Gata2 is a tissue-specific post-mitotic selector gene for midbrain GABAergic neurons

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Midbrain GABAergic neurons control several aspects of behavior, but regulation of their development and diversity is poorly understood. Here, we further refine the midbrain regions active in GABAergic neurogenesis and show their correlation with the expression of the transcription factor Gata2. Using tissue-specific inactivation and ectopic expression, we show that Gata2 regulates GABAergic neuron development in the mouse midbrain, but not in rhombomere 1, where it is needed in the serotoninergic lineage. Without Gata2, all the precursors in the embryonic midbrain fail to activate GABAergic neuron-specific gene expression and instead switch to a glutamatergic phenotype. Surprisingly, this fate switch is also observed throughout the neonatal midbrain, except for the GABAergic neurons located in the ventral dopaminergic nuclei, suggesting a distinct developmental pathway for these neurons. These studies identify Gata2 as an essential post-mitotic selector gene of the GABAergic neurotransmitter identity and demonstrate developmental heterogeneity of GABAergic neurons in the midbrain.

KEY WORDS: GABA, Interneuron, Midbrain, Neurotransmitter, Mouse, Neurogenesis, Ventral tegmental area (VTA), Serotonin, Dorsal raphe, Rhombomere 1

INTRODUCTION

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain. Neurons producing GABA are located in almost all parts of the central nervous system (CNS) and are highly variable in their morphology, gene expression and projection patterns.

GABAergic neurons are found in several regions of the midbrain. They are thought to operate both as local inhibitory interneurons and as projection neurons with targets elsewhere in the brain. GABAergic neurons are abundant in the dorsal superior colliculi and periaqueductal gray matter, where they are involved in multiple processes, including saccadic eye movements, nociception and defensive behavior (Behbehani et al., 1990; Kaneda et al., 2008). In the ventral midbrain, GABAergic neurons are thought to regulate the activity of the dopaminergic (DA) neurons in the substantia nigra pars compacta and ventral tegmental area (VTA) (Laviolette and van der Kooy, 2004; Tepper and Lee, 2007). In addition to controlling the DA neurons, GABAergic neurons located in the VTA and substantia nigra pars reticulata (SNpr) send their axons to other nuclei in the midbrain, prefrontal cortex and other limbic areas (Laviolette et al., 2004; Fields et al., 2007). Thus, midbrain GABAergic neurons are crucial for neural processes such as the regulation of voluntary and involuntary movements, mood, motivation and addiction.

Despite the functional importance of the midbrain GABAergic neurons, their development remains poorly understood. There appear to be both similarities and differences in the mechanisms that control the development of GABAergic neurons in distinct brain regions. Proliferative progenitor cells of the GABAergic neurons are located in the ventricular zone of both ventral and dorsal midbrain (Tsuneoka et al., 2005). This is in contrast to the forebrain, where the GABAergic neurons are generated in ventral neuroepithelium and reach the cortex by dorsal tangential migration. As in ventral forebrain and other regions of GABAergic neurogenesis, the proneural bHLH transcription factor Ascl1 (Mash1) is expressed in the ventricular zone of the midbrain and plays an important role in GABAergic neuron development (Horton et al., 1999; Casarosa et al., 1999; Miyoshi et al., 2004; Mizuguchi et al., 2006). Helt (Heslike, Megane, Mgn) is a bHLH-Orange family transcription factor that is coexpressed with Ascl1 in the midbrain ventricular zone (Miyoshi et al., 2004). Mice lacking Helt function show impaired development of midbrain GABAergic neurons, especially in the dorsal region (Guinera et al., 2006; Nakatani et al., 2007). Furthermore, Helt has been shown to regulate the GABAergic versus glutamatergic neuron identity by repressing the proneural genes Ngn1 and Ngn2 (Neurog1 and Neurog2), which in turn promote glutamatergic neuron development (Nakatani et al., 2007).

In addition to genes controlling the proliferation, identity and neurogenesis of the ventricular zone progenitor cells, studies of other parts of the CNS have revealed transcription factors that are activated only in the post-mitotic neural precursors. Some of these factors do not regulate the neuronal differentiation process itself, but are needed to select a particular neuronal phenotype from among distinct alternatives and are therefore called selector genes. The bHLH transcription factor Ptf1a appears to act as a selector of the GABAergic, as opposed to glutamatergic, fate in the spinal cord and cerebellum (Glasgow et al., 2005; Hoshino et al., 2005; Cheng et al., 2005). However, no such selector gene for the midbrain GABAergic neurons has been identified so far.

