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ARTICLES

Ascl1/Mash1 is required for the development of central serotonergic neurons

Alexandre Pattyn[1], Nicolas Simplicio[2], J Hikke van Doorninck[3,4], Christo Goridis[1], François Guillemot[2] & Jean-François Brunet[1]

The transcriptional control of the differentiation of central serotonergic (5-HT) neurons in vertebrates has recently come under scrutiny and has been shown to involve the homeobox genes *Nkx2-2* and *Lmx1b*, the *Ets*-domain gene *Pet1* (also known as *Fev*) and the zinc-finger gene *Gata3*. The basic helix-loop-helix (bHLH) gene *Ascl1* (also known as *Mash1*) is coexpressed with *Nkx2-2* in the neuroepithelial domain of the hindbrain, which gives rise to 5-HT neurons. Here we show in the mouse that *Ascl1* is essential for the birth of 5-HT neurons, both as a proneural gene for the production of postmitotic neuronal precursors and as a determinant of the serotonergic phenotype for the parallel activation of *Gata3*, *Lmx1b* and *Pet1*. Thus *Ascl1*, which is essential for noradrenergic differentiation, is also a determinant of the serotonergic phenotype.

The central 5-HT system in mammals consists of clusters of neurons, which were numbered B1 to B9 in the original description[1] but are now more commonly named after the cytoarchitectonic groups that include them[2]. They are born[3] and located[4] exclusively in the ventral rhombencephalon (even the so-called dorsal raphe nucleus is ventral relative to embryological coordinates). This complex of cells, which is less clearly partitioned than the 'B' nomenclature and nuclear description suggest, is subdivided by a region that lacks any 5-HT neurons into a rostral (pontine) and caudal (medullary) group. This subdivision is paralleled by distinct patterns of projections—the rostral group projects to the cortex and hypothalamus, and the caudal group projects to the spinal cord—and by developmental pathways that are at least partially distinct (see below). Through extensive projections and collateralization, 5-HT neurons supply most regions of the CNS with serotonin and are thought to modulate many diverse neurological phenomena ranging from locomotion and pain to mood[5].

5-HT neurons are born between embryonic day (E)10.5 and E12 from the ventral-most neuroepithelial domain of the hindbrain right after it has produced branchiomotor and visceromotor (BM/VM) neuronal precursors[6,7]. (This region is topologically equivalent to the p3 domain of the spinal cord and is called pMNv throughout rhombomeres r2 to r7.) This temporal pattern varies at two locations. In r4, the generation of facial branchial motor neurons carries on until E12.5 and consequently no serotonergic differentiation occurs, hence the gap between the rostral and caudal 5-HT nuclei[7]. In ventral r1, the first rhombomere in which 5-HT neurons appear, no BM/VM neuronal differentiation ever takes place.

Transcriptional determinants of 5-HT differentiation have recently been identified. In r2–r3 and r5–r7, at the transition from motor to 5-HT neuron production, the ventral part of pMNv switches its homeobox gene code from *Nkx2-2*$^+$/*Nkx2-9*$^+$/*Phox2b*$^+$ to *Nkx2-2*$^+$/*Nkx2-9*$^-$/*Phox2b*$^-$. Loss-of-function experiments have demonstrated that *Nkx2-2* expression and the downregulation of *Phox2b* are actually required for 5-HT neuron production[6,7]. In r1, *Phox2b* is irrelevant (because it is never expressed) and the generation of at least one cluster of 5-HT neurons (the anlage of the dorsal raphe nucleus) is independent of *Nkx2-2* expression[6,8]. In r4, the BM/VM to 5-HT switch is prevented by the maintenance of *Phox2b* expression by *Hoxb1* (whose expression is in turn maintained by *Nkx6-1/6-2* and *Hoxb2*)[7]. Postmitotically, 5-HT differentiation is marked by the expression of the zinc-finger, homeodomain and Ets-domain transcription factors Gata2, Gata3, Lmx1b and Pet1. Three of these have been implicated in 5-HT differentiation by loss-of-function analyses. Chimeras derived from wild-type and *Gata3*-null embryonic stem cells showed a much reduced contribution of mutant cells to caudal, but not rostral, 5-HT nuclei[9]. The inactivation of *Pet1* led to a general 70% decrease in 5-HT cells[10]. Finally, *Lmx1b* homozygous knockouts are practically devoid of 5-HT neurons[8,11]. Epistatic analysis clearly places *Nkx2-2* upstream of *Lmx1b* and *Pet1*, which are activated in parallel[11].

Here we characterize the contribution of *Ascl1*, the only known classical proneural gene expressed in pMNv, to the differentiation of 5-HT neurons. We show that it controls 5-HT differentiation at two levels: it is required for the generation of postmitotic precursors from pMNv after the shutdown of *Phox2b*, and it has a type-specification role in serotonergic precursors. These data, together with a re-examination of *Gata3*-null mutants, led to a model, whereby *Ascl1* together with *Nkx2-2* activates in parallel the three determinants of 5-HT differentiation, *Lmx1b*, *Pet1* and *Gata3*. Thus *Ascl1*, which is also required for 5-HT differentiation in the enteric nervous system[12]

[1]CNRS UMR 8542, Ecole Normale Supérieure, 75005 Paris, France. [2]Molecular Neurobiology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK. [3]Department of Cell Biology, Erasmus MC, Box 1738, 3000DR Rotterdam, The Netherlands. [4]Present address: Department of Neurosciences, Erasmus MC, Box 1738, 3000DR Rotterdam, The Netherlands. Correspondence should be addressed to J.-F.B. (jfbrunet@biologie.ens.fr).

