Distinct developmental origins and regulatory mechanisms for GABAergic neurons associated with dopaminergic nuclei in the ventral mesodiencephalic region

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SUMMARY
GABAergic neurons in the ventral mesodiencephalic region are highly important for the function of dopaminergic pathways that regulate multiple aspects of behavior. However, development of these neurons is poorly understood. We recently showed that molecular regulation of differentiation of the GABAergic neurons associated with the dopaminergic nuclei in the ventral midbrain (VTA and SNpr) is distinct from the rest of midbrain, but the reason for this difference remained elusive. Here, we have analyzed the developmental origin of the VTA and SNpr GABAergic neurons by genetic fate mapping. We demonstrate that the majority of these GABAergic neurons originate outside the midbrain, from rhombomere 1, and move into the ventral midbrain only as postmitotic neuronal precursors. We further show that Gata2, Gata3 and Tal1 define a subpopulation of GABAergic precursors in ventral rhombomere 1. A failure in GABAergic neuron differentiation in this region correlates with loss of VTA and SNpr GABAergic neurons in Tal1 mutant mice. In contrast to midbrain, GABAergic neurons of the anterior SNpr in the diencephalon are not derived from the rhombomere 1. These results suggest unique migratory pathways for the precursors of important GABAergic neuron subpopulations, and provide the basis for understanding diversity within midbrain GABAergic neurons.

KEY WORDS: GABAergic neuron, Ventral tegmental area (VTA), Substantia nigra pars reticulata (SNpr), Dopaminergic neuron, Gata2, Tal1, Mouse

INTRODUCTION
GABAergic neurons are the primary source of inhibitory signals in the mammalian brain. Like the central nervous system in general, functionally distinct subpopulations of GABAergic neurons are distributed in several regions of the midbrain. Of special interest are the GABAergic neurons associated with the dopaminergic nuclei in ventral tegmental area (VTA) and substantia nigra (SN) in the ventral midbrain. These GABAergic neurons regulate the activity of dopaminergic pathways, and are involved in the control of mood, motivation and voluntary movements (Laviolle and van der Kooy, 2004; Tepper and Lee, 2007). In addition to direct regulation of the dopaminergic neurons themselves, a substantial proportion of the ascending projections from the dopaminergic nuclei are in fact GABAergic in nature (Fields et al., 2007). As a result, ventral midbrain GABAergic neurons regulate processing of appetitive and aversive stimuli and are targets of treatment for mental disorders as well as drugs of abuse (Cohen et al., 2012; Jhou et al., 2009; Vargas-Perez et al., 2009). Thus, understanding the composition and development of the ventral midbrain GABAergic neurons is of great importance.

Although the various subpopulations of GABAergic neurons share their primary neurotransmitter, they are different in their molecular composition, morphology and function. Different GABAergic neuron subpopulations also use distinct developmental regulatory mechanisms. Indeed, in midbrain, the VTA and SN pars reticulata (SNpr) GABAergic neurons appear molecularly distinct, as they develop independently of the known transcriptional regulators of midbrain GABAergic neurogenesis, e.g. Ascl1, Helt or Gata2 (Guimera et al., 2006; Kala et al., 2009; Nakatani et al., 2007; Peltopuro et al., 2010). Most strikingly, in the conditional Gata2 mutants, GABAergic neurons are specifically retained in the VTA and SNpr region, despite complete failure of GABAergic neurogenesis in the embryonic midbrain and fate transformation in the presumptive GABAergic precursor populations. In these embryos, GABAergic neurons are still generated in the diencephalon, which has normal Gata2 expression, and in rhombomere 1 (r1), where GABAergic neurogenesis does not require Gata2 (Kala et al., 2009). Thus, it is possible that the GABAergic neurons in the VTA and SNpr are derived from brain regions that flank the ventral midbrain.

To clarify the developmental origin of the midbrain GABAergic neurons, we performed a series of tissue-specific mutagenesis and genetic fate-mapping analyses. We demonstrate that, in the midbrain, the VTA and SNpr GABAergic neurons are derived from r1, whereas the diencephalic SNpr GABAergic neurons have a distinct origin. Our results show that the VTA and SNpr GABAergic precursors exit the proliferative neuroepithelium around the same time as other midbrain GABAergic neuron populations, but assume their final locations only much later during post-mitotic differentiation. Finally, we demonstrate transcriptional regulatory mechanisms of GABAergic neuron differentiation in r1 and show that a failure

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in GABAergic neuron production in a specific domain of ventral r1 correlates with a later loss of VTA and SNpr GABAergic neurons in the midbrain.

MATERIALS AND METHODS

**Mice**

En1\(^{Cre}\) (Kimmel et al., 2000), Foxg1\(^{Cre}\) (Hebert and McConnell, 2000), Gad67\(^{GFP}\) (Tamamaki et al., 2003), Gata2\(^{2lox}\) (Haugas et al., 2010), Gata3\(^{3lox}\) (Kurek et al., 2007), Gbx2\(^{CreERT2}\) (Chen et al., 2009), R26R reporter (Soriano, 1999), Rosa26\(^{Tdtomato}\) reporter (Jackson Laboratories stock 007914) (Madisen et al., 2010), Shh\(^{Cre}\) (Harfe et al., 2004) and Tal1\(^{lox}\) (Bradley et al., 2006) alleles have been described previously. For staging, the day of vaginal plug was counted as embryonic day 0.5 (E0.5). The activity of Gbx2\(^{CreERT2}\) activity was induced by a single administration of tamoxifen (4 mg/30 g body weight, dissolved in corn oil) either by intraperitoneal (IP) injection at E8.5, or by oral injection at E9.5. All results were reproduced with at least three embryos per genotype. All experiments were approved by the committee of experimental animal research in Finland.

