Transcriptional regulatory mechanisms underlying the GABAergic neuron fate in different diencephalic prosomeres

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SUMMARY
Diverse mechanisms regulate development of GABAergic neurons in different regions of the central nervous system. We have addressed the roles of a proneural gene, Ascl1, and a postmitotic selector gene, Gata2, in the differentiation of GABAergic neuron subpopulations in three diencephalic prosomeres: prethalamus (P3), thalamus (P2) and pretectum (P1). Although the different proliferative progenitor populations of GABAergic neurons commonly express Ascl1, they have distinct requirements for it in promotion of cell-cycle exit and GABAergic neuron identity. Subsequently, Gata2 is activated as postmitotic GABAergic precursors are born. In P1, Gata2 regulates the neurotransmitter identity by promoting GABAergic and inhibiting glutamatergic neuron differentiation. Interestingly, Gata2 defines instead the subtype of GABAergic neurons in the rostral thalamus (pTh-R), which is a subpopulation of P2. Without Gata2, the GABAergic precursors born in the pTh-R fail to activate subtype-specific markers, but start to express genes typical of GABAergic precursors in the neighbouring P3 domain. Thus, our results demonstrate diverse mechanisms regulating differentiation of GABAergic neuron subpopulations and suggest a role for Gata2 as a selector gene of both GABAergic neuron neurotransmitter and prosomere subtype identities in the developing diencephalon. Our results demonstrate for the first time that neuronal identities between distinct prosomeres can still be transformed in postmitotic neuronal precursors.

KEY WORDS: Diencephalon, Thalamus, Pretectum, Prethalamus, vLGN, IGL, Neurogenesis, GABAergic neuron, Transcription factor, Gata2, Ascl1, Mouse, Cre-recombinase, Proneural gene, Terminal selector gene

INTRODUCTION
The thalamic complex in the diencephalon contains nuclei primarily involved in relay and processing of sensory information. These nuclei are located in three main anatomical regions: prethalamus (former ventral thalamus), thalamus (former dorsal thalamus) and pretectum (Jones, 2007). The nuclei in the prethalamus and pretectum contain abundant GABAergic neurons and project to extracortical targets. By contrast, the thalamic nuclei are mainly glutamatergic and involved in direct information exchange between the sensory systems (auditory, somatosensory, visual) and the cortex. The GABAergic diencephalic nuclei include the ventral lateral geniculate nucleus (vLGN) and the intergeniculate leaflet (IGL), which are located near the prethalamus-thalamus boundary. The vLGN and IGL are closely related structures and have been considered to be an unseparated complex in several studies (Kataoka and Shimogori, 2008; Kitamura et al., 1997). They receive input from the retina and other sources and regulate circadian clock and visuomotor responses (Harrington, 1997). According to a traditional view, vLGN and IGL are prethalamic nuclei because they are GABAergic, do not project to the cortex and are located adjacent to the prethalamus (Jones, 2007). However, recent studies have shown that some of the IGL and vLGN neurons have an origin distinct from other prethalamic nuclei.

The developing diencephalon also consists of three units called prosomeres (P1/pretectum, P2/thalamus and P3/prethalamus) and a narrow zona limitans interthalamica (Zli) domain located between P2 and P3 (Puelles and Rubenstein, 2003; Scholpp and Lumsden, 2010) (Fig. 1A). The identities of the prosomeres are established early during patterning of the neuroepithelium and are manifested by the expression of domain-specific transcription factors, including Fez (Fezf1/2; in P3), Irx3 and Otx2 (in P2). Later, Zli is an important source of the morphogen Shh, which is crucial for further patterning of both the developing prethalamus and thalamus (Hashimoto-Torii et al., 2003; Jeong et al., 2011; Kiecker and Lumsden, 2004; Scholpp et al., 2007; Vieira et al., 2005; Vue et al., 2009). Other local signals regulating thalamic development include fibroblast growth factors and Wnts (Braun et al., 2003; Kataoka and Shimogori, 2008; Martinez-Ferre and Martinez, 2009; Zhou et al., 2004).

Together with the domain-specific transcription factors, Zli-derived signals regulate the activity of proneural genes Ngn1 (Neurog1), Ngn2 (Neurog2) and Ascl1 in the neural progenitor cells of the ventricular zone (VZ) (Scholpp and Lumsden, 2010; Vue et al., 2007). These transcriptional networks are thought to regulate both cell-cycle exit and neuronal identity at the level of proliferative progenitor cells. Ngn1 and Ngn2 are expressed in the progenitors of a large caudal domain of P2/thalamus (pTh-C), which gives rise to the thalamic glutamatergic neurons (Chen et al., 2009; Garcia-Lopez et al., 2004; Vue et al., 2007). In turn, Ascl1 is expressed in the progenitors of P3/prethalamus, P1/pretectum, and a narrow domain in the most rostral part of P2 (pTh-R; also called Rim, P2ZL) (Kataoka and Shimogori, 2008; Vue et al., 2007). Progenitors in P1 and pTh-R produce postmitotic neuronal precursors expressing genes encoding the GABAergic marker Gad1 and the transcription factors Tal1, Tal2 and Sox14 (Hashimoto-Torii et al., 2003; Kataoka and Shimogori, 2008). P1-derived precursors contribute to pretectal nuclei, whereas the pTh-R-derived precursors were recently shown to contribute to the IGL and vLGN (Jeong et al., 2011; Vue et al., 2007). In addition, vLGN
has contributions from Zli and P3 (Delaunay et al., 2009; Kitamura et al., 1997; Suzuki-Hirano et al., 2011). P3 also produces GABAergic neuron precursors but their molecular identity is distinct from pTh-R and P1. The GABAergic precursors born in P3 express genes encoding the transcription factors Dlx1, Dlx2, Dlx5 and Arx, but are negative for Tal1, Tal2 and Sox14 (Panganiban and Rubenstein, 2002; Kataoka and Shimogori, 2008; Kitamura et al., 1997). By contrast, the organizer region Zli expresses Ngn2 and produces a small population of glutamatergic neurons.