Gata2 and Gata3 are related C4 zinc-finger transcription factors. They are involved in the development of several organs and tissues, and have been extensively studied, especially in the hematopoietic system (Tsai et al., 1994). In the developing CNS, Gata2 and Gata3 are expressed in similar patterns in distinct brain regions and Gata2 is often required for the expression of Gata3 (Nardelli et al., 1999). Gata2 has been suggested to play a role in the correct development of the GABAergic lineage in the midbrain, suggesting a distinct developmental pathway for these neurons. Without Gata2, all the precursors in the embryonic midbrain fail to activate GABAergic neuron-specific gene expression and instead switch to a glutamatergic phenotype. Surprisingly, this fate switch is also observed throughout the neonatal midbrain, except for the GABAergic neurons located in the ventral dopaminergic nuclei, suggesting a distinct developmental pathway for these neurons. These studies identify Gata2 as an essential post-mitotic selector gene of the GABAergic neurotransmitter identity and demonstrate developmental heterogeneity of GABAergic neurons in the midbrain.
of cranial motoneurons (Nardelli et al., 1999), rostral serotonergic neurons in rhombomere 1 (Craven et al., 2004) and spinal V2 interneurons (Zhou et al., 2000; Karunarathne et al., 2002). However, studies of Gata2 function in neuronal development have been hampered by the early death of Gata2-null embryos.

We show here that Gata2 is specifically expressed in the developing midbrain GABAergic neurons as they exit the cell cycle and differentiate. Using conditional mutagenesis and ectopic expression experiments, we show that Gata2 acts as an essential post-mitotic selector gene of the GABAergic identity without affecting proliferating neural progenitors or early neurogenic processes in the embryonic midbrain. We further map the GABAergic progenitor domains in the mouse embryonic midbrain and demonstrate region-specific interactions between Helt and Gata2. Interestingly, our results suggest that GABAergic neurons associated with the ventral DA nuclei in the midbrain use distinct regulatory mechanisms for their development.

MATERIALS AND METHODS

Mice

En1Cre (Kimmel et al., 2000), HeltCre [Mgniz (Guimera et al., 2006)] and Ascl1Cre [Mash1Cre (Guillemot et al., 1993)] alleles have been described previously. The conditional Gata2 allele (Gata2flox) will be described elsewhere (M.H., K.L. and M.S., unpublished). Briefly, in Gata2flox, exons 1-3, encoding the N-terminal half of Gata2, are flanked by loxP sites. Their recombination by Cre recombinase is expected to produce a null allele of Gata2. Gata2flox embryos were generated from En1Cre; Gata2flox × Gata2flox mouse crosses. For staging, the day of vaginal plug was counted as embryonic day 0.5 (E0.5). For BrdU-incorporation analysis, pregnant females were given an intraperitoneal injection of BrdU (3 mg/100 g body weight) 1-2 hours before dissecting the embryos. All experiments were approved by the Committee of Experimental Animal Research of the University of Helsinki, Finland.

In ovo electroporation

In ovo electroporation was performed at Hamburger-Hamilton stage (HH) 14-16. For Gata2 overexpression, pAdRSVGata2HA plasmid (El Wakil et al., 2006) was microinjected into the embryonic midbrain (third ventricle) together with the EGFP expression vector pEGFP-N3 (Clontech). As a control, embryos were electroporated with the EGFP expression vector only. Electroporation was performed at 20 µmV; 10 × 20 millisecond pulses were applied with 500 millisecond intervals. The embryos were harvested 24 or 48 hours later (at HH20-22) and EGFP-expressing embryos were embedded in paraffin for analysis. Five embryos electroporated with pAdRSVGata2HA and two control embryos were analyzed.

In situ mRNA hybridization and immunohistochemistry

Whole-mount mRNA in situ hybridization (ISH) analysis of E10.5 embryos was performed by a modified protocol (Henrique et al., 1995) using a digoxigenin-labeled antisense Gata2 cRNA probe. For ISH and immunohistochemistry (IHC) on sections, embryos or embryonic brains were fixed in 4% paraformaldehyde (PFA) at room temperature for 1-5 days. Samples were dehydrated and mounted into paraffin. Sections were cut at 5 µm, and adjacent sections were collected on separate slides for parallel stainings. mRNA ISH analyses on paraffin sections were performed as described (Wilkinson and Green, 1990) using 35S- or digoxigenin-labeled cRNA probes. Mouse cDNA probes used for ISH analysis were: Gata2, Gata3 (Lillevali et al., 2004), Ascl1, Fev (Pet1), Ngn2 (Jukkola et al., 2006), Gad1 (Gad67), Helt, Slc17a6 (Vglut2), Pax2 (Gumera et al., 2006), Pou4f1 (gift from Siew-Lan Ang, National Institute of Medical Research, London, UK), Nkx2-2 (IMAGE clone 480100), Pax6 (gift from P. Gruss, Max-Planck-Institute for Biophysical Chemistry, Goettingen, Germany), Isl1 (gift from V. Pachnis, National Institute of Medical Research, London, UK) and Lmx1b (gift from H. Simon, Interdisciplinary Centre for Neuroscience, Universität, Heidelberg, Germany). In addition, probes for chicken Gata3 (Lillevali et al., 2007), Ngn2 (Matter-Sadzinskii et al., 2001), Slc17a6 and Gad1 (Cheng et al., 2004) were used.

