12.5 (Fig. 2D,L,P). Moreover, the ventral Nkx6-1$^+$ progenitor domain was still lost and the remaining Nkx6-1$^+$ cells colocalized with Isl1$^+$ OM neurons in the triple mutant midbrain (Fig. 2D,H,L). The Isl1$^+$ OM might only be partially affected in *En1$^{+/Cre}$*; *Otx2$^{flox/flox}$* mutants, as the birth of OM neurons (between E9.2 and E9.7) precedes that of RN neurons (between E10.2 and 10.7) (see Fig. S1 in the supplementary material), and most OM neurons are thus born before full inactivation of Otx2 in these mutants (Puelles et al., 2004). We concluded that the absence of the ventral Nkx6-1 expression domain in *En1$^{+/Cre}$*; *Otx2$^{flox/flox}$* and *En1$^{+/Cre}$*; *Otx2$^{flox/flox}$*; *Nkx2-2$^{-/-}$* mice is due to the lack of Otx2, although a repressive effect of the dorsally expanded Shh domain cannot be ruled out completely in these mutants (Prakash et al., 2006; Puelles et al., 2004).

Otx2 inactivation also affected the ventral, but not dorsal, *Nkx6-2* expression domain in the midbrain of conditional and compound triple mutants at E12.5 (Fig. 2Q-T), indicating that maintenance of *Nkx6-2* expression in the midbrain BP also depends either directly or indirectly on Otx2. The loss of Nkx6-1 and *Nkx6-2* expression in the VM of these mutants, however, did not affect the expression of the dorsal marker *Dbx1* (Fig. 2U-X).

Otx2 is sufficient for the ectopic induction of Nkx6-1 and Pou4f1$^+$ RN neurons

The preceding loss-of-function (LOF) data suggested a causal relationship between Otx2 and Nkx6-1 expression and the generation of RN neurons in the VM. To assess if the ectopic expression of Otx2 leads to the ectopic induction of Nkx6-1, and concomitantly to the ectopic generation of Pou4f1$^+$ RN neurons, we used a previously characterized mouse model in which ectopic expression of *Wnt1* under the control of the *En1* promoter (*En1$^{+/Wnt1}$* knock-in mice) induces ectopic expression of Otx2 in the Shh$^+$ rostral hindbrain FP (Prakash et al., 2006). In this mutant, ectopic expression of Otx2 in the hindbrain FP was first detected at E9.5, concomitant with ectopic Nkx6-1$^+$ cells, some of which coexpressed Otx2 (Fig. 3A,G) (Prakash et al., 2006). At E12.5, the ectopic Nkx6-1$^+$ cells were located in the MZ lateral to the Otx2$^+$ and Shh$^+$ domain (Fig. 3H-J). This result indicated that Otx2 is sufficient for the ectopic induction of Nkx6-1, although a certain level of Shh signaling might be required in addition. Notably, ectopic Pou4f1$^+$, but not Isl1$^+$, cells were detected at E12.5 in the MZ at this caudal level of the *En1$^{+/Wnt1}$* rostral hindbrain FP (Fig. 3K,L; see Fig. S2 in the supplementary material). These cells probably arose from the ectopic Otx2$^+$ and Nkx6-1$^+$ domain, thus suggesting that Otx2 and Nkx6-1 might be sufficient for the generation of Pou4f1$^+$ RN neurons but not of Isl1$^+$ OM neurons.

Nkx6-1 is required for the generation of Pou4f1$^+$ RN neurons

Our previous results strengthened the hypothesis that Nkx6-1 is necessary for the generation of Pou4f1$^+$ RN neurons in the VM. To test this, we analyzed the midbrain phenotype of an *Nkx6-1* null mutant mouse (Sander et al., 2000b). Although gross morphological defects were not apparent in the *Nkx6-1$^{-/-}$* midbrain, a drastic reduction of the VM *Pou4f1$^+$* domain was already discernible at E12.5 (Fig. 4A-D), 1 day after Pou4f1$^+$ cells are first detected in the midbrain tegmentum (Fedtsova and Turner, 1995). The loss of *Pou4f1* expression was most pronounced in the rostral and caudal third of the corresponding wild-type (WT) domain (Fig. 4A-D). At E18.5, the Pou4f1$^+$ RN was strongly reduced in the mutants (Fig. 4E-H). RN neurons express the homeobox gene *Hb9* (also known as *Mnx1*) at this stage (Fig. 4I). The *Hb9$^+$* RN domain was also strongly reduced at E18.5, and the corresponding Nissl-stained sections confirmed the hypoplasia of the RN and revealed the absence of the OM in the *Nkx6-1$^{-/-}$* midbrain (Fig. 4I-L). Quantification of Pou4f1$^+$ RN neurons at E18.5 showed that only 27% of these neurons remained in the mutants (Fig. 4M). The loss of Pou4f1$^+$ RN neurons could be due to reduced progenitor proliferation, increased apoptosis or defective neurogenesis in the *Nkx6-1$^{-/-}$* VM. In this context, the number of phosphorylated

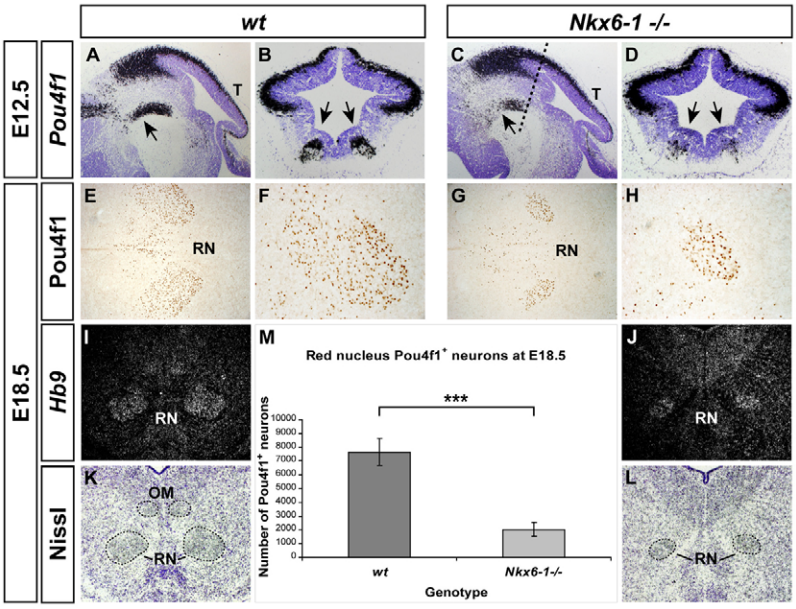

Fig. 4. Nkx6-1 is required for the generation of Pou4f1⁺ RN neurons. (**A-D**) Representative parasagittal (A,C) and coronal (B,D) midbrain sections of E12.5 WT (A,B) and *Nkx6-1*$^{-/-}$ (C,D) embryos. *Pou4f1* expression is strongly reduced in the rostral (arrow in A,C) and caudal (arrows in B,D) *Nkx6-1*$^{-/-}$ VM. Dashed line in C indicates the plane of coronal sections shown in B,D and all subsequent displays. (**E-H**) Representative horizontal midbrain sections of E18.5 WT (E,F) and *Nkx6-1*$^{-/-}$ (G,H) embryos. The Pou4f1⁺ RN is strongly reduced in *Nkx6-1*$^{-/-}$ embryos. F and H are higher magnifications of E and G, respectively. (**I-L**) Representative coronal midbrain sections of E18.5 WT (I,K) and *Nkx6-1*$^{-/-}$ (J,L) fetuses. Reduction in *Hb9* expression (I,J) and Nissl staining (K,L) reveals the RN hypoplasia and absence of the OM in mutant mice. (**M**) The number of Pou4f1⁺ RN neurons is significantly reduced in E18.5 *Nkx6-1*$^{-/-}$ brains (mean±s.d: WT: 7641±994, *n*=5; *Nkx6-1*$^{-/-}$: 2033±515, *n*=6; Student's *t*-test: ***P<0.0005). OM, oculomotor nucleus; RN, red nucleus; T, tectum.

