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Distinct requirements for *Ascl1* in subpopulations of midbrain GABAergic neurons

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ABSTRACT

Midbrain GABAergic neurons regulate multiple aspects of behavior and play important roles in psychiatric and neurological disease. These neurons constitute several anatomical and functional subpopulations, but their molecular heterogeneity and developmental regulatory mechanisms are poorly understood.

Here we have studied the involvement of the proneural gene *Ascl1* in the development of the midbrain GABAergic neurons. Analysis of Ascl1 mutant mice demonstrated highly region-specific requirements for *Ascl1* for development of different GABAergic neuron subpopulations. Ascl1 is dispensable for the development of the ventral-most midbrain GABAergic neurons associated with dopaminergic nuclei substantia nigra pars reticulata (SNpr) and ventral tegmental area (VTA) GABAergic neurons. In the ventrolateral midbrain, loss of *Ascl1* results in markedly delayed neurogenesis in the midbrain domains m3–m5. Within this region, Ascl1 has a unique role in m4, where it also regulates glutamatergic neurogenesis. Our results suggest that the m3–m5 midbrain neuroepithelium gives rise to the GABAergic neuron groups located in the midbrain reticular formation and ventrolateral periaqueductal gray. In contrast to m3–m5, *Ascl1* is absolutely required in the dorsal midbrain domains m1–m2, for generation of the GABAergic neurons populating the superior and inferior colliculi as well as dorsal periaqueductal gray.

These studies demonstrate different molecular regulatory mechanisms for the distinct midbrain GABAergic neuron subpopulations. Also, our results have implications on understanding the origins of the various midbrain GABAergic neuron groups in the embryonic neuroepithelium.

Introduction

GABAergic neurons are the primary inhibitory neurons in the mature brain. Midbrain GABAergic neurons are important components of neuronal circuitries regulating multiple aspects of behavior. They can be divided into anatomical and functional subpopulations. The ventrally located GABAergic neurons in the SNpr and VTA are involved in regulation of the dopaminergic pathways. These neurons are thought to act as both local interneurons controlling dopaminergic neuron activity and projection neurons contacting other brain regions (Laviolette and van der Kooy, 2004; Fields et al., 2007; Tepper and Lee, 2007). As a result, the SNpr and VTA GABAergic neurons are very important in the regulation of motor activity, sensing reward and motivation. GABAergic neuron subpopulations located in the medial midbrain include the deep mesencephalic reticular formation, a functionally and biochemically incompletely analyzed group of neurons, and periaqueductal gray, which regulates fear and aggression. The most dorsal GABAergic neurons are located in the superior and inferior colliculi and are involved in functions such as the processing and integration of sensory information.

In contrast to their functional diversity, rather little is known about the molecular and developmental heterogeneity of the GABAergic neuron subpopulations. In the mouse, both ventro-lateral and dorsal regions of the embryonic midbrain produce GABAergic neurons at E10.5–E14.5 (midbrain dorso-ventral domains m1–m5) (Tsunekawa et al., 2005; Nakatani et al., 2007; Kala et al., 2009). Transcription factors (TFs) Ascl1, Helt and Gata2 have been shown to be more or less selectively required for the development of midbrain GABAergic neurons. Zinc-finger transcription factor Gata2 is a post-mitotic selector gene of the GABAergic neurotransmitter phenotype specifically in the midbrain (Kala et al., 2009). Tissue-specific inactivation of *Gata2* in the midbrain-rhombomere 1 (r1) region (*Gata2*^{cko}) results in the complete transformation of the midbrain-derived post-mitotic GABAergic neuron precursors into a glutamatergic fate. In a striking contrast, r1 GABAergic neurons develop normally in *Gata2*^{cko}, although Gata2 also is expressed in the wild-type r1. Interestingly, despite the phenotypic transformation during midbrain neurogenesis, the most ventral GABAergic neurons associated with the dopaminergic nuclei in the mature SN and VTA are still found in the *Gata2*^{cko} mutants at birth. This suggests a distinct developmental regulatory mechanisms for the SNpr and VTA GABAergic neurons (Kala et al., 2009).

Basic helix–loop–helix (bHLH) transcription factor Helt is required for Gata2 expression in the midbrain, except for the ventrolateral m5 domain (Kala et al., 2009). Consistently, GABAergic neurons are only produced in the m5 domain of the *Helt* mutant embryos (Guimera et

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al., 2006b; Nakatani et al., 2007). In addition to the ventral GABAergic neurons of SNpr and VTA, some more medial GABAergic neurons of the midbrain reticular formation also develop in the *Helt* mutants. *Helt* is not expressed in the r1 and, consistently, it is not required for GABAergic neurogenesis there.

The proneural bHLH transcription factor *Ascl1* appears to regulate midbrain GABAergic neurogenesis together with *Helt* and *Gata2*, but through another pathway (Miyoshi et al., 2004; Kala et al., 2009). Similar to its role in the ventral forebrain, *Ascl1* regulates Delta-Notch signaling in the developing midbrain (Casarosa et al., 1999). In the forebrain, there are different requirements for *Ascl1* in medial and lateral ganglionic eminences but although defective, GABAergic neurogenesis can still take place without *Ascl1* (Casarosa et al., 1999). In contrast, *Ascl1* was reported to be essential for the development of midbrain GABAergic neurons (Miyoshi et al., 2004).

Here we have analyzed the requirement for *Ascl1* in development of distinct midbrain GABAergic neuron subpopulations in more detail. Our results demonstrate highly region-specific requirements for *Ascl1* in the midbrain GABAergic neurogenesis, further underscoring the developmental heterogeneity of the midbrain GABAergic neurons. Our results also have implications for understanding the developmental origins of these cells.

Materials and methods

Mice

Wild-type and homozygous $Ascl1^{KO}$ embryos were obtained from intercrosses of *Ascl1* +/- mice (Guillemot et al., 1993). $Ascl1^{KO}$ and $Gata2^{cko}$ mutant mice were genotyped as previously described (Guillemot et al., 1993; Kala et al., 2009). For staging, the day of vaginal plug was counted as embryonic day 0.5 (E0.5). All experiments were approved by the Committee of Experimental Animal Research of the University of Helsinki, Finland.

In situ mRNA hybridization

For *in situ mRNA hybridization* (ISH) on sections embryos were fixed in 4% paraformaldehyde (PFA) at room temperature for 2–7 days. Samples were dehydrated and mounted into paraffin. 5 µm adjacent sections were cut and collected on separate slides for parallel stainings. mRNA ISH analyses on paraffin sections were performed as described previously (Wilkinson and Green, 1990) using ^{35}S-labeled RNA probes. Mouse cDNA probes used for ISH analysis were: *Gad1 (Gad67), Slc17a6 (Vglut2)* (Guimera et al., 2006), *Ascl1, Ngn2* (Jukkola et al. 2006), Nkx2.2 (IMAGE clone 480100), *Delta1* (IMAGE p968BO7112D6), *Hes5* (Gift from I. Thessleff) and *Ngn1* (IMAGE p968GO3104D).