IHC was performed as described (Kala et al., 2008). The following antibodies were used: guinea pig anti-Heslike (Helt, 1:500; gift from R. Kageyama, Institute for Virus Research, Kyoto University, Japan), goat anti-HA probe (Santa Cruz sc-805-G, 1:500) and anti-Olig2 (Neuromics GT15132, 1:200), mouse anti-BrdU (GE Healthcare RPN20AB, 1:400), anti-HuC/D (Molecular Probes A21271, 1:500), anti-Lim1/2 [Lhx1, Developmental Studies Hybridoma Bank (DShB) 4F2, 1:10], anti-Mash1 (Ascl1, BD Biosciences 556604, 1:200), anti-Nkx2-2 (DShB 745A5, 1:250), anti-Nkx6-1 (DShB F55A10, 1:500) and anti-Pax6 (DShB PAX6, 1:100), rabbit anti-caspase 3 active (R&D Systems AF835, 1:500), anti-Gata2 (Santa Cruz sc-9008, 1:250), anti-5-HT (Immunostar 20080, 1:5000), anti-p57 (NeoMarkers RB-1637-P0, 1:500), anti-phospho-histone H3 (Upstate 60-570, 1:500), anti-Sox2 (Millipore AB5603, 1:500) and anti-TH (Millipore AB152, 1:500).

For combined ISH and IHC, additional primary antibodies were added together with the anti-DIG-POD Fab fragments (Roche). The TSA Fluorescence Palette System (PerkinElmer) was used to visualize ISH signal. Detailed ISH and IHC protocols are available upon request.

Microscopy and quantification

Whole-mount stainings were visualized with a Leica MZFLIII microscope and photographed using an Olympus DP50-CU camera. Stainings on paraffin sections were visualized with an Olympus AX70 microscope and photographed using an Olympus DP70 camera. Images were processed and assembled using Adobe Photoshop software. Red pseudo-color images representing the ISH results were produced by replacing the white signal in dark-field images with red, and overlaying the resulting image with the respective bright-field image.

Confocal images were acquired using the Leica TCS SP5 confocal system and LAS-AF software. Confocal stacks and images were processed and deconvoluted using Imaris 6.1 (Bitplane) and AutoQuantX (AutoQuant) software.

For quantification, cells were counted only from the Helt expression domain. For BrdU incorporation, cells from whole neuroepithelium were counted. For phospho-histone H3 expression, only cells lining the ventricle were counted. A standard Student’s t-test was used for comparing the mean values of the data sets.

RESULTS

Gata2 and Gata3 are expressed in the regions of GABAergic neurogenesis in the embryonic mouse midbrain

Gata2 and Gata3 are expressed in the developing mouse midbrain, but they have not been associated with any particular neuronal lineage (Nardelli et al., 1999; Zhou et al., 2000). We compared the pattern of expression of Gata2 and Gata3 with that of the neural subtype markers Lmx1b (DA neurons), Isl1 (Isl1et1, motoneurons), Pou4f1 (glutamatergic neurons) and glutamic acid decarboxylase 1 (Gad1, GABAergic neurons) in the mouse midbrain from E10.5 to E12.5 by in situ hybridization (ISH) (Fig. 1; see Fig. S1 in the supplementary material). Throughout development, expression of both Gata2 and Gata3 coincided with that of Gad1 and was flanked by Pou4f1 expression domains (Fig. 1A-L). Strong Gata2 and Gata3 ISH signal was detected in the intermediate and marginal zones. Weaker expression of Gata2, but no expression of Gata3, was also observed in the ventricular zone. We detected no Gata2 transcripts in Lmx1b- or Isl1-expressing regions in the mouse midbrain (Fig. 1A-C,M-R). From E12.5 onwards, scattered expression of Gata2, Gata3 and Gad1 appeared in the dorsal midbrain (Fig. 1C,F,I) correlating with the timing of GABAergic neurogenesis in this region (Tsunekawa et al., 2005).

In order to understand in detail the identity of cells expressing Gata2, we compared the expression of Gata2 protein with homeodomain transcription factors expressed in progenitor and precursor cells of the developing midbrain. Lhx1 (Lim1) is
expressed in post-mitotic precursor cells of all midbrain GABAergic neurons in domains m1-m5, as well as in glutamatergic neurons of the red nucleus in m6 (for a description of the midbrain domains m1-m7, see Fig. 8 and Nakatani et al. (Nakatani et al., 2007)). At E12.5, immunohistochemistry (IHC) demonstrated coexpression of Gata2 and Lhx1 in the intermediate zone cells of domains m3-m5, where GABAergic neurons are produced at this stage of development (Fig. 1S,T). Nkx6-1 and Nkx2-2 are expressed in specific subsets of GABAergic neuron progenitors and precursors (Nakatani et al., 2007). Colocalization of Gata2 and Nkx6-1 was observed in the basal ventricular zone of domains m5 and m3 (Fig. 1U; see Fig. S1A in the supplementary material). Also, some progenitor/precursor cells in m4 expressed both Nkx2-2 and Gata2 (Fig. 1V; see Fig. S1B in the supplementary material). Thus, we found Gata2 expression in all the midbrain regions that give rise to GABAergic neurons.

We observed a small area that was negative for Gata2, Gata3 and Gad1 expression in the m4 domain expressing Nkx2-2 (Fig. 1B,C,F,H,I). Pax6 is also expressed near the Nkx2-2-positive region in the developing midbrain (Ahsan et al., 2007). By combining ISH and IHC, we showed that Pax6 is expressed in a subset of Nkx2-2-positive cells in the ventral m4 domain at E11.5 (see Fig. S1C in the supplementary material). Interestingly, no Gata2 protein was detected in the Pax6-expressing cells (Fig. 1W).