Regulation of postmitotic differentiation and mechanisms specifying the different GABAergic neuron subpopulations in the P1/prethalamus, pTh-R and P3/preptectum are still incompletely understood. Ascl1 is important for GABAergic neurogenesis in the telencephalon (Parras et al., 2002), and also appears to control it in the diencephalon. Development of ectopic GABAergic neurons in P2/thalamus of conditional Dlx2 mutants correlates with upregulation of Ascl1 expression (Puuelles et al., 2006). Also, changes in ascl1 expression due to modulation of Her6 or Ngn1 activity correlate with changes in GABAergic neurogenesis in zebrafish (Schlopp et al., 2009). Finally, inactivation of Ascl1 has been reported to lead to loss of GABAergic neurons in the posterior diencephalon (P2 and P1) (Miyoshi et al., 2004). However, Ascl1 might regulate multiple aspects of GABAergic neurogenesis (Guillemot, 2007; Peltopuro et al., 2010) and its exact roles in distinct regions of diencephalon remain unknown.

Determination of neuronal identities is best understood at the level of proliferative progenitor cells (Dessaud et al., 2008; Guillemot, 2007). However, the definitive markers of neuronal identity are only turned on as progenitors exit the cell cycle and become postmitotic neuronal precursors. Furthermore, there are examples of transcription factors, putative ‘terminal selector genes’ (Hobert, 2008), which are often activated only in the postmitotic precursors and are important determinants of either GABAergic or glutamatergic identity in the spinal cord, cerebellum and telencephalon (Cheng et al., 2005; Glasgow et al., 2005; Hoshino et al., 2005; Rouaux and Arlotta, 2010). We showed previously that the zinc-finger transcription factor Gata2 is an essential postmitotic selector of the GABAergic neuron identity in the embryonic midbrain (Kala et al., 2009). Gata2 expression is induced when the midbrain GABAergic neuron precursors leave the cell cycle and differentiate. In tissue-specific Gata2 mutants, the midbrain neural precursors fail to activate genes characteristic for GABAergic precursors but start to express genes typical for adjacent glutamatergic neurons. Gata2 cooperates with Tal transcription factors during development of the haematopoietic system, V2b spinal interneurons and midbrain GABAergic neurons (Joshi et al., 2009; Osada et al., 1995) (our unpublished results). As Tal1 and Tal2 mark some of the diencephalic GABAergic neuron precursors (see above), it is possible that a Gata-Tal complex also regulates their differentiation.

In the present study, we have studied the functions of Ascl1 and Gata2 during GABAergic neuron development in the diencephalon. Our results demonstrate distinct transcriptional regulatory mechanisms controlling differentiation of prethalamic, pTh-R and preptectal GABAergic neurons. Furthermore, we suggest that Gata2 is a terminal selector gene, which can specify both the neurotransmitter and neuronal subtype identities during postmitotic differentiation.

MATERIALS AND METHODS

Mice

Foxg1<sup>Cre</sup> (Hebert and McConnell, 2000), Gata2<sup>Slox</sup> (Haugas et al., 2010), Ascl1<sup>XO</sup> (Guillemot et al., 1993) and Gad1<sup>GFP</sup> (Gad67<sup>GFP</sup>) (Tamamaki et al., 2003) alleles have been described previously. For staging, the day of vaginal plug was counted as embryonic day (E) 0.5. Embryos were fixed in 4% paraformaldehyde in PBS, and transferred to polymer wax (Merck). As controls, we used wild-type (WT), Foxg1<sup>Cre</sup>; Gata2<sup>Slox</sup>, Foxg1<sup>Cre</sup>; Gata2<sup>Slox</sup> or Ascl1<sup>XO</sup> littermates, which were phenotypically indistinguishable from each other. All the results were reproduced with at least three embryos per genotype. Samples were sectioned at 5 μm for immunohistochemistry (IHC) and in situ hybridization (ISH) analyses. All the experiments were approved by the committee of experimental animal research of Finland.

Brdu labelling

To label the actively proliferative cells, a single dose of BrdU (3 mg/100 g body weight) was administered intraperitoneally to the pregnant females 1 or 12 hours before dissection of embryos.