In situ mRNA hybridization and immunohistochemistry

mRNA in situ hybridization analyses on paraffin sections were performed essentially as described (Wilkinson and Green, 1996) using digoxigenin- or \(^{35}S\)-labeled eRNA probes. Mouse cDNA probes used for in situ hybridization analysis were Gata2, Gata3 (Lillevalti et al., 2004), Gad1 (Gad67) (Guimerà et al., 2006), Corin (Ono et al., 2007) and Tal1 (IMAGE 6826611). For combined in situ hybridization and immunohistochemistry, additional primary antibodies were added together with the anti-DIG-POD Fab-fragments (Roche). TSA Fluorescence Palette System (PerkinElmer) was used to visualize the in situ hybridization signal.

Immunohistochemistry was performed as described previously (Kala et al., 2008). The following antibodies were used in this study: goat anti-GFP (Abcam ab6673, 1:500), anti-Otx2 (R&D Systems BAF1979, 1:200), mouse anti-BrdU (GE Healthcare RPN20AB, 1:400), anti-Gata3 (Santa Cruz SC-268, 1:200), anti-Pou4f1 (Santa Cruz sc-8429, 1:400), anti-Nkx2.2 (1:400, DSHB 74.5A), anti-Nkx6.1 (1:400, DSHB F55A10) and anti-TH (Millipore MAB318, 1:300); rabbit anti-β-gal (MP Biomedicals 55976, 1:1000), anti-5-HT (Immunostar 20080, 1:500), anti-GFP (Abcam ab290, 1:600), anti-Lmxa1 (a gift from Michael German, University of California at San Francisco, San Francisco, CA, 1:750), anti-RFP (Rockland 600-401-379, 1:500) and anti-TH (Millipore AB152, 1:500). X-gal staining was performed as described previously (Kala et al., 2008).

**Birth dating by BrdU labeling**

Two different BrdU labeling regimes were used: (1) for the birth-date endpoint analysis, pregnant females were given IP injection of BrdU (3 mg/100 g body weight) every 24 hours for the indicated periods of time, the last injection given at E17.5; (2) for detection of the peak of neurogenic proliferation, pregnant females were given IP injection of BrdU (3 mg/100 g body weight) every 24 hours for the indicated periods of time, the last injection given at E11.5 (Fig. 1K). Altogether, these results showed that the post-mitotic GABAergic neuron progenitors are produced first in the mRF, with a labeling peak at E10.5. The majority of last mitoses of the progenitors of SNpr and SC GABAergic neurons occur later, with labeling peaks at E11.5 (Fig. 1K). Altogether, these results show that production of post-mitotic precursors of the SNpr GABAergic neurons is completed by E13.5 and that they are produced around the same developmental stages as other midbrain GABAergic neurons, but with slightly different kinetics.

Next, we analyzed the timing of GABAergic neurogenesis in SNpr of the En1\(^{Cre}\);Gata2\(^{2lox}\) (En1\(^{Cre}\);Gata2\(^{2lox}\)) embryos in which Gata2 has been inactivated in midbrain and r1 (Kala et al., 2009). For this, pregnant females received BrdU once a day, starting either at E10.5 or at E13.5, and the SNpr region was analyzed at E18.5. The results in these experiments were highly similar to the ones obtained earlier in wild-type embryos only (Fig. 1L). Approximately half of Gad1-expressing cells in SNpr were BrdU labeled when injections were started at E10.5, whereas starting the labeling 2 days later resulted in less than 10% of the cells being BrdU positive. No statistically significant difference was detected between mutant and wild-type embryos. These data suggest that SNpr neurons are born at the same time in both wild-type and En1\(^{Cre}\);Gata2\(^{2lox}\) embryos.

We then compared expression of Gad1 in En1\(^{Cre}\);Gata2\(^{2lox}\) and wild-type midbrain at E13.5 and E16.5. As at the earlier stages of embryogenesis (Kala et al., 2009), no Gad1 expression could be detected in E13.5 mutant midbrain (Fig. 1H,l). However, 3 days later Gad1 expression was detected in the mutant SNpr (Fig. 1M,N). Thus, the post-mitotic precursors of VTA and SNpr GABAergic neurons are produced with unique kinetics, but at the time when the VTA and SNpr GABAergic neurons are born, no GABAergic precursors can be detected in the Gata2 mutant midbrain.

**RESULTS**

**VTA and SNpr GABAergic neurons are born with unique kinetics, but their early precursors are not detected in the midbrain**

To analyze the developmental origin of the VTA and SNpr GABAergic neurons, we wanted to establish (1) when the VTA and SNpr GABAergic neurons exit the cell cycle and (2) whether GABAergic precursors can be detected in the Gata2 mutant midbrain at this stage.