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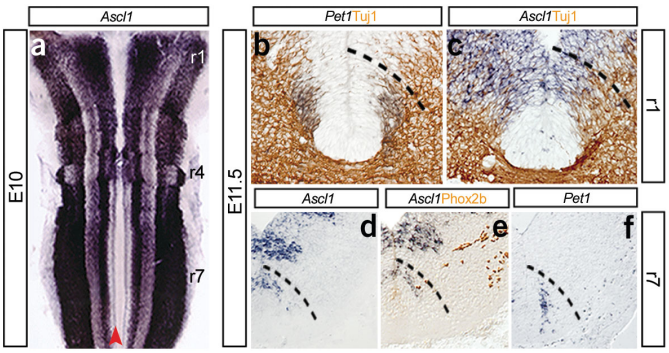


Figure 1 Expression pattern of *Ascl1*, *Pet1*, Phox2b and βIII-tubulin (Tuj1) in the ventral hindbrain. (**a**) Flatmount of the hindbrain at E10 hybridized with an *Ascl1* probe showing (arrowhead) the narrow ventral stripe of expression corresponding to the pMNv neuroepithelial domain. (**b**,**c**) Cross-section through ventral r1 at E11.5 showing that *Ascl1*, as shown in **c**, is expressed in the ventral-most domain (dorsally delineated by a dashed line), which gives rise to *Pet1*$^{+}$ neurons, as shown in **b**. (**d**–**f**) Cross-sections through r7 at E11.5, showing that pMNv maintains *Ascl1* expression (**d**,**e**) as it downregulates Phox2b (**e**) and gives rise to *Pet1*$^{+}$ postmitotic precursors (**f**).

and parafollicular cells of the thyroid[13], emerges as an essential and general determinant of the serotonergic phenotype.

RESULTS

Ascl1 mutants are devoid of serotonergic neurons

The pMNv domain of r2–r7 and the ventral-most domain of r1 express *Ascl1* (**Fig. 1a**,**c**,**d**), but no other proneural bHLH gene that is normally detected in the basal plate (see **Supplementary Fig. 1** online) throughout the period of motor and 5-HT neuron generation. At E11.5, pMNv has ceased expressing *Phox2b* (**Fig. 1e**) and gives rise to *Pet1*$^{+}$ postmitotic 5-HT precursors (**Fig. 1b**,**f**).

Ascl1 is not required for a grossly normal generation of BM/VM motor neurons[14]. We therefore asked whether it was required for the second wave of neurogenesis from the same neuroepithelial region, which produces 5-HT neurons. At E14.5, 5-HT immunohistochemistry on flatmounts of the hindbrain and on sections through the rostral pons (r1) and caudal medulla (r7) showed 5-HT neurons to be missing in *Ascl1* homozygous mutants, save for a few scattered ones close to the midbrain-hindbrain junction (**Fig. 2a**). We then examined E11.5 embryos to test whether early differentiation steps in the 5-HT lineage were preserved in *Ascl1* mutants. At all rostrocaudal levels, expression of *Pet1*, *Gata2*, *Gata3*, *Lmx1b* and the presence of 5-HT abutting the floorplate, which were seen in heterozygous mutants, were undetectable in homozygous mutants (**Fig. 2b**). This shows that from the earliest stage on, 5-HT differentiation fails in *Ascl1*-null mutants.

