Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors

Alexandre Pattyn,[1,2] Anna Vallstedt,[1] José M. Dias,[1] Omar Abdel Samad,[3] Robb Krumlauf,[4] Filippo M. Rijli,[3] Jean-Francois Brunet,[2] and Johan Ericson[1,5]

[1]Department of Cell and Molecular Biology, Karolinska Institute S-171 77 Stockholm, Sweden; [2]CNRS UMR8542 Ecole Normale Supérieure, Département de Biologie 75005 Paris, France; [3]Institut de Génétique et de Biologie Moléculaire et Cellulaire CNRS/INSERM/ULP, Collège de France BP 163-67404 Illkirch Cedex, CU de Strasbourg, France; [4]Stowers Institute, Kansas City, Missouri 64110, USA

Neural progenitor cells often produce distinct types of neurons in a specific order, but the determinants that control the sequential generation of distinct neuronal subclasses in the vertebrate CNS remain poorly defined. We examined the sequential generation of visceral motor neurons and serotonergic neurons from a common pool of neural progenitors located in the ventral hindbrain. We found that the temporal specification of these neurons varies along the anterior-posterior axis of the hindbrain, and that the timing of their generation critically depends on the integrated activities of Nkx- and Hox-class homeodomain proteins. A primary function of these proteins is to coordinate the spatial and temporal activation of the homeodomain protein Phox2b, which in turn acts as a binary switch in the selection of motor neuron or serotonergic neuronal fate. These findings assign new roles for Nkx, Hox, and Phox2 proteins in the control of temporal neuronal fate determination, and link spatial and temporal patterning of CNS neuronal fates.

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Neuronal cell diversity is established by mechanisms that operate in space and over time during central nervous system (CNS) development. Insight has been obtained regarding the initial steps of spatial patterning of neurons along the dorsal-ventral (DV) and anterior-posterior (AP) axes of the neural tube (Lumsden and Krumlauf 1996; Jessell 2000). Local inductive signals determine the spatial pattern of expression of transcription factors along both these axes, so that neural progenitors at different positions acquire distinct molecular identities. In the ventral neural tube, neuronal fate along the DV axis depends on the Shh-mediated patterning of Nkx-, Dbx-, Pax-, and Irx-class homeodomain (HD) proteins (Briscoe et al. 2000). Along the AP axis, the overlapping, or nested, expression pattern of Hox HD proteins provides positional values that influence the fate of neurons (Lumsden and Krumlauf 1996). Despite significant advances, however, DV and AP patterning have generally been analyzed independently, leaving open the issue as to what degree these orthogonal patterning mechanisms are integrated (Davenne et al. 1999; Gaufo et al. 2000). Compared to spatial patterning, little is known about the mechanisms that underlie how neural progenitors produce distinct types of neurons in a specific temporal order. Studies of the retina (Livesey and Cepko 2001) and developing neo-cortex (Monuki and Walsh 2001) suggest that the sequential production of different neuronal subtypes reflects temporal changes in neural progenitors, either in response to extrinsic cues or mechanisms intrinsic to neural progenitor cells. Recent data indicate that modulation of Notch signaling by the bHLH protein Mash1 and the HD proteins Dlx1/2 may control the sequential specification of progenitors in subcortical areas of the telencephalon (Yun et al. 2002). Apart from this, few molecular determinants that influence these temporal processes in the vertebrate CNS have been identified to date.

[5]Corresponding author.
E-MAIL Johan.Ericson@cmb.ki.se; FAX 46-8-30-83-74.
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Results

To address how spatial and temporal aspects of cell patterning are integrated during development, we examined the sequential generation of visceral motor neurons

(vMNs) and serotonergic (S) neurons from Nkx2.2$^+$ progenitors (Briscoe et al. 1999) in the ventral hindbrain. S neurons are initially detected as two distinct cell groups, one rostral and one caudal (Lidov and Molliver 1982; Aitken and Tork 1988), indicating that the generation of these neurons is interrupted along the AP axis of the hindbrain. We localized the gap between these two groups of S neurons to rhombomere (r) 4, by mapping the exclusion of *pet1* expression, an early marker for S neurons (Hendricks et al. 1999), to the r4-specific expression of *Hoxb1* (Fig. 1a,b; Studer et al. 1996). S neurons were excluded from r4, whereas they could be detected in a position ventral to vMNs at all other levels of the hindbrain at embryonic day 11.5 (E11.5; Fig. 1c,d). BrdU birthdating analyses revealed that in r4, vMNs are produced at a high rate between E9.5 and E11.5, whereas at other axial levels most vMNs have been generated prior to E10.5 (Fig. 1e; see also Fig. 2a). These data reveal that the exclusion of S neurons from r4 is accompanied by a prolonged generation of vMNs (Fig. 1v).

The generation of vMNs precedes that of S neurons (Taber-Pierce 1973; Briscoe et al. 1999), and we next examined the precise spatial and temporal generation of these neuronal subtypes in relation to Nkx2.2$^+$ progenitors. Phox2b, an HD protein required for the generation of hindbrain vMNs (Pattyn et al. 2000), is expressed in Nkx2.2$^+$ vMN progenitors and in postmitotic vMNs that also express Isl1 (Ericson et al. 1997). At early stages (E9–E9.5), numerous vMNs but no S neurons are produced (Fig. 1f), and Phox2b expression was detected in most Nkx2.2$^+$ progenitors, independent of axial level (Fig. 1p). At this stage, essentially all Nkx2.2$^+$ progenitors expressed the HD proteins Nkx6.1 (Sander et al. 2000), Nkx6.2 (Vallstedt et al. 2001), and Nkx2.9 (Fig. 1g,j,m; Briscoe et al. 1999). These data show that Nkx2.2$^+$ progenitors initially represent a largely uniform progenitor population, and that all or most cells are devoted to produce vMNs. Subsequently, the expression of Phox2b and Nkx2.9 became, within the Nkx2.2$^+$ domain, dorsally restricted at all axial levels except r4 (see below). At E10.5, only the dorsal half of the Nkx2.2$^+$ domain expressed Phox2b and Nkx2.9 (Fig. 1h,n,q), and this dorsal restriction correlated with a cessation of vMN production and the initiation of S-neuron generation (Fig. 1f). This observation suggests that only the dorsal Nkx2.2$^+$/Nkx2.9$^+$/Phox2b$^+$ subpopulation continues to produce motor neurons at E10.5 and raises the possibility that S neurons, in turn, derive from ventral Nkx2.2$^+$ progenitors that have ceased to express Phox2b and Nkx2.9. In support for this, the initial expression of *pet1* at E10.75 was detected in a position immediately ventral to Phox2b$^+$/Isl1$^+$ motor neurons and dorsal to the Shh$^+$ floor plate (Fig. 1t). Because the first S neurons to be generated have completed their final round of DNA synthesis by E10.5 (Fig. 1f) and newly-born neurons initially migrate in a strict medial-to-lateral fashion (Leber and Sanes 1995), these data strongly suggest that S neurons derive from ventral Nkx2.2$^+$/Nkx2.9$^-$/Phox2b$^-$ progenitors that by E10.5 no longer produce vMNs (Fig. 1v). Moreover, although Nkx2.9 became restricted to dorsal Nkx2.2$^+$ progenitors in r4, the progenitor expression of Phox2b continued to span the entire width of the Nkx2.2$^+$ domain up to E11.5 at this level (Fig. 1i,o,r; data not shown). These data show that the exclusion of S neurons and the extended phase of vMN production observed in r4 correlate with an extended temporal and spatial progenitor expression of Phox2b (Fig. 1v).