Histone H3 (pH3)⁺ mitotic cells and cleaved caspase 3 (cCasp3)⁺ apoptotic cells was not significantly altered in the *Nkx6-1*$^{-/-}$ VM between E9.0 and E12.5 (see Fig. S3 in the supplementary material; data not shown), indicating that the reduction of Pou4f1⁺ RN neurons is not due to impairments in proliferation or survival. Furthermore, the normal expression of the two proneural genes neurogenin 1 and 2 (*Neurog1* and *Neurog2*) in the *Nkx6-1*$^{-/-}$ VM (see Fig. S3 in the supplementary material) suggests that neurogenesis was not impaired in the mutants. Altogether, these data suggest that Nkx6-1 is intrinsically required for the generation and/or identity of VM Pou4f1⁺ RN neurons.

Midbrain BP progenitors acquire a partial dorsal fate in the absence of *Nkx6-1*

Based on the previous conclusion, the loss of Pou4f1⁺ RN neurons might be due to an altered cell fate of the mutant progeny. To test this, we analyzed the expression of a number of markers to assess abnormalities in D/V patterning, as well as progenitor and postmitotic identity, in the *Nkx6-1*$^{-/-}$ midbrain.

In the absence of *Nkx6-1*, the transcription of *Nkx6-2* was upregulated and dorsally expanded in the VZ/SVZ corresponding to the dorsal *Nkx6-1*⁺ progenitor domain in the E12.5 WT (Fig. 5A-D′). Nkx6-2 upregulation was already evident at earlier embryonic stages (E9.5-E11.0, data not shown), when Nkx6-1 is not expressed in this region (Fig. 1), suggesting that Nkx6-1 initially regulates Nkx6-2 expression non-cell-autonomously in the lateral midbrain. By contrast, the expression of Nkx6-2 in postmitotic cells or the expression of Nkx2-2 was not altered in the *Nkx6-1*$^{-/-}$ VM (Fig. 5C-D′; see Fig. S5 in the supplementary material). However, *Dbx1* and *Otx2* were induced ectopically in the SVZ/MZ of the mutant VM at the same caudal level where *Pou4f1* expression was strongly reduced (Fig. 5E-J′). Ectopic induction of *Dbx1* was first detected at E10.5 within the *Phox2a*⁺ domain of the *Nkx6-1*$^{-/-}$ VM (Fig. 1; see Fig. S4 in the supplementary material). The *Dbx1* paralog *Dbx2* was not expressed in the WT midbrain at E9.5-E10.5, and *Dbx2* was not induced ectopically in the mutants (see Fig. S4 in the supplementary material). At E12.5, Dbx1 was expressed ectopically in cells other than Isl1⁺ OM neurons (Fig. 5K,M). Otx2, by contrast, was expressed at low levels in Isl1⁺ OM neurons of the WT embryo and might be slightly upregulated in these neurons in the mutants (Fig. 5L,N). Nevertheless, ectopic Otx2⁺ cells that did not coexpress Isl1 were intermingled with OM neurons in the mutant VM (Fig. 5N). Some ectopic Otx2⁺ cells coexpressed Nkx6-2 (see Fig. S9 in the supplementary material), indicating that Nkx6-2 was not sufficient to repress *Otx2* in these cells. Although the fate of the ectopic Otx2⁺ cells was difficult to assess at later stages due to Otx2 expression in other midbrain neurons, ectopic *Dbx1*⁺ cells were found adjacent to mDA neurons in the *Nkx6-1*$^{-/-}$ VM at E18.5 (data not shown).

The ectopic expression of Dbx1 suggested a partial dorsalization of the *Nkx6-1*$^{-/-}$ VM. To support this conclusion, we analyzed the expression of *Pax7* in *Nkx6-1*$^{-/-}$ embryos. At E12.5, *Pax7* was ectopically activated in the VZ/SVZ of the mutant BP corresponding to the ventral *Nkx6-1*⁺ progenitor domain of WT embryos, but *Pax7* expression was also strongly upregulated within and around the Isl1⁺ OM (Fig. 5O-R). The transcription of *Shh*, *Foxa2*, *Wnt1*, *Wnt5a*, *Lmx1a*, *Lmx1b* and *Emx2*, however, was unchanged in the mutant VM (see Fig. S5 in the supplementary material; data not shown). Next, we studied whether the glutamatergic phenotype of Pou4f1⁺ cells and the GABAergic identity of Helt-derived neurons (Nakatani et al., 2007) were abnormal in *Nkx6-1*$^{-/-}$ embryos. Expression of the vesicular glutamate transporter 2 (*Vglut2*, also known as *Slc17a6*), as well as that of glutamic acid decarboxylase 1 (*Gad1*) and *Helt*, were apparently not affected (see Fig. S6 in the supplementary material), and mDA neurons developed normally until E18.5 in the mutants (not shown). Thus, the neurotransmitter and general identity of the ectopic *Dbx1*⁺ and *Otx2*⁺ cells in the mutant VM remains to be established.

We concluded that in the absence of Nkx6-1, midbrain BP progenitors acquired a partial dorsal (*Pax7*⁺) identity and probably generated ectopic Dbx1⁺ and Otx2⁺ neurons. In this context, Nkx6-1 might be cell autonomously required to repress *Pax7* in progenitors and/or *Dbx1* and *Otx2* in postmitotic neurons.