In situ mRNA hybridization and immunohistochemistry

mRNA in situ hybridization (ISH) analyses on paraffin sections were performed as described (Wilkinson and Green, 1990) using digoxigenin- or 35S-labelled antisense cRNA probes. The two different detection methods were used owing to variation in sensitivity between the probes. Mouse cDNA probes used for ISH analysis were: Arx (Kataoka and Shimogori, 2008), Ascl1 (previously known as Mash1), Dlx1 (from J. L. Rubenstein, University of California San Francisco, USA), cyclin D1 (IMAGE 3155470), Fov (also known as Pet1), Gad1 (also known as Gad67), Gata2, Gata3 (Lilevalvi et al., 2004), Helt (also known as Mgn) (Guimera et al., 2006b), Mab21l1 (IMAGE 4526962), Ngn2 (Jukkola et al., 2006), Nkx2-2 (IMAGE480100), Npy (IMAGE 482891), Penk1 (IMAGE 6432610), Ptx2 (Guimera et al., 2006b), Pou4f1 (from S.-L. Ang, NIMR, UK), Rora (Vue et al., 2009), Shh (from A. McMahon, Harvard University, USA), Sst (IMAGE 761326), Slt1a6 (Vglut1) (Guimera et al., 2006b), Sox14 (IMAGE 6822889), Tal1 (IMAGE 6826611), Tal2 (IMAGE 40051579).

Immunohistochemistry (IHC) was performed as described (Kala et al., 2008) with the following antibodies: rabbit anti-Arx [from Kunio Kitamura, 1:500 (Kitamura et al., 2002)], mouse anti-Brdu (GE Healthcare RP200AB, 1:400), rabbit anti-cyclin D1 (LabVision, Neomarkers RM-9104-SO, 1:400), rabbit anti-Gata2 (Santa Cruz sc-9008, 1:250), mouse anti-Gata3 (Santa Cruz sc-268, 1:200), goat anti-GFP (Abcam ab6673, 1:500), rabbit anti-GFP (Abcam ab290, 1:600), guinea pig anti-Hes-like (Helt, 1:500; from R. Kageyama, Kyoto University, Japan), mouse anti-HuC/D (Molecular Probes A22171, 1:800), mouse anti-Mash1 (Ascl1, BD Biosciences 556604, 1:200), mouse anti-Nck2-2 [Developmental Studies Hybridoma Bank (DSHB) 74.5A5, 1:250], rabbit anti-NPY (ImmunoStar 22940, 1:800), rabbit anti-p53<sup>ß</sup> (Neomarkers RB-1637-P0, 1:500), mouse anti-Pax7 (DSHB, 1:300), mouse anti-proliferating cell nuclear antigen (PCNA) (Dako Cytomation M-0878, 1:800), mouse anti-phosphoistone 3 (PH3) (Cell Signaling Technology #9706S, 1:250), mouse anti-Sox2 (Millipore AB5603, 1:400) rabbit anti-tyrosine hydroxylase (Millipore MAB318, 1:300) and mouse anti-Slt1a6 (Vglut2; Sigma V2514, 1:1000).

Alexa Fluor 488- and Alexa Fluor 568-conjugated goat anti-rabbit IgG and anti-mouse IgG, and donkey anti-rabbit IgG, anti-mouse IgG and anti-goat IgG (1:400, Invitrogen) were used as secondary antibodies. For combined ISH and IHC, primary antibodies were added together with the anti-DIG-POD Fab fragments (Roche). The TSA Fluorescence Palette System (PerkinElmer) was used to visualize the ISH signal. Images were processed using Adobe Photoshop or CorelDraw X4 software. Red pseudocolour images were produced by replacing the dark-field images with processed using Adobe Photoshop or CorelDraw X4 software. Red pseudocolour images were produced by replacing the dark-field images with pseudocolour images were produced by replacing the dark-field images with pseudocolour images. Red pseudocolour images were produced by replacing the dark-field images with pseudocolour.
specificity in the diencephalon. Therefore, we compared first the expression patterns of Gata2 and Gata3 and their putative co-factors, Tal1 and Tal2, with markers of GABAergic progenitors, Ascl1 and Helt, as well as to glutamic acid decarboxylase 1 (Gad1) in postmitotic GABAergic neurons. Gata2 and Gad1 expression was first detected in the postmitotic zone of P1 at ~E10.5 (supplementary material Fig. S1A-C). At E11.5-12.5, we detected co-expression of Gata2/3 and Tal1/2 in P1 and pTh-R, which give rise to Gad1+ GABAergic neurons (Fig. S1D-H). Our IHC analyses further demonstrated co-expression of Gata2 and their putative co-factors, Ascl1- and Helt-positive GABAergic progenitors (supplementary material Fig. S2A-D). We also detected Gata2 and Gata3 expression in Gad1+ GABAergic neurons by co-IHC for Ascl1- and Helt-positive GABAergic progenitors (Fig. 1C-G; supplementary material Fig. S1D-H). Our IHC analyses further demonstrated co-expression of Gata2 protein in Ascl1- and Helt-positive GABAergic progenitors (supplementary material Fig. S2A-D). We also detected Gata2 and Gata3 expression in Gad1+ GABAergic neurons by co-IHC for Gad1 (supplementary material Fig. S2A-D). At E12.5, we observed Gata2 in the ventricular, intermediate and mantle zones (VZ, IZ and MZ, respectively; Fig. 1C,I,M), whereas the expression of Gata3 was restricted to the Gad1-expressing cells in the MZ (Fig. 1E,K,O). Like Gata2, Tal2 was observed mainly in the VZ and IZ (Fig. 1D,J), whereas Tal1 appeared to be expressed strongly in the IZ and MZ (Fig. 1F,L). Co-labelling with the cyclin-dependent kinase (CDK) inhibitor p21cip1 and p27, markers of early IZ precursors (Gui et al., 2007; Nguyen et al., 2006), showed that Gata2 and Tal1/2 are expressed in the early postmitotic cells in VZ and IZ (Fig. 1I,J,L; supplementary material Fig. S2G,H), whereas Gata3 is confined to the differentiating cells in MZ (Fig. 1K). No co-labelling of Gata2 and BrdU was detected after a short (1 hour) BrdU administration pulse, which should label nuclei of progenitors in the S phase (supplementary material Fig. S2E,F). Upon a longer labelling pulse (12 hours), some BrdU-labelled nuclei started expressing Gata2 and could be traced to the IZ/MZ (supplementary material Fig. S2M,N). Furthermore, Gata2-positive nuclei were mostly devoid of PCNA, a marker of proliferative cells (supplementary material Fig. S2K,L) and never expressed phospho-histone H3, a marker of G2/M phase nuclei (supplementary material Fig. S2O,P). In addition, we observed several cells co-expressing Gata2 and the postmitotic neuron marker HuC/D, indicating that Gata2 is
expressed in postmitotic neuronal precursors (supplementary material Fig. S1C and Fig. S2L).