Immunohistochemistry

For BrdU-incorporation analysis, pregnant females were given intraperitoneal injection of BrdU (3 mg/100 g body weight; GE Healthcare) 1 h before dissecting the embryos.

For immunohistochemistry embryos were fixed for 2–7 days in 4% PFA at room temperature, dehydrated and embedded in paraffin. Embryos were sectioned coronally at 5 µm. After deparaffination, the antigen retrieval was done by heating the sections in 10 mM sodium-citrate buffer, pH 6.0, in a microwave oven for 12 min. The sections were

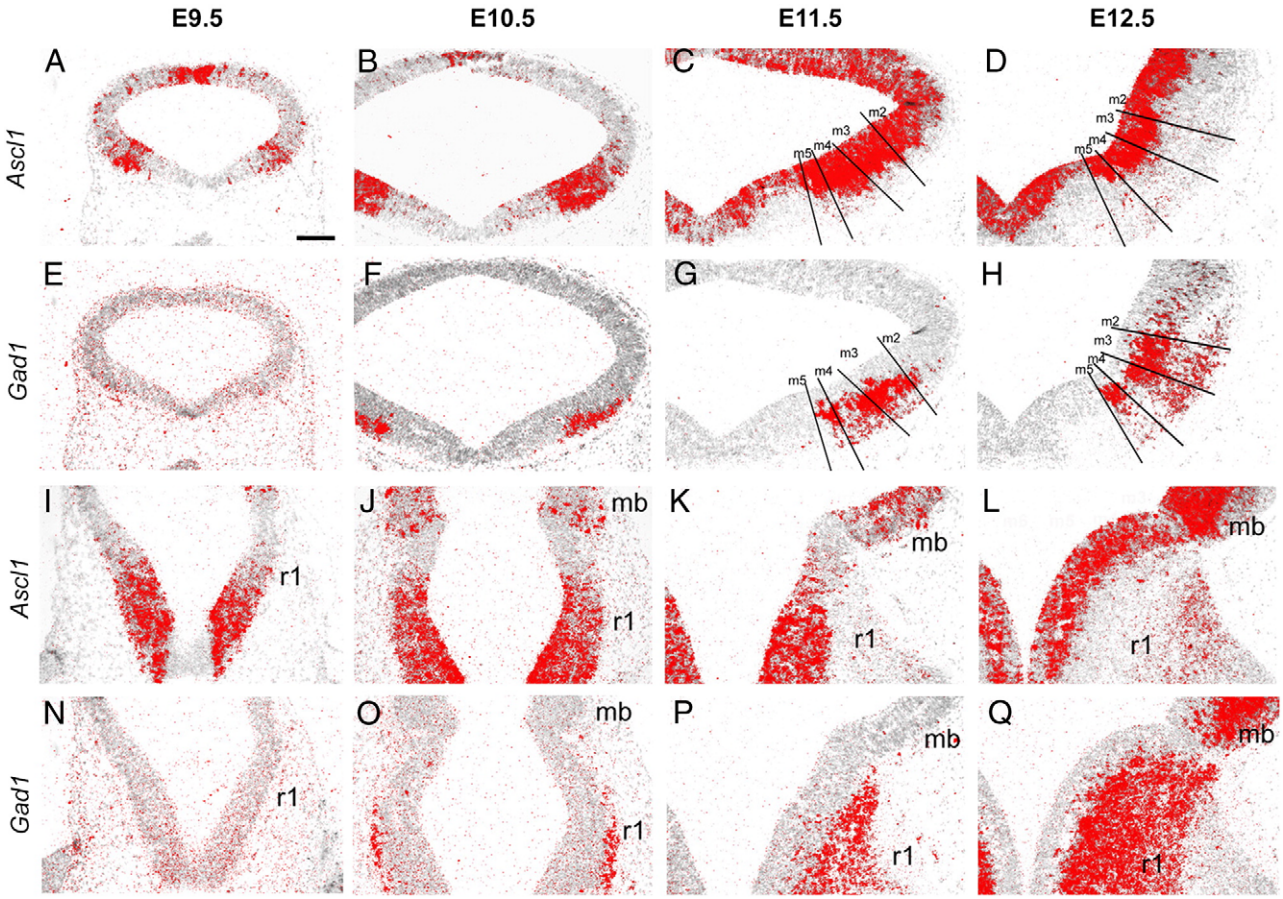

Fig. 1. Expression of Ascl1 correlates with GABAergic neurogenesis in the midbrain and rhombomere 1. *Ascl1* and *Gad1* expression was analyzed by in situ hybridization (ISH) on coronal sections from E9.5 to E12.5 wild-type midbrain (A–H) and r1 (I–Q). The borders of midbrain domains m2–m5 were deduced from immunohistochemistry (IHC) staining with Nkx2.2 and Nkx6.1 on parallel section (not shown). r1, rhombomere 1; mb, midbrain. Scale bar 100 µm.

washed in TBT (0.3% Triton-X) and blocked for 60 min in Blocking solution (TBS + 4% BSA + 10% goat serum + 0.1% Triton-X). The samples were incubated in the primary antibody in TBS + 2% BSA + 0.1% Triton-X 1–3 nights at +4 °C, rinsed in TBT (0.1% Triton-X) and placed in the secondary antibody in TBS + 2% BSA + 0.1% Triton-X for 3 h at RT. Sections were rinsed twice in TBS and stained with DAPI, rinsed again in TBS and mounted in Mowiol.

Antibodies used for immunohistochemistry were: rabbit-anti Sox2 (1:400 Millipore AB5603), guinea pig anti-Heslike (Helt 1:400; gift from R. Kageyama, Institute for Virus Research, Kyoto University, Japan), mouse anti-BrdU (1:400,Ge Healtcare RPN20AB) mouse anti-HuC/D (1:800, Molecular probes A21271), mouse anti-Lim1/2 (1:100, Developmental Studies Hybridoma Bank (DSHB) 4F2), mouse anti-Nkx2.2 (1:400, DSHB 74.5A5), mouse anti-Nkx6.1 (1:400, DSHB F55A10), mouse anti-Pax6 (DSHB), rabbit anti-caspase (R&D systems AF835), mouse anti-Gata2 (1:400, SantaCruz sc-9008), rabbit anti-p57 (1:400, Neomarkers RB-1637-P0), mouse anti-p27 (1:800, BD Transduction laboratories 610241), rabbit anti-p27 (1:100, Thermo Scientific Rb-006-P0), mouse anti-Pou4f1 (1:400, Santa Cruz sc-8429 Clone nr. 14A6), rabbit anti-cyclinD1 (1:400, Lab-vision RM-9104-SO), mouse anti-TH (1:400, Chemicon MAB318). Goat anti-rabbit IgG (1:400, Alexa-488 and Alexa-568, Invitrogen), goat anti-mouse IgG (1:400, Alexa-488 and Alexa-568, Invitrogen) were used as secondary antibodies.