Nkx6-1 controls the proper migration and axon outgrowth of OM and TN neurons

Some of the reported alterations in the *Nkx6-1*$^{-/-}$ VM might also affect the Isl1⁺ OM and TN neurons, as Nkx6-1 is expressed in a significant proportion of these neurons (Fig. 1) (Müller et al., 2003), and the OM was not distinguishable on Nissl-stained sections at

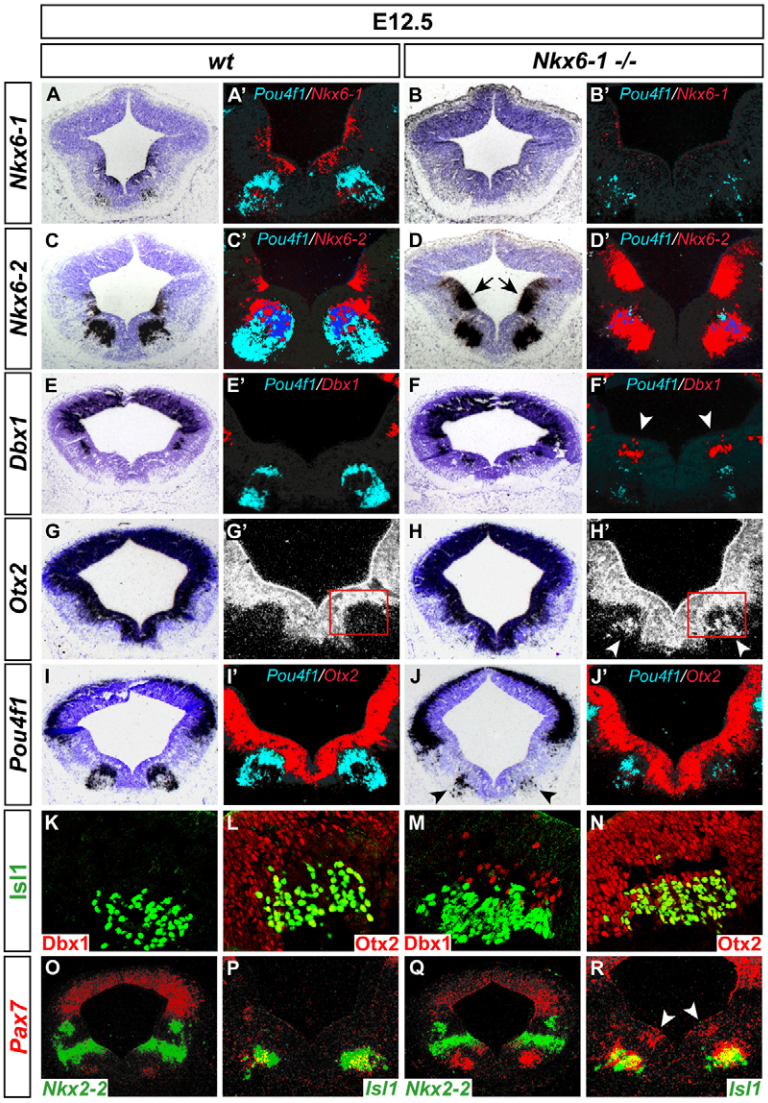

Fig. 5. Midbrain BP progenitors acquire a partial dorsal fate in the absence of *Nkx6-1*. (**A-J′**,**O-R**) Representative coronal sections from the caudal midbrain of E12.5 WT (A,A′,C,C′,E,E′,G,G′,I,I′,O,P) and *Nkx6-1*$^{-/-}$ (B,B′,D,D′,F,F′,H,H′,J,J′,Q,R) embryos. (A-B′) *Nkx6-1* is not expressed in *Nkx6-1*$^{-/-}$ mutants. (C-D′) *Nkx6-2* expression is upregulated (arrows in D) in the *Nkx6-1*$^{-/-}$ lateral midbrain. There is ectopic induction of *Dbx1* (E-F′; white arrowheads in F′) and of *Otx2* (G-H′; white arrowheads in H′) in the BP of the mutant midbrain. (I-J′) Ventral *Pou4f1* expression is strongly reduced (black arrowheads in J) at this level of the mutant midbrain. (**K-N**) Close-up views of the OM (red boxed area in G′,H′) in caudal midbrain sections of E12.5 WT (K,L) and *Nkx6-1*$^{-/-}$ (M,N) embryos. The ectopic Dbx1^{+} cells do not coincide with Isl1^{+} OM neurons (K,M), but ectopic Otx2^{+} cells intermingle with these neurons (L,N). (O-R) *Pax7* is induced ectopically (white arrowheads in R) in the VZ/SVZ and upregulated in the MZ of the mutant BP. (A′-J′,O-R) Pseudo-colored overlays of consecutive sections hybridized with probes for *Pou4f1* (light blue) and *Nkx6-1* (A′,B′), *Nkx6-2* (C′,D′), *Dbx1* (E′,F′) or *Otx2* (I′,J′) (red); with overlapping expression domains in dark blue, and for *Pax7* (red; O-R) and *Nkx2-2* (O,Q) or *Isl1* (P,R) (green); with overlapping expression domains in yellow. G′ and H′ are enlarged darkfield images of brightfield images in G and H.

E18.5 (Fig. 4). The *Phox2a*$^{+}$ OM/TN progenitor domain, however, was established correctly in *Nkx6-1*$^{-/-}$ embryos at E9.5 (see Fig. S4 in the supplementary material), and *Phox2a* expression persisted in postmitotic OM/TN neurons until E12.5 (see Fig. S7 in the supplementary material). These neurons also expressed Isl1, but the appearance of the Isl1^{+} OM/TN was less compact in *Nkx6-1*$^{-/-}$ embryos, and several Isl1^{+} cells were misplaced and dispersed throughout the domain that would normally be occupied by the Pou4f1^{+} RN neurons (Fig. 6A-P; see Fig. S7 in the supplementary material). Furthermore, expression of the vesicular acetylcholine transporter *Vacht* (also known as *Slc18a3*), a marker for these cholinergic MNs, was reduced markedly throughout development (Fig. 6Q-X; see Fig. S7 in the supplementary material). Therefore, these findings indicate that Nkx6-1 is also required for the proper migration/positioning of the Isl1^{+} OM/TN neurons in the VM/rostral hindbrain, and prompted us to examine other aspects of OM/TN development in *Nkx6-1*$^{-/-}$ embryos.

Neurofilament staining at E12.5 revealed that the nIII (oculomotor nerve) failed to grow out properly and stalled after a distance from its central nervous system (CNS) exit point, and that the nIV (trochlear nerve) was entirely missing in the mutants (Fig. 7A-D). Before stalling, the mutant nIII displayed a characteristic rostrodorsal deflection instead of its normal ventral turning towards the eye, growing back in the direction of the neural tube (Fig. 7C). Defasciculation of the nIII was evident before most of its axons stopped to grow, although single axons were seen to grow beyond the stalling point into different directions (Fig. 7C). In one out of five *Nkx6-1*$^{-/-}$ embryos analyzed at E12.5, the left nIII displayed an initially normal trajectory but became progressively thinner and the remaining axons stalled at a distance (Fig. 7D; data not shown). In the same mutant embryo, a few axons exited the ventral neural tube close to the mid-/hind-brain boundary (MHB) and projected in a similar manner as the nIV before stalling on their trajectory towards the eye (data not shown), and some axons projected dorsally within the neural tube at a position that might correspond to the nIV (Fig. 7D). No difference in the aberrant projection of the nIII was observed between the left and right side in all other mutant embryos, and a dorsal decussation of the nIV was never detected (Fig. 7D).