In summary, our results suggest that Gata2 expression is activated in early postmitotic GABAergic precursors in P1 and pTh-R. The expression of Gata2 was probably initiated very soon after cell-cycle exit when precursor cell nuclei still resided in the VZ and coincided with expression of Tal2. By contrast, other markers of the GABAergic lineage, including Gata3, Tal1, Sox14, Six3, Npy, and Gad1, are activated after the onset of Gata2 expression (Fig. 1B).

**Gata3 is expressed in the pretectum and IGL/vLGN in the prenatal brain**

To study the putative derivatives of Gata2/3-expressing P1 and pTh-R GABAergic precursors, we first mapped the expression of Gata and Tal factors in the GABAergic nuclei in caudal diencephalon of E18.5 brain. We detected no expression of Gata2 or Tal2 in the diencephalon at E18.5 (data not shown). Gata3 was expressed in the Gad1\(^+\) anterior pretectal nucleus (APN; Fig. 2B,C), IGL and in scattered cells in lateral vLGN (Fig. 2E,F), in agreement with an earlier report (Zhao et al., 2008). Consistent with its expression in more differentiated postmitotic precursors (see above), we also found Tal1 in the same regions as Gata3 (Fig. 2K; data not shown). IGL contains two functionally distinct populations of GABAergic neurons expressing either Npy or enkephalin (Penk1) as co-neurotransmitter (Jones, 2007). In IGL, Gata3/Tal1 expression was seemingly uniform and overlapped with Nkx2-2 as well as both Npy\(^+\) and Penk1\(^+\) neurons (Fig. 2E,H-L). Also in the lateral vLGN we found Gata3 and Tal1 in regions expressing Nkx2-2 and Penk1 (Fig. 2E,K,L), but not Arx or Dlx1, which are expressed in ventral and medial vLGN (Fig. 2M and Fig. 7J; data not shown). In the lateral vLGN, Gata3\(^+\) neurons appeared to intermingle with Gata3-negative cells. In summary, restricted Gata3/Tal1 expression in the GABAergic neurons suggests that Gata/Tal-expressing cells in embryonic P1 and pTh-R might specifically contribute to the GABAergic nuclei in the caudal diencephalon, APN, IGL and vLGN.

**Analysis of conditional Gata2 mutants: Gata2 regulates GABAergic neuron differentiation in P1 and pTh-R**

To study the function of Gata2 in diencephalic development, we used a conditional Gata2 mutant strain (Foxg1\(^{cre}\); Gata2\(^{F/F}\)) in order to circumvent the early haematopoietic defect seen in the Gata2-null mutants (Tsai et al., 1994). The Foxg1\(^{cre}\) allele has been reported to catalyse recombination primarily in the telencephalon and inner ear (Hebert and McConnell, 2000). However, genetic background was shown to have a major effect on Foxg1\(^{cre}\)-mediated recombination and, thus, the recombination pattern can be much wider than the detectable Foxg1 expression. In the Foxg1\(^{cre}\); Gata2\(^{F/F}\) embryos, Gata2 was widely and efficiently inactivated at an early stage. However, presumably owing to incomplete recombination in the yolk sac, haematopoiesis is rescued and the mutants survive until birth (Haugas et al., 2010). We detected no Gata2 expression in the brains of Foxg1\(^{cre}\); Gata2\(^{F/F}\) embryos at E12.5 (supplementary material Fig. S3).

We examined first the expression of genes associated with GABAergic fate in Foxg1\(^{cre}\); Gata2\(^{F/F}\) diencephalon. No defects in GABAergic neuron differentiation were observed in P3 (supplementary material Fig. S4B). By contrast, complete loss of Gad1, Gata3, Six3, Npy, Tal1 and Sox14 expression was detected in the mutant P1 and pTh-R at E12.5 (Fig. 3A-D and Fig. 4A,B,Q,R; supplementary material Fig. S4A,B,G-J,M-R). Despite the failure in GABAergic neuron differentiation, the GABAergic progenitor markers Ascl1 and Helt (Fig. 3G,H; supplementary material Fig. S4E,F,U,V), as well as other regional markers such as Shh, Ngn2 and Nkx2-2, were normally expressed in the VZ throughout the P1-P3 (supplementary material Fig. S4S,T,X,Y; data not shown). Interestingly, expression of Tal2, a putative Gata2 co-factor, also appeared to be unaffected in the VZ and IZ of the mutants (Fig. 5A,B,I,J; supplementary material Fig. S4K,L). In summary, in the absence of Gata2, postmitotic GABAergic markers were downregulated, but gene expression in GABAergic progenitors remained unaffected in P1 and pTh-R.
Gata2 selects the GABAergic neurotransmitter identity in P1