Microscopy

Staining on paraffin sections were visualized with an Olympus AX70 microscope with Olympus DP70 camera. Images were processed and assembled with Adobe Photoshop software. Red pseudo-color images representing the ISH results were produced by replacing the white signal in the dark-field images with red and overlaying the resulting image with the respective bright field image.

Results

Expression of Ascl1 correlates with midbrain GABAergic neurogenesis

First, we studied how *Ascl1* mRNA expression relates to GABAergic neurogenesis during midbrain and r1 development. We compared the expression of *Ascl1* and glutamic acid decarboxylase 1 (*Gad1*, GABAergic neurons) in the mouse midbrain and r1 from E9.5 to E12.5 by *in situ* hybridization (ISH). At E9.5 *Ascl1* expression was first observed in the ventrolateral midbrain and r1 region of the hindbrain (Fig. 1A, I). Subsequently, ventral expression of *Ascl1* becomes more intense between domains m5 to m2 and expands throughout the midbrain neuroepithelium both dorsally and ventrally by E11.5 (Fig. 1B–C). At the same time postmitotic GABAergic neurons start to express *Gad1* in the marginal layer, indicating GABAergic differentiation (Fig. 1F–G). At E12.5 both *Ascl1* and *Gad1* are expressed strongly in ventrolateral and dorsal midbrain and in the r1 region of the hindbrain (Fig. 1D, H, L, Q). Thus, although not absolutely specific for neuroepithelium undergoing GABAergic neurogenesis (Kim et al., 2008), strong *Ascl1* expression is detected in the midbrain and r1 regions giving rise to GABAergic neurons.

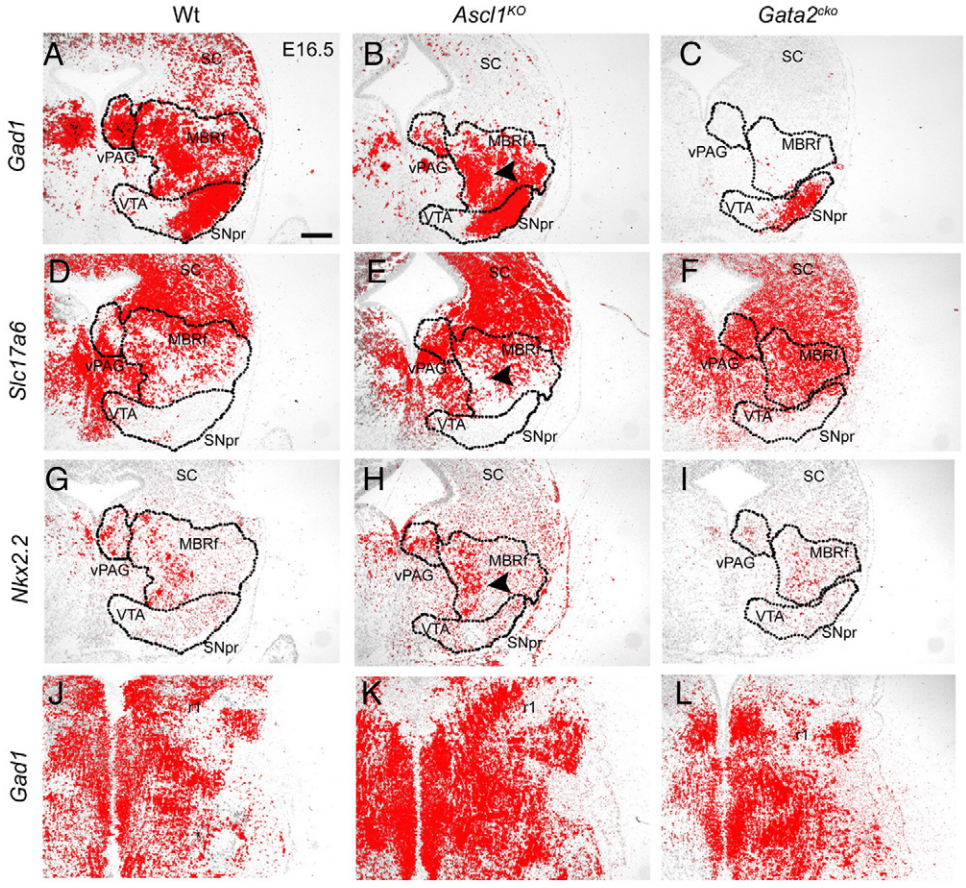

Fig. 2. Loss of specific GABAergic neuron groups in the Ascl1 mutant midbrain at E16.5. (A-I), ISH with *Gad1, Slc17a6* and *Nkx2.2* probes on adjacent coronal sections of E16.5 wild-type (Wt), *Ascl1*^{KO} and *Gata2*^{cko} midbrains. The dotted line delineates subpopulations of ventral midbrain GABAergic neurons. Black arrows (B, H) point to the Nkx2.2 positive GABAergic neurons derived from m4 region. VTA and SNpr area was deduced from TH-staining on adjacent section. (J–L) *Gad1* ISH on coronal sections from Wt, *Ascl1*^{KO} and *Gata2*^{cko} r1 region. vPAG, ventrolateral periaqueductal gray, MBRf, midbrain reticular formation, SC, superior colliculus; VTA, ventral tegmental area, SNpr, substantia nigra pars reticulata; r1, rhombomere 1. Scale bar 200 µm.

Loss of specific midbrain GABAergic neuron subpopulations in Ascl1 mutants

GABAergic neurogenesis was reported to be completely blocked in the midbrain of *Ascl1* mutant embryos (Miyoshi et al., 2004). However, in that study embryos were analyzed at E11.5, when GABAergic neurogenesis has only just initiated. We looked at GABAergic neurons in *Ascl1* mutants at E16.5–E18.5 (Fig. 2A–B, J–K and data not shown). In agreement with the study by Miyoshi et al., *Gad1* expressing GABAergic neurons were lost in the *Ascl1* mutant dorsal midbrain, including superior and inferior colliculi as well as dorsal periaqueductal gray (Fig. 2B). However, GABAergic neurons were still present in the medial and ventral midbrain. In the ventral periaqueductal gray and midbrain reticular formation, the number of GABAergic neurons was reduced compared to wild-type (Fig. 2A–B). In contrast, *Gad1* was expressed at a level comparable to wild type in *Ascl1* deficient VTA and SNpr. Also at E13.5, GABAergic neurons were produced in the ventromedial midbrain (m3–m5), but not dorsally (m1–m2; Fig. 3B). The phenotype of the *Ascl1* mutants is clearly different from the *Gata2*^{cko} mutants, in which no GABAergic neurons are found at E13.5 and only the GABAergic neurons associated with the ventral dopaminergic nuclei in VTA and SNpr are present at E16.5 (Fig. 2C). In summary, our observations of the *Ascl1* mutant phenotype indicate that different midbrain GABAergic neuron subpopulations have variable requirements for *Ascl1*.