We expected the OM/TN neuron migration and axon outgrowth defects to be a cell-autonomous consequence of the *Nkx6-1* LOF. Analysis of the expression patterns of different receptors and ligands involved in axon guidance, cell migration and/or cell adhesion revealed three striking changes in the *Nkx6-1*$^{-/-}$ VM. First, expression of the netrin 1 (Ntn1) receptor *Unc5c*, which is normally detected in a VM/rostral hindbrain domain corresponding to the position of the *Isl1*$^{+}$ OM/TN, was strongly increased in the mutants (Fig. 7E-H), and

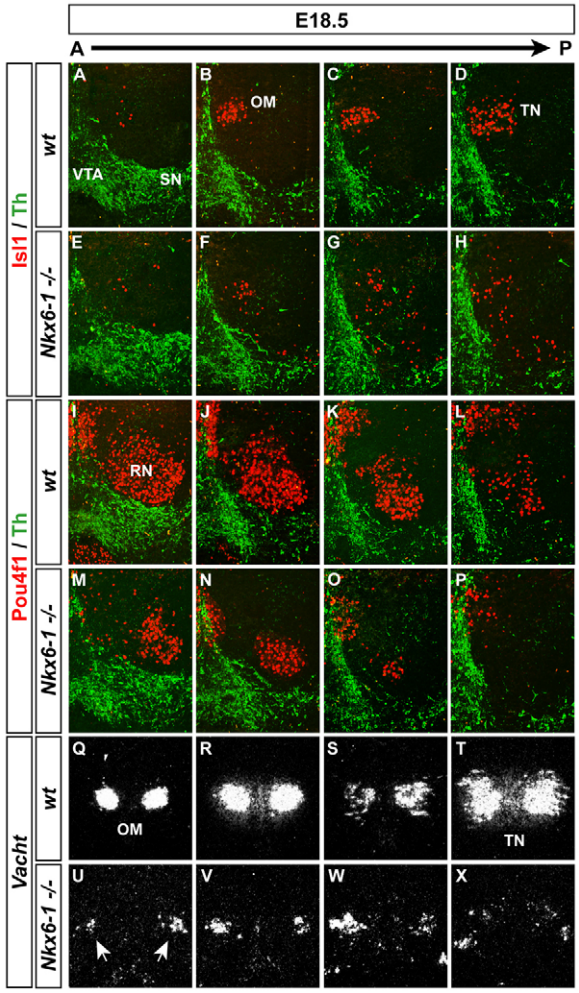

Fig. 6. Nkx6-1 controls the proper migration of Isl1⁺ OM/TN neurons. (**A-X**) Representative coronal sections at consecutive anterior-posterior (A→P, left to right) levels of the midbrain/rostral hindbrain of E18.5 WT (A-D,I-L,Q-T) and *Nkx6-1*$^{-/-}$ (E-H,M-P,U-X) brains. (A-P) The OM and TN cannot be distinguished in the E18.5 *Nkx6-1*$^{-/-}$ brain and the corresponding Isl1⁺ cells are dispersed throughout the strongly reduced Pou4f1⁺ RN domain but they do not intermingle with Th⁺ mDA neurons. (Q-X) Expression of *Vacht* in OM (arrows in U) and TN neurons is strongly reduced and scattered in the mutants. OM, oculomotor nucleus; RN, red nucleus; SN, Substantia nigra; TN, trochlear nucleus; VTA, Ventral tegmental area.

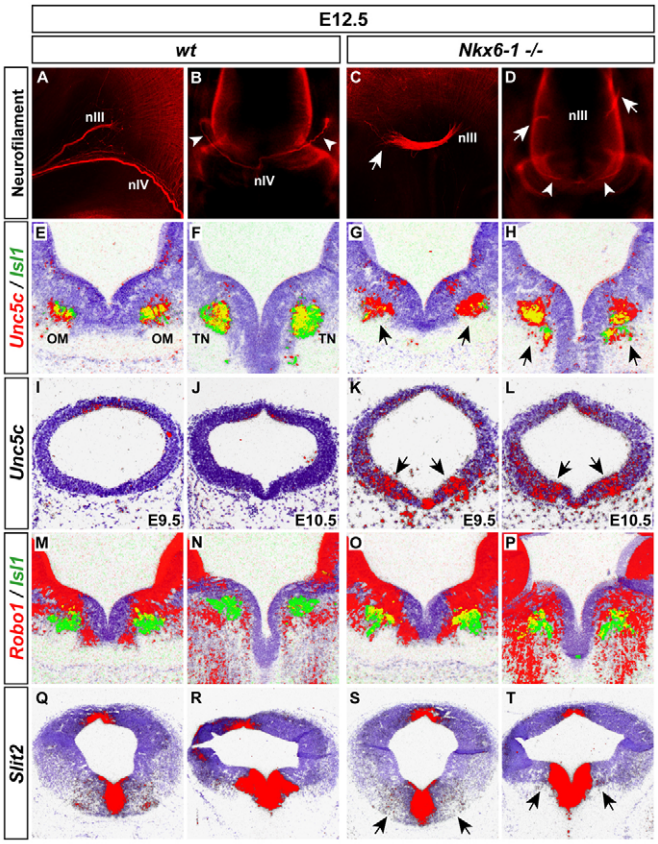

Fig. 7. Nkx6-1 controls axon outgrowth of OM/TN neurons. (**A-D**) Neurofilament staining of E12.5 WT (A,B) and *Nkx6-1*$^{-/-}$ (C,D) embryo heads (A,C: anterior left, dorsal top; B,D: dorsal views) shows a characteristic rostrodorsal deflection and truncation (white arrow in C) of the nIII and absence of the nIV in *Nkx6-1*$^{-/-}$ embryos. (D) Dorsal view of one of five mutants in which the left nIII (white arrow on the left) followed an initially normal trajectory before stalling at a distance, whereas the right nIII (white arrow on the right) showed a rostral deflection. White arrowheads in B point at the nIV projecting out of the CNS after its dorsal decussation and in D point at the few nIV axons projecting dorsally but failing to exit the CNS. (**E-T**) Representative coronal midbrain (E,G,I-L,M,O,Q,S) and rostral hindbrain (F,H,N,P,R,T) sections of WT (E,F,I,J,M,N,Q,R) and *Nkx6-1*$^{-/-}$ (G,H,K,L,O,P,S,T) embryos at E9.5 (I,K), E10.5 (J,L) and E12.5 (E-H,M-T). Images were pseudo-colored for better visualization of ISH signals, with overlapping domains in yellow. (E-H) *Unc5c* expression in E12.5 *Nkx6-1*$^{-/-}$ mutants is upregulated in a region overlapping with *Isl1*⁺ OM/TN neurons (black arrows in G,H). (I-L) Premature and ectopic (black arrows in K,L) transcription of *Unc5c* in the E9.5 and E10.5 mutant VM. (M-P) *Robo1* expression overlaps only partly with *Isl1*⁺ OM/TN neurons but is upregulated in the *Nkx6-1*$^{-/-}$ VM/rostral hindbrain. (Q-T) Partial loss (black arrows in S,T) of *Slit2* expression in E12.5 mutants. nIII, third cranial nerve; nIV, fourth cranial nerve; OM, oculomotor nucleus; TN, trochlear nucleus.