Next, we analyzed the neurotransmitter identities in the m4 marginal zone. Both ISH and IHC demonstrated that the Pax6-positive cells express Slc17a6 (vesicular glutamate transporter 2 (Vglut2)) and are negative for Gad1 (see Fig. S1D-G in the supplementary material). Thus, the m4 domain can be further divided into a GABAergic Gata2-positive dorsal part (m4-D) and a glutamatergic Pax6-positive ventral part (m4-V). In addition to the m4-V, putative glial progenitors expressing Olig2 were also devoid of Gata2 expression at E12.5 (Fig. 1X). In conclusion, the expression of Gata2 mRNA and protein correlates with the spatial and temporal appearance of the GABAergic neuron lineage in the developing mouse midbrain.

Gata2 is activated in the developing GABAergic neurons as the progenitor cells exit the cell cycle and start to differentiate

To gain insight to the possible role of Gata2 in GABAergic neurogenesis, we compared the expression of the bHLH transcription factors Ascl1 and Helt with that of Gata2 in the m3-m5 ventricular zone by IHC (Fig. 2A-C). As reported, extensive coexpression of Ascl1 and Helt was observed in the progenitors (Fig. 2D) (Miyoshi et al., 2004). Notably, we observed coexpression mostly at the apical and medial regions of the ventricular zone, while on the basal side, the Ascl1-expressing nuclei were negative for Helt. Also, Gata2 was detected in the Ascl1-positive cells (Fig. 2F), but, in contrast to Helt, its expression was detected primarily in the nuclei on the basal side of the ventricular zone (Fig. 2E,F). In the medial ventricular zone, nuclei coexpressing Helt and Gata2 were detected (Fig. 2E). The gradual upregulation of Gata2 in the Ascl1- and Helt-expressing cells/nuclei as they move further away from the ventricular surface suggested that Gata2 expression might be switched on in the GABAergic progenitors that are about to exit the ventricular zone. To confirm this, we analyzed the Gata2-expressing cells for expression of HuC/D (Elavl3/4), a marker of post-mitotic neurons, and for DNA

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**Fig. 1.** Gata2 and Gata3 expression in embryonic mouse midbrain compared with expression of neural subtype markers. (A-R) In situ hybridization (ISH) with Gata2, Gata3, Gad1, Pou4f1, Isl1 and Lmx1b probes on coronal sections of wild-type embryos. The embryonic stages are indicated at the top of each column. Black arrows point to the negative areas within the expression domains of Gata2, Gata3 and Gad1. (S-X) Co-immunostaining for Gata2 (red) and Lhx1, Nkx6-1, Nkx2-2, Pax6 or Olig2 (green). The boxed area in S indicates the region enlarged in T-X. White arrows indicate examples of Gata2 and Nkx6-1 (U), and Gata2 and Nkx2-2 (V), coexpressing cells. Scale bars: 100 μm.
To study the role of embryonic midbrain loss of GABAergic neuron precursors in the inactivated [Mgk^{pp2} (Guimera et al., 2006)] for Gata2 mRNA expression. Interestingly, Gata2 was undetectable in E11.5 and E13.5 Helt^{ko} midbrain, except for the ventral-most GABAergic neurons (Fig. 3A,B; data not shown). The remaining Gata2 expression correlated with Gad1 expression (Fig. 3C,D, arrow). We detected no Gata2 or Gad1 expression in the dorsal midbrain of the E13.5 Helt^{ko} embryos (data not shown). At the same time, Ascl1 expression was unaffected by Helt inactivation (Fig. 3E,F), as shown previously (Nakatani et al., 2007).

To test whether Ascl1 also regulates Gata2 expression, we analyzed E11.5 Ascl1-null mutants (Guillemot et al., 1993). In these embryos, expansion of the Sox2- and Helt-expressing layer suggested a failure in cell cycle exit of GABAergic progenitors (Fig. 3I-L). However, Gata2 was still expressed in regions where post-mitotic precursors were produced (Fig. 3G,H). Altogether, except for the ventral-most GABAergic precursors, activation of Gata2 requires Helt, but does not require Ascl1.

Conditional Gata2 inactivation leads to specific loss of GABAergic neuron precursors in the embryonic midbrain

To study the role of Gata2 in GABAergic neuron development, we inactivated Gata2 in the mouse midbrain and rhombomere 1 (r1) by crossing mice carrying a conditional allele of Gata2 (M.H., K.L. and M.S., unpublished) with those carrying the En1^{Cre} allele (Kimmel et al., 2000). In the En1^{Cre} mouse strain, the Cre recombinase activity has been demonstrated as early as the 5-10 somite stage (Chi et al., 2003; Trokovic et al., 2003), well before the expression of Gata2 in the midbrain-r1 region. In the En1^{Cre}; Gata2^{flx/flx} (Gata2^{cko}) mice, no Gata2 expression was detected in the midbrain-r1 region at E10.5 or E11.5 (see Fig. S2 in the supplementary material).