First, we determined the developmental stage when the neurogenesis of distinct midbrain GABAergic neuron subpopulations is completed. For this, BrdU was injected into pregnant females once a day between E10.5 and E17.5, E11.5 and E17.5, E12.5 and E17.5, E13.5 and E17.5, and E14.5 and E17.5. BrdU incorporation was analyzed in Gad1-expressing midbrain GABAergic neurons in the SNpr, midbrain reticular formation (mRF) and superior colliculus (SC) at E18.5 (Fig. 1A-E,J). Of these three GABAergic neuron populations, neurogenesis was completed first in the mRF (by E12.5) followed by SNpr (by E13.5) and SC (by E14.5).

Second, we aimed to determine the stage of most active cell-cycle exit for the precursors of distinct midbrain GABAergic neuron populations. For this, pregnant females were given six BrdU injections at 3 hours intervals, starting at E9.5, E10.5, E11.5, E12.5 or E13.5. Embryonic brains were dissected at E18.5 and the numbers of GABAergic neurons with robust BrdU incorporation was analyzed in the SNpr, mRF and SC (Fig. 1F,G,K). Because of dilution of the BrdU-containing DNA during cell proliferation, we estimate that this labeling scheme allows the detection of direct progeny of the BrdU-incorporating cells after one or two rounds of cell division. The results showed that the post-mitotic GABAergic neuron progenitors are produced first in the mRF, with a labeling peak at E10.5. The majority of last mitoses of the progenitors of SNpr and SC GABAergic neurons occur later, with labeling peaks at E11.5 (Fig. 1K).

For quantification, the nuclei of the Gad1+ or TH-positive cells were counted from the areas indicated. β-Gal or BrdU-positive cells were counted among these marked nuclei. At least four different E18.5 brains were analyzed per labeling regime and stage (BrdU labeling) or genotype (genetic fate-mapping).
SNpr is composed of both Gata2 dependent and Gata2 independent GABAergic neurons

As GABAergic neuron markers are absent from the Gata2-deficient midbrain at E13.5 (Fig. 1I) when the VTA/SN GABAergic neurons are born, yet appear in the VTA and SNpr by E16.5 (Fig. 1M,N, arrows), we considered the possibility that these cells originate from flanking brain regions. The developing dopaminergic neurons are derived from the diencephalon and would thus be spared in the midbrain/r1 mutants (Kala et al., 2009). Therefore, we reasoned that GABAergic neurons in VTA and SNpr could be derived from the diencephalon and would thus be spared in the midbrain/r1-specific En1Cre;Gata2flox/flox mutants. To test this, we inactivated the Gata2flox allele by crossing with the Foxg1Cre mouse strain, which expresses Cre-recombinase in the forebrain and also in other tissues in a pattern dependent on the genetic background (Hebert and McConnell, 2000). In our hands, Foxg1Cre-mediated widespread inactivation of Gata2flox allele, including diencephalon, midbrain and r1 of the Foxg1Cre;Gata2flox/flox (Foxg1Cre;Gata2flox/flox) embryos already at E10.5 (Haugas et al., 2010) (data not shown). In the Foxg1Cre;Gata2flox/flox mutants, Gadjl expressing GABAergic neurons failed to develop in posterior diencephalon and midbrain (S. M. Virolainen, K.A., P.P., M.S. and J.P., unpublished). However, only a partial loss of SNpr GABAergic neurons was observed at E18.5, restricted to the anterior region of SN (Fig. 2B-F,J-N).

Fig. 1. VTA and SNpr GABAergic neurons are born by E13.5, but are absent in the En1Cre;Gata2flox/flox midbrain at that stage. (A) Schematic of E18.5 mouse brain showing GABAergic regions analyzed. (B,C) Examples of the SNpr region of E18.5 wild-type midbrain used in the proliferation end-point analysis (see J). Intrapерitoneal BrdU injections were given to pregnant females once a day for the indicated periods of time. Dotted line indicates the position of TH+ neurons, detected on a parallel section. (D,E) High-magnification images of boxed regions in B,C. (F, G) High-magnification images of wild-type SNpr used in the neurogenic proliferation peak analysis (see K). BrdU was administered six times, at 3-hour intervals, starting at the indicated time points. (J) Proliferation end-point analysis. (K) Neurogenic proliferation peak analysis. The charts represent average ratios of the Gad1+ BrdU positive cells, counted from the brain regions indicated in A. Data points are presented as mean percentage±s.d. Statistical significance in the difference between the labeling ratios respective to the SNpr is indicated when P<0.05 by paired Student’s t-test. (L) End-point analysis of SNpr neurons in wild-type and En1Cre;Gata2flox/flox embryos, from BrdU labeling (one per day) E10.5-E17.5 and E13.5-E17.5, with P-values indicated. (H, I, M, N) Gad1 expression (in situ hybridization) in wild-type and En1Cre;Gata2flox/flox midbrains at E13.5 (H, I) and E16.5 (M, N). GABAergic neurons are lost in mutants (arrows in H, I), whereas SNpr neurons appear in the midbrain by E16.5 (arrows in M, N). *P<0.05; **P<0.005; ***P<0.001. di, diencephalon; fb, forebrain; mb, midbrain; mRF, midbrain reticular formation; r1, rhombomere 1; SC, superior colliculus; SNpr, substantia nigra pars reticulata. Scale bars: 200 μm in B; 50 μm in D, F, 100 μm in H.