To study the origins of the GABAergic neurons less affected by the loss of *Ascl1*, we analyzed *Nkx2.2* expression in the *Ascl1* mutant embryos. At E13.5, *Nkx2.2* is expressed in the neurons derived from m2 and m4 in wild-type embryos (Fig. 3E). We detected no *Nkx2.2* expression in m2 of the *Ascl1* mutants, correlating with the loss of GABAergic precursors in this domain (Fig. 3F, arrow). In contrast, *Nkx2.2* was still expressed in m4 in the *Ascl1* mutants. In both wild-type and *Ascl1* mutant embryos at E16.5, *Nkx2.2* was expressed in the medial part of the midbrain reticular formation as well as the ventral region of the periaqueductal gray (Fig. 2G–H). *Nkx2.2* marks both GABAergic and glutamatergic neurons derived from m4 (Kala et al., 2009). However, analyses of *Gad1* and *Vglut2* expression on adjacent sections suggested that a major proportion of the *Nkx2.2* positive neurons at least in the midbrain reticular formation are GABAergic (Fig. 2A, D, G, B, E, H and arrowhead). Thus, our results suggest that the medial subpopulation of GABAergic neurons in the midbrain reticular formation is derived from the m4 domain and some of these neurons are still generated in the *Ascl1* mutant embryos.

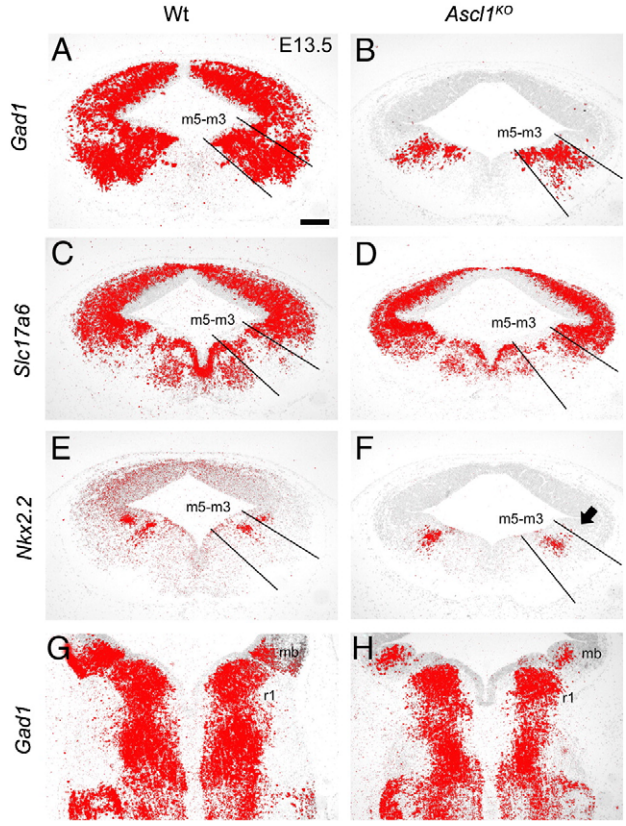

Fig. 3. Loss of specific GABAergic neuron groups in the Ascl1 mutant midbrain at E13.5. (A–F), ISH with *Gad1*, *Slc17a6* (*Vglut2*) and *Nkx2.2* probes on adjacent coronal sections of E13.5 wild-type (Wt) and *Ascl1*^{KO} midbrain. Black arrow (F) points to Nkx2.2 negative area in the *Ascl1* deficient m2, indicating the loss of GABAergic precursors in this domain. (G–H), *Gad1* expression in the r1 region of E13.5 Wt and *Ascl1*^{KO} embryos. Scale bar 200 µm.

Development of GABAergic neurons in r1 of Ascl1 mutants

Ascl1 has been shown to be expressed in the hindbrain rhombomeres (Pattyn et al., 2004). To gain insight to the possible role of *Ascl1* in the GABAergic neurogenesis in the r1 region, we analysed the expression of *Gad1* in *Ascl1* mutant embryos at E13.5 (Fig. 3G–H) and at E16.5 (Fig. 2J–K) by *in situ* hybridization. Similar to the wild-type and *Gata2*^{cko} embryos, we observed abundant *Gad1* expression in the r1 region of the *Ascl1* mutant embryos, demonstrating that *Ascl1* is not required for the GABAergic neurogenesis in the r1.

Region-specific delay in GABAergic neurogenesis in Ascl1 mutants

To understand the mechanisms how *Ascl1* regulates GABAergic neuron development in the different cell populations, we next analyzed the expression of genes characteristic to proliferative progenitors and post-mitotic precursors of the GABAergic neurons.

At E11.5, the layer of HuC/D positive post-mitotic neurons was markedly reduced in the m3–m5 of the *Ascl1* mutants (Fig. 4A–B). Conversely, the thickness of the Sox2 positive progenitor cell layer was increased, indicating a failure in cell cycle exit and onset of differentiation. Helt is a specific marker of the GABAergic progenitor cells in the ventricular zone. Consistent with earlier studies, Helt expression was still observed in the *Ascl1* mutants (Fig. 4F, Supplementary Fig. 1B, D). Notably, the relative amount of Helt positive cells was greatly increased and only few Helt negative cells could be found in the *Ascl1* mutant ventricular zone domains m3–m5 (Fig. 4J), while in the m1–m2 domains Helt expression was completely unaffected (Supplementary Fig. 1B, D). Gata2 is a Helt dependent marker of the post-mitotic GABAergic precursors, which are about to exit the ventricular zone and differentiate. Markedly fewer Gata2 positive cells could be observed in the *Ascl1* mutant midbrains at this stage (Fig. 4N). In addition to GABAergic neurons, m4 domain also gives rise to glutamatergic neurons in its ventral part. The post-mitotic precursors of these neurons are characterized by Pax6 expression (Kala et al., 2009). In the *Ascl1* mutants, the amount of m4-derived Pax6 positive precursors was reduced as compared to the WT at E11.5 (Fig. 4M–N). Thus, the progenitors of m3–m5 are severely impaired in their ability to give rise to post-mitotic precursors of both GABAergic and glutamatergic neurons at E11.5. Glutamatergic neurogenesis may also be affected in the *Ascl1* deficient dorsal midbrain as we observed a slight reduction of Pou4f1-positive cells in m2–m1 region at E13.5 (Supplementary Fig. 1D).