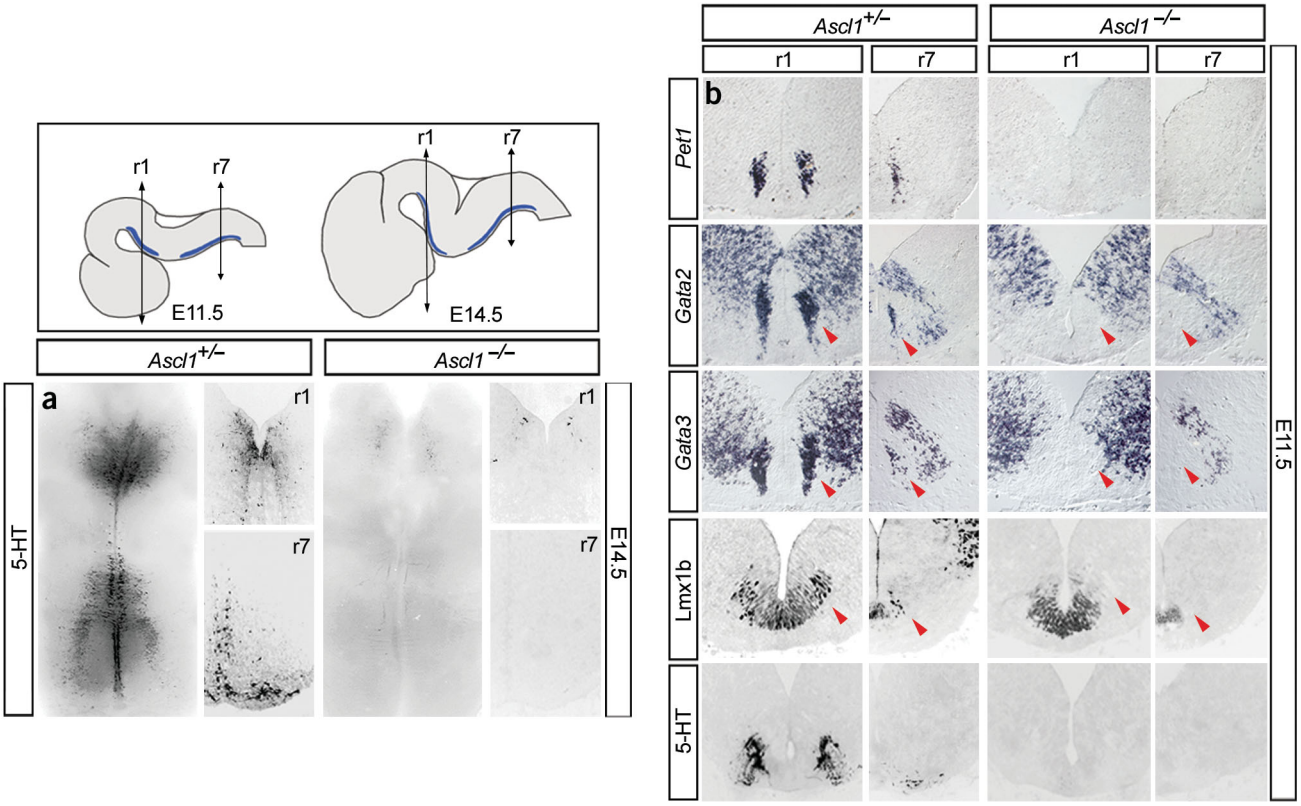


Figure 2 Serotonergic differentiation is virtually abolished in the hindbrain of *Ascl1* mutants. (**a**) Anti-5-HT immunohistochemistry on flatmounts of the hindbrain (left) and sections through the rostral (r1) and caudal (r7) regions of the hindbrain (right) in *Ascl1* heterozygous and homozygous mutants at E14.5. (**b**) At E11.5, *Pet1* expression, the ventral expression of *Gata2*, *Gata3*, *Lmx1b* (arrowheads) and the presence of 5-HT are abolished in *Ascl1* mutants. Note that *Lmx1b* is also expressed in another *Ascl1*-dependent population of neurons and in the floorplate.

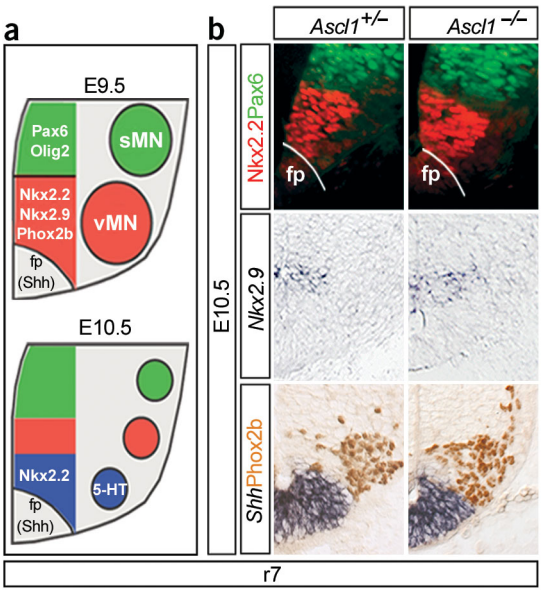
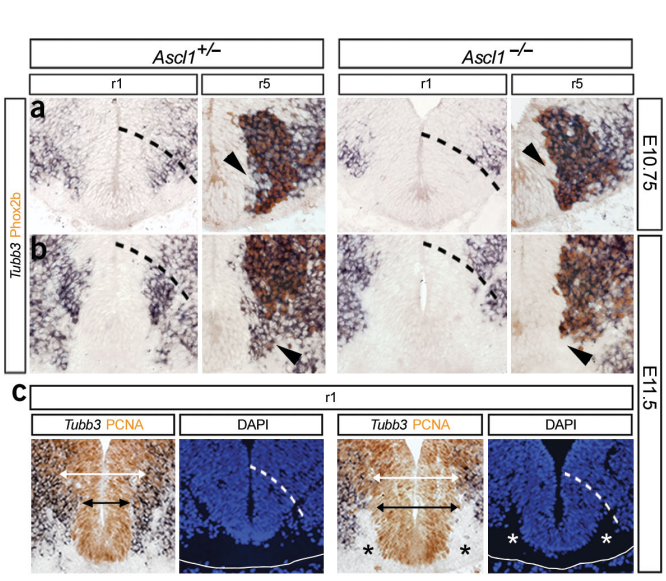


Figure 3 Expression of other progenitor determinants is not affected in the ventral hindbrain of *Ascl1* mutants. (**a**) Schematic of the transcriptional code in pMNv and pMNs (from which somatic motor neurons arise) at E9.5, during visceral motor neuron generation (top) and at E10.5, at the outset of 5-HT neuron generation (bottom). fp, floorplate. (**b**) Immunostaining for Nkx2.2, Pax6, Phox2b and *in situ* hybridization with *Nkx2-9* and *Shh* as indicated, showing that their expression patterns are unchanged by the *Ascl1* mutation.

We tested whether the inactivation of *Ascl1* had broader consequences for progenitor identity in the pMNv domain (**Fig. 3a**) that could explain the block of 5-HT differentiation. At E10.5, which is at the outset of 5-HT differentiation, the homeobox proteins Pax6 and Nkx2-2 and the genes *Nkx2-9* and *Shh* (*sonic hedgehog*) had kept their normal levels and dorso-ventral boundaries of expression in homozygous versus heterozygous mutants (**Fig. 3b**). Similarly, the pattern of Phox2b expression was unchanged: it was in the process of fading out from neuroepithelial cells, while being maintained in postmitotic BM/VM precursors (**Fig 3b**). Finally, the exclusion from pMNv of *Neurog1* (also known as *Ngn1*), *Neurog2* (*Ngn2*) and *Neurog3* (*Ngn3*), the three other known proneural bHLH genes expressed in the basal plate, was maintained in homozygous mutants (**Supplementary Fig. 1** online). This latter observation raised the possibility that neurogenesis stops altogether after E10.5 in pMNv of *Ascl1* mutants.

