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 Ericson1,5

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

Keywords: CNS; development; motor neuron; 5HT; patterning; homeodomain

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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; Gauf 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.

Results

To address how spatial and temporal aspects of cell patterning are integrated during development, we examined the sequential generation of visceral motor neurons...
possibility that S neurons, in turn, derive from ventral vMN 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 r4, 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 birth-dating 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. 1f].

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. 1f]. 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 Aitken 1988], 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. 1f]. 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. 1f].

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–l]. 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. 1f]. 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. 2h–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].
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-
pressed at high levels in the Nkx2.2+ domain in r4 at (F. Rijli, unpubl.). In Hoxb2 expression is down-regulated at a late stage in these mice (Fig. 3g,i). Strikingly, S neurons were also detected in r4 of Nkx6 mutants (cf. Figs. 2y and 3o). The progenitor expression of Phox2b is reduced and primarily detected in dorsal Nkx2.2+ progenitors in r4 in Nkx6 mutants at E10.5 compared to controls (b). (i–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 (i,k). At E11.5, 5HT expression is excluded in r4 of controls (l) but is detected ventral to IsIl1+ 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,v). 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 [Karlis 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. 3i,j,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...
and Nkx6.2 was unaffected and the expression pattern of Nkx2.9 and Phox2b was similar to controls at early stages. The subsequent dorsal restriction of Nkx2.9 and Phox2b expression in controls failed to occur in Nkx2.2 mutants. The loss of S neurons was further accompanied by a total increase in vMN numbers \((33 \pm 6\% \text{ S.D.}, n = 4)\) in r5 at E11, and many vMNs occupied a position at which S neurons are normally detected. 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 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, 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; Patterson 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 coexpress 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.
Nkx6.1 and Nkx6.2 is indirect and reflects a requirement paired. We provide evidence that the AP-specific role for are generated in r4 and the production of vMNs is im-fate. In both influences the spatial and temporal control of neuronal cell

between Nkx6 proteins and Hoxb1 in r4 that directly in-

vert Nkx2.2+/Nkx2.9+/Phox2b+ vMN progenitors into

activity of such a signal would be predicted to induce,
of Nkx2.2 expression is unchanged over time, a key

the floor plate may be involved. Because the pattern

Nkx2.9 expression indicates that a signal provided by

but the progressive dorsal restriction in Phox2b and

vMN to S-neuron generation is initiated is still unclear,

these changes reflect a conversion of vMN progenitors

Hoxb1 for these proteins to sustain Hoxb1 expression. Hoxb1,
in turn, promotes vMN generation and suppresses S neu-

expression observed in Nkx2.2 mutants (i–l). (m, n) Summary of vMN and S-cell generation in r5 in controls (m) and Nkx2.2 mu-

for the generation of S neurons. Indeed, the suppression

Discussion

In this study, we examined the sequential generation of

mRNAs and S neurons from a common pool of Nkx2.2 progenitor cells in the developing mouse hindbrain. We

expression and in this way simply overrules the estab-

lishment 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 tem-

poral requirement for Hoxb1 to confer r4-positional iden-

ity in the hindbrain. The initial phase of Hoxb1 expres-

sion 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,

mutants (Studer et al. 1996;Gaufo et al. 2000), these obser-

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 mu-

for these proteins to sustain Hoxb1 expression. Hoxb1, in turn, promotes vMN generation and suppresses S neu-

FiguRe 4. Nkx2.2 is required for the tem-

poral establishment of S-neuron progeni-

ors. (a–f) 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 restric-

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Hoxb1 mutants [Studer et al. 1996;Gaufo et al. 2000], vMNs generated prior to E10.5 retain r4 characteristics,

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+ progeni-

ors.
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).

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.
Immunohistochemistry and in situ hybridization histochemistry

Immunohistochemical localization of proteins was performed as described [Briscoe et al. 2000] using the following antibodies: mouse [ml], rabbit [r], and guinea [gg] Ig [g] 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 [SHT] 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, SHT, Gata3 antibodies.

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Alexandre Pattyn, Anna Vallstedt, José M. Dias, et al.

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