Development 139, 2360-2370 (2012) doi:10.1242/dev.076380
© 2012. Published by The Company of Biologists Ltd

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

Kaia Achim[1,2], Paula Peltopuro[1,2], Laura Lahti[1,2], James Li[3], Marjo Salminen[4] and Juha Partanen[1,2,*]

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 (Laviolette 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.

[1]Department of Biosciences, PO Box 56, Viikinkaari 5, FIN00014-University of Helsinki, Helsinki, Finland. [2]Institute of Biotechnology, FIN00014-University of Helsinki, Helsinki, Finland. [3]Department of Genetics and Developmental Biology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA. [4]Department of Veterinary Biosciences, PO Box 66, FIN00014-University of Helsinki, Helsinki, Finland.

*Author for correspondence (juha.m.partanen@helsinki.fi)

Accepted 11 April 2012

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

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*^{Flox} (Haugas et al., 2010), *Gata3*^{Flox} (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*^{flox} (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, 1990) using digoxigenin- or ^{35}S-labeled cRNA probes. Mouse cDNA probes used for in situ hybridization analysis were *Gata2*, *Gata3* (Lillevali et al., 2004), *Gad1* (*Gad67*) (Guimera 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 74.5A5), 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, 1:5000), anti-Gata2 (Santa Cruz SC-9008 1:200), anti-GFP (Abcam ab290, 1:600), anti-Lmx1a (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 end-point 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 3 hours, for a period of 15 hours (six injections in total) starting at desired stages. Embryonic brains were dissected at E18.5 and sectioned at 4 μm for analyses.

Microscopy and quantification

Whole-mount staining was visualized under Leica MZFLIII microscope and photographed using Olympus DP50-CU camera. The staining on paraffin sections was visualized with Olympus AX70 microscope and photographed using Olympus DP70 camera. Images were processed and assembled using Adobe Photoshop software.

For quantification, the nuclei of the *Gad1*- or TH-positive cells were marked and 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).

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 (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). 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*$^{flox/flox}$ (*En1*^{Cre};*Gata2*$^{flox/flox}$) 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*$^{flox/flox}$ embryos.

We then compared expression of *Gad1* in *En1*^{Cre};*Gata2*$^{flox/flox}$ 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,I). 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.

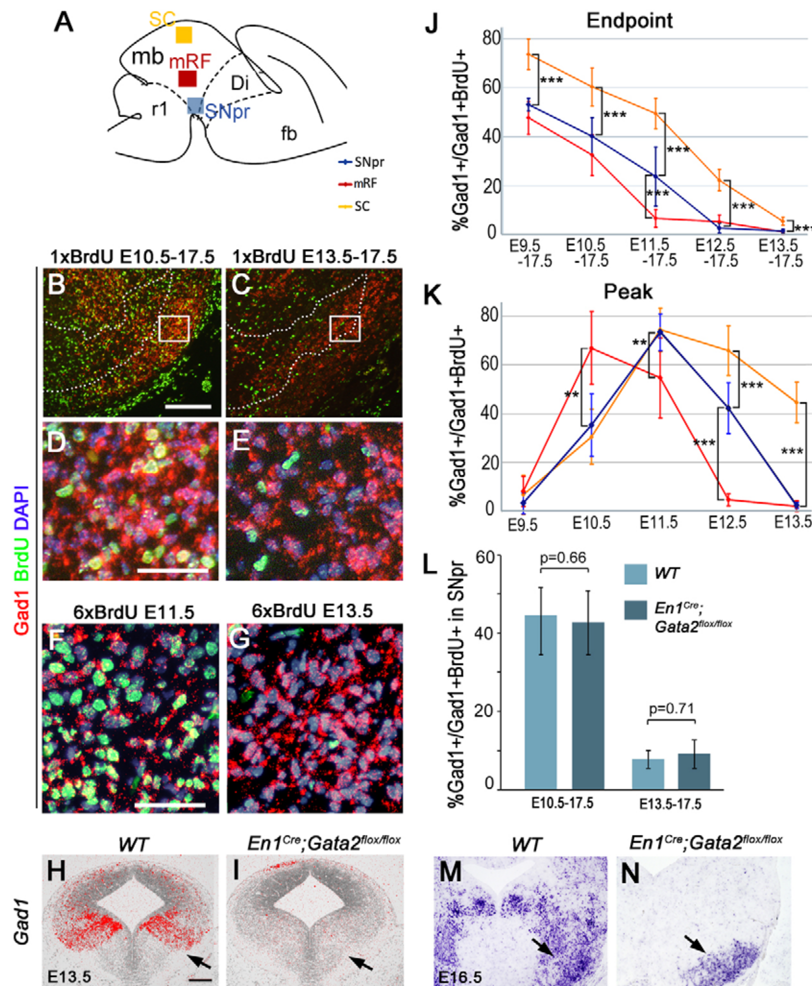

Fig. 1. VTA and SNpr GABAergic neurons are born by E13.5, but are absent in the *En1*^{Cre}*;Gata2*$^{flox/flox}$ midbrain at that stage. (**A**) Schematic of E18.5 mouse brain showing GABAergic regions analyzed. (**B**,**C**) Examples of the SNpr region of E18.5 wild-type midbrain used in the proliferation end-point analysis (see J). Intraperitoneal 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 double-positive cells from the total *Gad1*-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 *En1*^{Cre}*;Gata2*$^{flox/flox}$ 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 *En1*^{Cre};*Gata2*$^{flox/flox}$ 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.