Interestingly, we observed a complete loss of Gad1, Gad2 and Gata3 expression in Gata2^{cko} midbrain at E11.5 and E13.5 (Fig. 4A,A',B,B',E,F; see Fig. S3A-H in the supplementary material). No GABAergic neuron precursors were detected at any dorsoventral or anteroposterior level in the midbrain of Gata2^{cko} mutants at these stages. These results suggested an early and absolute requirement for Gata2 in midbrain GABAergic neuron development that cannot be compensated for over time.

Unaltered progenitor cell proliferation, patternning, neurogenesis, survival and cell cycle exit in the Gata2^{cko} mutant midbrain

Next, we investigated which steps in midbrain GABAergic neuron development are affected by the loss of Gata2. Despite the loss of GABAergic precursors, there were no overt anatomical defects in the embryonic Gata2^{cko} mutant midbrain. Nevertheless, we analyzed in more detail the properties of the proliferative progenitor cells that give rise to the midbrain GABAergic precursors. In the Gata2^{cko} mutants, the ventricular zone progenitors still expressed Sox2 and there was no difference in the thickness of the Sox2-positive ventricular zone or HuC/D-positive marginal zone compared with the wild type (see Fig. S4A-E in the supplementary material). Consistently, we detected no major changes in the numbers of phospho-histone H3-positive mitotic nuclei or BrdU-incorporating S-phase nuclei in the GABAergic progenitor domain of the Gata2^{cko} mutants (see Fig. S4F-K in the supplementary material). Also, we observed no increase in apoptotic cell numbers (see Fig. S4N,O in the supplementary material). Thus, we conclude that the loss of GABAergic neurons is not due to impaired proliferation or survival of their progenitors.
We then characterized patterned and neurogenesis in the GABAergic neuron progenitors of the Gata2cko mutants. All the analyzed transcription factors, including Nkx6-1, Nkx2-2 and Pax7, involved in the patterning of the ventricular zone progenitor cell layer were correctly expressed (Fig. 4K,L,Q,T; data not shown). The transcription factors showing normal expression in the progenitors also included Ascl1 and Helt (Fig. 4M,N,I,J), as well as Ngn2, which has been suggested to be involved in glutamatergic neurogenesis in the midbrain (Fig. 4O,P). In addition, we observed no change in the expression of p57 (Cdkn1c), a cell cycle inhibitor upregulated at withdrawal from the cell cycle, demonstrating that Gata2-deficient progenitors are still able to exit from the cell cycle and become post-mitotic precursors (see Fig. S4L,M in the supplementary material). Our results suggest that progenitor cell patterning and neurogenic cell cycle exit are not disturbed by the loss of Gata2 in the midbrain.

GABAergic-to-glutamatergic fate transformation of the post-mitotic precursors in the midbrain of Gata2cko mutants

Since the GABAergic progenitors were unaffected in Gata2cko midbrain, we analyzed the characteristics of the post-mitotic neuronal precursors. In contrast to the normal appearance of the GABAergic progenitor cell layer, all the post-mitotic derivatives of these cells had transdifferentiated into a glutamatergic phenotype in the Gata2cko midbrain. In E11.5 Gata2cko mutants, the expression of Slc17a6 expanded to cover the entire marginal zone, including the regions where the GABAergic precursors should reside (Fig. 4C,C’,D,D’). In addition, we detected ectopic expression of Pou4f1 in the post-mitotic cells of the m3 domain (Fig. 4G,H), indicating that in the absence of Gata2, differentiating neurons in m3 could have acquired a glutamatergic neuron fate typical for the adjacent m2. Ventrally in the m5 domain, Nkx6-1 is downregulated during post-mitotic differentiation of wild-type GABAergic precursor cells. We noticed continued Nkx6-1 expression in the m5 marginal zone in the E11.5 Gata2cko mutants, similar to that in the neighboring glutamatergic domain m6 (Fig. 4S,T). However, the fate transformation is incomplete because Pou4f1, another marker of m6, was not activated in the mutant m5 domain (data not shown).

In addition to Gad1, Gad2 and Gata3, other genes characteristic of the post-mitotic GABAergic neuron precursors were also downregulated in the Gata2cko mutants. At E11.5, Lhx1 was downregulated in the m5 and m3 domains, but persisted in the m4 (Fig. 4U,V). In addition, the glutamatergic domain m6 continued to express Lhx1, as expected. Although some Lhx1-positive cells appeared in the marginal zone of m5 (Fig. 4V), they might have originated from neighboring domains, as the intermediate zone of m5 is negative for Lhx1 but both m4 and m6 continued to express it. The m4 domain also appeared to retain its identity as judged by continued Nkx2-2 expression and lack of Pou4f1 expression in the Gata2cko mutants (Fig. 4K,L,G,H). Despite continued Lhx1 and Nkx2-2 expression, there was complete loss of GABAergic markers and uniform glutamatergic marker gene expression in the m4 domain of the mutants. This discrepancy could be explained by an uncoupling of regional patterning and neurotransmitter selection, as suggested by Nakatani et al. (Nakatani et al., 2007). Alternatively, because m4 produces both GABAergic and glutamatergic precursors (see above), there could be a transformation within m4 whereby the GABAergic m4 precursors (m4-D) assume an identity of glutamatergic m4 precursors (m4-V, Fig. 4A,C). To test this, we analyzed Pax6 expression in the Gata2cko mutants. Ectopic Pax6-expressing cells were observed in the m4-D region (Fig. 4W,X) indicating that in the absence of Gata2, the whole m4 domain acquired a fate similar to m4-V glutamatergic neurons. Thus, Gata2 is essential for activating gene expression patterns typical for post-mitotic GABAergic precursor cells in the midbrain. Without Gata2, the precursor cells appear to adopt a phenotype resembling, but not necessarily identical to, that of the adjacent glutamatergic regions.