What factors control the sequential generation of vMNs and S neurons in the hindbrain? Previous studies showed that Nkx6.1 and Nkx6.2 have a central role in DV patterning and in the specification of somatic MNs, which are generated in a position immediately dorsal to vMNs (Sander et al. 2000; Vallstedt et al. 2001). Nkx6.1 and Nkx6.2 are coexpressed in all Nkx2.2$^+$ progenitors in the hindbrain (Fig. 1g–l), and we therefore investigated whether these HD proteins also influence the generation of vMNs and S neurons. Because these proteins have overlapping functions (Vallstedt et al. 2001), we focused our analysis on *Nkx6.1* and *Nkx6.2* compound mutant mice (*Nkx6* mutants). The number of Nkx2.2$^+$/Phox2b$^+$ vMN progenitors and Isl1$^+$/Phox2b$^+$ neurons was similar in *Nkx6* mutants and control embryos at most hindbrain levels between E9 and E10.5 (Fig. 2a). Thus, in contrast to somatic MNs (Vallstedt et al. 2001), Nkx6 proteins are dispensable for the initial specification of vMN fate. We noticed, however, that the number of Nkx2.2$^+$/Phox2b$^+$ vMN progenitors, and the total number of vMNs generated, were drastically reduced at r4 levels in *Nkx6* mutants (Fig. 2h–m). Quantification of Phox2b expression in Nkx2.2$^+$ progenitors over time indicated that vMN generation in r4 was prematurely arrested at approximately E10.5, and the remaining expression of Phox2b was largely confined to dorsal Nkx2.2$^+$/Nkx2.9$^+$ progenitors (Fig. 2a–i). These data suggested that r4-progenitors in *Nkx6* mutants adopt a profile of vMN generation similar to that of other hindbrain levels, and that the loss of Nkx6 function primarily affects the late phase of vMN generation unique to r4 (Fig. 1v). Strikingly, the reduced production of vMNs in *Nkx6* mutants in r4 was accompanied by ectopic generation of S neurons, as indicated by a continuous expression of *pet1* along the AP axis of the hindbrain and the detection of S neurons ventral to vMNs in r4 at E11.5 (Fig. 2j–q).

The selective requirement for Nkx6 proteins to promote vMN and suppress S neuron generation in r4 uncovers an unanticipated role for these HD proteins in AP patterning. Because Nkx6.1 and Nkx6.2 are coexpressed by all Nkx2.2$^+$ progenitors in the hindbrain (Fig. 1g–l), we reasoned that the AP-specific mode of action of these proteins must be indirect. We therefore examined the expression of *Hox* genes implicated in the establishment of r4 identity of the hindbrain, and found that the expression of *Hoxb1* was extinguished in the ventral half of r4 at E11.5 in *Nkx6* mutants (Fig. 2r,s). Several other *Hox* genes appeared unaffected, indicating that the overall AP identity of the hindbrain is not perturbed in these mice (Fig. 2t,u; data not shown). Analysis of *Hoxb1* expression in *Nkx6* mutants at earlier stages revealed a normal expression pattern at E9.5, and a reduction of *Hoxb1* expression levels was first detected at E10.5 (Fig. 2v–y).