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 GABAn 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 nuclei span the midbrain-diencephalon region in the embryonic brain, and in the *En1*^{Cre};*Gata2*$^{flox/flox}$ mutants abundant *Gata2* expression and GABAergic neurogenesis was still observed in the diencephalon (Kala et al., 2009). Therefore, we reasoned that the GABAergic neurons in VTA and SNpr could be derived from the diencephalon and would thus be spared in the midbrain/r1-specific *En1*^{Cre};*Gata2*$^{flox/flox}$ mutants. To test this, we inactivated the *Gata2*^{flox} allele by crossing with the *Foxg1*^{Cre} mouse strain, which expresses *Cre-recombinase* in the forebrain but also in other tissues in a pattern dependent on the genetic background (Hebert and McConnell, 2000). In our hands, Foxg1^{Cre} mediated widespread inactivation of *Gata2*^{flox} allele, including diencephalon, midbrain and r1 of the *Foxg1*$^{Cre/+}$;*Gata2*$^{flox/flox}$ (*Foxg1*^{Cre};*Gata2*$^{flox/flox}$) embryos already at E10.5 (Haugas et al., 2010) (data not shown). In the *Foxg1*^{Cre};*Gata2*$^{flox/flox}$ mutants, *Gad1*-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). A less prominent, but clear reduction in *Gad1*$^{+}$ cells was also detected in the anterior SNpr of *En1*^{Cre}*;Gata2*$^{flox/flox}$ brains (Fig. 2T-V). By contrast, the GABAergic neurons in more posterior SNpr and the whole VTA area appeared normal both in the *Foxg1*^{Cre};*Gata2*$^{flox/flox}$ and *En1*^{Cre};*Gata2*$^{flox/flox}$ mutants (Fig. 2G,H,O,P,W,X). In addition, the GABAergic neurons in the r1 were found in the both mutants (Fig. 2I,Q,Y). Thus, the GABAergic neurons at different anteroposterior levels of SNpr have distinct requirements for *Gata2*. This might reflect distinct developmental origins for anterior and posterior SNpr GABAergic neurons.

VTA and SNpr GABAergic neurons do not require Gata3

In *En1*^{Cre};*Gata2*$^{flox/flox}$ 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 *En1*^{Cre};*Gata3*$^{flox/flox}$ 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*.

DEVELOPMENT

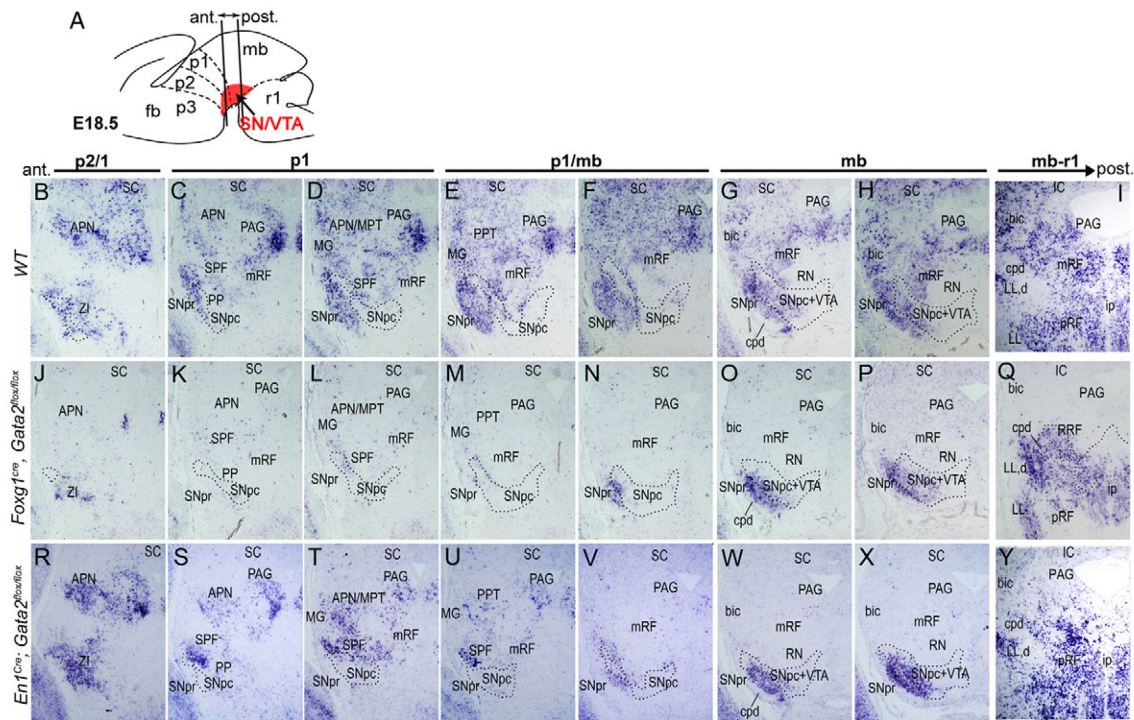

Fig. 2. Gata2 is required for the development of diencephalic SNpr GABAergic neurons, but dispensable for the midbrain SNpr and VTA GABAergic neurons. *Gad1* expression analysis covering the anteroposterior axis of SNpr-VTA. (**A**) Schematic of E18.5 mouse brain, sagittal view; SNpr-VTA complex is marked 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-I), $Foxg1^{Cre}$;$Gata2^{flox/flox}$ (J-Q) and $En1^{Cre}$;$Gata2^{flox/flox}$ (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; LLd, LL 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.