As GABAergic-to-glutamatergic fate transformation takes place in midbrain neuronal precursors in the absence of Gata2 (Kala et al., 2009), we hypothesized that this would happen also in the developing diencephalon. Indeed, we detected ectopic expression of the glutamatergic neuron marker Slc17a6 in the putative GABAergic domain of P1 in E12.5 Foxg1cre; Gata2F/F embryos (Fig. 3A,B,E,F; supplementary material Fig. S4A-D, arrows). We detected no apparent changes in tissue morphology, apoptosis or expression of the glutamatergic neuron marker Slc17a6 in the putative GABAergic domain of P1 in E12.5 Foxg1cre; Gata2F/F embryos (data not shown). Therefore, the ectopic Slc17a6 expression in P1 probably indicates fate transformation in Foxg1cre; Gata2F/F mutants. Thus, inactivation of Gata2 appears to result in a switch from GABAergic to glutamatergic neurotransmitter phenotype in the diencephalic P1 precursors.

Gata2 selects the GABAergic neuron subtype in pTh-R

Strikingly, despite the loss of GABAergic neuron markers in pTh-R (Fig. 4A,B, arrow), we detected no upregulation of Slc17a6 in this area in Foxg1cre; Gata2F/F mutants at E12.5 (Fig. 4E,F, arrow). To determine whether the fate transformation in pTh-R occurs later in development, we analysed the expression of GABA- and glutamatergic neuron markers a day later, in E13.5 embryos. However, similar to E12.5 pTh-R, we detected no upregulation of Slc17a6 in the mutant pTh-R at E13.5 either (Fig. 4G,H, arrow; supplementary material Fig. S5A-B). Instead, we found that Gad1 expression was now restored in the intermediate cell population of pTh-R (Fig. 4C,D, arrow; supplementary material Fig. S5C-D). These data suggest that in pTh-R, Gata2-deficient GABAergic precursors activate a GABAergic differentiation pathway characteristic to P3 progenitors and thus acquire a more rostral GABAergic neuron phenotype.

Alternatively, Axr-expressing cells from lateral P3 could move to populate pTh-R in the Foxg1cre; Gata2F/F mutants. We find this unlikely for the following reasons: first, we always observed a narrow gap in the mantle zone between the Axr-expressing cells in pTh-R. (Supplementary material Fig. S5E-H; data not shown).

As in P1, we did not detect defects in production of postmitotic precursors or their survival in the mutants (data not shown). This suggested that in Foxg1cre; Gata2F/F diencephalon, the pTh-R precursors acquire a different GABAergic phenotype in which Gad1, Slc17a6 and Gata3 are not expressed. Such neurons develop in the adjacent P3 region. We therefore analysed expression of markers for P3 GABAergic neurons, Dlx1 and Arx. Indeed, Dlx1 and Arx expression was robustly upregulated in the pTh-R intermediate zone precursor population already at E12.5 (Fig. 4I-L, arrows). By E13.5, expression of Dlx1 and Arx was even more prominent and coincided with the Gad1-expressing region in the pTh-R of Foxg1cre; Gata2F/F mutants (Fig. 4M-P, arrows). These data suggest that in pTh-R, Gata2-deficient GABAergic precursors activate a GABAergic differentiation pathway characteristic to P3 progenitors and thus acquire a more rostral GABAergic neuron phenotype.

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P3 and ectopic Arx expression in pTh-R (Fig. 4L); second, Dlx1 is not expressed in the lateral P3 yet is ectopically induced in the pTh-R in Foxg1\textsuperscript{cre}; Gata2\textsuperscript{2F/F} mutants (Fig. 4O,P); and third, Gad1 is not expressed in ectopic Arx- and Dlx-positive cells at E12.5 (Fig. 4B,J,N). Furthermore, Zli is a lineage-restricted compartment between pTh-R and P3 (Garcia-Lopez et al., 2004; Kiecker and Lumsden, 2004; Larsen et al., 2001). Nevertheless, we addressed this possibility by analysing whether the Arx- and Dlx1-expressing cells in pTh-R represent recently born cells in the intermediate zone indicative of local origin in pTh-R. Indeed, the early IZ precursor marker pS7\textsuperscript{kip2} was co-expressed with Arx and Dlx1 in the pTh-R of the Foxg1\textsuperscript{cre}; Gata2\textsuperscript{2F/F} mutants (Fig. 4U-X). By contrast, the differentiating GABAergic neurons in the neighbouring P3 had already lost pS7 expression (Fig. 4V, arrowhead). This further supports the conclusion that the local pTh-R GABAergic precursors undergo a transformation from Gata3/Tal2- to Arx/Dlx1-expressing cell identity in the absence of Gata2.