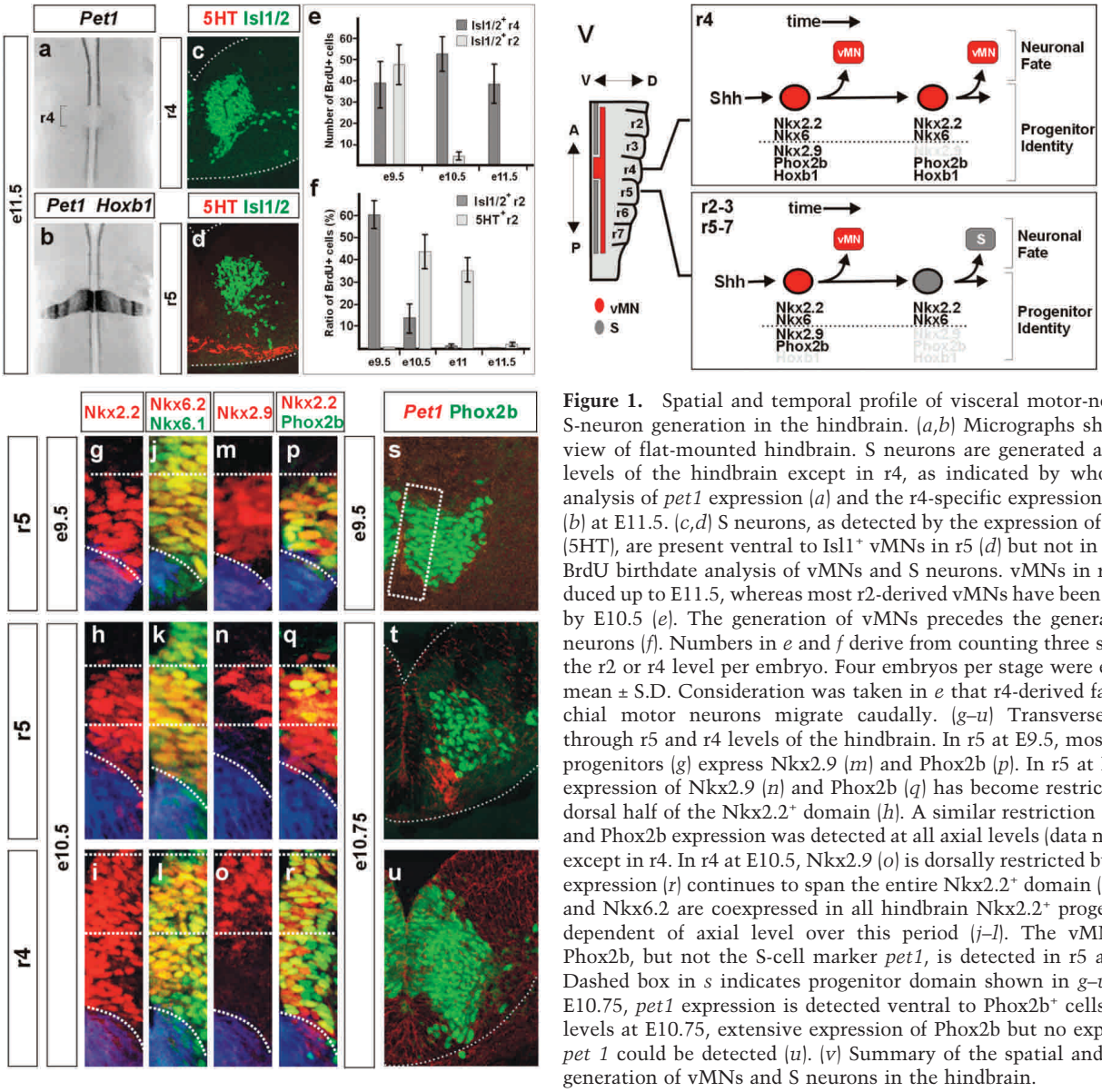

Figure 1. Spatial and temporal profile of visceral motor-neuron and S-neuron generation in the hindbrain. (*a*,*b*) Micrographs show dorsal view of flat-mounted hindbrain. S neurons are generated at all axial levels of the hindbrain except in r4, as indicated by whole-mount analysis of *pet1* expression (*a*) and the r4-specific expression of *Hoxb1* (*b*) at E11.5. (*c*,*d*) S neurons, as detected by the expression of serotonin (5HT), are present ventral to Isl1$^+$ vMNs in r5 (*d*) but not in r4 (*c*). (*e*,*f*) BrdU birthdate analysis of vMNs and S neurons. vMNs in r4 are produced up to E11.5, whereas most r2-derived vMNs have been generated by E10.5 (*e*). The generation of vMNs precedes the generation of S neurons (*f*). Numbers in *e* and *f* derive from counting three sections at the r2 or r4 level per embryo. Four embryos per stage were examined, mean ± S.D. Consideration was taken in *e* that r4-derived facial branchial motor neurons migrate caudally. (*g*–*u*) Transverse sections through r5 and r4 levels of the hindbrain. In r5 at E9.5, most Nkx2.2$^+$ progenitors (*g*) express Nkx2.9 (*m*) and Phox2b (*p*). In r5 at E10.5, the expression of Nkx2.9 (*n*) and Phox2b (*q*) has become restricted to the dorsal half of the Nkx2.2$^+$ domain (*h*). A similar restriction of Nkx2.9 and Phox2b expression was detected at all axial levels (data not shown) except in r4. In r4 at E10.5, Nkx2.9 (*o*) is dorsally restricted but Phox2b expression (*r*) continues to span the entire Nkx2.2$^+$ domain (*i*). Nkx6.1 and Nkx6.2 are coexpressed in all hindbrain Nkx2.2$^+$ progenitors independent of axial level over this period (*j*–*l*). The vMN marker Phox2b, but not the S-cell marker *pet1*, is detected in r5 at E9.5 (*s*). Dashed box in *s* indicates progenitor domain shown in *g*–*u*. In r5 at E10.75, *pet1* expression is detected ventral to Phox2b$^+$ cells (*t*). At r4 levels at E10.75, extensive expression of Phox2b but no expression of *pet 1* could be detected (*u*). (*v*) Summary of the spatial and temporal generation of vMNs and S neurons in the hindbrain.

Thus, Nkx6 proteins are dispensable for the initial phase of ventral *Hoxb1* expression, but are necessary to maintain high levels of *Hoxb1* expression in the ventral half of r4 from E10.5.

AP positional values in the hindbrain are established soon after neural tube closure (Guthrie et al. 1992; Simon et al. 1995), and the role of *Hoxb1* in conferring r4 identity is well documented (Studer et al. 1996; Bell et al. 1999; Gaufo et al. 2000). The finding that *Hoxb1* expression in r4 depends on Nkx6 proteins reveals a regulatory interaction between HD proteins previously implicated in DV and AP patterning, and implies that Nkx6 proteins operate upstream of *Hoxb1* to promote vMN and suppress S neurons in r4. Indeed, the reduction of Phox2b expression in progenitors (Gaufo et al. 2000) and the transformation of r4-derived MNs into an r2-like identity (Studer et al. 1996) observed in *Hoxb1* mutants point towards an altered profile of vMN production in these mice. In r4 of *Hoxb1* mutants, we observed a reduction and premature arrest of vMN generation that was associated with a complementary generation of ectopic S neurons (Fig. 3a,b,d,e,j,k). Because the progenitor expression of Nkx6.1 and Nkx6.2 was unaffected by the loss of *Hoxb1* (Fig. 3g,h), these findings favor the idea that a primary role for Nkx6 proteins in AP patterning is to sustain *Hoxb1* expression in r4.