its transcription was also prematurely and ectopically initiated at E9.5 (Fig. 7I-L). Second, expression of the roundabout receptor *Robo1*, which normally overlaps only partly with the *Isl1*⁺ OM/TN domain, was upregulated in these regions in *Nkx6-1*$^{-/-}$ embryos (Fig. 7M-P). Third, transcription of the secreted molecule *Slit2*, a ligand of Robo receptors, was reduced or even abolished in the MZ of the midbrain/rostral hindbrain BP, but remained unaffected in the FP of the mutant embryos (Fig. 7Q-T). Expression of the Ntn1 receptor deleted in colorectal carcinoma (*Dcc*), the Plexin A semaphorin receptor family (*Plxna1-4*), their coreceptor neuropilin2 (*Nrp2*), and the repulsive guidance molecule receptor neogenin (*Neo1*), by contrast, did not appear abnormal in the *Nkx6-1*$^{-/-}$ VM/rostral hindbrain (not shown). We concluded that in *Nkx6-1*$^{-/-}$ mutants, the aberrant migration and axon pathfinding defects of the OM/TN neurons are a consequence of the misexpression of at least three cell migration/axon guidance molecules in the OM/TN neurons themselves, or in their close vicinity.

Nkx6-2 is not required for RN and OM/TN neuron development

Finally, we reasoned that Nkx6-2 might also contribute to the patterning of the VM and to the proper development of RN neurons, as ~63% of the postmitotic Nkx6-1⁺ cells and ~15% of the Pou4f1⁺

RN neurons coexpressed Nkx6-2 (Fig. 1). Thus, we analyzed the midbrain phenotype of single *Nkx6-2* (*Nkx6-2*$^{tlz/tlz}$, hereafter termed *Nkx6-2*$^{-/-}$) and double *Nkx6* (*Nkx6-1*$^{-/-}$; *Nkx6-2*$^{-/-}$) null mutants (Vallstedt et al., 2001). The generation of Pou4f1$^+$ RN and Isl1$^+$ OM/TN neurons was not affected in *Nkx6-2*$^{-/-}$ embryos (see Fig. S8 in the supplementary material). Moreover, the RN and OM/TN phenotype of *Nkx6-1*$^{-/-}$; *Nkx6-2*$^{-/-}$ double mutants did not differ from that of *Nkx6-1*$^{-/-}$ single mutants (see Fig. S8 in the supplementary material). In line with this finding, Otx2 and *Dbx1* were induced ectopically in the VM of *Nkx6-1*$^{-/-}$; *Nkx6-2*$^{-/-}$ double, but not of *Nkx6-2*$^{-/-}$ single, mutants (see Fig. S9 in the supplementary material). Notably, the dorsal *Dbx1*$^+$ domain expanded ventrally in both *Nkx6-2*$^{-/-}$ single and *Nkx6-1*$^{-/-}$; *Nkx6-2*$^{-/-}$ double mutants (see Fig. S9 in the supplementary material), indicating that Nkx6-2 is required for the repression of *Dbx1*. These data demonstrated that in the midbrain, Nkx6-2 is dispensable for the normal development of Pou4f1$^+$ RN and Isl1$^+$ OM/TN neurons but is necessary for the dorsal antagonism of *Dbx1*.

DISCUSSION

We identified the HD TF Nkx6-1 as a key factor controlling the development of the most rostral MNs and related neurons in the mouse midbrain/rostral hindbrain. Nkx6-1 is required for the generation of proper numbers of Pou4f1$^+$ RN neurons, for the correct migration of Isl1$^+$ OM/TN neurons in the VM/rostral hindbrain and for the proper outgrowth of nIII and nIV towards their extraocular target muscles. Nkx6-1 thus exhibits functional similarity in the VM/rostral hindbrain and in the caudal hindbrain/spinal cord. Therefore, these data reinforce the notion that the ontogenetic profile of the OM/TN neurons has more similarities to special (branchio-) vMNs than to the sMNs located in the caudal hindbrain/spinal cord. We also identified the HD TF Otx2 as necessary and sufficient for the maintenance of Nkx6-1 expression and Pou4f1$^+$ RN neurons in the VM, and for their ectopic induction in the rostral hindbrain.

Otx2 confers positional information for the generation of RN neurons

Although earlier reports showed that Shh is both necessary and sufficient for the generation of Pou4f1$^+$ and Isl1/Phox2a$^+$ neurons in the ventral and dorsal midbrain, respectively (Agarwala and Ragsdale, 2002; Bayly et al., 2007; Blaess et al., 2006; Fedtsova and Turner, 2001; Fogel et al., 2008; Watanabe and Nakamura, 2000), our study indicates that Shh alone is not sufficient to maintain Nkx6-1 expression and to instruct VM progenitors with the Pou4f1$^+$ RN neuron fate. Otx2 is additionally required for the maintenance of the VM Nkx6-1 expression domain and subsequent generation of postmitotic Pou4f1$^+$ neurons. In the hindbrain, A/P positional information is conferred by the Hox factors for the generation of the different cranial motor nuclei (Cordes, 2001; Gaufo et al., 2003; Jungbluth et al., 1999). In the *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ mutant midbrain, only the most ventral progenitors of Th/Pitx3$^+$ mDA and Pou4f1$^+$ RN neurons adopted a rostral hindbrain identity, whereas the overall VM identity remained unchanged as judged by the fact that this territory in the mutants is rostral to the Fgf8$^+$ domain at the MHB, expresses *Otx1* and exhibits normal development of OM/TN neurons (Puelles et al., 2004). Removal of the ectopic Nkx2-2 in the compound *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$; *Nkx2-2*$^{-/-}$ mutants restored the VM identity of mDA progenitors (Prakash et al., 2006), but notably this rescue did not include the VM Nkx6-1$^+$ progenitor domain and Pou4f1$^+$ RN neurons. Apart from imparting midbrain identity, Otx2 is thus required cell autonomously for the maintenance of Nkx6-1 expression either directly (direct activation) or indirectly (inactivation of another repressor) in BP progenitors, and subsequently for the generation of Pou4f1$^+$ RN neurons. Consistent with this finding, the ectopic activation of *Otx2* in the Shh$^+$ rostral hindbrain FP of *En1*$^{+/Wnt1}$ mice was sufficient to induce Nkx6-1 expression and the ectopic generation of Pou4f1$^+$ neurons. Shh and Otx2 might thus act synergistically for the induction and maintenance of the ventral Nkx6-1 expression domain in the developing mouse midbrain (Fig. 8). However, the fact that *Shh* gain-of-function (GOF) or LOF did not affect *Otx2* transcription (Fogel et al., 2008; Watanabe and Nakamura, 2000), but the expression of Shh was altered in *Otx2* GOF or LOF mutants