Proneural role of *Ascl1* in the pMNv domain

To assess the production of postmitotic neuronal precursors from pMNv in *Ascl1* mutants, we used *in situ* hybridization with the gene encoding βIII-tubulin (*Tubb3*), a pan-neuronal marker. In heterozygous mutants at E10.75 (right after the BM/VM to 5-HT switch), a small cluster of Phox2b^{-}/*Tubb3*$^{+}$ 5-HT precursors had formed. These precursors were just dorsal to the floorplate in r1 and, more caudally, were sandwiched between the pMNv neuroepithelial domain on the ventricular side and postmitotic Phox2b^{+} BM/VM precursors on the pial side (**Fig. 4a**). By E11.5, this cluster had increased in size (**Fig. 4b**). In homozygous *Ascl1* mutants, it was not detectable at any rostro-caudal level, neither at E10.75 nor at E11.5, with the ventral-most postmitotic cells in r5 being Phox2b^{+}



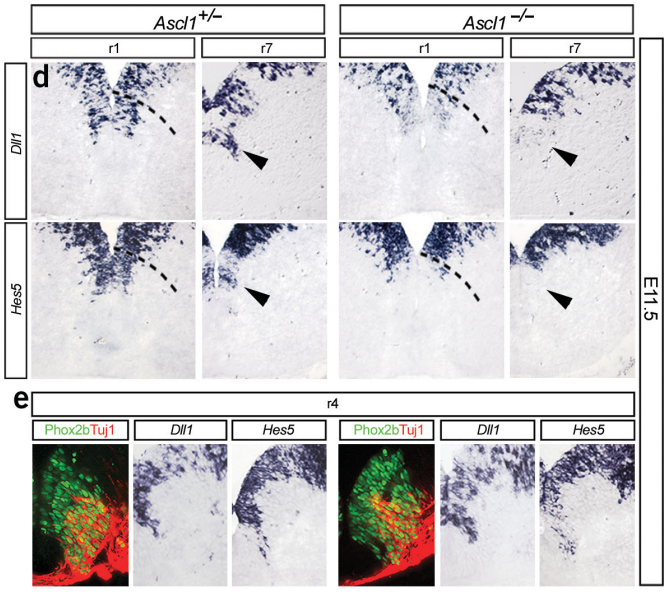
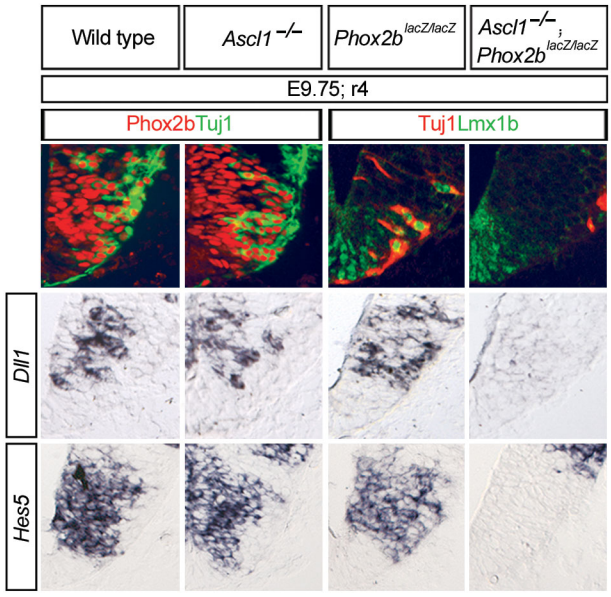


Figure 4 Neurogenesis stops after E10.5 in pMNv of *Ascl1*$^{-/-}$ mutants. (**a**) E10.75 and (**b**) E11.5 embryos. The ventral cluster of *Tubb3*$^{+}$ cells that is visible in wild-type r1 (dorsal to the floorplate and ventral to the broken line) and the cluster of Phox2b^{-}/*Tubb3*$^{+}$ cells in r5 on the pial side of pMNv (arrowhead) is absent in the homozygous mutant. (**c**) The neuroepithelium, labeled by PCNA immunohistochemistry (double arrows), is narrower at the level of ventral r1 (black double arrow) in heterozygous mutants than it is in homozygous ones. The adjacent mantle layer, which is normally occupied by *Tubb3*$^{+}$ cells and is visualized, together with the neuroepithelium, by DAPI staining, is replaced in *Ascl1*$^{-/-}$ mutants by an acellular zone (asterisks). The thin white outline in the DAPI-stained sections delineates the ventral edge of the neural tube. (**d**) The salt-and-pepper pattern of *Dll1* and *Hes5* expression at E11.5, which is visible in heterozygous mutants in ventral r1 (ventral to the broken line in left panels) and in pMNv of r7 (arrowhead in right panels), is markedly reduced and absent, respectively, in homozygous mutants. (**e**) Cross-sections through pMNv in r4 at the same stage show that the ongoing production of facial motor neurons, as reflected by the expression of Phox2b, *Dll1* and *Hes5*, is unaffected in an *Ascl1*$^{-/-}$ background.



Summary

	Wild type	*Ascl1* −/−	*Phox2b* −/−	dKO
Neurogenesis:	+	Grossly normal	Reduced	Blocked
Neuronal identity:	VM/BM	VM/BM	5-HT (premature)	-

Figure 5 Proneural activities of *Phox2b* and *Ascl1* in pMNv at E9.75. Immunohistochemistry with Tuj1 (βIII-tubulin) and Phox2b or Lmx1b at the level of r4 pMNv (top row), show that pMNv produces Phox2b$^+$ motor neurons in wild-type and *Ascl1* mutants, Lmx1b$^+$ 5-HT neurons in *Phox2b* mutants and no neurons at all in *Phox2b*/*Ascl1* double mutants. *In situ* hybridization with *Dll1* and *Hes5* (middle panels) showing that their salt-and-pepper expression is preserved in single *Ascl1* or *Phox2b* mutants but is abrogated in double mutants. Summary table (bottom row) of the type and amount of neurogenesis from pMNv before E10 in single and double mutants in r2–r7.