In Nkx6 mutants, *Hoxb1* expression is gradually lost and a vMN-to-S neuron switch is observed. These data imply that Hoxb1 is required continuously throughout development in order for progenitors to retain their r4 identity. A prediction from such a hypothesis is that ventral r4-derived neurons generated prior to the loss of Hoxb1 in *Nkx6* mutants should retain their r4 identity, whereas such neurons should be ablated in *Hoxb1* mu-

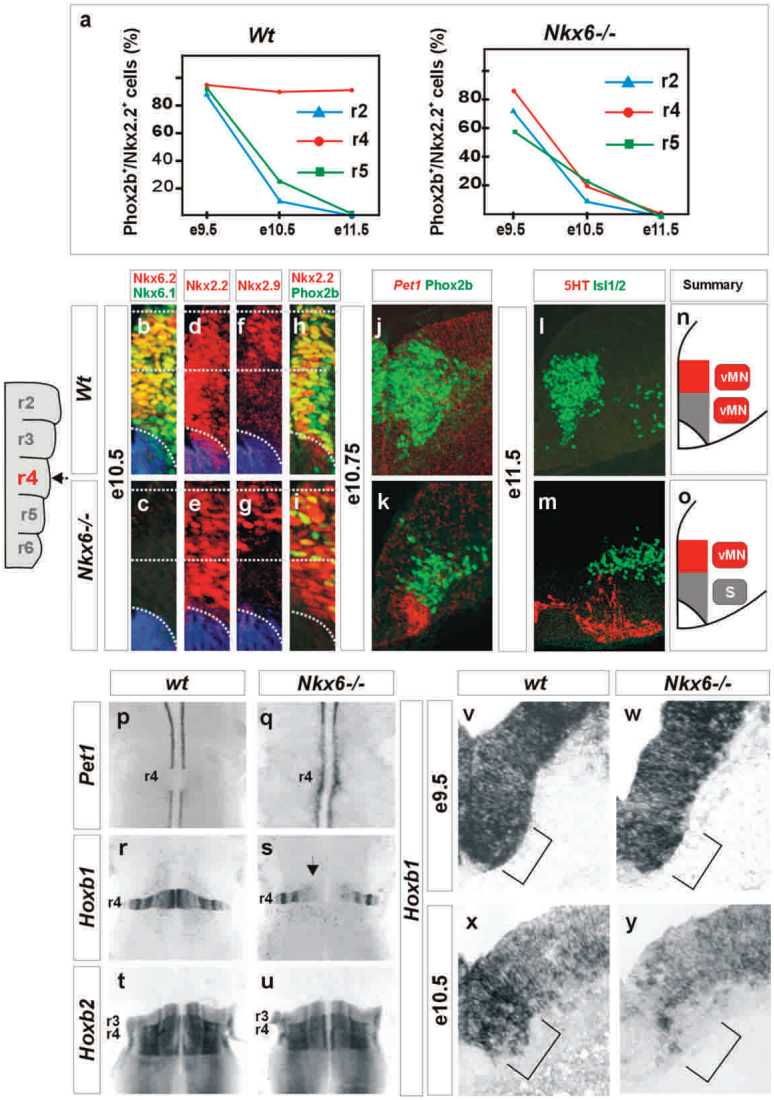

Figure 2. Nkx6 proteins promote motor neuron generation and suppress S neurons in r4. (*a*) Graph showing the temporal profile of Phox2b expression in Nkx2.2$^+$ progenitors in r2, r4, and r5 in wild-type (wt) and *Nkx6* mutant mice. Note the reduced Phox2b expression in r4 of *Nkx6* mutants. (*b–i*) Micrographs through r4 at E10.5 in controls (*b,d,f,h*) and *Nkx6* mutants (*c,e,g,i*). The expression of Nkx2.2 (*d,e*) and Nkx2.9 (*f,g*) is similar in controls and *Nkx6* mutants. The progenitor expression of Phox2b is reduced and primarily detected in dorsal Nkx2.2$^+$ progenitors in r4 in *Nkx6* mutants at E10.5 (*i*) compared to controls (*h*). (*j–o*) Ectopic generation of S neurons in r4 of *Nkx6* mutants. In r4 at E10.75, the reduction and ventral loss of Phox2b expression in *Nkx6* mutants are accompanied by ectopic expression of *pet1* (*j,k*). At E11.5, 5HT expression is excluded in r4 of controls (*l*) but is detected ventral to Isl1$^+$ vMNs in *Nkx6* mutants (*m*). (*n,o*) Summary of vMN and S-cell generation in r4 of wild-type (wt; *n*) and *Nkx6* mutant mice (*o*). (*p–y*) Nkx6 proteins are required to maintain *Hoxb1* expression in r4. (*p–u*) Micrographs showing expression of *pet1* (*p,q*), *Hoxb1* (*r,s*), and *Hoxb2* (*t,u*) in flat-mounted hindbrains at E11.5 in wild-type (wt; *p,r,t*) and *Nkx6* mutants (*q,s,u*). Note that the continuous expression of *pet1* in the hindbrain in *Nkx6* mutants (*q*) is associated with a ventral loss of *Hoxb1* expression in r4 (*s*, arrowhead). (*v–y*) Transverse sections showing *Hoxb1* expression in r4. The expression of *Hoxb1* is similar in controls (*v*) and *Nkx6* mutants (*w*) at E9.5. A ventral down-regulation of *Hoxb1* expression is observed in r4 at E10.5 in *Nkx6* mutants (*y*) compared to controls (*x*). Brackets indicate Nkx2.2$^+$ progenitor domain.

tants. In line with this idea, we found that Phox2b$^+$/Gata3$^+$ inner ear efferent (iee) neurons (Karis et al. 2001), which are selectively generated from Nkx2.2$^+$ progenitors in r4 prior to E10.5 (data not shown), are still detected in *Nkx6* mutants (albeit in reduced numbers), but are completely missing in *Hoxb1* mutants (Fig. 3q–s).