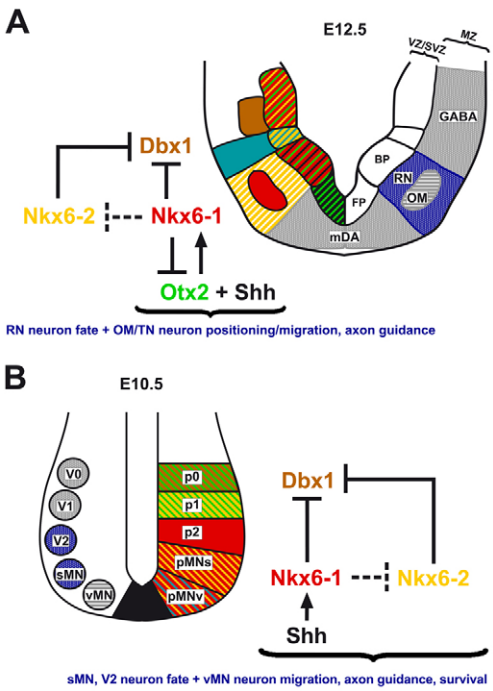

Fig. 8. A conserved function of Nkx6-1 in RN/OM and MN development in the mouse. (**A**) Schematic coronal section of the ventrolateral midbrain at E12.5. The left half of the schematic illustrates the different expression and progenitor domains at this stage, giving rise to the neuronal populations depicted on the right. The genetic interactions identified in this report, and controlling the generation of RN neurons and the migration and axon guidance of OM/TN neurons, are shown to the left of the scheme. See text for details. Otx2, green; Shh, black; Nkx6-1, red; Nkx6-2, yellow; Dbx1, brown; Nkx2-2, turquoise; Pou4f1$^+$ RN neurons, blue; and Isl1$^+$ OM neurons, gray stripes. (**B**) Schematic coronal section of the ventral caudal hindbrain at E10.5. The left half of the schematic depicts the neuronal populations arising from the different progenitor domains shown on the right. For details see Pattyn et al., Sander et al. and Vallstedt et al. (Pattyn et al., 2003; Sander et al., 2000a; Vallstedt et al., 2001). Nkx6-1, red; Nkx6-2, yellow; Nkx2-2, turquoise; Dbx1, brown; Dbx2, green; Isl1$^+$/Hb9$^+$ sMNs and Chx10$^+$ V2 INs, blue; and Isl1$^+$/Phox2b$^+$ vMNs, gray stripes. Black bars indicate repression, black arrows indicate activation, broken lines indicate indirect (non-cell-autonomous) repression (midbrain) or repression is not demonstrated (hindbrain). BP, basal plate; FP, floor plate; GABA, γ-aminobutyric acid-synthesizing neurons; mDA, midbrain dopaminergic neurons; MNs, motoneurons; MZ, mantle zone; OM, oculomotor nucleus; p, progenitor domain; RN, red nucleus; s, somatic; SVZ, subventricular zone; V0-2, interneurons; v, visceral; VZ, ventricular zone.

(Prakash et al., 2006; Puelles et al., 2003; Puelles et al., 2004), suggests that *Otx2* might even be upstream of *Shh* in this genetic cascade as a factor controlling the positioning of Shh expression.

Nkx6-1 directs BP progenitors to ventral neuronal fates in the midbrain

Nkx6-1 expression is confined mostly to a domain within the midbrain BP from where Pou4f1$^+$ RN and Isl1$^+$ OM neurons arise during development. The loss of ~73% Pou4f1$^+$ RN neurons and the developmental deficits of the Isl1$^+$ OM neurons (reduced *Vacht* expression, faulty migration and defective nIII outgrowth) in *Nkx6-1*$^{-/-}$ embryos indicate that Nkx6-1 is indeed required in BP progenitors for the proper specification of these two VM neuronal populations. The function of Nkx6-1 in these progenitors can be either permissive (by repressing alternative neural fates) or instructive (by initiating the corresponding developmental program). Our findings show that *Pax7*, a marker of dorsal progenitors, is ectopically induced in BP progenitors of the *Nkx6-1*$^{-/-}$ midbrain. This is indicative of a partial dorsalization, as several other ventral genes were still expressed normally in these progenitors, and suggests a permissive function of Nkx6-1. The ectopic expression of Dbx1, Otx2 and *Pax7* in postmitotic cells of the *Nkx6-1*$^{-/-}$ VM suggests that these cells also acquired dorsal features. In this context, the ectopic Dbx1$^+$ and some of the ectopic Otx2$^+$ cells represented a separate subpopulation that did not overlap with Isl1$^+$ OM neurons, suggesting that at least part of the RN progeny adopted an alternative (dorsal) fate. The activation of Otx2 and *Pax7* in Isl1$^+$ OM neurons and/or their precursors might also have contributed to their developmental defects. Although an instructive role of Nkx6-1 in the generation of RN and OM/TN neurons remains to be established, the loss of only ~73% Pou4f1$^+$ RN neurons suggests that Nkx6-1 should not be the only fate determinant for these neurons. In this regard, a participation of Nkx6-2 in the specification of Pou4f1$^+$ RN and Isl1$^+$ OM/TN neuron fates can be ruled out: Nkx6-2 is not expressed in the ventral Nkx6-1$^+$ progenitor domain; it is not activated in this domain in *Nkx6-1*$^{-/-}$ embryos; and a loss of Pou4f1$^+$ RN or Isl1$^+$ OM/TN neurons was not detected in *Nkx6-2*$^{-/-}$ mutants. Although the Forkhead homeobox genes *Foxa1* and *Foxa2* were reported to control RN neuron development (Ferri et al., 2007), and *Foxa2* expression was normal in the *Nkx6-1*$^{-/-}$ VM, a cell-autonomous and direct requirement of Foxa1 and Foxa2 for RN generation still remains to be established, as Nkx6-1 expression was also reduced in the *Foxa1 Foxa2* double mutant midbrain (Ferri et al., 2007). However, high temporal resolution fate-mapping of Nkx6-1$^+$ progenitors would certainly provide more conclusive data on the Nkx6-1 contribution to RN and OM/TN neuron development.

Postmitotic control of OM/TN neuron migration and axon guidance by Nkx6-1

Apart from the BP progenitors, Nkx6-1 is expressed in a significant proportion of Isl1$^+$ OM/TN neurons, the migration and axon outgrowth of which was strongly affected in *Nkx6-1*$^{-/-}$ embryos, suggesting that Nkx6-1 also plays a role in the terminal differentiation of these neurons. Nkx6-1 is a transcriptional repressor (Mirmira et al., 2000; Muhr et al., 2001), and most of our data also point towards such a function of Nkx6-1 in the VM. Indeed, ectopic expression of *Dbx1* and *Otx2* in *Nkx6-1*$^{-/-}$ postmitotic neurons is consistent with a repression of these genes by Nkx6-1 (Fig. 8). Supporting this possibility is the fact that Nkx6-1 binding sites (BSs) in the *Dbx1* and *Otx2* promoters are particularly conserved among mammalian species (see Table S1 in the supplementary material). A further abnormality was the activation or derepression of two genes involved in migration and axon guidance, the Ntn1 receptor *Unc5c* and the Slit receptor *Robo1*, in the mutant OM/TN neurons. It is noteworthy that Unc5c mediates nIV axon guidance and overexpression of Unc5 may cause a short- and long-range repulsion of axons (Burgess et al., 2006; Keleman and Dickson, 2001), but Slit1/2 may also have a repulsive effect on TN axons (Hammond et al., 2005).