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 $En1^{Cre}$ 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, $Rosa26^{TdTomato}$ (Madisen et al., 2010)] reporter gene upon Cre-mediated site-specific recombination. For easier identification of GABAergic neurons, we used the $Gad67^{GFP}$ allele, which produces GFP in all *Gad1*-expressing GABAergic neurons in the CNS (Tamamaki et al., 2003). In the midbrains of E16.5 $En1^{cre/+}$;$Rosa26^{TdTomato/+}$; $Gad67^{GFP/+}$ ($En1^{Cre}$;$Rosa26^{TdTomato}$;$Gad67^{GFP}$) 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′,G-H′,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′,H′,K,L-L‴, arrowhead), suggesting that $En1^{Cre}$ activity can extend into posterior diencephalon in the ventral brain. To address this hypothesis, we analyzed the recombination pattern in the $En1^{Cre/+}$;$R26R^{/+}$ ($En1^{Cre}$;*R26R*) embryos at E12.5. Consistent with the labeling of dopaminergic/TH-positive precursors born in the p1 and p2 by $En1^{cre}$ (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 $En1^{Cre}$-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 $Gad67^{GFP}$ (Fig. 3P,Q-Q″; supplementary material Fig. S2C-E‴). 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 $Shh^{Cre/+}$; $Rosa26^{TdTomato/+}$;$Gad67^{GFP/+}$ (Shh^{Cre};$Rosa26^{TdTomato}$;$Gad67^{GFP}$) mouse embryo at E11.5 (supplementary material Fig. S2F-J), supporting their origin outside *Shh*-expressing basal plate. These data show that $En1^{Cre}$ 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

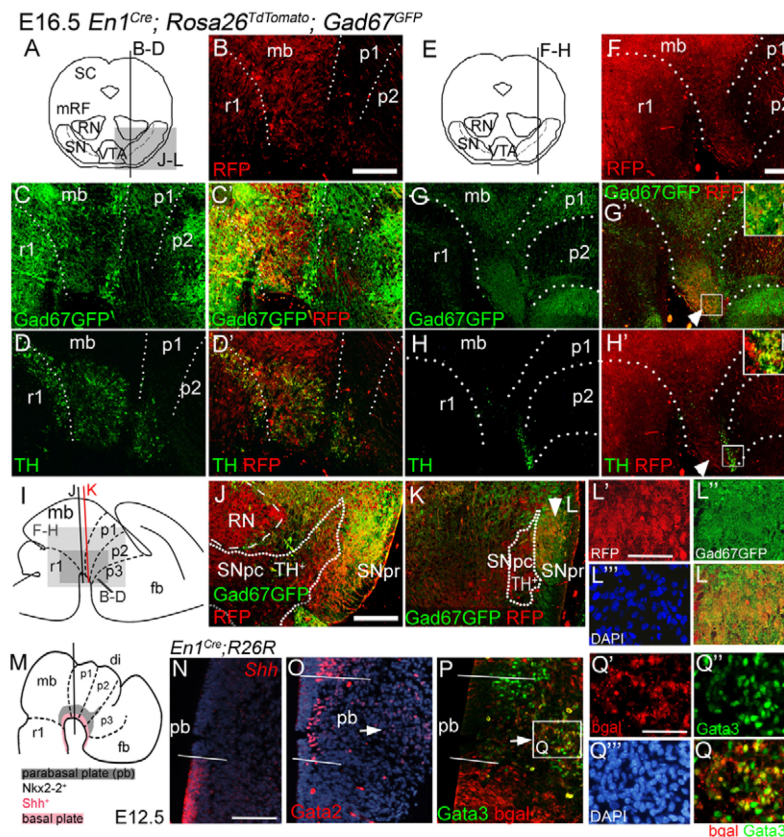

Fig. 3. SNpr and VTA GABAn are labeled by En1^{Cre}. (**A-L‴**) Immunohistochemistry on sagittal and coronal sections of E16.5 *En1$^{Cre/+}$;Rosa26$^{TdTomato/+}$;Gad67$^{GFP/+}$* mouse brains. Sectioning planes are indicated with lines and imaged areas by gray shaded boxes in the schemes A,E,I. (A-H′) Sagittal sections stained for RFP (representing recombined cells, B,F) and GFP (Gad67GFP, representing GABAergic cells; C,G); merged images are shown in C′,G′, respectively. Higher magnification image of RFP,GFP double-labeled cells in anterior SNpr is shown in inset g (area of higher magnification is indicated by white box in G′). Adjacent sections were stained for RFP and TH (D,H; merged images in D′,H′). Inset h presents a higher magnification image of RFP$^+$ TH$^+$ cells in anterior SNpc (boxed area in H′). The borders of diencephalon prosomeres 1 and 2 (p1 and p2), midbrain (mb) and rhombomere 1 (r1) are indicated by white dotted lines. The p1-p2 border was defined by the position of the GFP$^+$ GABAergic prethalamic nuclei (G), p1-mb by the RFP expression border and mb-r1 border by the localization of Otx2 (adjacent section, not shown) and caudal limit of TH (D,H) expression. (J-L‴) Co-immunohistochemistry for RFP and GFP on coronal sections; levels across midbrain (J) and from posterior diencephalon (K) are presented. Dotted line delineates TH$^+$ area. (L-L‴) Higher magnification of the GFP$^+$/RFP$^+$ staining in the anterior SNpr. Dashed line marks red nucleus (RN) in J. White arrowheads (G′,H′,K) indicate the diencephalic part of SNpr. (**M-Q‴**) En1^{Cre} recombination pattern in the ventral diencephalon at E12.5. The sectioning plane is indicated with a line in M. Coronal sections of the *En1$^{Cre/+}$;R26R$^{/+}$*mouse embryo analyzed for *Shh* (N, in situ hybridization), Gata2 (O, immunohistochemistry), and Gata3 and β-gal (P, co-immunohistochemistry). White arrows indicate Gata2 expression in the parabasal plate (pb) in O and β-gal$^+$ Gata3$^+$ cells in P. (Q-Q‴) Higher magnification image of the β-gal (Q′) and Gata3 (Q″) staining in pb; area of higher magnification is indicated by the box in P. DAPI (Q‴) shows the location of cell nuclei. di, diencephalon; fb, forebrain; SNpr, substantia nigra pars reticulata. Scale bars: 200 μm in B,F,J,N; 50 μm in L′,Q′.