To examine further the control of vMN and S neuron fate in r4, we also examined the patterns of neurogenesis in *Hoxb2* mutants (Davenne et al. 1999), because *Hoxb1* expression is down-regulated at a late stage in these mice (F. Rijli, unpubl.). In *Hoxb2* mutants, *Hoxb1* was expressed at high levels in the Nkx2.2$^+$ domain in r4 at E10.5, and extensive down-regulation was not detected until E11.5 (Fig. 3m–p). Thus, a significant ventral loss of *Hoxb1* expression occurs later in *Hoxb2* mutants than in *Nkx6* mutants (cf. Figs. 2y and 3o). The progenitor expression of Nkx6.1 and Nkx6.2 was unaffected in these mice (Fig. 3g,i). Strikingly, S neurons were also detected in r4 in *Hoxb2* mutants, but the number of S neurons was considerably lower than that observed in both *Nkx6* and *Hoxb1* mutants (Fig. 3a–f; Fig. 2m,q). Moreover, the profile of vMN generation in *Hoxb2* mutants appeared largely unaffected in r4 at E10.5 (Fig. 3j,l,q,t), and a significant reduction of Phox2b$^+$/Nkx2.2$^+$ vMN progenitors was not observed until E11.5 (data not shown). These data link the profile of vMN and S neuronal generation in r4 of *Hoxb2* mutants to the temporal loss of *Hoxb1* expression, rather than to the genetic ablation of *Hoxb2*, and they provide additional, albeit indirect, support for the idea that expression of *Hoxb1* is necessary to promote vMN generation and to suppress S neurons at this axial level.

We next turned our attention to the sequential production of vMNs and S neurons observed at all axial levels, except in r4. The finding that the down-regulation of Phox2b and Nkx2.9 in Nkx2.2$^+$ progenitors anticipates the establishment of S-neuron progenitors (Fig. 1v) prompted us to characterize the loss of S neurons in Nkx2.2 mutant mice (Briscoe et al. 1999) in more detail. In *Nkx2.2* mutants, the progenitor expression of Nkx6.1