Ectopic Gata2 is sufficient to induce GABAergic differentiation in embryonic chicken midbrain

We next examined whether Gata2 alone is sufficient to switch on the GABAergic differentiation pathway. For this, we employed ectopic expression of Gata2 in chicken embryos. First, we studied the pattern of endogenous chicken Gata2 (cGata2) expression and its relationship with the development of GABAergic neurons in chick midbrain. As in the mouse embryo at the equivalent stage, expression of Helt, Gata2, cGata3, Lhx1 and cGad1 coincided in the ventrolateral chick midbrain, and was flanked by cNgn2- and cSlc17a6-expressing glutamatergic domains at HH22-24 (Fig. 5A-I). Similar to in
E12.5 mouse midbrain, we observed scattered cGad1 expression in the chick dorsal midbrain at a slightly later stage (HH22-24, Fig. 5H). Unlike in the mouse, dorsal cNgn2 expression was detected primarily in the marginal zone and might mark the differentiated glutamatergic neurons rather than their progenitors (Fig. 5C). However, our results demonstrate that overall, the pattern, timing and regulatory mechanisms of midbrain GABAergic neuron production are likely to be conserved between these two species.

We then used tissue electroporation to overexpress Gata2 in the dorsal chick midbrain, which normally gives rise to both GABAergic and glutamatergic neurons. Abundant Lhx1-, cGata3- and cGad1-positive cells were observed in the regions expressing ectopic Gata2, 24 and 48 hours after electroporation (Fig. 5J-M,O,P; data not shown). No ectopic expression of GABAergic markers was observed on the control side not expressing ectopic Gata2 or in embryos electroporated with the GFP-expressing control vector only (data not shown). At the same time, expression of cNgn2 was reduced dorsally (Fig. 5N,Q). Given the pattern of cNgn2 expression (see above), this might reflect a specific failure in the production of glutamatergic neurons, rather than a defect in their ventricular zone progenitors. However, general developmental retardation of the Gata2-electroporated tissue might also contribute to cNgn2 downregulation. In all, these results suggest that Gata2 is sufficient to induce a GABAergic phenotype, as judged by cGad1, cGata3 and Lhx1 expression, in the midbrain neuroepithelium.

Normal development of GABAergic, but loss of serotonergic, neurons in rhombomere 1 of Gata2cko mutants

To study GABAergic neuron development in r1, we analyzed E11.5 wild-type and Gata2cko mouse embryos for Gata3 and Gad1 expression. In striking contrast to the midbrain, where both genes were completely downregulated, Gata3 and Gad1 expression in the GABAergic precursors of r1 was not altered in the mutant (Fig. 6A,B,E,F). Thus, Gata2 is dispensable for the early development of GABAergic neurons in r1.

Previous studies have suggested a role for Gata2 and Gata3 in the development of other neuronal populations in the midbrain-r1 region, including cranial motoneurons and serotonergic neurons (Nardelli et al., 1999; Craven et al., 2004; Pattyn et al., 2004). Consistent with the pattern of Gata2 expression, we observed no defects in the cranial Isl1-expressing motoneurons of nIII and nIV in the Gata2cko mutants (see Fig. S3I,J in the supplementary material). Thus, the nIII/nIV defects in the Gata2-null mutants were likely to be secondary to other developmental abnormalities in these embryos. By contrast, almost no serotonin (5-HT)-, Lmx1b- or Fev (Pet1)-positive serotonergic neurons were detected in r1 of E11.5 and E13.5 Gata2cko mutants (Fig. 6C,D,G-L). Despite its continued expression in the GABAergic precursors, Gata3 was specifically downregulated in the serotonergic neuron precursors in r1 (Fig. 6A,B). These results confirm that Gata2 is required for the development of rostral serotonergic neurons. They also further demonstrate the loss of Gata2 function in r1 of Gata2cko mutants.
Analysis of midbrain GABAergic neuron subpopulations in perinatal Gata2cko mutants demonstrates differential requirements for Gata2

To study whether the cells with the transformed neurotransmitter identity contribute to the maturing brain, we analyzed Gata2cko mutants shortly before birth. At E17.5 and E18.5, the gross brain morphology of the Gata2cko mutants was similar to that of the wild type. In sagittal sections, we observed loss of expression of the GABAergic markers Gad1, Gata3 and Pitx2 in the Gata2 cko midbrain, in both the dorsal and ventral brain regions (see Fig. S5 in the supplementary material). By contrast, abundant expression of GABAergic marker genes was detected in r1. Thus, consistent with the loss of GABAergic precursor cells in the midbrain at earlier stages of development, inactivation of Gata2 leads into a specific loss of GABAergic neurons in the perinatal midbrain but not in r1.