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 in the following text) 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 shown 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 in fact derived from r1 progenitors, we crossed the reporter mice with mice carrying the *Gbx2^{CreERT2}* 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^{TdTomato}*). 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 *Gbx2^{CreERT2}*-expressing cells are genetically labeled between E8.5 and E11.0. At the time of labeling, at E10.5, both *Gbx2^{CreERT2};R26R* and *Gbx2^{CreERT2};Rosa26^{TdTomato}* embryos showed widespread recombination in the *Gbx2*-expressing r1 (Fig. 4A-D; supplementary material Fig. S3F,I). In addition, *Gbx2^{CreERT2}*-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

DEVELOPMENT

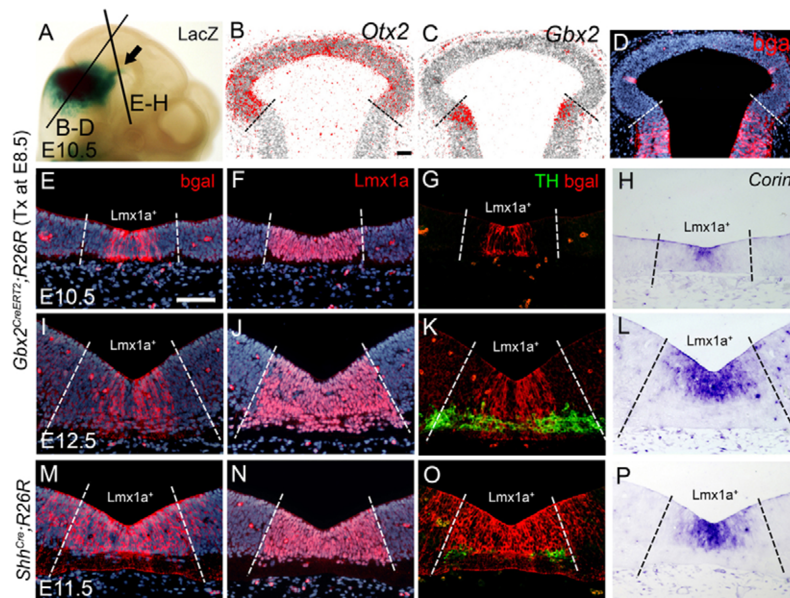

Fig. 4. Recombination by *Gbx2^{CreERT2}* in the r1 and in a ventralmost subdomain of *Shh^{Cre}*-expressing midbrain floor plate. (**A-L**) Recombinant cells in E10.5 (A-H) and E12.5 (I-L) *Gbx2$^{CreERT2/+}$;R26R/$^{+}$* mouse embryo were visualized by whole-mount X-gal staining (A) or β-gal immunohistochemistry on sections (D,E,G,I,K). Sectioning planes are indicated by lines in A. β-Gal expression was compared with that of *Otx2* (B) and *Gbx2* (C) in the midbrain-r1 sections. In the midbrain, β-gal expression was compared with Lmx1a (F,J; immunohistochemistry on adjacent sections), TH (G,K; double immunohistochemistry) and *Corin* (H,L; in situ hybridization). (**M-P**) Recombinant cells in E11.5 *Shh$^{Cre/+}$;R26R$^{/+}$* embryo were visualized by β-gal immunohistochemistry (M), and compared with Lmx1a (N), TH (O) and *Corin* (P) expression. Dashed lines mark the borders of Lmx1a-positive area. Arrow in A indicates the recombined floor-plate domain in ventral midbrain. Scale bars: 100 μm.

plate, we used Shh^{Cre}, 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, Shh^{Cre} 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 Gbx2^{CreERT2} and Shh^{Cre}, whereas the r1-derived neurons are labeled only by Gbx2^{CreERT2}.

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 *Gbx2^{CreERT2};Rosa26^{TdTomato};Gad67^{GFP}* 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 Gad67^{GFP} 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 *Gbx2^{CreERT2};Rosa26^{TdTomato};Gad67^{GFP}* midbrains (Fig. 5C-C′,F,G; data not shown). Importantly, the most anterior SNpr GABAergic neurons in p1-p2 were not labeled by the Gbx2^{CreERT2}, 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 *Gbx2^{CreERT2}* 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 Shh^{Cre}-labeled cells in E16.5 *Shh^{Cre};Rosa26^{TdTomato}* midbrain (supplementary material Fig. S4). Consistent with Joksimovic et al. (Joksimovic et al., 2009a), we observed RFP in the TH^{+} midbrain dopaminergic neurons and neurons of the red nucleus (supplementary material Fig. S4B,D,G,I). 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 Shh^{Cre} either, consistent with earlier studies (Joksimovic et al., 2009a). We further quantified labeling of *Gad1*$^{+}$ vMB cells in *Gbx2^{CreERT2};R26R*, *En1^{Cre};R26R* and *Shh^{Cre};R26R* embryos at E18.5 (Fig. 6A). Both En1^{Cre} and Gbx2^{CreERT2} labeled the vMB GABAn efficiently (Fig. 6A). The slightly lower labeling percentage in the case of the Gbx2^{CreERT2} when compared with the En1^{Cre} could reflect the mosaic nature of the Tx-induced recombination. As expected, labeling efficiency with Shh^{Cre} 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 En1^{Cre} as well as Shh^{Cre} (Fig. 6B; Fig. 3D′, inset h; supplementary material Fig. S4D). About 16-22% of the TH^{+} cells in vMB were recombined by *Gbx2^{CreERT2}* (Fig. 6B). We find these cells to be likely descendants of the *Gbx2^{CreERT2}*-expressing ventral midline domain in midbrain (Sunmonu et al., 2011). We did not observe a major difference in the numbers of Gbx2^{CreERT2}-labeled TH^{+} cells in the VTA and SNpc (Fig. 6B). Thus, in contrast to recently published results with Shh^{CreERT2} (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 *En1^{Cre};Gata2$^{flox/flox}$* embryos. However, the vMB GABAn could be found perinatally