**Tal2-expressing precursors switch their fate in Gata2 mutants**

Tal2 is co-expressed together with Gata2 in precursors giving rise to GABAergic neurons, yet the expression of Tal2 does not require Gata2 function (Fig. 5A,B,K,O; supplementary material Fig. S4K,L) (K.A., P.P., J.P., unpublished results). This allowed us to use Tal2 as a marker of GABAergic neurogenesis and analyse the fate of Tal2-expressing precursors in the absence of Gata2 function. For analysis of P1, we performed fluorescence ISH for Tal2, Slc17a6 and Mab2111 (a marker of IZ precursors in the glutamatergic precommissural and commissural parts of P1) (Vue et al., 2007) (Fig. 5A-H,N-L) as well as IHC for Slc17a6 and Pax7 (strongly expressed in the commissural part of P1) (Merchán et al., 2011) (Fig. 5C-F,L). Analyses of adjacent sections suggested that the Tal2-expressing cells in P1 of the Foxg1\textsuperscript{cre}; Gata2\textsuperscript{2F/F} mutants gave rise to MZ progenitors expressing Slc17a6 (Fig. 5C-F,L,M). Furthermore, Mab2111 expression coincided with the domain positive for Tal2 in the IZ of Foxg1\textsuperscript{cre}; Gata2\textsuperscript{2F/F} mutants (Fig. 5D,F,L). This was most clearly seen in the IZ of juxtacommissural P1, which in WT embryos uniformly expresses Tal2 and gives rise only to GABAergic neurons. Thus, in the absence of Gata2, the Tal2-positive precursors appear to adopt the glutamatergic identity of precommissural or commissural P1. For analysis of pTh-R, we combined fluorescence ISH for Tal2, Slc17a6 and Gad67GFP (K.A., P.P., J.P., unpublished results) with IHC for Pax7 (E,F), Arx (I,J), Slc17a6 (C,D,L) and Gad67GFP (K,O) in WT, Gad67GFP\textsuperscript{cre} and Foxg1\textsuperscript{cre}; Gata2\textsuperscript{2F/F} embryos at E12.5. ISH signals on adjacent sections analysed for expression of Pax7 and Slc17a6 (M), or Tal2 and Mab2111 (N) are overlaid. White arrows and white square bracket indicate the JcP region where the fate transformation is most apparent in P1; white arrowhead indicates the domain of identity switch in pTh-R. Dotted lines indicate the prosomere boundaries. CoP, commissural pretectum; JcP, juxtacommissural pretectum; PcP, precommissural pretectum. Scale bars: in A, 100 \(\mu\)m for A-J; in K, 50 \(\mu\)m for K-O.

**Distinct requirements for Ascl1 in P1 and pTh-R**

Ascl1 is differentially required in the dorsal (m1-2) and ventral (m3-5) GABAergic progenitors in the developing midbrain (Peltopuro et al., 2010) and is a potential regulator of various aspects of neuronal progenitor proliferation, cell-cycle exit and migration (Castro et al., 2011; Pacary et al., 2011). To study how Ascl1 regulates GABAergic neuron development in different diencephalic progenitor populations, we analysed expression of GABAergic and glutamatergic markers in Ascl1\textsuperscript{KO} diencephalon at E12.5. We observed complete loss of Gad1\textsuperscript{+}, Gata2\textsuperscript{+} and Gata3\textsuperscript{+} postmitotic cells in Ascl1-deficient P1 (Fig. 6A,B,I-L, arrow). Furthermore, we found upregulation of Ngn2 in the P1 VZ and consequent ectopic Slc17a6\textsuperscript{+} glutamatergic neuron differentiation (Fig. 6E-H). By contrast, Ngn2 was not upregulated in the pTh-R and P3. Instead, we detected upregulation of the Ngn repressor Helt (Nakatani et al., 2007) in the VZ (Fig. 6C,D). Consistently, Gad1\textsuperscript{+}, Gata2\textsuperscript{+} and Gata3\textsuperscript{+} cells were produced in the pTh-R of Ascl1 mutants (Fig. 6A,B,I-L). However, thickening of the Sox2\textsuperscript{+} VZ and reduction in the HuC/D\textsuperscript{+} MZ indicated delayed cell-cycle exit in the mutant P1 and especially in P3 at E12.5 (Fig. 6M,N; data not shown). By contrast, upregulation of HuC/D and p27 in the VZ as well as downregulation of cyclin D1 and mastic Sox2 expression indicated premature cell-cycle exit and reduced progenitor cycling in the pTh-R (Fig. 6M,N; supplementary material Fig. S6A-H), suggesting that Ascl1 might regulate progenitor proliferation specifically in this region. Changes in cell-cycle exit might also explain the observed increase in the Gata2\textsuperscript{+} Gata3\textsuperscript{+} double-positive cells in the Ascl1 mutant pTh-R (Fig. 6K,L, insets). However, the...
defects in neurogenesis appeared to be reversible as by E13.5 the Sox2- and HuC/D-expressing cell layers had acquired a more normal appearance throughout the diencephalon in Ascl1KO mutants (Fig. 6O,P). In summary, the proneural gene Ascl1 appears to play distinct and even opposing roles in the diencephalic neuronal progenitors, promoting cell-cycle exit in the P1 and P3, and supporting progenitor proliferation in the pTh-R. At the same time, Ascl1-deficient P1 undergoes a GABAergic-to-glutamatergic fate transformation concomitant with upregulation of proneural gene Ngn2 expression, whereas in the more rostral diencephalon, pTh-R-P3, the fate selection process is unaltered.