To examine the development of the GABAergic neurons in the ventral midbrain, we performed ISH with Gad1 and Slc17a6 probes and IHC with anti-tyrosine hydroxylase (TH) antibodies on adjacent coronal sections of E18.5 embryos. Loss of Gad1 and Gata3 expression in the ventrally located VTA and SNpr was unaltered. No increase in Slc17a6 expression was observed in these nuclei either. Therefore, unlike in the rest of the midbrain, GABAergic neurons associated with the ventral DA nuclei appear normal in the Gata2cko mutants.

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To examine the development of the GABAergic neurons in the ventral midbrain, we performed ISH with Gad1 and Slc17a6 probes and IHC with anti-tyrosine hydroxylase (TH) antibodies on adjacent coronal sections of E18.5 embryos. Loss of Gad1 and Gata3 expression in the ventrally located VTA and SNpr was unaltered. No increase in Slc17a6 expression was observed in these nuclei either. Therefore, unlike in the rest of the midbrain, GABAergic neurons associated with the ventral DA nuclei appear normal in the Gata2cko mutants.

To test the hypothesis that the GABAergic neurons of VTA and SNpr were derived from the midbrain neuroepithelium late in development, we analyzed Gad1 and Gata3 expression in the...
ventral midbrain at E13.5 and E15.5, after the neurogenic period of this brain region (Altman and Bayer, 1981). Few Gad1- or Gata3-expressing cells were detected at E15.5 in the VTA-SNpr area of wild-type or Gata2^cko embryos (Fig. 7I-L,M-P; see Fig. S3 in the supplementary material). Thus, GABAergic neurons associated with the DA nuclei appear late in development.

**DISCUSSION**

Despite their importance in multiple aspects of behavior (Behbehani, 1995; Fields et al., 2007; Brandao et al., 2008), development of the midbrain GABAergic neurons is poorly understood. Here, we mapped the regions of the embryonic midbrain that give rise to GABAergic neurons. We identified Gata2 as the first post-mitotic selector gene required for GABAergic identity in the midbrain. Finally, our results demonstrate differences in the regulatory pathways and developmental history of the GABAergic neurons in the midbrain-r1 region.

**Gata2 marks regions of midbrain GABAergic neurogenesis**

A useful dorsoventral fate map of the developing mouse midbrain was recently presented by Nakatani et al. (Nakatani et al., 2007). We detected Gata2 and Gata3 expression in all midbrain domains where GABAergic neurons are produced. Our studies suggest a further refinement to the map of Nakatani et al. We propose that the Nkx2-2-positive m4 domain gives rise to both GABAergic and glutamatergic neurons, the latter being negative for Gata2 and positive for Pax6 expression (Fig. 8A). In the future, it will be important to further define the mature brain structures to which these neural progenitor and precursor subpopulations contribute.

In the ventricular zone, the pattern of colocalization of Ascl1, Helt and Gata2 suggests that during their maturation, the Ascl1^-Helt^ progenitors turn into Ascl1^-Gata2^ precursors. Consistent with this, inactivation of Helt resulted in a dramatic reduction in Gata2 expression. Our results thus suggest that Gata2 expression is activated in a Helt-dependent fashion as the neural progenitor cells turn into post-mitotic GABAergic neuron precursors and leave the ventricular zone. The ventral m5 domain appears to be an exception, as here Helt was not essential for Gata2 expression (Fig. 8B). Regulation of Gata2 expression by an as yet unidentified mechanism in m5 is likely to explain the more severe phenotype of Gata2^cko, as compared with Helt, mutants.

**Gata2 is a selector gene for GABAergic neuron identity of the post-mitotic midbrain precursors**

Gata2 has been proposed to inhibit neural progenitor cell proliferation and promote post-mitotic differentiation, a model consistent with its pattern of expression (El Wakil et al., 2006). However, our results suggest that there are no marked changes in embryonic brain morphology and layering, progenitor proliferation, or neurogenic cell cycle exit in conditional Gata2 mutants. Thus,
although Gata2 may still contribute to the cell cycle exit of neuronal progenitors, it is dispensable in this respect at least in the midbrain.

By contrast, the Gata2 mutant neural precursors in the midbrain appear to undergo a complete cell fate transformation. Several genes, the expression of which is normally activated in the post-mitotic GABAergic precursors, failed to be expressed in the Gata2 mutants. In addition, Nkx6-1, the expression of which is normally downregulated upon GABAergic differentiation in the m5 domain, continued to be expressed in the conditional Gata2 mutants. Instead of the GABAergic phenotype, the Gata2-deficient midbrain precursor cells activate expression of glutamatergic marker genes. In the Gata2 mutants, Slc17a6 expression was observed throughout the midbrain, whereas Pou4f1 expression was not ectopically activated in m4. By contrast, expression of Pax6 expanded in the m4 domain of Gata2 mutants. As we showed that Pax6 is specifically expressed in glutamatergic precursors in m4, these results together suggest that in the conditional Gata2 mutants, the identity of m4 is retained but the GABAergic m4 precursors switch to a glutamatergic fate. Also, in other parts of the midbrain, the Gata2-deficient precursors still have regional characteristics despite the transformation of their neurotransmitter identity.