BM/VM precursors (**Fig. 4a**,**b**). In the mutants, the space normally occupied by postmitotic 5-HT precursors was filled by an enlarged neuroepithelium and an acellular gap (as shown by the absence of *Tubb3* expression or DAPI staining; **Fig. 4c**). This gap was filled by fibers (data not shown). We then examined the expression of two Notch signaling components, *Dll1* and *Hes5*, whose expression in the neuroepithelium depends on proneural activity. Dll1, the earliest known marker of cell cycle exit in the neuroepithelium, is a ligand of Notch and is probably a direct transcriptional target of proneural proteins[15], whereas Hes5, an effector of Notch signaling, is induced when newborn neurons activate Notch in neighboring cells[16]. *Dll1*, which is normally expressed in a salt-and-pepper pattern throughout the ventricular zone[17], was almost undetectable in the homozygous *Ascl1* mutants at the times and locations of 5-HT neuron generation, and *Hes5* was abolished altogether ventrally (**Fig. 4d**). Therefore, in *Ascl1* mutants, neurogenesis fails ventrally in r1 and, after the extinction of *Phox2b*, in pMNv of r2–r3 and r5–r7.

BM/VM neurons are grossly normal in *Ascl1*$^{-/-}$ embryos[14], indicating that neurogenesis proceeds in the mutants during the period of BM/VM neuron production. Accordingly, in r4, the only rhombomere where *Phox2b* remains on after E10.5 in pMNv, neurogenesis was unperturbed in *Ascl1* mutants at E11.5, as assessed by the expression of βIII-tubulin, *Dll1* and *Hes5* (**Fig. 4e**). Similarly, 2 d earlier (while BM/VM neurons are still being produced at all rostro-caudal levels), βIII-tubulin, *Dll1* and *Hes5* expression were grossly intact in *Ascl1*$^{-/-}$ embryos throughout r2–r7 (**Fig. 5** and data not shown). This *Ascl1*-independent neurogenesis was driven by *Phox2b*, because in *Phox2b*/*Ascl1* double mutants, *Dll1* expression was barely detectable, *Hes5* was undetectable and no postmitotic (βIII-tubulin$^+$) cell was born from pMNv (**Fig. 5** and data not shown). The spatial extent of *Shh* expression was unchanged (data not shown), arguing that pMNv, which was rendered non-neurogenic by the combined inactivation of *Phox2b* and *Ascl1*, had not acquired a floorplate identity. Together, these data show that between E9.5 and E12.5, *Phox2b* and then *Ascl1* are essential for any postmitotic precursor to arise from pMNv (see Discussion).

Type-specification role of *Ascl1* in the pMNv domain

We then asked whether *Ascl1*, in addition to driving pMNv neuroepithelial cells to become postmitotic, specifies them as 5-HT precursors. We first examined whether *Ascl1* could ectopically trigger 5-HT differentiation. *Ascl1*, when expressed in the chick spinal cord after electroporation, is capable of switching on *Gata3* expression[18], demonstrating that *Gata3* (a marker of 5-HT neurons, although not a specific one) is a direct or indirect target of *Ascl1*. Electroporation of *Ascl1*, either alone or in combination with *Nkx2-2* in chick, failed, however, to switch on other 5-HT markers such as *Pet1* and *Lmx1b* or 5-HT synthesis itself (data not shown).

To uncouple the proneural action of *Ascl1* from a putative specification function, we examined the progeny of the pMNv domain in embryos whose *Ascl1* coding sequences had been replaced by those of the bHLH gene *Neurog2*[19]. Indeed, *Neurog2* and *Ascl1* have equivalent proneural but divergent neuronal type-specification capacities, for example in the cortex[20]. At E14, after all 5-HT differentiation has normally taken place, there were very few 5-HT cells (about 15% of that observed in wild type) in these homozygous knock-in (*Ascl1*^{KINeurog2}) embryos at any rostro-caudal level (**Fig. 6a**). Thus, *Neurog2* has a very limited ability to rescue 5-HT differentiation in an *Ascl1*-null background. To ascertain whether the persistent defect in 5-HT differentiation was due to a persistent defect in neurogenesis, we examined pan-neuronal markers at E11.5. A wild-type salt-and-pepper pattern of *Dll1* expression was restored in pMNv and ventral r1 of *Ascl1*^{KINeurog2} embryos (**Fig. 6b**), indicating that neurogenesis was taking place at a normal rate. Indeed, *in situ* hybridization for the gene encoding βIII-tubulin showed that a normal-sized cluster of postmitotic precursors had accumulated immediately adjacent to the floorplate in r1 (**Fig. 6c**; compare with **Fig. 4b**). Very few of those cells, however, expressed *Gata3* (**Fig. 6d**) or *Pet1* (**Fig. 6e**). Thus, although *Neurog2* shows the same proneural activity as *Ascl1* when misexpressed in pMNv, it lacks most of its 5-HT-specification ability. Surprisingly, *Neurog2* rescued to a somewhat larger, if very partial, extent the expression of Lmx1b, another early marker and determinant of 5-HT differentiation (**Fig. 6f**). Most of these Lmx1b$^+$ cells failed to synthesize 5-HT (**Fig. 6f**), as expected from the fact that they expressed neither *Gata3* nor *Pet1*.