**Neuronal derivatives of caudal diencephalon maintain their identity switch in perinatal brain**

To elucidate the consequences of observed fate transformations to the prenatal brain, we examined the derivatives of P1 and pTh-R at E16.5-18.5. Consistent with the phenotype in the E12.5 P1, the expression of Gad1 or Gata3 was completely absent from the Foxg1+; Gata2F/F neurons in the anterior GABAergic neurons in the P3. To our knowledge, this is the first demonstration of a neuronal fate transformation in the posterior diencephalon (P3). By contrast, loss of Gata2 function results in an apparent complete GABAergic-to-glutamatergic fate transformation of postmitotic neuronal precursors in the posterior diencephalon (P1). Yet in the medial diencephalon (pTh-R), Gata2 regulates the type of GABAergic neurons produced. In Gata2 mutants, the pTh-R GABAergic neurons do not switch neurotransmitter identity, but do start to express markers typical for more anterior GABAergic neurons in the P3. To our knowledge, this is the first demonstration of a neuronal fate transformation to

**DISCUSSION**

GABAergic neurons are important components of several diencephalic nuclei in prethalamus, thalamus and pretectum. However, relatively little is known about the factors that control their differentiation. Here, we demonstrate that neuronal identity and cell-cycle exit are differentially regulated in various GABAergic progenitor subpopulations of the developing diencephalon. In addition, we suggest that terminal selector genes activated upon cell-cycle exit are important for the identities of diencephalic neurons. Our results demonstrate distinct selector functions for Gata2 in different regions of the developing diencephalon (Fig. 8; supplementary material Fig. S7). The pTh-R-derived neurons contribute to the IGL/vLGN complex in the diencephalon (Jeong et al., 2011). Our analyses showed that vLGN and IGL contain numerous Gata2+ cells that are probably derivatives of Gata2-expressing pTh-R precursors to analyse the expression of the P2-type GABAergic neuron markers Npy, Penk1, Gata3 and Tal1 with that of the P3-type markers Arx and Dlx1 in the germinative complex of WT and Foxg1cre; Gata2F/F prenatal brains. Consistent with our observations at earlier developmental stages, we detected no expression of Gata3, Tal1, Npy or Penk1 in the E18.5 Foxg1cre; Gata2F/F brains (Fig. 7D,E,G,H; data not shown). However, we also did not observe any apparent changes in the expression of Gad1 and Slc17a6 (Fig. 7A,B; data not shown), or in the size of the vLGN/IGL. Instead, we detected downregulation of Nkx2-2 (Fig. 7M,N), which could indicate reduction in pTh-R contribution or reflect the transformation to P3-type cell fate as the P3 cells seem to lose Nkx2-2 expression as they move to the MZ (supplementary material Fig. S4S). Indeed, cells expressing the P3 GABAergic neuron markers Dlx1 and Arx were found in the IGL and lateral vLGN area in the Foxg1cre; Gata2F/F mutants (Fig. 7J,K; data not shown). In conclusion, the vLGN complex maintains a GABAergic neurotransmitter phenotype in the absence of Gata2, but the neuronal subtype composition in the complex is altered.

Because we observed a defect in neurogenesis but not in the cell-type identity in the pTh-R of developing Ascl1KO embryos, we also analysed the expression of the IGL/vLGN markers Gad1, Gata3, Npy, Penk1, Dlx1 and Arx in prenatal Ascl1KO brains. In these brains, we could detect the expression of all these markers characteristic to WT vLGN/IGL, although the spatial organization of the cells expressing different markers seemed to be disrupted (Fig. 7C,F,I,L). Overall, unlike in the Foxg1cre; Gata2F/F brain, the vLGN/IGL seems to display a WT gene expression profile in Ascl1KO, indicating unaltered subtype composition. In summary, the cell-fate changes observed in the embryos undergoing neurogenesis are reflected in the prenatal brain structures both in the Foxg1cre; Gata2F/F and Ascl1KO mice.
Ascl1 differently regulates GABAergic neuron differentiation in distinct GABAergic neuron subpopulations in the diencephalon

We demonstrate that Ascl1 has distinct functions in the different GABAergic neuron progenitor populations of the diencephalon. In P1, Ascl1 regulates neuronal identity through repression of Ngn2. Loss of Ascl1 resulted in upregulation of Ngn2 in the VZ progenitors and glutamatergic markers in postmitotic precursors resulting in a complete failure of GABAergic neurogenesis in P1 (supplementary material Fig. S7). These observations are consistent with earlier studies, which showed that the proneural genes Ascl1 and Ngn2 cross-repress each other in the forebrain and that Otx2-mediated repression of Ascl1 is required for glutamatergic differentiation in the caudal thalamus (P2, pTh-C) (Parras et al., 2002; Puelles et al., 2006). In contrast to P1, GABAergic neurons were still produced in the pTh-R and posterior P3 in the Ascl1KO. A recent study offers a possible explanation for the different effects of Ascl1 inactivation in P1 and pTh-R. In zebrafish, the basic helix-loop-helix (bHLH) transcription factor Her6 represses ngn2 (neurog3 – Zebrafish Information Network) expression affecting the balance between the proneural genes ascl1 and ngn2 and allowing GABAergic neuronogenesis (Scholpp et al., 2009). In mouse, as Her6 is expressed in rostral P2 but not in P1, loss of the Ngn2 repressor Ascl1 might lead to Ngn2 upregulation in P1 but might not be sufficient to induce it in pTh-R. Another bHLH-Orange (bHLH-O) transcription factor Hel, an Ngn repressor in the embryonic midbrain (Nakatani et al., 2007), is also expressed in the diencephalon and is required for normal GABAergic neuron development in the posterior pretectum (Guimera et al., 2006a; Guimera et al., 2006b). Interestingly, loss of Ascl1 inhibited cell-cycle exit in P3, but appeared to promote it in pTh-R. A recent study identified both cell-cycle repressors and promoters as Ascl1 targets (Castro et al., 2011). Our results suggest that Ascl1 might have distinct targets in different neuroepithelial domains. In summary, the proneural gene networks in the proliferative progenitors appear to be wired differently in various GABAergic progenitor populations in the diencephalon (Fig. 8).