VTA and SNpr GABAergic neurons do not require Gata3

In En1Cre;Gata2flox/flox mutants, Gata3 is normally expressed in the VTA and SNpr GABAergic neurons. Thus, it is possible that distinct Gata3 or Gata2-dependent GABAergic precursor populations contribute to VTA/SNpr and other midbrain GABAergic nuclei, respectively. To address this possibility, we analyzed GABAergic neuron development in En1Cre;Gata2flox/flox embryos. Gad1+ cells appeared normal both in the midbrain and r1 at early stages of GABA neurogenesis without Gata3 function (supplementary material Fig. S1B, D). At E16.5, Gad1+ GABAergic neurons were detected in the SNpr region (supplementary material Fig. S1F). Considering also their timing of birth before E13.5, we find that the VTA and SNpr GABAergic neurons are unlikely to represent a late-born midbrain GABA neuron population dependent on Gata3 rather than Gata2.
VTA and SNpr GABAergic neurons are derived from the En1-expressing cells

To study the origin of the VTA and SNpr GABAergic neurons more directly, we used Cre-recombinase-based genetic cell marking. To label the cells in the midbrain and r1, we crossed mice carrying the En1Cre allele, which drives Cre-recombinase expression in these brain regions already at E8.5 (Kimmel et al., 2000; Trokovic et al., 2003), with mice carrying universal Cre reporter alleles, which express β-galactosidase [β-gal; R26R reporter (Soriano, 1999)] or TdTomato red fluorescent protein [RFP, Rosa26TdT omato (Madisen et al., 2010)] reporter gene upon Cre-mediated site-specific recombination. For easier identification of GABAergic neurons, we used the Gad67GFP allele, which produces GFP in all Gad1-expressing GABAergic neurons in the CNS (Tamamaki et al., 2003). In the midbrains of E16.5 En1cre/+;Rosa26TdT omato/+;Gad67GFP/+ (En1Cre;Rosa26TdT omato;Gad67GFP) embryos, RFP expression could be widely detected with an anti-RFP antibody (Fig. 3B,F) and included the GABAergic and dopaminergic neurons in the VTA and throughout the SN (Fig. 3C-D/H11032, G-H/H11032, J,K). GABAergic neurons in dorsal prosomere 1 (p1) did not express RFP (Fig. 3F). However, we detected recombination in the anterior SNpr neurons, apparently located in the ventralmost p1-p2 (Fig. 3G/H11032, H/H11032, K,L) and suggesting that En1Cre activity can extend into posterior diencephalon in the ventral brain. To address this hypothesis, we analyzed the recombination pattern in the En1Cre/+;R26R/+ (En1Cre;R26R) embryos at E12.5. Consistent with the labeling of dopaminergic/TH-positive precursors born in the p1 and p2 by En1cre (Lahti et al., 2012), we observed efficient recombination throughout the diencephalic basal plate expressing sonic hedgehog (Shh, Fig. 3N). In posterior diencephalon, GABAergic neurons are generated in the parabasal and alar plates (Garcia-Lopez and Martinez, 2010). We detected some En1Cre-labeled cells in the alar and parabasal ventricular zones in the diencephalon at E11.5 (supplementary material Fig. S2A-D). Indeed, En1 was weakly expressed in the ventral parabasal region at E9.5, after which it was soon downregulated (supplementary material Fig. S2K-M). Postmitotic cells in the parabasal plate showed more abundant recombination at E11.5. However, at this stage En1 was not expressed in the region (data not shown). Many, but not all, of the recombinant cells co-expressed Gata3 and Gad67GFP (Fig. 3P,Q-Q/H11630; supplementary material Fig. S2C-E/H11630). In addition, Gata2 was expressed in the parabasal plate MZ (Fig. 3O, arrow). Gata3+/Gad1+ cells in the parabasal plate were not recombined in the ShhCre/+;Rosa26TdT omato/+;Gad67GFP/+ (ShhCre;Rosa26TdT omato;Gad67GFP) mouse embryo at E11.5 (supplementary material Fig. S2F-J), supporting their origin outside Shh-expressing basal plate. These data show that En1Cre can label the Gata2/3-positive GABAergic neurons in the diencephalic parabasal plate. It is possible that these neurons contribute to the diencephalic region of the SNpr in the mature brain. Altogether, the results above suggest that the anterior SNpr