We verified whether the lack of 5-HT differentiation in *Ascl1*^{KINeurog2} embryos was paralleled by an abnormally prolonged BM/VM neuron production. In r2 at E11 (half a day after the termination of BM/VM and at the peak of 5-HT neuron production in wild-type embryos), *Phox2b* expression had been switched off in pMNv in *Ascl1*^{KINeurog2} embryos, as was seen in wild-type embryos and in *Ascl1*$^{-/-}$ embryos (**Fig. 6g**). Moreover, a cluster of Phox2b$^-$, *Tubb3*$^+$ (hence postmitotic) neurons had accumulated at the ventricular side of the last Phox2b$^+$ BM/VM neurons produced, as was seen in wild-type embryos (**Fig. 6g**). Therefore, the neurons produced from *Ascl1*^{KINeurog2} pMNv after the normal window of BM/VM neuron production are not extra BM/VM neurons. Finally, we note that

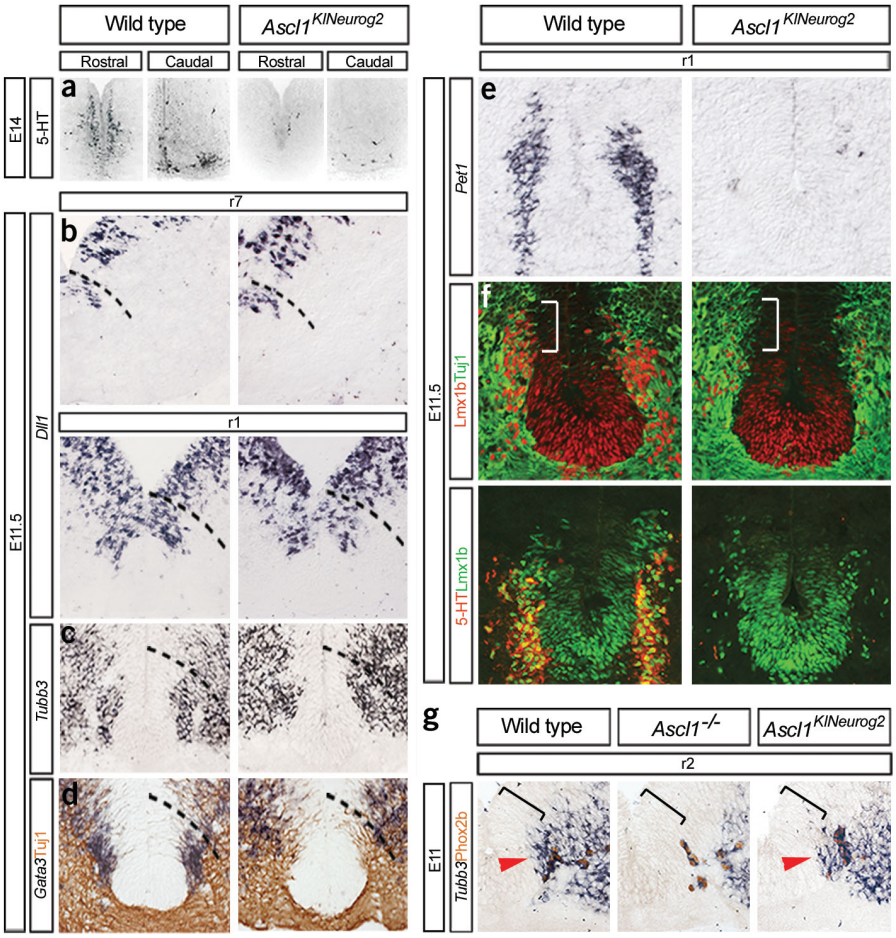


Figure 6 *Neurog2* can rescue the neurogenic but not the serotonergic deficit of *Ascl1* mutants. (**a**) Immunostaining for serotonin at E14. Few 5-HT neurons are generated in *Ascl1*^{KINeurog2} embryos as compared with wild-type embryos. (**b**) *In situ* hybridization with *Dll1*. Proneural activity in pMNv of r7 and ventral r1 is similar in wild-type and *Ascl1*^{KINeurog2} mutants. (**c**–**e**) *In situ* hybridizations at the level of r1. A normal accumulation of postmitotic progeny occurs in *Ascl1*^{KINeurog2} mutants as compared with wild type, as shown in **c**, but these progeny have lost expression of *Gata3*, as shown in **d**, and *Pet1*, as shown in **e**. (**f**) Co-immunostaining for Lmx1b and βIII-tubulin or 5-HT. The production of Lmx1b$^+$ precursors from ventral r1 (bracketed) in *Ascl1*^{KINeurog2} mutants is severely reduced as compared with wild type. In addition, all ventral Lmx1b$^+$ precursors synthesize 5-HT in wild-type embryos, whereas almost none synthesize it in the mutants. Note that the floorplate expresses Lmx1b. (**g**) Cross-sections through r2 at E11. Phox2b has already been downregulated in pMNv (bracket) of *Ascl1*^{KINeurog2}, similar to wild-type and *Ascl1*$^{-/-}$ embryos, and the neurons that have accumulated on the ventricular side of the last BM/VM neurons (arrowhead) are Phox2b$^-$ in *Ascl1*^{KINeurog2}, as they are in wild type.

the extinction of *Gata3* (normally expressed also in V2 interneurons, which dorsally abut 5-HT neurons in r1–r3) demonstrates that there is no dorsalization of neuronal fate by the ventral ectopic expression of *Neurog2* (**Fig. 6d**).