Gata2 is required for GABAergic identity in P1 and GABAergic subtype identity in pTh-R

Analyses of conditional Gata2 mutants suggest that in the absence of Gata2, neurogenic cell-cycle exit is undisturbed, but the cell-type specification process fails in GABAergic precursors of P1 and pTh-R. However, the P1 and pTh-R neuronal precursors respond differentially to the loss of Gata2. Similar to the midbrain (Kala et al., 2009), Gata2 is an essential postmitotic selector of the GABAergic versus glutamatergic neuron identity in P1. However, the fate transformation in Gata2 mutant P1 is not restricted to the neurotransmitter identity, as without Gata2 the Tal1-positive precursors also acquire other features of the glutamatergic P1.
By contrast, expression of GABAergic neuron-specific gene expression is not blocked but is delayed by the loss of Gata2 in pTh-R. Strikingly, our results demonstrate that Gata2 is a selector of the GABAergic neuron subtype identity in pTh-R. In the absence of Gata2, the pTh-R precursors adopt the phenotype of the adjacent GABAergic region in P3, where Gata2 is normally not expressed. During the normal development of GABAergic neurons in P3, expression of Arx and Dcx1 precedes the expression of Gad1. This might also be the reason why Gad1 activation is delayed in the Gata2 mutant pTh-R. We hypothesize that only after transformation of the neuronal identity and upregulation of the P3-specific GABAergic neuron regulators can the definitive GABAergic neuron markers be activated in pTh-R in the Gata2 mutants.

**Is Gata2 a terminal selector gene?**

Selection of neuronal identity is thought to depend progressively upon extracellular signal-regulated events in progenitor cells to cell-intrinsic mechanisms at the precursor stage (Edlund and Jessell, 1999). Although cell-cycle exit has been implicated as a crucial period in this transition, the mechanisms are still poorly understood. To date, several postmitotic transcription factors that regulate neurotransmitter identities have been identified. By contrast, the known determinants of regional/prosomatic identities, such as homeodomain transcription factors, operate already at the level of proliferative progenitors. Our results highlight the cell-cycle exit as a culmination point of cell-fate determination and demonstrate that postmitotic loss of a single transcription factor can result in a change of the neuronal identity to the fate of a different prosomere.

Studies of neuronal differentiation in *Caenorhabditis elegans* have led to the concept of ‘terminal selector genes’, which regulate neuron-type-specific gene batteries (Hobert, 2008). Terminal selector genes were suggested to be expressed throughout the life of a neuron, positively autoregulate themselves, regulate selector genes were suggested to be expressed throughout the life neuron-type-specific gene batteries (Hobert, 2008). Terminal have led to the concept of ‘terminal selector genes’, which regulate
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**Molecular and developmental heterogeneity of the GABAergic neurons in the IGL/vLGN**

IGL and vLGN are highly heterogenic nuclei, which consist of several types of (mainly) GABAergic neurons. IGL is a mixture of GABAergic neurons expressing one of the two co-neurotransmitters Npy and Penk1. In this study, Gata3 was abundantly expressed in both types of IGL neurons. In addition, Gata3 and Penk1 were expressed in the lateral part of vLGN in a mosaic pattern. Our results show that Gata2 is a selector gene for both Npy- and Penk1-positive GABAergic cells in the IGL and lateral part of the vLGN, whereas other factors control the specification of Gata3-negative GABAergic neurons in the vLGN. Our results support the recent conclusions on the origins of the IGL and vLGN neurons (Jeong et al., 2011; Vue et al., 2007) and further suggest that both the Npy- and Penk1-expressing neurons are derived from the pTh-R. The mechanisms that distinguish between these two neuronal subtypes remain unknown.

**Conclusions**

The identity of a developing neuron is regulated at multiple levels. Our results demonstrate that, similar to telencephalon, proneural genes are important regulators of neuronal identity in proliferative progenitors in the posterior diencephalon. However, we suggest that the proneural gene networks are different between the diencephalic domains giving rise to distinct GABAergic neuron subpopulations. Another important regulatory step affecting the identity of a developing neuron occurs at the point of cell-cycle exit. We show that the transcription factor Gata2 acts as a postmitotic cell-fate selector, which can be used differently in different GABAergic precursor populations in the developing diencephalon. During embryonic development, Gata factors have been proposed to act as pioneer transcription factors involved in early marking and opening of genes for expression (Zaret et al., 2008). Consistently, the successful and apparently complete cell-fate transformation upon the loss of Gata2 suggests that Gata2 acts relatively early in the transcriptional regulatory cascades leading to differentiation of GABAergic neurons and their specific GABAergic subtypes.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**References**


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