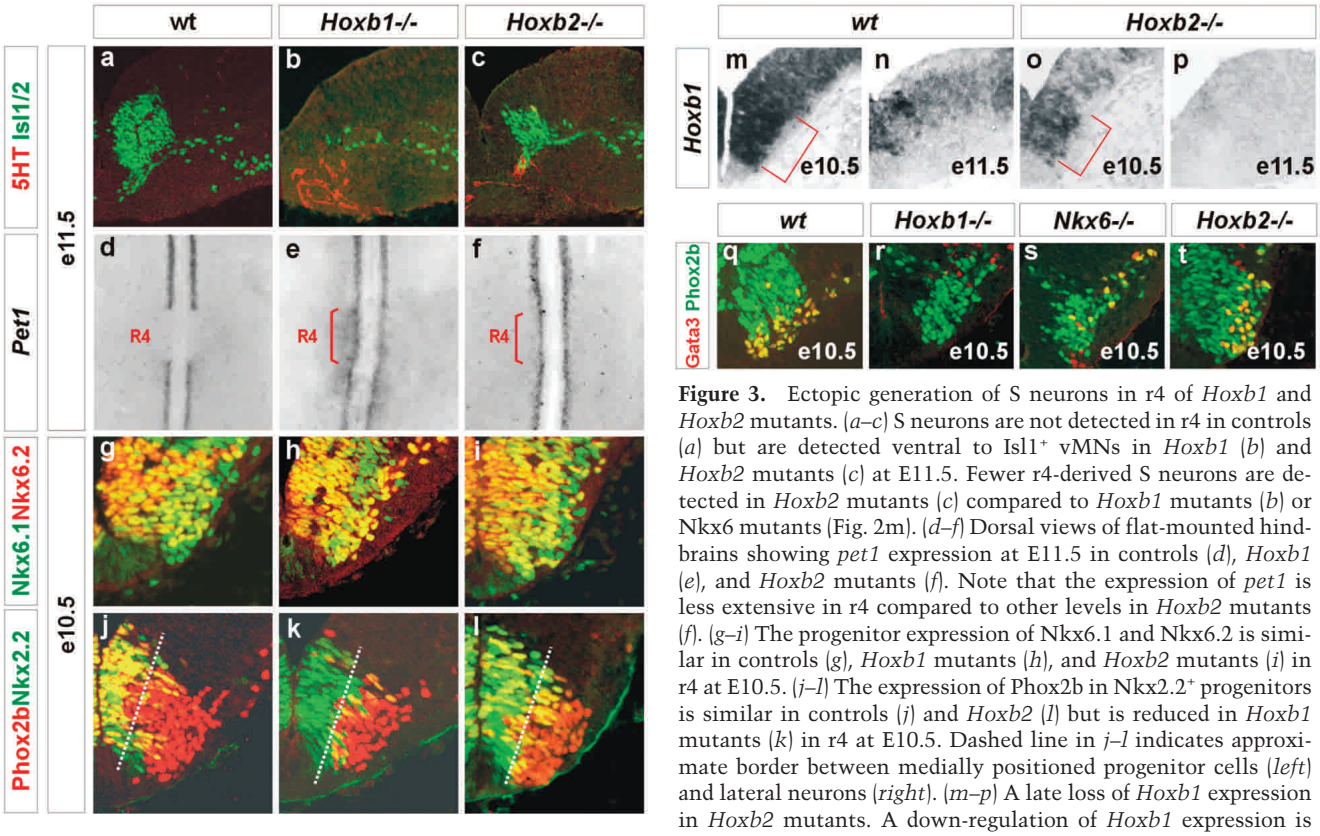

Figure 3. Ectopic generation of S neurons in r4 of *Hoxb1* and *Hoxb2* mutants. (*a–c*) S neurons are not detected in r4 in controls (*a*) but are detected ventral to Isl1$^+$ vMNs in *Hoxb1* (*b*) and *Hoxb2* mutants (*c*) at E11.5. Fewer r4-derived S neurons are detected in *Hoxb2* mutants (*c*) compared to *Hoxb1* mutants (*b*) or Nkx6 mutants (Fig. 2m). (*d–f*) Dorsal views of flat-mounted hindbrains showing *pet1* expression at E11.5 in controls (*d*), *Hoxb1* (*e*), and *Hoxb2* mutants (*f*). Note that the expression of *pet1* is less extensive in r4 compared to other levels in *Hoxb2* mutants (*f*). (*g–i*) The progenitor expression of Nkx6.1 and Nkx6.2 is similar in controls (*g*), *Hoxb1* mutants (*h*), and *Hoxb2* mutants (*i*) in r4 at E10.5. (*j–l*) The expression of Phox2b in Nkx2.2$^+$ progenitors is similar in controls (*j*) and *Hoxb2* (*l*) but is reduced in *Hoxb1* mutants (*k*) in r4 at E10.5. Dashed line in *j–l* indicates approximate border between medially positioned progenitor cells (*left*) and lateral neurons (*right*). (*m–p*) A late loss of *Hoxb1* expression in *Hoxb2* mutants. A down-regulation of *Hoxb1* expression is detected in r4 of *Hoxb2* mutants (*o*) compared to controls (*m*), but the level of expression is still relatively high in the ventral Nkx2.2$^+$ domain (indicated by brackets in *m*,*o*) compared to *Nkx6* mutants at the corresponding stage (cf. Fig. 2y). At E11.5, *Hoxb1* is expressed in controls (*n*) but is essentially extinguished in r4 of *Hoxb2* mutants (*p*). (*q–t*) Generation of iee neurons in r4 of wild-type (wt), *Hoxb2*, *Nkx6*, and *Hoxb1* mutants: iee neurons that coexpress Phox2b and GATA3 (yellow) can be detected at r4 levels at E10.5 in controls (*q*), *Hoxb2* (*t*), and *Nkx6* mutants (*s*), but these neurons are completely missing in *Hoxb1*mutants (*r*).

and Nkx6.2 was unaffected and the expression pattern of Nkx2.9 and Phox2b was similar to controls at early stages (E9.5; data not shown). Remarkably, the subsequent dorsal restriction of Nkx2.9 and Phox2b expression observed in controls failed to occur in *Nkx2.2* mutants (Fig. 4a–h). The loss of S neurons was further accompanied by a total increase in vMN numbers (33% ± 6% S.D., n = 4) in r5 at E11, and many vMNs occupied a position at which S neurons are normally detected (Fig. 4i–n; data not shown). These data reveal that Nkx2.2 is required for the temporal conversion of vMN progenitors into S-neuron progenitors. Nkx2.9 cannot compensate the loss of Nkx2.2 function, despite a ventral expansion of Nkx2.9 expression (Fig. 4e,f) at stages when S neurons are normally being specified. Thus, although Nkx2.2 and Nkx2.9 have redundant functions in other aspects of neural patterning (Briscoe et al. 1999, 2000), our analysis reveals a novel role for Nkx2.2 in the establishment of S-neuron progenitors.

In normal conditions, as well as in *Nkx2.2*, *Nkx6*, *Hoxb1*, and *Hoxb2* mutant mice, there is a strict correlation between the progenitor expression of Phox2b and the selection of vMN fate. This observation suggests that Phox2b may be a key mediator of the switch that determines whether Nkx2.2$^+$ progenitors will select a vMN or S fate. To examine this possibility, we analyzed Phox2b mutant mice (Pattyn et al. 2000). All vMNs are missing in *Phox2b* mutants, and many progenitors are arrested in an Nkx2.2$^+$ state, most likely reflecting the role of Phox2b to induce pro-neural bHLH proteins in the vMN pathway (Dubreuil et al. 2000; Pattyn et al. 2000). Importantly, not all cells fail to exit the cell cycle (Pattyn et al. 2000), and this allowed us to determine the identity of the neurons derived from Nkx2.2$^+$ progenitors in *Phox2b* mutants. Strikingly, the loss of vMNs observed in these mice was accompanied by premature expression of *pet1* and S-neuron generation at all axial levels of the hindbrain at E10.5, including r4 (Fig. 5a–d; data not shown). The production of ectopic S neurons was extensive in r4 at E11.5 (Fig. 5e,f), despite the fact that the progenitor expression of Nkx2.2, Nkx6.1, and Nkx6.2 at all axial levels, and *Hoxb1* in r4, appeared unaffected (Fig. 5g,h; data not shown). Moreover, cells that coexpressed serotonin (5HT) and *LacZ* driven by the *Phox2b* locus (Pattyn et al. 2000) could be detected (Fig. 5e,f), providing direct evidence that vMN progenitors, in the absence of Phox2b, give rise to S neurons. These findings establish a requirement for Phox2b to suppress S-neuronal fate in vMN progenitors, and predict that the progressive extinction of Phox2b in Nkx2.2$^+$ progenitors is necessary

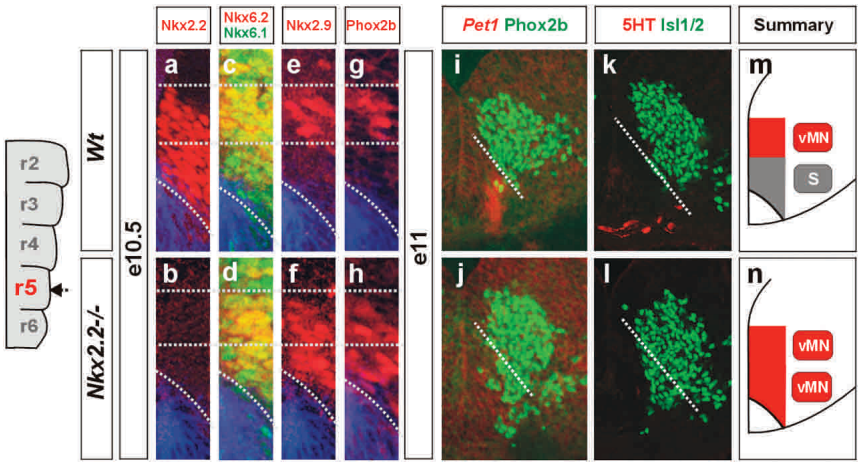

Figure 4. Nkx2.2 is required for the temporal establishment of S-neuron progenitors. (*a–j*) Transverse sections through the ventral hindbrain at the r5 level in wild-type (wt) and *Nkx2.2* mutant mice. The progenitor expression of Nkx6.1 and Nkx6.2 is similar in control embryos (*a*,*c*) and in *Nkx2.2* mutants (*b*,*d*) at E10.5. The dorsal restriction of Nkx2.9 and Phox2b observed in control embryos (*e*,*g*) fails to occur in *Nkx2.2* mutants (*f*,*h*), and the expression of these proteins extends ventrally to the dorsal boundary of Shh expression in the floor plate (*a–h*, blue staining). In r5 at E11, *Pet1* and 5HT expression is missing, and vMNs identified by Phox2b and Isl expression are increased in number and ventrally expanded (indicated by dashed line) in *Nkx2.2* mutants (*i–l*). (*m*,*n*) Summary of vMN and S-cell generation in r5 in controls (*m*) and *Nkx2.2* mutants (*n*).

for the generation of S neurons. Indeed, the suppression of S-neuronal fate in r4 provides direct support for this idea, because the extended production of motor neurons at the expense of S neurons at this level depends on the prolonged activation of Phox2b in all Nkx2.2$^+$ progenitors.