Position of *Gata3* relative to other 5-HT determinants

The *Ascl1* dependence of *Gata3* (together with all other known determinants of 5-HT precursors) led us to examine its involvement in 5-HT differentiation. We analyzed *Gata3* homozygous mutants (which normally die at around E10) that were rescued throughout the phase of 5-HT neuron generation by noradrenergic agonists[21]. In agreement with the analysis of *Gata3*$^{-/-}$/wild-type chimeras[9], there was a drastic reduction (80%) in 5-HT$^+$ neurons in the caudal-most region of the medulla, even though 5-HT precursors had been generated in normal numbers as detected by the expression of *lacZ* from the *Gata3* locus (**Fig. 7a**,**b**). Within the medulla, there was a gradient of diminishing requirement for *Gata3* from caudal to rostral, with only a 30% reduction in 5-HT differentiation in the pons in the absence of *Gata3* (**Fig. 7a**,**b**). Unexpectedly, the expression of *Pet1* and *Lmx1b* was virtually unchanged in *Gata3* mutants, even at caudal levels (r8) where few 5-HT$^+$ neurons arose (**Fig. 7**). This shows that 5-HT precursors have not globally switched to another identity and that *Gata3*, whose onset of expression is independent of both *Lmx1b*[11] and *Pet1*[8], does not control them either. Therefore *Gata3* is required downstream of *Ascl1* for 5-HT differentiation, in a pathway parallel to those of *Pet1* and *Lmx1b*.

DISCUSSION

Ascl1 is essential for noradrenergic differentiation in the CNS[14] and in the peripheral nervous system[12,22]. We now show that *Ascl1* is also a general determinant of another aminergic neurotransmitter phenotype, as it is required for the differentiation of central 5-HT neurons in addition to enteric 5-HT neurons[12] and the parafollicular cells of the thyroid[13].

A complex picture of the transcriptional control of neuronal differentiation in the pMNv neuroepithelial domain, from which 5-HT neurons arise, emerges from this and previous studies (**Supplementary Fig. 2** online). In a first phase from E9 to E10.5 (until E12.5 in r4), *Phox2b*—induced by *Nkx2-2*[23] and possibly *Nkx2-9*[24]—ensures the promotion of BM/VM identity[25], the repression of 5-HT identity[7] and the very production of postmitotic neurons (refs. 25,26 and **Fig. 5**). *Ascl1* is coexpressed with *Phox2b*[25] but its type-specification function (**Fig. 6**) is suppressed by *Phox2b* (ref. 7 and **Fig. 5**), whereas its proneural one[15] is largely dispensable (ref. 14 and **Fig. 5**) and is revealed only upon inactivation or downregulation of *Phox2b* (ref. 25 and **Fig. 5**). Comparison of single and double knockouts (**Fig. 5**) further shows that for any postmitotic neuronal production to occur in pMNv, either *Ascl1* or *Phox2b* is required. This places the homeobox gene *Phox2b* at the same operational level as conventional bHLH proneural genes in the transcriptional cascade of neural differentiation.

At around E10.5, *Phox2b* is downregulated in pMNv of r2–r3 and r5–r7 (ref. 7) and BM/VM neuron production switches to 5-HT neuron production, during which *Ascl1* is involved in type specification and proneural activity (**Figs. 4** and **6**). Both actions are mechanistically distinct because the latter but not the former can be carried out by *Neurog2* (**Fig. 6**). The situation is thus reminiscent of the inability of *Neurog2* to replace *Ascl1* function in the differentiation of spinal V2 interneurons and of the locus coeruleus[19], although generic and type-specific postmitotic differentiation could not be analyzed separately in these cell types. In contrast, *Neurog2* rescues[19] the early differentia-

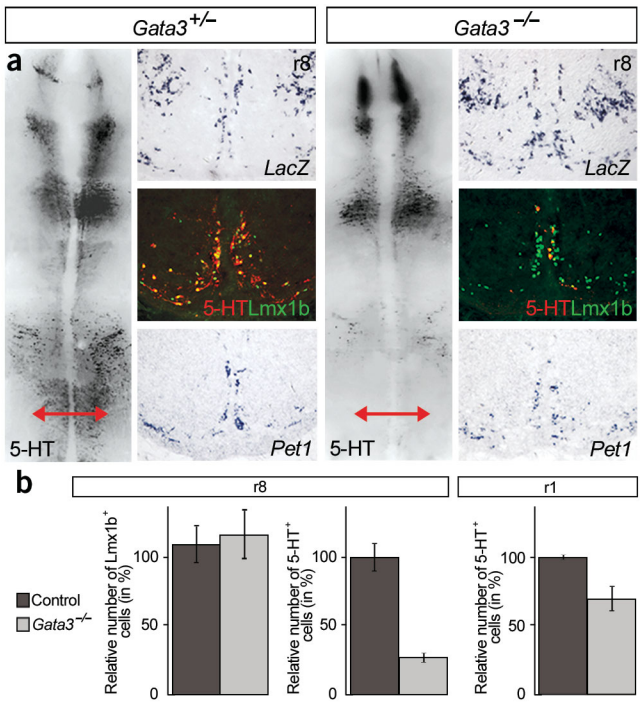


Figure 7 *Gata3* controls 5-HT differentiation independently of *Lmx1b* and *Pet1*. (**a**) Flatmounts (left) and cross-sections caudally through r8 (indicated by a double arrow on the flatmount; right) at E13.5 immunostained for 5-HT and Lmx1b or hybridized with *lacZ* (expressed from the *Gata3* locus) or *Pet1* in *Gata3* heterozygous and homozygous mutants. The synthesis of 5-HT in the caudal medulla of the mutants is greatly reduced even though 5-HT precursors (detected by *lacZ*) are generated that express Lmx1b and *Pet1*. (**b**) Quantification of Lmx1b$^+$ and 5-HT$^+$ neurons in r8 and of 5-HT$^+$ neurons in r1. Control, *Gata3* heterozygous embryos.

tion defect of *Ascl1*$^{-/-}$ noradrenergic sympathetic precursors[14,27], leaving uncertain whether *Ascl1* is involved in type specification in this lineage. The ability of ectopic *Neurog2* to activate one downstream determinant of the 5-HT phenotype (*Lmx1b*) slightly more efficiently than others (*Pet1* or *Gata3*) in pMNv indicates that *Ascl1* may normally cooperate with different transcription factors on the promoters of different type-specific target genes.