A conserved function of Nkx6-1 in RN/OM and MN development

Taken together, our data indicate that several aspects of Nkx6-1 function in hindbrain/spinal cord sMN and vMN development have been conserved in the midbrain/rostral hindbrain, despite obvious spatiotemporal, molecular and anatomical differences (Fig. 8). Findings from this and previous studies indicate that Nkx6-1 is required for the generation of most Pou4f1$^+$ RN neurons and spinal sMNs (Sander et al., 2000a; Vallstedt et al., 2001), as well as for the correct migration and axon outgrowth of Isl1$^+$ OM/TN and hindbrain (branchio-) vMNs (Müller et al., 2003; Pattyn et al., 2003). Moreover, Nkx6-1 represses *Dbx1* in the midbrain BP and in the hindbrain/spinal cord V2, sMN and vMN/V3 domains (Pattyn et al., 2003; Vallstedt et al., 2001), and Nkx6-1 represses *Nkx6-2* in the lateral midbrain and in the pMN/p2 domains of the spinal cord (Vallstedt et al., 2001). Based on these conserved features, we suggest that the developmental profile of the OM/TN neurons bears significantly more resemblance to special (branchio-) vMNs, as compared with sMNs located in the caudal hindbrain/spinal cord. In support of this, it has previously been reported that OM/TN neurons share additional features with vMNs rather than sMNs, such as the requirement of the presence of a *Phox2* gene for their generation (Coppola et al., 2005; Pattyn et al., 2000; Pattyn et al., 1997), the absence of *Hb9* expression (Thaler et al., 1999), and the dorsal CNS exit point and repulsion by Slit1 and Slit2 in the case of the nIV and vMN axons (Hammond et al., 2005).

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Supplementary material

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References

Acampora, D., Annino, A., Tuorto, F., Puelles, E., Lucchesi, W., Papalia, A. and Simeone, A. (2005). Otx genes in the evolution of the vertebrate brain. *Brain Res. Bull.* **66**, 410-420.

Agarwala, S. and Ragsdale, C. W. (2002). A role for midbrain arcs in nucleogenesis. *Development* **129**, 5779-5788.

Bayly, R. D., Ngo, M., Aglyamova, G. V. and Agarwala, S. (2007). Regulation of ventral midbrain patterning by Hedgehog signaling. *Development* **134**, 2115-2124.

Blaess, S., Corrales, J. D. and Joyner, A. L. (2006). Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. *Development* **133**, 1799-1809.