Fig. 2. Gata2 is required for the development of diencephalic SNpr GABAergic neurons, but dispensable for the midbrain SNpr and VTA GABAergic neurons. Gad1 expression analysis covering the anteroposterior axis of SNpr-VTA. (A) Schematic of E18.5 mouse brain, sagittal view; SNpr-VTA complex is marked in red. Lines indicate the anterior and posterior limits of analyzed area. The regional levels of sections are indicated at the top of images. fb, forebrain; p1, p2, p3, diencephalon prosomeres 1, 2 and 3; mb, midbrain; r1, rhombomere 1. (B-Y) Gad1 expression was analyzed by in situ hybridization on coronal sections of E18.5 wild-type (B-L), Foxg1Cre;Gata2flox/flox (J-Q) and En1Cre;Gata2flox/flox (R-Y) brains. Dotted lines delineate TH-positive area, determined by anti-TH staining on a parallel section (not shown). APN, anterior pretectal nucleus; bic, brachium of the inferior colliculus; cpd, cerebral peduncle; IC, inferior colliculus; ip, interpeduncular nucleus; LL, lateral lemniscus; Ld, L dorsal; MG, medial geniculate complex; MPT, medial pretectal nucleus; mRF, midbrain reticular formation; PAG, periaqueductal gray; PP, peripeduncular nucleus; PPT, posterior pretectal nucleus; prF, pontine reticular formation; RN, red nucleus; RRF, retrorubral field; SC, superior colliculus; SNpc, substantia nigra pars compacta; SNpr, SN pars reticulata; SPF, subparafascicular nucleus; VTA, ventral tegmental area; ZI, zona incerta.
GABAergic neurons are Gata2 dependent and might be derived from the parabasal plate of diencephalon, whereas the GABAergic cells in the posterior region of SNpr and in VTA (vMB GABAn) are Gata2 independent and have a distinct origin.

Genetic labeling of neural progenitors in the r1 and midbrain floor plate

The presence of vMB GABAn in the Gata2 mutant brains suggests that they are derived from a region where GABAn differentiation is Gata2 independent. Interestingly, we have previously showed that the generation of GABAn in the several dorsoventral regions of the r1 does not require Gata2 (Kala et al., 2009). To test whether the vMB GABAn could be fact derived from r1 progenitors, we crossed the reporter mice with mice carrying the Gbx2CreERT2 allele (Chen et al., 2009; Sunmonu et al., 2011). To induce the recombinase activity of the Cre-ERT2 fusion protein, we gave a single dose of tamoxifen (Tx) to the pregnant females at E8.5 (R26R) or at E9.5 (Rosa26 TdT omato). These induction protocols resulted similar recombination patterns in the different reporter lines (supplementary material Fig. S3). A single dose of Tx activates the Cre recombinase for up to 36 hours post-injection (Hayashi and McMahon, 2002), and thus in our experiments the Gbx2CreERT2-expressing cells are genetically labeled between E8.5 and E11.0. At the time of labeling, at E10.5, both Gbx2CreERT2;R26R and Gbx2CreERT2;Rosa26 TdT omato embryos showed widespread recombination in the Gbx2-expressing r1 (Fig. 4A-D; supplementary material Fig. S3F,I). In addition, Gbx2CreERT2-labeled cells were detected at the ventral midline of the midbrain, consistent with an earlier study (Sunmonu et al., 2011). These labeled cells were located in the Lmx1a and Corin-expressing floor-plate area of the midbrain (Fig. 4E-L). To distinguish the labeled cell populations in the r1 and midbrain floor...
plate, we used ShhCre, which labels ventral midbrain cells (Harfe et al., 2004; Joksimovic et al., 2009a). In contrast to the more posterior levels of central nervous system where Shh is confined to the non-neurogenic floor plate cells, the domain of Shh expression in the ventral midbrain is broader, comprising also the progenitors of dopaminergic neurons (Joksimovic et al., 2009b). In our experiments, ShhCre efficiently labeled the midbrain m6 and m7 domains, including the most ventral Corin-expressing domain of the midbrain (Fig. 4M-P). Thus, the medial midbrain floor-plate-derived cells are labeled both by Gbx2CreERT2 and ShhCre, whereas the r1-derived neurons are labeled only by Gbx2CreERT2.

**VTA and midbrain SNpr GABAergic neurons are derived from Gbx2-expressing cells in r1**

Interestingly, at E16.5, we detected abundant RFP+ cells in the ventral midbrains of Gbx2CreERT2;Rosa26-LCL;Gad67GFP embryos (Fig. 5B,F) ventral to the red nucleus (Pou4f1+, Fig. 5J) and overlapping with the TH+ dopaminergic nuclei (Fig. 5D’,H’,L). Although these cells were located in the Otx2+ and TH+ area (Fig. 5L,M), the majority of the RFP+ cells did not co-express Otx2, consistent with their possible r1 origins (Fig. 5M’), or TH (Fig. 5L’). Staining for Gad67GFP revealed that the recombined cells represent GABAergic neurons in the VTA and SNpr (Fig. 5C,G’,K,K’). No RFP-positive cells were found among the more dorsal GABAergic neurons in the mRF or SC of Gbx2CreERT2;Rosa26-LCL;Gad67GFP midbrains (Fig. 5C,G,F; data not shown). Importantly, the most anterior SNpr GABAergic neurons in p1-p2 were not labeled by the Gbx2CreERT2, suggesting their origin outside the r1 (arrowhead in Fig. 5F,G’,H’). Altogether, these results suggest that the vMB GABAn, unlike the rest of midbrain GABAergic neurons, are derived from the Gbx2-expressing cell lineage.