Interestingly, we observed a partial recovery of neurogenesis in the *Ascl1* mutant midbrain by E13.5. In m3–m5, large amounts of postmitotic precursors expressing GABAergic neuron markers Lim1 (Fig. 4G–H) or Gata2 (Fig. 4P) were observed. Also Pax6 expressing glutamatergic neuron precursors were generated from m4 (not shown). In wild-type, the GABAergic neurogenesis is already diminishing in m3–m5 at this stage, indicated by the down-regulation of Helt expression (Fig. 4G). In the *Ascl1* mutants, the characteristic salt-and-pepper expression pattern of Helt was restored in the E13.5 *Ascl1* mutants (Fig. 4L). Helt expression levels appeared to be decreasing, suggesting that the GABAergic neurogenesis ends at around the same stages in

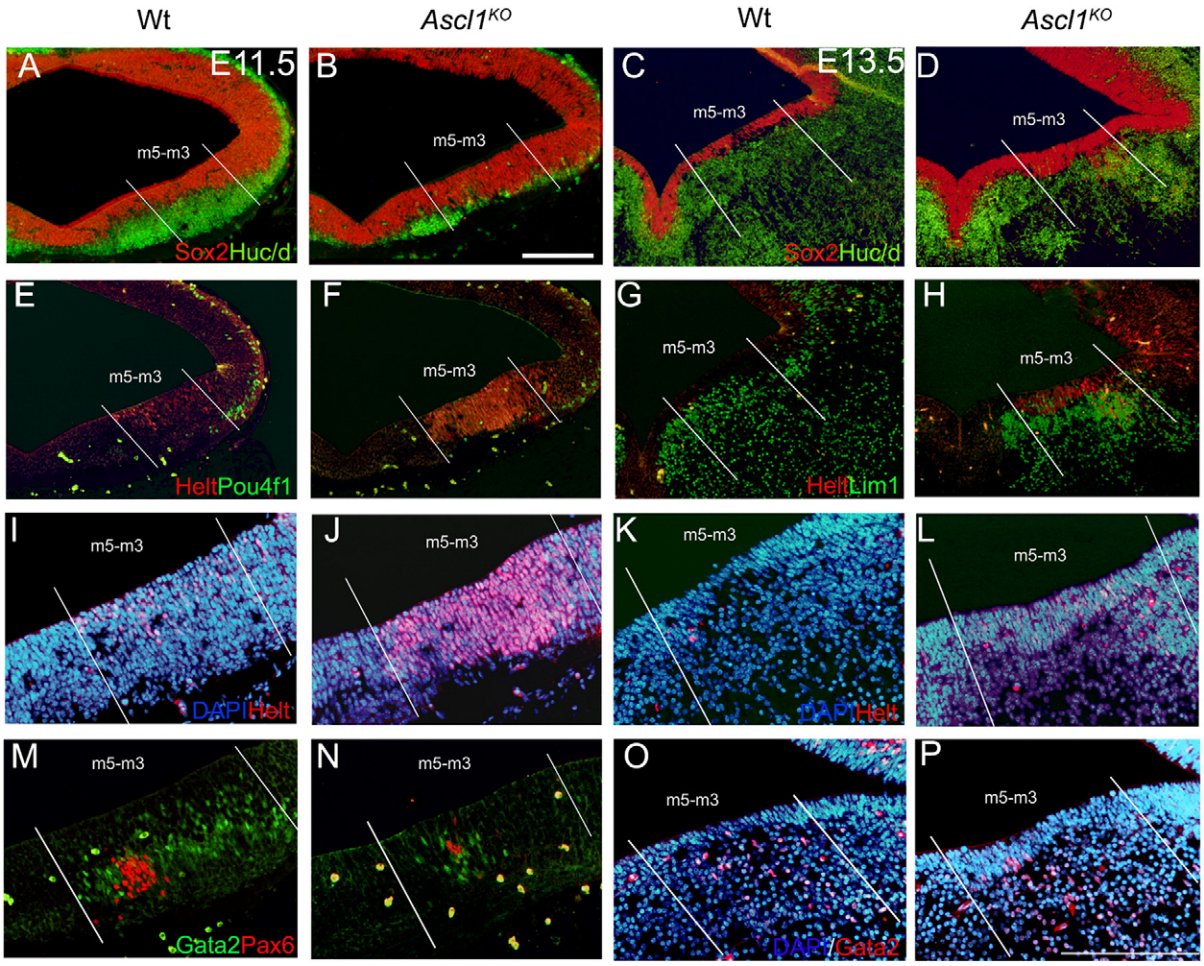

Fig. 4. Analysis of interneuron progenitor and precursor markers in the midbrain domains m3–m5. (A–D) Sox2 and HuC/D expression analyzed by co-IHC in wild-type (Wt) and *Ascl1*^{KO} embryos at E11.5 (A, B) and E13.5 (C, D). Midbrain m3–m5 borders are indicated in white lines. (E–P) Helt and Pou4f1 co-IHC (E–F), Helt (IHC, I–J), Gata2 and Pax6 (co-IHC, M–N) expression is shown in E11.5 embryos. Note the increased Helt expression levels in the Ascl1 mutant (J) and loss of the characteristic salt-and-pepper expression pattern (I). E13.5 wild-type (WT) and *Ascl1*^{KO} embryos were analyzed for Helt and Lim1 (co-IHC, G–H), Helt (IHC, K–L) and Gata2 (IHC, O–P). Scale bar 200 µm.

wild-type and *Ascl1* mutant embryos (data not shown). In conclusion, the initiation of GABAergic neurogenesis is delayed, but still takes place in midbrain m3–m5 regions without *Ascl1*.