DEVELOPMENT

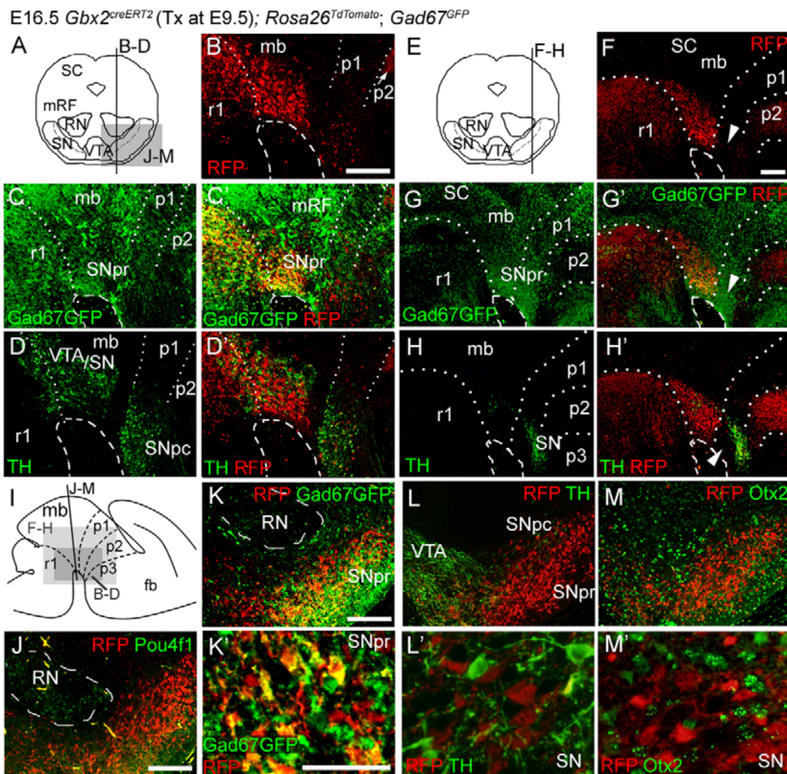

Fig. 5. Midbrain SNpr and VTA GABAn are labeled by *Gbx2*^{CreERT2}. The Cre recombinase was activated by tamoxifen (Tx) at E9.5. Immunohistochemistry on sagittal and coronal sections of E16.5 *Gbx2*$^{CreERT2/+}$;*Rosa26*$^{TdTomato/+}$;*Gad67*$^{GFP/+}$ mouse brains. (**A**,**E**,**I**) Sectioning planes are indicated with lines and imaged areas by gray shaded boxes. (**B**,**C-D′**,**F-H′**) Sagittal sections stained for RFP (recombined cells, B,F) and GFP (GABAergic cells; C,G); merged images in C′,G′. Adjacent sections were stained for RFP and TH (D,H; merged images in D′,H′). White arrowheads indicate the diencephalic part of the SN, where GABAergic cells are not labeled by *Gbx2*^{CreERT2}. White dotted lines indicate the compartment borders as follows: p2 borders were defined by the position of the RFP$^+$ cells in dorsal thalamus (B, arrow; F); p1-mb border is defined by the position of the GFP$^+$ GABAergic prethalamic nuclei (G); and the mb-r1 border is defined by the position of the RFP$^+$ cells in dorsal r1 (B,F), Otx2 immunohistochemistry (not shown) and caudal limit of TH (D,H) expression. (**J-M′**) Coronal sections across midbrain were co-stained for RFP and Pou4f1 (J), GFP (K,K′), TH (L,L′) or Otx2 (M,M′). Respective higher magnifications from the SN area are presented in K′,L′,M′. Broken lines in J,K delineate the red nucleus (RN). mb, midbrain; mRF, midbrain reticular formation; p1 and p2, diencephalon prosomeres 1 and 2; r1, rhombomere 1; RN, red nucleus; SC, superior colliculus; SNpc, substantia nigra pars compacta; SNpr, SN pars reticulata; VTA, ventral tegmental area. Scale bars: 200 μm in B-K,L-M′; 100 μm in K′,L′,M′.

(Fig. 1I,N). This suggests that the vMB GABAn move to their final locations in the ventral midbrain as post-mitotic precursors only after E13.5.

To follow the appearance of GABAergic neurons in the ventral midbrain, we analyzed the areas associated with the dopaminergic neurons (TH$^+$; supplementary material Fig. S5A-D) for *Gad1*-expressing cells in wild-type embryos (supplementary material Fig. S5E-H) as well as RFP$^+$/Gad67^{GFP+} cells in *Gbx2*^{CreERT2};*Rosa26*^{TdTomato};*Gad67*^{GFP} embryos (supplementary material Fig. S5I-L). *Gad1* and RFP appeared with similar developmental kinetics: we detected earliest *Gad1* mRNA-expressing as well as RFP$^+$/Gad67^{GFP+} cells in the VTA-SNpr area between E14.5 and E15.5 (supplementary material Fig. S5F,G,J,K, arrows), which is 24 hours after the last mitosis and 3 days after the peak neurogenesis in their progenitor pool (Fig. 1J,K). In addition, the first Gad67^{GFP+} cells in the *En1*^{Cre};*Gata2*$^{flox/flox}$,*Gad67*^{GFP} midbrains appeared at E14.5-E15.5 (supplementary material Fig. S5M-P, arrow). These data support the scenario in which r1-derived precursors migrate to vMB during post-mitotic differentiation.

GABAn differentiation in ventral rhombomere 1 and development of vMB GABAn require Tal1 function

Finally, we addressed the regulatory mechanisms of vMB GABAn 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 *Tal1*-expressing neurons in the r1, although the cell-type specificity and developmental mechanisms remain incompletely characterized (Bradley et al., 2006). Therefore, we analyzed the *En1*^{Cre};*Tal1*$^{flox/flox}$ 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 *Tal1* expression domain in wild-type embryos (Fig. 7A,I). At the same time, although *Tal1* 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 GABAn also express *Tal1* (Fig. 7Q). Thus, the *Tal1*-dependent precursors in the r1 may contribute to the vMB GABAn. Therefore, we analyzed the *En1*^{Cre};*Tal1*$^{flox/flox}$ prenatal brains for a possible vMB GABAn 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 GABAn development. These data further support the origin of vMB GABAn in r1 and suggest that *Tal1* is required for the differentiation of the vMB GABAn.