Loss-of-function experiments have established that *Nkx2-2*, in addition to *Ascl1*, is required in the neuroepithelium to specify the 5-HT phenotype[6,8]. Because *Ascl1* is also expressed with *Nkx2-2* in non-serotonergic progenitors of the spinal p3 domain (data not shown) and fails, even when ectopically coexpressed with *Nkx2-2*, to elicit 5-HT differentiation (data not shown), at least a third transcriptional determinant has to be posited to explain the restriction of 5-HT differentiation to ventral r1 and pMNv. At least three transcription factors lie downstream of *Ascl1*, *Nkx2-2* and this unknown factor. These three factors, *Gata3*, *Lmx1b* and *Pet1*, are switched on in postmitotic precursors and are all required for 5-HT synthesis[8–11]. They do not constitute a transcriptional cascade, as previously proposed[8], but rather are switched on in parallel (ref. 11 and **Fig. 7**). This scheme, however, is complicated by several observations. First, a degree of cross-regulation is suggested by the fact that *Pet1* and *Gata3* expression are secondarily lost in *Lmx1b* mutants (although the continuing presence of the cells was not assessed)[8]. Second, there is partial redundancy between these factors (or, alternatively, a cryptic heterogeneity of the 5-HT population), which is demonstrated by the loss of only 70% of 5-HT cells in *Pet1*-null mutants[10]. Third, there is a rostro-caudal gradient in the role of some transcription factors: *Nkx2-2* is not required in r1 (ref. 8), possibly because of redundancy with *Nkx2-9*[6], and the requirement for *Gata3* varies from moderate (rostrally) to strong (caudally; ref. 9 and **Fig. 7**). One may invoke partial redundancy with the highly related *Gata2* factor, which is expressed with *Gata3* in 5-HT precursors (**Fig. 2**) and is known to control *Gata3* expression in at least some neuronal types[28]. As for *Nkx2-2* and *Nkx2-9*, this redundancy could vary along the rostro-caudal axis. Unfortunately, *Gata2* knockout mice die from hematopoietic failure around the time of 5-HT neuron generation[29], making the involvement of this gene difficult to assess.

The 5-HT phenotype has become one of the best-understood neurotransmitter phenotypes at the transcriptional level. Still, the architecture of the underlying genetic network is far from elucidated. For example, it is unclear to what extent individual postmitotic determinants require the full combination of upstream progenitor factors (*Gata3*, for one, can be ectopically induced by *Ascl1* alone[18]). Downstream, the mode of convergence of the three (possibly more) postmitotic determinants on the individual promoters of effector genes remains to be established.

METHODS

Genotyping and maintenance of mutant mice. *Phox2b*, *Ascl1*, *Gata3* and *Ascl1*^{KINeurog2} mutant mice were produced and genotyped as previously described[9,19,22,30]. *Gata3* mutants were rescued from early embryonic lethality beyond E10.5 as previously described for *Phox2b* mutants[31].

Probes and antibodies. The following antisense RNA probes were used: *Tubb3*[32]; *Dll1*[33]; *Gata2*[34]; *Gata3*[35]; *Hes5*[36]; *Ascl1*[37]; *Neurog1*, *Neurog2* and *Neurog3*[38]; *Nkx2-2*[39]; *Nkx2-9*[40] and *Pet1* (kind gift of T. Jessell, Columbia University, New York). The antibodies used were raised against Phox2a[41], Phox2b[42], Lmx1b[43], Pax6 (kind gift of V. van Heyningen, MRC, Edinburgh), Tuj1 (antibody to βIII-tubulin; Covance), proliferating-cell nuclear antigen (PCNA; Chemicon) and 5-HT (Sigma).

***In situ* hybridization and immunohistochemistry.** These were carried out as described[41]. Double immunofluorescence experiments were analyzed on a TPS/SP2 Leica confocal microscope and pictures were superimposed in Photoshop (Adobe).

Whole-mount immunofluorescence. E13 or E14 embryos were fixed overnight at 4 °C in 4% paraformaldehyde/PBS. Brains were dissected out and incubated in PBS/10% FCS/0.1% Triton X-100 for 4 h. They were then incubated overnight at 4 °C with a rabbit antibody to 5-HT (Sigma; diluted 1:6,000), washed eight times for 1 h in blocking buffer at room temperature and then incubated overnight at 4 °C with a Cy3-conjugated anti-rabbit antibody (Jackson; diluted 1:1,000). They were washed for several hours at room temperature in blocking buffer and flat mounted in Vectashield mounting medium. The photographs in **Figures 2** and **7** are composites assembled in Photoshop from the negative of three black-and-white pictures taken using a Leica microscope.

Cell counts. 5-HT$^+$ cells in r1 and Lmx1b$^+$ and 5-HT$^+$ cells in r8 were counted on transverse sections through the pons (r1) and the caudal medulla (r8) of E13.5 wild-type embryos ($n = 8$ sections for r1 and $n = 14$ sections for r8, from two embryos) and *Gata3*$^{-/-}$ mutants ($n = 8$ sections for r1 and r8, from two embryos). The rostro-caudal level r8 was operationally defined in wild-type embryos as the caudal-most region of the medulla in which virtually all ventral Lmx1b$^+$ cells were 5-HT$^+$. Values are means ± s.e.m.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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