Ascl1 regulates separation of neurogenic and neural stem cell fates in m3–m5

To study the defect in neurogenesis in more detail, we analyzed expression of genes regulating the fate selection between neural stem/progenitor cells and differentiating post-mitotic neurons. The lateral inhibition via Delta-Notch pathway is central for the control of this process, as Notch ligand Delta expressed in the differentiating neurons is stimulating Notch receptor and activating its target gene expression in the adjacent cells. One such Notch target is *Hes5*, which is important for the maintenance of the neural stem cell identity in the neuronal progenitors. *Delta* expression has been shown to be regulated by proneural genes in several regions of the CNS. Consistently, we observed rather complete down-regulation of *Delta1* expression in the m3–m5 region of *Ascl1* mutants at E11.5 (Fig. 5A–B). Correlating with the down-regulation of *Delta1*, also *Hes5* expression was abolished in the m3–m5 region (Fig. 5E–F). In contrast to m3–m5, *Delta1* and *Hes5* were still expressed in the m1–m2 region of the *Ascl1* mutants. This is likely due to glutamatergic neurogenesis driven by Ngn2/Ngn1. No compensatory up-regulation of *Ngn2* or *Ngn1* was observed in the *Ascl1* mutants (Fig. 5I–P, Supplementary Fig. 1E–H). Nevertheless, in the E13.5 embryos we observed restored *Delta1* expression in the m5–m3, although in reduced levels (Fig. 5C–D). Simultaneously *Hes5* recovered to wild-type levels in *Ascl1* mutant midbrain (Fig. 5G–H).

Unique requirements for Ascl1 in m4

In addition to regulating the Delta-Notch pathway, proneural genes have been shown to affect cell cycle regulation through interaction with cyclin-dependent kinase inhibitors. Therefore, we analyzed the proliferative cell markers as well as the expression of cyclin dependent kinase inhibitors p27, p57 and cyclin D1 in the m3–m5 of wild-type and *Ascl1* mutant embryos at E11.5. The amounts of p27 positive post-mitotic precursors were reduced in the marginal zone of m3–m5, in contrast to the ventricular zone, where p27 seemed to be up-regulated (Fig. 6C–D). Interestingly, this up-regulation was clearly more prominent in the m4 when compared to the adjacent m3 and m5 domains (Fig. 6D, arrowhead). Similarly, p57 and HuC/D expression was observed closer to the ventricular surface in the m4 (Fig. 6F, arrowhead). Consistently, cyclinD1 expression was down-regulated in the m4 domain (Fig. 6G–H, arrowhead), indicating that in m4 cells are able to exit cell cycle. Despite the more prominent expression of the postmitotic precursor markers in m4, cell proliferation was still active also in m4 of the *Ascl1* mutants (Fig. 6B). These results suggest that without *Ascl1* the neural progenitors in m3–m5 fail to properly exit the ventricular zone. Paradoxically however, the progenitors also loose characteristics of the neural stem cells and precociously activate expression of some of

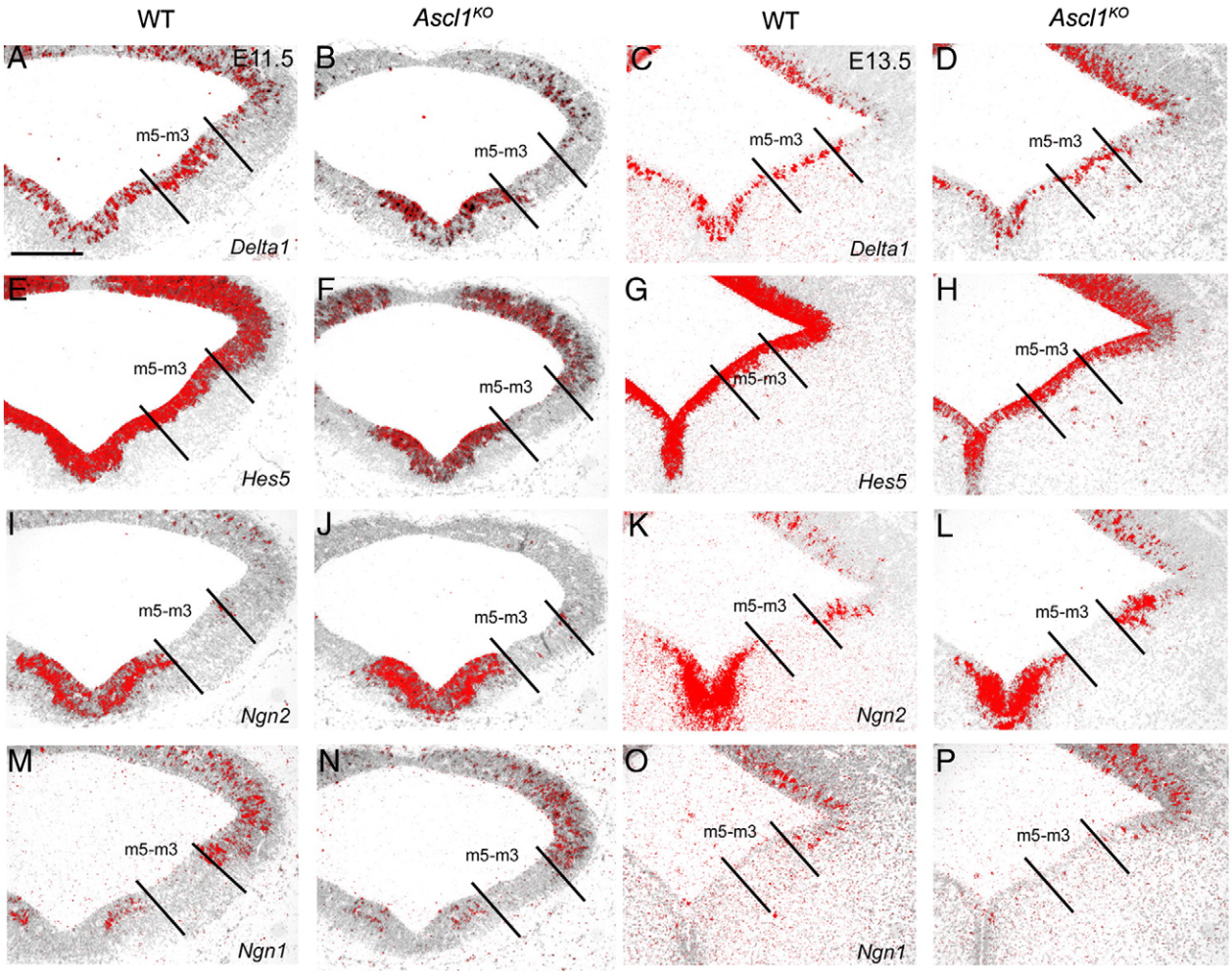

Fig. 5. Neurogenic and neural stem cell –specific gene expression. (A-P) ISH analysis of *Delta1*, *Hes5*, *Ngn2* and *Ngn1* at E11.5 and E13.5 in the wild-type (WT) and *Ascl1*^{KO} embryos. Scale bar 200 µm.

the genes typical for post-mitotic differentiation. The requirement of *Ascl1* is highly region specific, and there appears to be distinct requirements for it even within m3–m5 region.