Labeling by Gbx2CreERT2 could reflect either developmental origin in the r1 or in the medial midbrain floor plate (see above). To distinguish between these possibilities, we analyzed the distribution of ShhCre-labeled cells in E16.5 ShhCre;Rosa26-LCL midbrain (supplementary material Fig. S4). Consistent with Joksimovic et al. (Joksimovic et al., 2009a), we observed RFP in the TH+ midbrain dopaminergic neurons and dopaminergic neurons of the red nucleus (supplementary material Fig. S4B,D,G). By contrast, only very few GABAergic neurons in the VTA and SNpr were RFP+, both in the midbrain and diencephalon (supplementary material Fig. S4C,H). The more dorsal midbrain GABAergic neurons were not labeled by ShhCre either, consistent with earlier studies (Joksimovic et al., 2009a). We further quantified labeling of Gad1+ vMB cells in Gbx2CreERT2;R26R, En1Cre;R26R and ShhCre;R26R embryos at E18.5 (Fig. 6A). Both En1Cre and Gbx2CreERT2 labeled the vMB GABAn efficiently (Fig. 6A). The slightly lower labeling percentage in the case of the Gbx2CreERT2 when compared with the En1Cre could reflect the mosaic nature of the Tlx-induced recombination. As expected, labeling efficiency with ShhCre remained very low (Fig. 6A). Taken together, these results indicate that the two primary neuronal cell types in SN and VTA, the GABAergic and dopaminergic neurons, have distinct developmental origins and that the vMB GABAn are derived from r1, excluding its floor plate (Fig. 6C).

**Origins of the SNpc and VTA dopaminergic neurons**

We also addressed the origin of the midbrain dopaminergic cells using our experimental setup. Consistent with earlier studies, TH+ cells in vMB were efficiently labeled by both En1Cre as well as ShhCre (Fig. 6B; Fig. 3D’, inset h; supplementary material Fig. S4D). About 16-22% of the TH+ cells in vMB were recombined by Gbx2CreERT2 (Fig. 6B). We find these cells to be likely descendants of the Gbx2CreERT2-expressing ventral midline domain in midbrain (Summonu et al., 2011). We did not observe a major difference in the numbers of Gbx2CreERT2-labeled TH+ cells in the VTA and SNpc (Fig. 6B). Thus, in contrast to recently published results with ShhCre (Joksimovic et al., 2009a), the mediolateral regionalization of the Lmx1a+ ventral midbrain was not found to correlate strictly with the development of VTA or SNpc dopaminergic neurons. Therefore, the differences observed in the previous study might also be due to the timing of labeling in the medial Shh domain (Blaess et al., 2011).

**Migration of the precursors of VTA and SNpr GABAergic neurons**

Although the progenitors of the SNpr GABAergic neurons undergo their final mitoses by E13.5, at this stage no GABAergic precursors are detected in the ventral midbrains of En1Cre;Gata6flox/flox embryos. However, the vMB GABAn could be found perinatally...
GABAergic differentiation in ventral rhombomere 1 and development of vMB GABA neurons require Tal1 function

Finally, we addressed the regulatory mechanisms of vMB GABAergic differentiation. In the ventral spinal cord, a basic helix-loop-helix transcription factor Tal1 and Gata2 act together to specify ventral interneuron fate (Joshi et al., 2009; Karunaratne et al., 2002; Muroyama et al., 2005). We detected overlapping expression of Gata2, Gata3 and Tal1 specifically in a ventrolateral subdomain of GABAergic precursors in r1 (Fig. 7A–C; data not shown). This ventrolateral GABAergic domain was flanked ventrally by serotonergic neurogenesis domain and its dorsal boundary coincided with the border of Nkx6.1 expression (Fig. 7D; data not shown). Neither Gata2 nor Gata3 alone is required for GABAergic neuron differentiation in the r1 (supplementary material Fig. S1A–F) (Kala et al., 2009). However, Tal1 has been suggested to support the production of Tall-expressing neurons in the r1, although the cell-type specificity and developmental mechanisms remain incompletely characterized (Bradley et al., 2006). Therefore, we analyzed the En1Cre; Tal1fl/fl mouse mutants for a GABAergic neuron phenotype in the r1 and midbrain. At E11.5–E12.5, we detected loss of Gad1 expression in a ventral subpopulation of neuronal precursors in the mutant r1 (Fig. 7F,N, arrow). The affected region coincided with the Tall expression domain in wild-type embryos (Fig. 7A,I). At the same time, although Tall is also specifically expressed in the midbrain GABAergic precursors, GABAergic marker expression in the midbrain was completely unaffected (Fig. 7N, arrowhead; data not shown).

Mature vMB GABA neurons also express Tall (Fig. 7Q). Thus, the Tall-dependent precursors in the r1 may contribute to the vMB GABA neuron. Therefore, we analyzed the En1Cre; Tal1fl/fl prenatal brains for a possible vMB GABA neuron phenotype. Indeed, Gad1-expressing cells in the VTA/SN were specifically lost in the mutant midbrains (Fig. 7V,W), whereas other GABAergic nuclei appeared normal (Fig. 7V). In conclusion, failure in GABAergic differentiation in a ventral subdomain of r1 GABAergic precursors correlates with abnormal vMB GABA neuron development. These data further support the origin of vMB GABA neurons in r1 and suggest that Tall is required for the differentiation of the vMB GABA neuron.