Bonilla, S., Hall, A. C., Pinto, L., Attardo, A., Gotz, M., Huttner, W. B. and Arenas, E. (2008). Identification of midbrain floor plate radial glia-like cells as dopaminergic progenitors. *Glia* **56**, 809-820.
Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L. and Ericson, J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-627.
Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-445.
Broihier, H. T., Kuzin, A., Zhu, Y., Odenwald, W. and Skeath, J. B. (2004). Drosophila homeodomain protein Nkx6 coordinates motoneuron subtype identity and axonogenesis. *Development* **131**, 5233-5242.
Burgess, R. W., Jucius, T. J. and Ackerman, S. L. (2006). Motor axon guidance of the mammalian trochlear and phrenic nerves: dependence on the netrin receptor Unc5c and modifier loci. *J. Neurosci.* **26**, 5756-5766.
Cai, J., Qi, Y., Wu, R., Modderman, G., Fu, H., Liu, R. and Qiu, M. (2001). Mice lacking the Nkx6.2 (Gtx) homeodomain transcription factor develop and reproduce normally. *Mol. Cell. Biol.* **21**, 4399-4403.
Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M. and Werner, T. (2005). MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* **21**, 2933-2942.
Cheesman, S. E., Layden, M. J., Von Ohlen, T., Doe, C. Q. and Eisen, J. S. (2004). Zebrafish and fly Nkx6 proteins have similar CNS expression patterns and regulate motoneuron formation. *Development* **131**, 5221-5232.
Coppola, E., Pattyn, A., Guthrie, S. C., Goridis, C. and Studer, M. (2005). Reciprocal gene replacements reveal unique functions for Phox2 genes during neural differentiation. *EMBO J.* **24**, 4392-4403.
Cordes, S. P. (2001). Molecular genetics of cranial nerve development in mouse. *Nat. Rev. Neurosci.* **2**, 611-623.
De Marco Garcia, N. V. and Jessell, T. M. (2008). Early motor neuron pool identity and muscle nerve trajectory defined by postmitotic restrictions in Nkx6.1 activity. *Neuron* **57**, 217-231.
Dessaud, E., McMahon, A. P. and Briscoe, J. (2008). Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. *Development* **135**, 2489-2503.
Eisen, J. S. (1999). Patterning motoneurons in the vertebrate nervous system. *Trends Neurosci.* **22**, 321-326.
Ericson, J., Thor, S., Edlund, T., Jessell, T. M. and Yamada, T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. *Science* **256**, 1555-1560.
Evinger, C. (1988). Extraocular motor nuclei: location, morphology and afferents. In *Neuroanatomy of the Oculomotor System* (ed. J. A. Buttner-Ennever), pp. 81-117. Amsterdam: Elsevier.
Fedtsova, N. G. and Turner, E. E. (1995). Brn-3.0 expression identifies early post-mitotic CNS neurons and sensory neural precursors. *Mech. Dev.* **53**, 291-304.
Fedtsova, N. and Turner, E. E. (2001). Signals from the ventral midline and isthmus regulate the development of Brn3.0-expressing neurons in the midbrain. *Mech. Dev.* **105**, 129-144.
Ferri, A. L. M., Lin, W., Mavromatakis, Y. E., Wang, J. C., Sasaki, H., Whitsett, J. A. and Ang, S.-L. (2007). Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner. *Development* **134**, 2761-2769.
Fischer, T., Guimera, J., Wurst, W. and Prakash, N. (2007). Distinct but redundant expression of the Frizzled Wnt receptor genes at signaling centers of the developing mouse brain. *Neuroscience* **147**, 693-711.
Fogel, J. L., Chiang, C., Huang, X. and Agarwala, S. (2008). Ventral specification and perturbed boundary formation in the mouse midbrain in the absence of Hedgehog signaling. *Dev. Dyn.* **237**, 1359-1372.
Gaufo, G. O., Thomas, K. R. and Capecchi, M. R. (2003). Hox3 genes coordinate mechanisms of genetic suppression and activation in the generation of branchial and somatic motoneurons. *Development* **130**, 5191-5201.
Hammond, R., Vivancos, V., Naeem, A., Chilton, J., Mambitisaeva, E., Andrews, W., Sundaresan, V. and Guthrie, S. (2005). Slit-mediated repulsion is a key regulator of motor axon pathfinding in the hindbrain. *Development* **132**, 4483-4495.
His, W. (1904). Die Entwickelung des menschlichen Gehirns während der ersten Monate: Untersuchungsergebnisse. Leipzig: Hirzel.
Huber, A. B., Kania, A., Tran, T. S., Gu, C., De Marco Garcia, N., Lieberam, I., Johnson, D., Jessell, T. M., Ginty, D. D. and Kolodkin, A. L. (2005). Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. *Neuron* **48**, 949-964.
Hutchinson, S. A., Cheesman, S. E., Hale, L. A., Boone, J. Q. and Eisen, J. S. (2007). Nkx6 proteins specify one zebrafish primary motoneuron subtype by regulating late islet1 expression. *Development* **134**, 1671-1677.
Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.
Jungbluth, S., Bell, E. and Lumsden, A. (1999). Specification of distinct motor neuron identities by the singular activities of individual Hox genes. *Development* **126**, 2751-2758.
Jurata, L. W., Thomas, J. B. and Pfaff, S. L. (2000). Transcriptional mechanisms in the development of motor control. *Curr. Opin. Neurobiol.* **10**, 72-79.
Keifer, J. and Houk, J. C. (1994). Motor function of the cerebellorubrospinal system. *Physiol. Rev.* **74**, 509-542.
Keleman, K. and Dickson, B. J. (2001). Short- and long-range repulsion by the Drosophila Unc5 netrin receptor. *Neuron* **32**, 605-617.
Kennedy, P. R. (1990). Corticospinal, rubrospinal and rubro-olivary projections: a unifying hypothesis. *Trends Neurosci.* **13**, 474-479.
Kimmel, R. A., Turnbull, D. H., Blanquet, V., Wurst, W., Loomis, C. A. and Joyner, A. L. (2000). Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. *Genes Dev.* **14**, 1377-1389.
Kittappa, R., Chang, W. W., Awatramani, R. B. and McKay, R. D. G. (2007). The foxa2 gene controls the birth and spontaneous degeneration of dopamine neurons in old age. *PLoS Biology* **5**, e325.
McEvilly, R. J., Erkman, L., Luo, L., Sawchenko, P. E., Ryan, A. F. and Rosenfeld, M. G. (1996). Requirement for Brn-3.0 in differentiation and survival of sensory and motor neurons. *Nature* **384**, 574-577.
Mirmira, R. G., Watada, H. and German, M. S. (2000). Beta-cell differentiation factor Nkx6.1 contains distinct DNA binding interference and transcriptional repression domains. *J. Biol. Chem.* **275**, 14743-14751.
Muhr, J., Andersson, E., Persson, M., Jessell, T. M. and Ericson, J. (2001). Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. *Cell* **104**, 861-873.
Müller, M., Jabs, N., Lorke, D. E., Fritzsch, B. and Sander, M. (2003). Nkx6.1 controls migration and axon pathfinding of cranial branchio-motoneurons. *Development* **130**, 5815-5826.
Nakatani, T., Minaki, Y., Kumai, M. and Ono, Y. (2007). Helt determines GABAergic over glutamatergic neuronal fate by repressing Ngn genes in the developing mesencephalon. *Development* **134**, 2783-2793.
Omodei, D., Acampora, D., Mancuso, P., Prakash, N., Di Giovannantonio, L. G., Wurst, W. and Simeone, A. (2008). Anterior-posterior graded response to Otx2 controls proliferation and differentiation of dopaminergic progenitors in the ventral mesencephalon. *Development* **135**, 3459-3470.
Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., Hayashi, H. et al. (2007). Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development* **134**, 3213-3225.
Panhuysen, M., Vogt Weisenhorn, D. M., Blanquet, V., Brodski, C., Heinzmann, U., Beisker, W. and Wurst, W. (2004). Effects of Wnt1 signaling on proliferation in the developing mid-/hindbrain region. *Mol. Cell. Neurosci.* **26**, 101-111.
Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J. F. (1997). Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. *Development* **124**, 4065-4075.
Pattyn, A., Hirsch, M., Goridis, C. and Brunet, J. F. (2000). Control of hindbrain motor neuron differentiation by the homeobox gene Phox2b. *Development* **127**, 1349-1358.
Pattyn, A., Vallstedt, A., Dias, J. M., Sander, M. and Ericson, J. (2003). Complementary roles for Nkx6 and Nkx2 class proteins in the establishment of motoneuron identity in the hindbrain. *Development* **130**, 4149-4159.
Pfaff, S. L., Mendelsohn, M., Stewart, C. L., Edlund, T. and Jessell, T. M. (1996). Requirement for LIM homeobox gene *Isl1* in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. *Cell* **84**, 309-320.
Prakash, N., Brodski, C., Naserke, T., Puelles, E., Gogoi, R., Hall, A., Panhuysen, M., Echevarria, D., Sussel, L., Weisenhorn, D. M. V. et al. (2006). A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. *Development* **133**, 89-98.
Puelles, E., Rubenstein, J. L. R. and Puelles, L. (2001). Chicken Nkx6.1 expression at advanced stages of development identifies distinct brain nuclei derived from the basal plate. *Mech. Dev.* **102**, 279-282.
Puelles, E., Acampora, D., Lacroix, E., Signore, M., Annino, A., Tuorto, F., Filosa, S., Corte, G., Wurst, W., Ang, S. L. et al. (2003). Otx dose-dependent integrated control of antero-posterior and dorso-ventral patterning of midbrain. *Nat. Neurosci.* **6**, 453-460.
Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S. L., Wurst, W. and Simeone, A. (2004). Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* **131**, 2037-2048.
Puelles, E., Acampora, D., Gogoi, R., Tuorto, F., Papalia, A., Guillemot, F., Ang, S.-L. and Simeone, A. (2006). Otx2 controls identity and fate of glutamatergic progenitors of the thalamus by repressing GABAergic differentiation. *J. Neurosci.* **26**, 5955-5964.

Qiu, M., Shimamura, K., Sussel, L., Chen, S. and Rubenstein, J. L. (1998). Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development. *Mech. Dev.* **72**, 77-88.

Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T. M. and Rubenstein, J. L. R. (2000a). Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. *Genes Dev.* **14**, 2134-2139.

Sander, M., Sussel, L., Conners, J., Scheel, D., Kalamaras, J., Dela Cruz, F., Schwitzgebel, V., Hayes-Jordan, A. and German, M. (2000b). Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. *Development* **127**, 5533-5540.

Sussel, L., Kalamaras, J., Hartigan-O'Connor, D. J., Meneses, J. J., Pedersen, R. A., Rubenstein, J. L. and German, M. S. (1998). Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development* **125**, 2213-2221.

Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J. and Pfaff, S. L. (1999). Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. *Neuron* **23**, 675-687.

Turner, E. E., Jenne, K. J. and Rosenfeld, M. G. (1994). Brn-3.2: A Brn-3-related transcription factor with distinctive central nervous system expression and regulation by retinoic acid. *Neuron* **12**, 205-218.

Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M. and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. *Neuron* **31**, 743-755.

Watanabe, Y. and Nakamura, H. (2000). Control of chick tectum territory along dorsoventral axis by Sonic hedgehog. *Development* **127**, 1131-1140.

Xiang, M., Gan, L., Zhou, L., Klein, W. H. and Nathans, J. (1996). Targeted deletion of the mouse POU domain gene Brn-3a causes a selective loss of neurons in the brainstem and trigeminal ganglion, uncoordinated limb movement, and impaired suckling. *Proc. Natl. Acad. Sci. USA* **93**, 11950-11955.