Discussion

Midbrain GABAergic neurons are involved in functions such as regulation of mood, movement and nociception and thus comprise clinically highly important targets. However, their developmental regulatory mechanisms are largely unknown. Here we show that various subpopulations of GABAergic neurons in the midbrain and r1 have distinct requirements for *Ascl1*. GABAergic neurons associated with the dopaminergic nuclei in the VTA and SN do not require *Ascl1*. Also, *Ascl1* is dispensable for GABAergic neurogenesis in the r1. In contrast, loss of *Ascl1* results in delayed neurogenesis in the lateromedial midbrain (m3–m5), and reduced numbers of GABAergic neurons populating mesencephalic reticular formation and ventral periaqueductal gray. The dorsal midbrain (m1–m2) has the strictest requirement for *Ascl1,* as no GABAergic neurons populating superior and inferior colliculi as well as dorsal periaqueductal gray are generated in *Ascl1* mutant midbrain.

In an interesting similarity to the *Ascl1* mutant mice, GABAergic neurons of the VTA and SNpr are also unaffected in *Helt* and *Gata2*^{cko} mutants. This suggests that these neurons are very different from the rest of the midbrain GABAergic neurons. The phenotype of the *Gata2*^{cko} mutants is the most clear-cut: despite complete loss of GABAergic gene expression in the embryonic midbrain at the time of neurogenesis (E11.5–E13.5) and transformation of the neurotransmitter phenotype of all other midbrain GABAergic neurons, the VTA and SNpr GABAergic neurons were apparently normal (Kala et al., 2009). This finding suggested that these neurons may have their developmental origin outside the midbrain. This hypothesis proves to be correct, as our recent fate mapping results show that the VTA and SNpr GABAergic neurons are derived from the r1 (K.K, J.P., unpublished). Consistent with this, the GABAergic neurons in r1 do not require *Gata2* (Kala et al., 2009), *Helt* (Guimera et al., 2006b; Nakatani et al., 2007), or *Ascl1* (present study) for their development. Our findings *in vivo* are also in agreement with a recent study demonstrating that *Ascl1* can promote GABAergic neurogenesis in *in vitro* cultured neural progenitors derived from the midbrain but not from the ones derived from the r1 (Jo et al., 2007). Thus, our results suggest that the VTA and SNpr GABAergic neurons are spared in the *Ascl1* mutant midbrain because these neurons are born in the r1 where *Ascl1* is not required for GABAergic neurogenesis.

Unlike in *Gata2*^{cko} midbrain, GABAergic neurons are still produced in the m3–m5 domains of the *Ascl1* mutants. This is in contrast to a previous study, which concluded that essentially no GABAergic neurons are produced in the *Ascl1* mutant midbrain (Miyoshi et al., 2004). A likely explanation is that these authors analyzed only a relatively early time point of GABAergic neurogenesis in the midbrain. Our results showed that although post-mitotic GABAergic neuron precursors were still generated from *Ascl1* deficient m3–m5, the neurogenesis was markedly delayed. This correlated with loss of *Delta1* and *Hes5* expression in m3–m5 at E11.5, consistent with the function of *Ascl1* in the ventral forebrain (Casarosa et al., 1999).

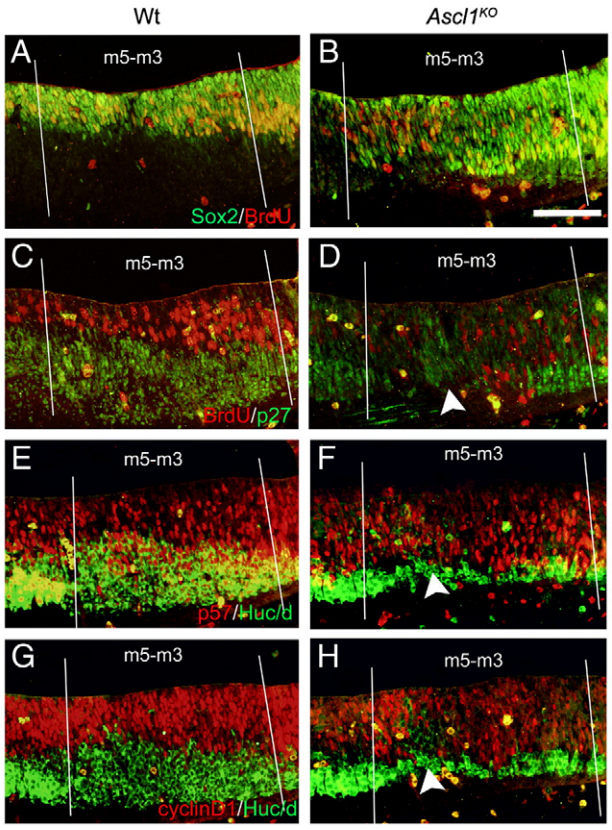

Fig. 6. Markers of cell cycle progression and onset of differentiation in the midbrain domains m3–m5. co-IHC analysis of Sox2 and BrdU (A–B), p27 and BrdU (C–D), p57 and HuC/D (E–F), cyclinD1 and HuC/D (G–H) in the wild-type and *Ascl1*^{KO} embryos at E11.5. White arrowheads demonstrate ventricular up-regulation of p27, p57 and HuC/D in the *Ascl1*^{KO} m4 region. Scale bar 100 µm.

Loss of *Ascl1* results in expanded expression of gene products characteristic to the cellular intermediates between the neuroepithelial stem cells and post-mitotic neurons. One of these genes may be *Helt*, which is rather ubiquitously expressed in m3–m5 neuroepithelium in the E11.5 *Ascl1* mutants compared to the salt-and-pepper expression pattern observed in the wild-type. Previous studies have shown that *Helt* is expressed in the ventricular zone where the proliferative neural progenitors are located (Miyoshi et al., 2004; Guimera et al., 2006a) and that the Helt expressing cells likely give rise to Gata2 positive post-mitotic precursors (Kala et al., 2009). Whether Helt-positive cells are still active in the cell cycle or represent an early stage of post-mitotic neural differentiation has not been conclusively demonstrated. Since the m3–m5 neuroepithelial cells in *Ascl1* mutants ubiquitously express *Helt* yet still are proliferative, this strongly argues that at least some of the *Helt* expressing cells represent proliferative intermediates between neuroepithelial stem cells and post-mitotic GABAergic precursors.

Other indicator of an intermediate cell differentiation phenotype in the *Ascl1* mutants is the expanded expression of cell cycle regulators p27 and p57, which was especially prominent in the m4 domain. The midbrain domain m4 also differs from the surrounding midbrain neuroepithelium as it gives rise to both GABAergic and glutamatergic neurons (Kala et al., 2009). Interestingly, unlike other midbrain regions giving rise to glutamatergic neurons, m4 does not express proneural genes *Ngn1* or *Ngn2*. Instead, our results indicate that *Ascl1* also promotes glutamatergic neurogenesis in m4.