DISCUSSION

Distinct origins of GABAergic and dopaminergic neurons in the VTA and SN

Dopaminergic pathways originating from the ventral midbrain control several important brain functions. The SN dopaminergic neurons regulate voluntary movements and their degeneration leads to Parkinson’s disease. The VTA dopaminergic neurons process...
markers were used in this study. Furthermore, consistent with others (Blaess et al., 2011; Joksimovic et al., 2009a; Zervas et al., 2004), the results presented here clearly indicate midbrain/diencephalic origin for the dopaminergic neurons. Later studies on Gad gene expression in the mouse and human midbrain led to the suggestion that the VTA and SNpr GABAergic neurons are derived from the ventrolateral midbrain (Katarova et al., 2000; Verney et al., 2001). However, this hypothesis has not been experimentally tested.

Here, we used genetic fate-mapping approaches to pinpoint the origin, timing of birth and migration of the GABAergic neurons in the ventral midbrain. Our results showed that, in midbrain, the VTA and SNpr GABAergic neurons greatly differ from the rest of the GABAergic cells in this brain region as they are derived from the r1 neuroepithelium and move to midbrain as postmitotic precursors (Fig. 8). Furthermore, the anterior diencephalic SNpr GABAan appear to have distinct developmental origins, possibly in the diencephalic parabasal plate. Correlating with this subdivision, loss of Gata2 function differentially affects SNpr GABAergic neurons. The GABAergic neurons that make up the diencephalic SNpr require Gata2 for their development, as they were specifically affected in the Foxg1^Cre;Gata2^−/− mutants. Although the diencephalic SNpr is Gata2 dependent and appears fully labeled with En1^Cre, the GABAergic phenotype in this domain appears milder in the En1^Cre;Gata2^−/− mutants. A possible explanation could be the timing of En1^Cre expression. Our results suggest that some diencephalic parabasal progenitors express En1 briefly at early developmental stage. However, we detected En1^Cre mediated recombination only in the postmitotic precursors in the diencephalon. This expression dynamics might explain the partial rescue of GABAergic progenitors in the En1^Cre;Gata2^−/− diencephalon. On the other hand, it is possible that these postmitotic recombined cells represent midbrain-derived migratory cells and thus some of the GABAergic neurons in the diencephalic region of SNpr originate in the midbrain. Unambiguous demonstration of the Gata2 dependence and diencephalic origin of this cell population would require specific cell marking and ablation of Gata2 function in the diencephalic (p1) parabasal plate. Unfortunately, suitable Cre lines for this purpose are unavailable at the moment.

**Differentiation of the vMB GABAan within r1**

r1 can be subdivided into smaller developmental units (Aroca and Puuelles, 2005; Joyner and Zervas, 2006), which appear to independently give rise to GABAergic neurons. In one unit, Pitf1a-expressing precursors in the dorsal r1 give rise to cerebellar GABAergic neurons (Hoshino et al., 2005). These neurons are negative for Gata2/3 expression (K.A., P.P. and J.P., unpublished). By contrast, we identified a Gata2/3 and Tal1 expressing GABAergic precursor domain in ventrolateral r1. As the mature VTA and SNpr GABAergic neurons express Gata3 and Tal1, we hypothesize that the vMB GABAan originate from this ventrolateral, Gata2/3- and Tal1-expressing domain of r1 neuroepithelium. This hypothesis is strongly supported by the Tal1 mutant phenotype, in which an early defect in the differentiation of the ventrolateral GABAergic precursors in the r1 correlates with a later loss of vMB GABAan.

To our knowledge, the current study is one of the first to demonstrate molecular regulatory mechanisms of GABAergic neurogenesis in the ventral r1. Unlike Tal1, inactivation of Gata2, a primary regulator of GABAergic neuron differentiation in the midbrain, does not affect the expression of Gad1 and Gata3 in appetitive and aversive stimuli, and have been associated with depression, addiction and schizophrenia. Therefore, the midbrain dopaminergic neurons and their development have been under intensive research. However, importance of the VTA- and SN-associated GABAergic neurons for the activity of dopaminergic pathways and behavioral control has become increasingly evident (Fields et al., 2007; Vargas-Perez et al., 2009).

In contrast to the dopaminergic neurons, very little is known about the development of ventral midbrain GABAergic neurons. Importantly, transcription factors Ascl1, Helt and Gata2, which regulate differentiation of other midbrain GABAergic neurons, have not been shown to participate in the development of VTA and SNpr GABAergic neurons (Guimera et al., 2006; Kala et al., 2009; Nakatani et al., 2007; Peltopuro et al., 2010). Because of their widespread distribution and lack of specific molecular markers, the developmental origins and migration patterns of the midbrain GABAergic neuron subpopulations are poorly understood. An early study of the rat SN development suggested that all neurons of the SN are derived from the isthmus (rostralmost r1) (Marchand and Poirier, 1983). However, no region- or cell type-specific
ventrolateral r1 (Kala et al., 2009). In addition, we did not observe defects in the early GABAergic neuron development in the Gata3 mutant embryos. Although dispensable individually, Gata2 and Gata3 are co-expressed in the developing r1 GABAergic precursors and thus might redundantly support Tal1 function in the r1 GABAergic neuron differentiation. Analysis of conditional Gata2;Gata3 double mutant phenotype should clarify this issue.