Discussion

In this study, we examined the sequential generation of vMNs and S neurons from a common pool of Nkx2.2$^+$ progenitor cells in the developing mouse hindbrain. We obtained evidence that Nkx2.2$^+$ progenitors undergo changes in progenitor cell identity over time, and that these changes reflect a conversion of vMN progenitors into S-neuron progenitors. Nkx2.2 is required in this process, and the loss of S neurons in Nkx2.2 mutants (Briscoe et al. 1999) is associated with an extended production of vMNs and a failure to suppress the progenitor expression of Phox2b and Nkx2.9. How the switch from vMN to S-neuron generation is initiated is still unclear, but the progressive dorsal restriction in Phox2b and Nkx2.9 expression indicates that a signal provided by the floor plate may be involved. Because the pattern of Nkx2.2 expression is unchanged over time, a key activity of such a signal would be predicted to induce, or activate, a cofactor necessary for Nkx2.2 to convert Nkx2.2$^+$/Nkx2.9$^+$/Phox2b$^+$ vMN progenitors into Nkx2.2$^+$/Nkx2.9$^-$/Phox2b$^-$ S neuron progenitors (Fig. 5i).

Our analysis further reveals a close regulatory link between Nkx6 proteins and Hoxb1 in r4 that directly influences the spatial and temporal control of neuronal cell fate. In both *Nkx6* and *Hoxb1* mutant mice, S neurons are generated in r4 and the production of vMNs is impaired. We provide evidence that the AP-specific role for Nkx6.1 and Nkx6.2 is indirect and reflects a requirement for these proteins to sustain *Hoxb1* expression. Hoxb1, in turn, promotes vMN generation and suppresses S neurons by extending the spatial and temporal activation of Phox2b expression in Nkx2.2$^+$ progenitors. In this respect, it is possible that Hoxb1 directly activates Phox2b expression and in this way simply overrules the establishment of S-neuron progenitors evident at other axial levels of the hindbrain (Fig. 5i).

The down-regulation of *Hoxb1* expression in *Nkx6* and *Hoxb2* mutants also provides insight into the temporal requirement for Hoxb1 to confer r4-positional identity in the hindbrain. The initial phase of *Hoxb1* expression is unaffected in *Nkx6* mutants, and a significant ventral loss of *Hoxb1* expression is detected first at approximately E10.5. In this situation, and in contrast to *Hoxb1* mutants (Studer et al. 1996; Gaufo et al. 2000), vMNs generated prior to E10.5 retain r4 characteristics, whereas late-born vMNs are missing because r4 progenitors instead adopt an S-neuronal fate. Moreover, we find that the even later loss of *Hoxb1* expression observed in *Hoxb2* mutants correlates with a mild phenotype in which only a few r4 progenitors adopt an S-neuronal fate. In addition to an early requirement to establish r4 identity (Studer et al. 1996; Gaufo et al. 2000), these observations indicate that the expression of *Hoxb1* must be maintained over time in order for progenitors to retain their r4-positional identity and select a fate appropriate to their developmental history.

The sequential production of distinct classes of neurons represents a central strategy to establish cell diversity in the developing CNS, but few molecular determinants involved in this process have been identified (Livesey and Cepko 2001; Monuki and Walsh 2001). Our data establish that the sequential production of vMNs and S neurons in the hindbrain critically depends on HD proteins previously implicated in DV and AP patterning, assigning new roles for these proteins in the temporal