Taken together, in the *Ascl1* mutant midbrain m3–m5 domains, separation of the neuroepithelium into stem cell -like cells and differentiating progenitors appears to fail at the start of neurogenesis. Our results are consistent with suggestions that proneural genes have dual functions: Their oscillatory expression is needed for the maintenance of the neural progenitors, whereas sustained expression drives neuronal differentiation (Kageyama et al., 2009). However, later in development, the defect in *Ascl1* mutants is overcome as *Delta1* and *Hes5* expression was restored and we also observed abundant GABA- and glutamatergic neurogenesis. Due to the initial delay in the start of neurogenesis, the m3–m5 derived neurons are born during shorter time period and thus possibly in reduced numbers. This appears similar to the function of *Ascl1* in the generation of the neurons of the nucleus of the solitary tract and symphatetic chain (Pattyn et al., 2006).

In contrast to the other regions of the midbrain, there was a strict requirement for *Ascl1* in GABAergic neuron progenitors in the dorsal midbrain (m1–m2). The loss of GABAergic neurogenesis in m1–m2 correlated with complete loss of GABAergic neurons in the superior

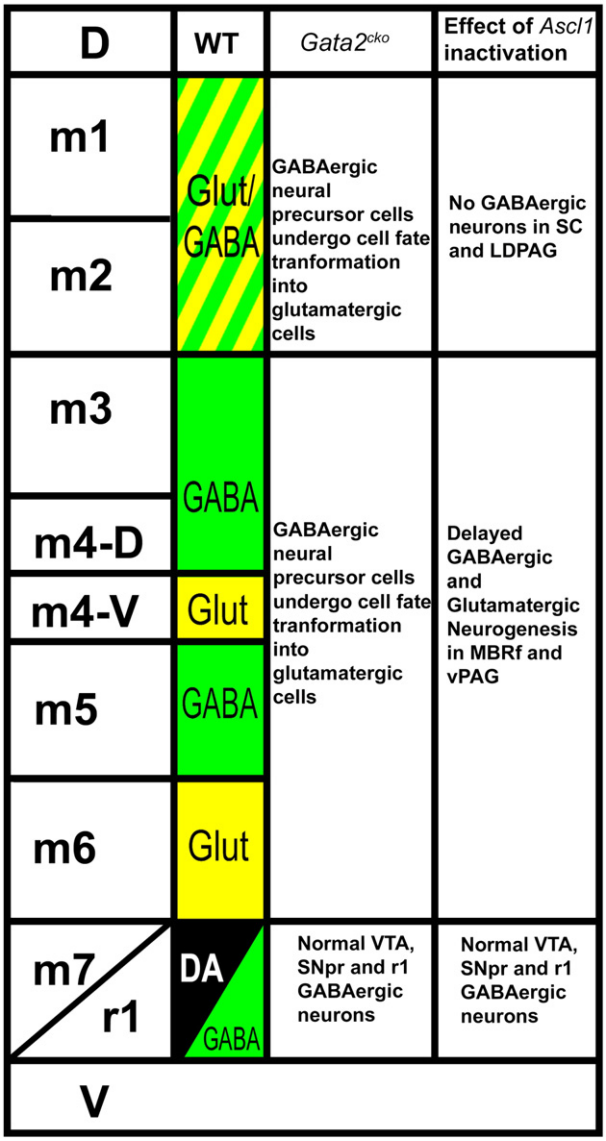

Fig. 7. *Ascl1*^{KO} phenotype in comparison with *Gata2*^{cko}. Different subpopulations of GABAergic neurons in the midbrain have distinct requirements for Ascl1. In the dorsal midbrain domains m1–m2, Ascl1 function is absolutely required. In domains m3–m5, loss of Ascl1 results in delayed neurogenesis. Unlike in *Gata2*^{cko}, there is no evidence of cell fate transformation in the GABAergic neurons of *Ascl1*^{KO}. GABAergic neurons associated with dopaminergic nuclei in VTA, SNpr and r1 do not require Ascl1 function, similar to the Gata2. Also the glutamatergic neurogenesis in m6 is not affected by the loss of either *Ascl1* or *Gata2*. IC, inferior colliculus; MBRf, midbrain reticular formation; LDPAG, dorsolateral periaqueductal gray; SC, superior colliculus; SNpr, substantia nigra pars reticulata; r1, rhombomere 1; vPAG, ventrolateral periaqueductal gray; VTA, ventral tegmental area.

and inferior colliculi as well as dorsal periaqueductal gray, demonstrating that these GABAergic subpopulations are likely completely derived from the dorsal m1–m2 domains. This result is consistent with previous studies demonstrating GABAergic neurogenesis in the dorsal midbrain and analyses of the Helt mutant mice (Tan et al., 2002; Tsunekawa et al., 2005; Guimera et al., 2006b; Nakatani et al., 2007).

Despite the specific loss of the dorsal GABAergic neurons, expression of *Delta1* and *Hes5* is clearly less affected in the dorsal midbrain (m1–m2) compared to the ventrolateral midbrain (m5–m3) in the *Ascl1* mutants. This is likely due to continued glutamatergic neurogenesis driven by *Ngn1* and *Ngn2* in the dorsal midbrain. As discussed above, no GABAergic neurons are produced in the dorsal m1–m2 midbrain domains even at later stages. This may represent cell-intrinsic differences between m1–m2 and m3–m5. However, sustained expression of Helt in the *Ascl1* mutants argues against a phenotypic transformation of the ventricular zone progenitors. Alternatively, the ongoing glutamatergic neurogenesis may further repress GABAergic neurogenesis in m1–m2 through lateral inhibition. Consequently, combinatory effects of *Ascl1* inactivation and continued Delta-Notch signaling may lead to a complete block of GABAergic neurogenesis in m1–m2.

There is no rigorous fate map of how the different regions of the midbrain neuroepithelium contribute to the various GABAergic neuron subpopulations. Comparison of the phenotypes of the $Ascl1^{KO}$ and $Gata2^{cko}$ mutant phenotypes together with persistent Nkx2.2 expression in postmitotic precursors suggest the following model (Fig. 7): the most ventral GABAergic neurons of VTA and SNpr are born outside the midbrain (see discussion above). The GABAergic neurons of the midbrain reticular formation and ventral periaqueductal gray are derived from domains m3–m5. Finally, the GABAergic neurons of the superior colliculi and laterodorsal periaqueductal gray are completely derived from the domains m1–m2.

Conclusion

In the various subpopulations of midbrain GABAergic neurons, the requirements for proneural gene *Ascl1* function are different during neurogenesis. Partly, these differences reflect distinct developmental origins of these subgroups of clinically highly important neurons. Our results also support a dual role of proneural genes in supporting neural stem cells and promoting neurogenic cell cycle exit.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.04.015.

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