Migration of r1-derived vMB GABA

Proliferative neural progenitor cells in the midbrain and r1 respect the midbrain-r1 boundary and do not move across it (Summonu et al., 2011; Zervas et al., 2004). Establishment of a specific boundary cell population in the midbrain-r1 border neuroepithelium is likely to contribute to this developmental compartmentalization (Kala et al., 2008; Trokovic et al., 2005). However, floor-plate cells might circumvent these rules, because, in addition to r1, we observed abundant cells labeled by the hindbrain indicator Gbx2CreERT2 in the midbrain floor plate. Furthermore, it is noteworthy that the expression of Fgf8 and some of the other genes characteristic for the midbrain-hindbrain boundary is not continuous but excludes the floor plate (Crossley and Martin, 1995). Nevertheless, our results with the floor-plate and midbrain basal-plate indicator ShhCre exclude floor-plate cells as progenitors of the VTA and SNpr GABAergic neurons.

In contrast to the majority of progenitor cells in the neuroepithelium, some of the post-mitotic neural precursors are apparently not as restricted by the midbrain-r1 compartment border and thus there are more cell movements between the adjacent brain regions in the mantle zone. In addition to the VTA and SNpr GABAergic neurons, serotonergic neurons of the dorsal raphe nuclei are also born in r1 but later move to the mature midbrain (Jensen et al., 2008; Scott and Deneris, 2005). Interestingly, movement of the postmitotic precursors from the r1 to the midbrain involves neural populations close to the ventral midline, which might thus play a role in the guidance of these cell migrations. It will be of interest to investigate whether the molecular mechanisms directing the movements of GABAergic precursors to ventral midbrain are similar to the ones regulating long-distance migrations of postmitotic GABAergic precursor in the forebrain (Marin and

Fig. 7. Tal1 is required for normal GABAn differentiation in the r1 and development of SNpr and VTA GABA

In situ hybridization and immunohistochemical analysis on transverse and coronal sections of E12.5 wild-type and En1Cre:Tal1flox/fox mouse embryos and E18.5 brains. (E,M,U) Sectioning planes are indicated with lines. (A-D,F-H) In situ hybridization for Gad1, Tal1 and Gata2 on transverse sections (A,C,F,G) and anti-SHT immunohistochemistry on parallel sections (D,H). The dotted lines in A-C and F,G delineate Nkx6.1+ domain. Arrows in F,G indicate the region of lost Gad1 expression in ventrolateral r1. (I-L,N-P) In situ hybridization with Tal1 and Gad1 probes; immunohistochemistry for Gad67-GFP (representing GABAergic cells, K,O), Gata2 (K-M,O-P) and Otx2 (representing midbrain). Arrows in N,O indicate loss of Gad1* and Gad67GFP* cells in ventrolateral r1. (Q-T,V-X) Coronal sections of E18.5 midbrain, analyzed for Tal1 (Q), Gad1 (in situ hybridization, R,S,V,W) and TH (immunohistochemistry, T,X). Arrow in W indicates loss of Gad1+ cells in the SNpr. TH+ area is delineated with a broken line. di, diencephalon; fb, forebrain; MB/mb, midbrain; mRF, midbrain reticular formation; p1, p2, p3, diencephalon prosomeres 1, 2 and 3; PAG, periaqueductal gray; R1/r1, rhombomere 1; RN, red nucleus; SNpc, substantia nigra pars compacta; SNpr, SN pars reticulata; VTA, ventral tegmental area. Scale bars: 200 μm.

Fig. 8. Distinct origins of dopaminergic and GABAergic neurons in VTA and SNpr

Both genetic fate mapping and analysis of Tal1 mutant mice suggest that GABAergic neurons in the midbrain region of SN and VTA originate from the r1 compartment (r1, red), whereas in the anterior, diencephalic part of SN, GABAergic neurons have a distinct origin, possibly the diencephalon (di, green), although contribution from midbrain (mb, gray) cannot be excluded. Dopaminergic neurons in these nuclei are also indicated (black).
Rubenstein, 2003; Metin et al., 2008). Of particular relevance are the recent findings demonstrating that activation of dopamine receptors controls tangential migration of GABAergic neuron precursors in the telencephalon (Crandall et al., 2007).

Conclusions

This study describes developmental heterogeneity of the midbrain GABAergic neurons, and emphasizes the unique regulatory mechanisms of the vMB GABAergic. In the future, it will be of importance to characterize further the molecular mechanisms responsible for neurogenesis and migration of the r1-derived GABAergic neuron precursors. Genetic variation in these processes might result in different behavioral traits and predisposition to psychiatric disease. Understanding the diverse origins and developmental regulatory mechanisms also offers approaches to modulation of the distinct GABAergic subpopulations and studies of their functions.

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