Importantly, all the observed changes in gene expression take place in the post-mitotic precursors and neurons in the intermediate and marginal zones, whereas the proliferative progenitor cells and proneural gene expression in the ventricular zone are unaffected. This is in a clear contrast to the function of Helt in the ventricular zone, which has been shown to support GABAergic neurogenesis partly by repressing Ngn2 expression in the progenitors and thereby glutamatergic neuron production (Nakatani et al., 2007). In addition to repressing Ngn2, Helt activates GABAergic gene expression (Miyoshi et al., 2004; Guimera et al., 2006; Nakatani et al., 2007), and Gata2 appears to be important for this function. Thus, the expression and function of Gata2 in the midbrain are analogous to those of the bHLH transcription factor Ptf1a, which acts as a post-mitotic selector gene of the GABAergic identity in the developing cerebellum and spinal cord (Glasgow et al., 2005; Hoshino et al., 2005; Mizuguchi et al., 2006).

Developmental diversity of the midbrain GABAergic neurons

In the perinatal Gata2 mutants, all the midbrain GABAergic neuron subpopulations were transformed to a glutamatergic phenotype, except for the GABAergic neurons associated with the DA neurons in the VTA and SNpr. This was highly unexpected because no GABAergic precursor cells were found at any dorsolateral level of the midbrain at the earlier stages of development. There are at least three possible explanations for this. Firstly, there could be incomplete recombination of the Gata2\textsuperscript{flox} allele by En\textsuperscript{Cre}. We find this unlikely because we could not see any Gata2 transcripts or protein in the Gata2\textsuperscript{cko} mutants analyzed at several stages. Also, no evidence for mosaic inactivation of Gata2 was observed in VTA, SNpr, or other regions of the midbrain. Secondly, the remaining GABAergic neurons could be born in a region of the midbrain that does not require Gata2 and was missed in our analysis. We cannot completely rule out this possibility, but nor is there any evidence for it, as no Gad1- or Gata3-positive cells were observed at any anteroposterior or dorsolateral level of the mutant midbrain at E11.5-15.5. Finally, the GABAergic neurons of the VTA and SNpr could be derived from neuroectoderm outside the midbrain. The late appearance of the VTA and SNpr GABAergic neurons (~E15.5) and their strikingly normal development in the Gata2\textsuperscript{cko} mutants are consistent with this hypothesis. Possible sources of the VTA and SNpr GABAergic neurons include r1 and the diencephalon. However, cell lineage-tracing experiments are needed to unambiguously determine the origin of these ventral-most GABAergic neurons.

Gata2 is dispensable for GABAergic, but essential for serotonergic, neuron development in rhombomere 1

Similar to in the midbrain, Gata2 and Gata3 are expressed in the GABAergic precursors of r1. Strikingly, however, we observed abundant GABAergic neurons in r1 of the Gata2 conditional mutants, despite the complete loss of Gata2 in this brain region. Interestingly, the hindbrain GABAergic precursors continued to express Gata3. This suggests that Gata3 expression is, perhaps, not directly regulated by Gata2 in the GABAergic precursors in r1. Analyses of Gata3 mutants and, possibly, Gata2; Gata3 double mutants, are needed to determine whether Gata3 can possibly compensate for the loss of Gata2 specifically in the r1 GABAergic precursor cells.

The development of rostral serotonergic neurons has also been shown to be regulated by Gata2. Craven et al. used explant cultures of Gata2-null mutant neuroepithelium to demonstrate a requirement for Gata2 for the differentiation of serotonin-positive cells in r1 (Craven et al., 2004). Consistent with this, our results show loss of serotonergic neurons in the conditional Gata2 mutants. In contrast to the Gata2 mutant explant cultures, our results suggest that loss of Gata2 also leads to downregulation of Gata3 in the serotonergic neuron precursors. This discrepancy might be explained by distorted tissue architecture of the cultured explants and by the continued expression of Gata3 in the nearby GABAergic precursors in r1. It will be of interest to determine whether Gata2 also acts as a post-mitotic selector gene in the serotonergic neuron lineage, similar to its role in the developing midbrain GABAergic neuron precursors.

Conclusion

GABAergic versus glutamatergic neuron identity appears to be regulated by different genetic cascades in different regions of the CNS. Here we show specific requirement for the transcription factor Gata2 as a cell-fate selector, and locate it in the gene hierarchy regulating development of the GABAergic neurons in the midbrain (Fig. 8B). Our study also further elucidates how regional identities are generated in the distinct midbrain GABAergic neuron subpopulations. Understanding the development, molecular identity and functional characteristics of these diverse neurons might lead to better diagnostics and treatment of several forms of neurological and psychiatric disease.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/253/DC1

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