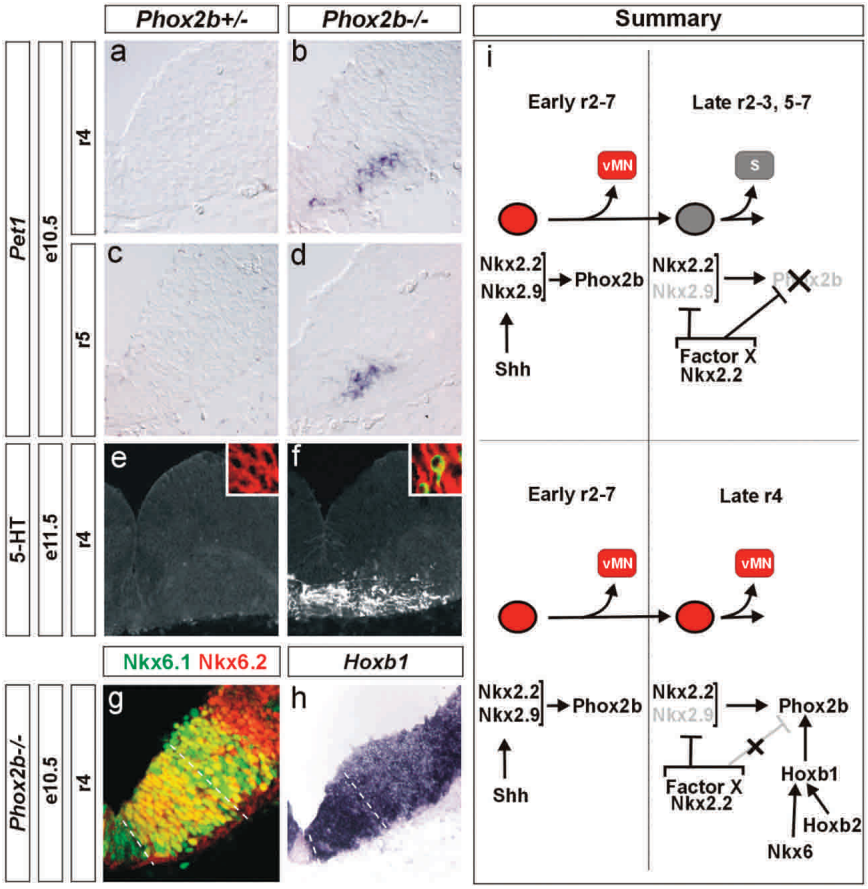

Figure 5. Phox2b is required to suppress the premature generation of S neurons in the hindbrain. (*a–f*) *Pet1* is prematurely and ectopically expressed in *Phox2b* mutants. No *pet1* expression is detected in r4 or r5 of *Phox2b*$^{+/-}$ control embryos at E10.5 (*a*,*c*), whereas a premature and ectopic expression of *pet1* was detected at this stage in *Phox2b*$^{-/-}$ embryos (*b*,*d*). 5HT expression could also be detected at these levels at E10.5 (data not shown). 5HT is not detected in r4 in controls (*e*) but is extensively expressed at this level in *Phox2b*$^{-/-}$ embryos at E11.5 (*f*). Inserted micrographs in *e* and *f* show *LacZ* expression under control of the Phox2b locus (red) and the expression of 5HT (green). (*g*,*h*) The progenitor expression of Nkx6.1 and Nkx6.2 (*g*) and *Hoxb1* (*h*) is unaffected in r4 of *Phox2b*$^{-/-}$ embryos at E10.5. (*i*) Model of vMN and S-neuron generation in the hindbrain. At early stages at all axial levels (r2–r7), Shh signaling induces vMN progenitors that express Nkx2.2, Nkx2.9, Nkx6.1, Nkx6.2, and Phox2b. The expression of Phox2b promotes vMNs and suppresses S neurons. At later stages (at all levels except r4), Nkx2.9 and Phox2b are suppressed in ventral Nkx2.2^{+} progenitors, converting vMN progenitors into S-neuron progenitors. In the absence of Phox2b, cells select the S-neuronal fate. The establishment of S-neuron progenitors may be mediated by a signal produced by the floor plate, that induces or activates a factor (Factor X) that is necessary for Nkx2.2 to suppress Nkx2.9 and Phox2b expression. In r4, all Nkx2.2 progenitors produce vMNs also at late stages, and the generation of S neurons is blocked. At this level, Hoxb1 ensures that all Nkx2.2^{+} progenitors express Phox2b. The sustained expression of Hoxb1 in r4, in turn, depends on Nkx6 and Hoxb2 proteins. Factor X is predicted to be induced also in r4 (because Nkx2.9 is suppressed and S neurons are generated if *Hoxb1* is missing or is down-regulated). Hoxb1 must therefore override the establishment of S-neuron progenitors evident at other levels, possibly by directly activating Phox2b expression in all Nkx2.2 progenitors. For further details, see text.

control of neuronal fate determination. We show that the primary role for Nkx and Hox HD proteins is to coordinate the temporal and spatial expression of Phox2b in neural progenitors, and that Phox2b in turn acts as a molecular switch that determines whether progenitors select a vMN or S-neuronal fate. The role of Phox2b to promote early-born neurons and suppress late-born neurons shows a high degree of similarity to the temporal determinant Hunchback in the *Drosophila* CNS (Isshiki et al. 2001). In *Drosophila*, a cell cycle-dependent clock mechanism has been proposed to underlie the regulation of temporal determinants (Isshiki et al. 2001). The variable generation of vMN and S neurons along the AP axis implies that the temporal control of Phox2b expression in the hindbrain is uncoupled from the cell cycle and, as discussed above, appears instead to rely on the integrated activity of Nkx and Hox proteins. Recent studies in the spinal cord have implicated that the switch from generating somatic MNs to produce oligodendrocytes may be triggered by an expansion of Nkx2.2 expression into the neighboring Olig2^{+} domain (Zhou et al. 2001). However, the precise role of these proteins in this switch remains unclear, because a neuron-to-glial switch is still observed in both Nkx2.2 (Qi et al. 2001) and Olig2 (Lu et al. 2002; Zhou and Anderson 2002) mutant mice. Nevertheless, data begin to suggest that determinants that control spatial patterning generally may be associated with temporal aspects of neural fate determination. In this view, the sequential control of neuronal fate specification would be mechanistically analogous to spatial patterning, but with the notion that the expression pattern of intrinsic determinants is dynamic and modulated over time.

Materials and methods

Mouse strains

The generation and genotyping of mouse mutants have been reported: Nkx6.1 (Sander et al. 2000), Nkx6.2 (Vallstedt et al. 2001), Hoxb1 (Studer et al. 1996), Hoxb2 (Davenne et al. 1999), Nkx2.2 (Briscoe et al. 1999), and Phox2b (Pattyn et al. 2000).

Immunohistochemistry and in situ hybridization histochemistry

Immunohistochemical localization of proteins was performed as described (Briscoe et al. 2000) using the following antibodies: mouse (m), rabbit (r), and guinea pig (gp) Isl1/2, gp Nkx2.9 (Briscoe et al. 2000), gp Nkx6.2 (Vallstedt et al. 2001), m Gata3 (Santa Cruz Biotechnology), m and r Nkx2.2 (Ericson et al. 1997), r Phox2b (Pattyn et al. 2000), and r Nkx6.1 (Briscoe et al. 1999). Serotonergic (S) neurons were detected by r Serotonin (5HT) antibody (Sigma). In situ hybridization histochemistry on sections or as whole mounts were performed as described (Wilkinson 1992; Schaeren-Wiemers and Gerfin-Moser 1993) using *pet1*, *Hoxb1*, *Hoxb2*, *Isl1*, *Hoxb4*, *Hoxa1*, and *Hoxa2* probes.

BrdU labeling

BrdU (Sigma) was injected intraperitoneally into pregnant mice (0.1 mg/g of body weight) at E8.5, E9.5, E10, E10.5, E11, and E11.5. Embryos were harvested at E12.5 and analyzed for incorporation of BrdU in motor neurons and S neurons using BrdU antibodies in combinations with Phox2b, Isl1/2, 5HT, Gata3 antibodies.

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