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

DEVELOPMENT

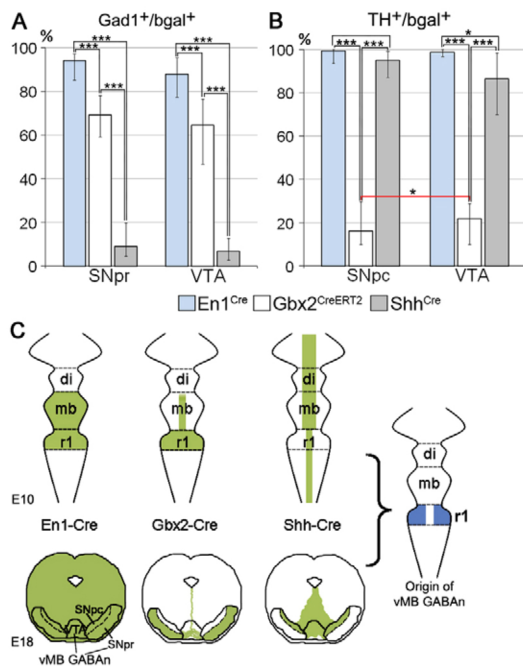

Fig. 6. Summary of the fate mapping of vMB GABAn. (**A**,**B**) Quantification of the fate-mapping data. β-Gal+ cells among ventral midbrain *Gad1*+ GABAergic (A) and TH+ dopaminergic (B) neurons were counted from E18.5 *En1*$^{Cre/+}$*;R26R*$^{/+}$, *Gbx2*$^{CreERT2/+}$*;R26R*$^{/+}$ (Tx at E8.5) and *Shh*$^{Cre/+}$*;R26R*$^{/+}$ embryos ($n \geq 4$ per genotype). For each sample, coronal sections selected from at least two different anteroposterior levels within the vMB GABAn domain were double stained for *Gad1*/β-gal or TH/β-gal. Cells were counted from representative 0.034 mm^2 areas within VTA and SN nuclei from each section. Error bars indicate variance. *$P<0.05$; **$P<0.005$; ***$P<0.001$ by paired Student's *t*-test. (**C**) Schematic summary of the fate-mapping data. The ventral midbrain GABAergic neurons (vMB GABAn) originate from the ventrolateral r1 region (blue). Schematic representations of E10 brains (top) and E18 midbrains (coronal view, bottom) are marked with green to indicate the recombination patterns for each Cre-expressing mouse line. di, diencephalon; mb, midbrain; r1, rhombomere 1; SNpc and SNpr substantia nigra pars compacta and pars reticulata, respectively; VTA, ventral tegmental area.

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 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 GABAn 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*$^{flox/flox}$ 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*$^{flox/flox}$ 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*$^{flox/flox}$ 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 GABAn within r1

r1 can be subdivided into smaller developmental units (Aroca and Puelles, 2005; Joyner and Zervas, 2006), which appear to independently give rise to GABAergic neurons. In one unit, *Ptf1a*-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 GABAn 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 GABAn.

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

DEVELOPMENT

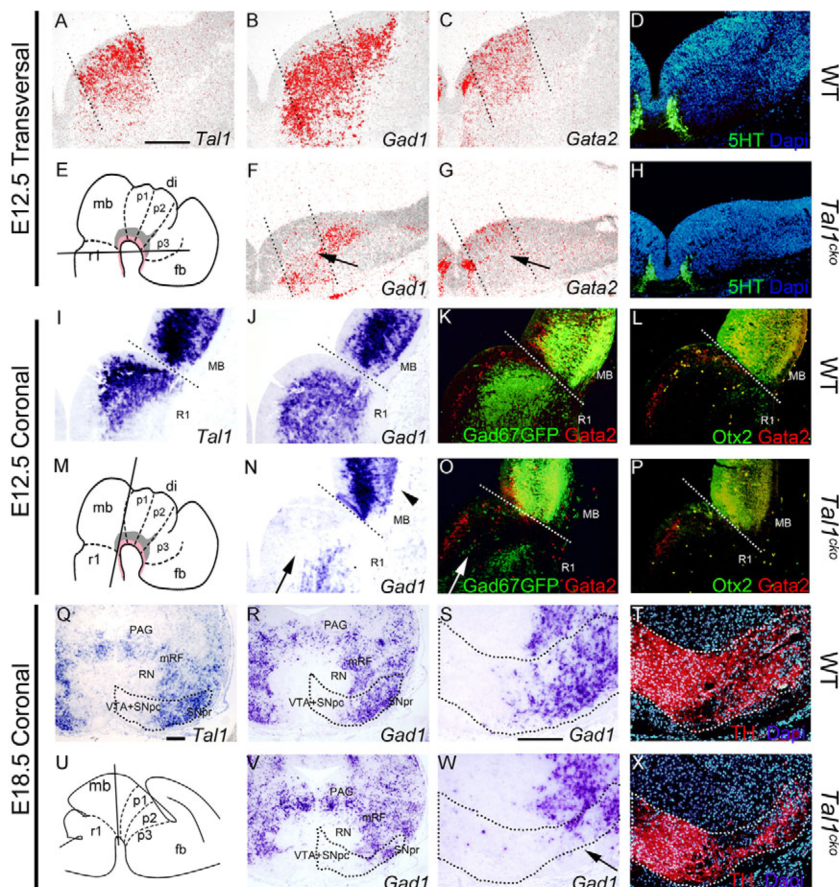

Fig. 7. Tal1 is required for normal GABAn differentiation in the r1 and development of SNpr and VTA GABAn. In situ hybridization and immunohistochemical analysis on transverse and coronal sections of E12.5 wild-type and *En1*^{Cre};*Tal1*$^{flox/flox}$ mouse embryos and E18.5 brains. (**E**,**M**,**U**) 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-5HT 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-L,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.

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 GABAn

Proliferative neural progenitor cells in the midbrain and r1 respect the midbrain-r1 boundary and do not move across it (Sunmonu 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 Gbx2^{CreERT2} 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 Shh^{Cre} 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

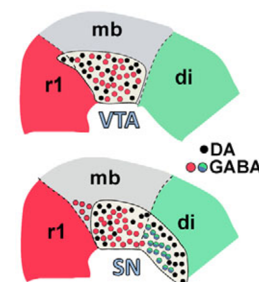

Fig. 8. Distinct origins of dopaminergic and GABAergic neurons in VTA and SNpr. Both genetic fate mapping and analysis of Tal1 mutant mice suggest that GABAergic neurons in the midbrain region of SN and VTA originate from the r1 compartment (r1, red), whereas in the anterior, diencephalic part of SN, 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).

DEVELOPMENT

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 GABAn. In the future, it will be of importance to characterize further the molecular mechanisms responsible for neurogenesis and migration of the r1-derived GABAergic 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.

Acknowledgements

We thank Eija Koivunen and Outi Kostia for expert technical assistance. We also thank Michael German for the Lmx1a antibody, Yuichi Ono for the Corin cDNA, Wolfgang Wurst for the *En1*^{Cre}, Cliff Tabin for the *Shh*^{Cre}, John Hebert and Sue McConnell for the *Foxg1*^{Cre}, and Frank Grosveld for the *Gata3*^{flox} and David Curtis for the *Tal1*^{flox} mice. We are grateful to Mart Saarma for critical comments on the manuscript.

Funding

This work was supported by the Academy of Finland, Sigrid Juselius Foundation and University of Helsinki.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.076380/-/DC1

References

Aroca, P. and Puelles, L. (2005). Postulated boundaries and differential fate in the developing rostral hindbrain. *Brain Res. Brain Res. Rev.* **49**, 179-190.
Blaess, S., Bodea, G. O., Kabanova, A., Chanet, S., Mugniery, E., Derouiche, A., Stephen, D. and Joyner, A. L. (2011). Temporal-spatial changes in sonic hedgehog expression and signaling reveal different potentials of ventral mesencephalic progenitors to populate distinct ventral midbrain nuclei. *Neural Dev.* **6**, 29.
Bradley, C. K., Takano, E. A., Hall, M. A., Gothert, J. R., Harvey, A. R., Begley, C. G. and van Eekelen, J. A. (2006). The essential haematopoietic transcription factor Scl is also critical for neuronal development. *Eur. J. Neurosci.* **23**, 1677-1689.
Chen, L., Guo, Q. and Li, J. Y. (2009). Transcription factor Gbx2 acts cell-nonautonomously to regulate the formation of lineage-restriction boundaries of the thalamus. *Development* **136**, 1317-1326.
Cohen, J. Y., Haesler, S., Vong, L., Lowell, B. B. and Uchida, N. (2012). Neuron-type-specific signals for reward and punishment in the ventral tegmental area. *Nature* **482**, 85-88.
Crandall, J. E., McCarthy, D. M., Araki, K. Y., Sims, J. R., Ren, J. Q. and Bhide, P. G. (2007). Dopamine receptor activation modulates GABA neuron migration from the basal forebrain to the cerebral cortex. *J. Neurosci.* **27**, 3813-3822.
Crossley, P. H. and Martin, G. R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.
Fields, H. L., Hjelmstad, G. O., Margolis, E. B. and Nicola, S. M. (2007). Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. *Annu. Rev. Neurosci.* **30**, 289-316.
Garcia-Lopez, R. and Martinez, S. (2010). Oligodendrocyte precursors originate in the parabasal band of the basal plate in prosomere 1 and migrate into the alar prosencephalon during chick development. *Glia* **58**, 1437-1450.
Guimera, J., Weisenhorn, D. V. and Wurst, W. (2006). Megane/Heslike is required for normal GABAergic differentiation in the mouse superior colliculus. *Development* **133**, 3847-3857.
Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P. and Tabin, C. J. (2004). Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* **118**, 517-528.
Haugas, M., Lillevali, K., Hakanen, J. and Salminen, M. (2010). Gata2 is required for the development of inner ear semicircular ducts and the surrounding perilymphatic space. *Dev. Dyn.* **239**, 2452-2469.
Hayashi, S. and McMahon, A. P. (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of cre: A tool for temporally regulated gene activation/inactivation in the mouse. *Dev. Biol.* **244**, 305-318.
Hebert, J. M. and McConnell, S. K. (2000). Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. *Dev. Biol.* **222**, 296-306.
Hoshino, M., Nakamura, S., Mori, K., Kawauchi, T., Terao, M., Nishimura, Y. V., Fukuda, A., Fuse, T., Matsuo, N., Sone, M. et al. (2005). Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. *Neuron* **47**, 201-213.
Jensen, P., Farago, A. F., Awatramani, R. B., Scott, M. M., Deneris, E. S. and Dymecki, S. M. (2008). Redefining the serotonergic system by genetic lineage. *Nat. Neurosci.* **11**, 417-419.
Jhou, T. C., Fields, H. L., Baxter, M. G., Saper, C. B. and Holland, P. C. (2009). The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. *Neuron* **61**, 786-800.
Joksimovic, M., Anderegg, A., Roy, A., Campochiaro, L., Yun, B., Kittappa, R., McKay, R. and Awatramani, R. (2009a). Spatiotemporally separable Shh domains in the midbrain define distinct dopaminergic progenitor pools. *Proc. Natl. Acad. Sci. USA* **106**, 19185-19190.
Joksimovic, M., Yun, B. A., Kittappa, R., Anderegg, A. M., Chang, W. W., Taketo, M. M., McKay, R. D. and Awatramani, R. B. (2009b). Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. *Nat. Neurosci.* **12**, 125-131.
Joshi, K., Lee, S., Lee, B., Lee, J. W. and Lee, S. K. (2009). LMO4 controls the balance between excitatory and inhibitory spinal V2 interneurons. *Neuron* **61**, 839-851.
Joyner, A. L. and Zervas, M. (2006). Genetic inducible fate mapping in mouse: establishing genetic lineages and defining genetic neuroanatomy in the nervous system. *Dev. Dyn.* **235**, 2376-2385.
Kala, K., Jukkola, T., Pata, I. and Partanen, J. (2008). Analysis of the midbrain-hindbrain boundary cell fate using a boundary cell-specific cre-mouse strain. *Genesis* **46**, 29-36.
Kala, K., Haugas, M., Lillevali, K., Guimera, J., Wurst, W., Salminen, M. and Partanen, J. (2009). Gata2 is a tissue-specific post-mitotic selector gene for midbrain GABAergic neurons. *Development* **136**, 253-262.
Karunaratne, A., Hargrave, M., Poh, A. and Yamada, T. (2002). GATA proteins identify a novel ventral interneuron subclass in the developing chick spinal cord. *Dev. Biol.* **249**, 30-43.
Katarova, Z., Sekerkova, G., Prodan, S., Mugnaini, E. and Szabo, G. (2000). Domain-restricted expression of two glutamic acid decarboxylase genes in midgestation mouse embryos. *J. Comp. Neurol.* **424**, 607-627.
Kimmel, R. A., Turnbull, D. H., Blanquet, V., Wurst, W., Loomis, C. A. and Joyner, A. L. (2000). Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. *Genes Dev.* **14**, 1377-1389.
Kurek, D., Garinis, G. A., van Doorninck, J. H., van der Wees, J. and Grosveld, F. G. (2007). Transcriptome and phenotypic analysis reveals Gata3-dependent signalling pathways in murine hair follicles. *Development* **134**, 261-272.
Lahti, L., Peltopuro, P., Piepponen, T. P. and Partanen, J. (2012). Cell-autonomous FGF signaling regulates anteroposterior patterning and neuronal differentiation in the mesodiencephalic dopaminergic progenitor domain. *Development* **139**, 894-905.
Laviolette, S. R. and van der Kooy, D. (2004). The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat. Rev. Neurosci.* **5**, 55-65.
Lillevali, K., Matilainen, T., Karis, A. and Salminen, M. (2004). Partially overlapping expression of gata2 and gata3 during inner ear development. *Dev. Dyn.* **231**, 775-781.
Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R. et al. (2010). A robust and high-throughput cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* **13**, 133-140.
Marchand, R. and Poirier, L. J. (1983). Isthmic origin of neurons of the rat substantia nigra. *Neuroscience* **9**, 373-381.
Marin, O. and Rubenstein, J. L. (2003). Cell migration in the forebrain. *Annu. Rev. Neurosci.* **26**, 441-483.
Metin, C., Vallee, R. B., Rakic, P. and Bhide, P. G. (2008). Modes and mishaps of neuronal migration in the mammalian brain. *J. Neurosci.* **28**, 11746-11752.
Muroyama, Y., Fujiwara, Y., Orkin, S. H. and Rowitch, D. H. (2005). Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. *Nature* **438**, 360-363.
Nakatani, T., Minaki, Y., Kumai, M. and Ono, Y. (2007). Helt determines GABAergic over glutamatergic neuronal fate by repressing Ngn genes in the developing mesencephalon. *Development* **134**, 2783-2793.

Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., Hayashi, H. et al. (2007). Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development* **134**, 3213-3225.

Peltopuro, P., Kala, K. and Partanen, J. (2010). Distinct requirements for Ascl1 in subpopulations of midbrain GABAergic neurons. *Dev. Biol.* **343**, 63-70.

Scott, M. M. and Deneris, E. S. (2005). Making and breaking serotonin neurons and autism. *Int. J. Dev. Neurosci.* **23**, 277-285.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.

Sunmonu, N. A., Li, K., Guo, Q. and Li, J. Y. (2011). Gbx2 and Fgf8 are sequentially required for formation of the midbrain-hindbrain compartment boundary. *Development* **138**, 725-734.

Tamamaki, N., Yanagawa, Y., Tomioka, R., Miyazaki, J., Obata, K. and Kaneko, T. (2003). Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J. Comp. Neurol.* **467**, 60-79.

Tepper, J. M. and Lee, C. R. (2007). GABAergic control of substantia nigra dopaminergic neurons. *Prog. Brain Res.* **160**, 189-208.

Trokovic, R., Trokovic, N., Hernesniemi, S., Pirvola, U., Vogt Weisenhorn, D. M., Rossant, J., McMahon, A. P., Wurst, W. and Partanen, J. (2003). FGFR1 is independently required in both developing mid- and hindbrain for sustained response to isthmic signals. *EMBO J.* **22**, 1811-1823.

Trokovic, R., Jukkola, T., Saarimaki, J., Peltopuro, P., Naserke, T., Weisenhorn, D. M., Trokovic, N., Wurst, W. and Partanen, J. (2005). Fgfr1-Dependent boundary cells between developing mid- and hindbrain. *Dev. Biol.* **278**, 428-439.

Vargas-Perez, H., Ting-A Kee, R., Walton, C. H., Hansen, D. M., Razavi, R., Clarke, L., Bufalino, M. R., Allison, D. W., Steffensen, S. C. and van der Kooy, D. (2009). Ventral tegmental area BDNF induces an opiate-dependent-like reward state in naive rats. *Science* **324**, 1732-1734.

Verney, C., Zecevic, N. and Puelles, L. (2001). Structure of longitudinal brain zones that provide the origin for the substantia nigra and ventral tegmental area in human embryos, as revealed by cytoarchitecture and tyrosine hydroxylase, calretinin, calbindin, and GABA immunoreactions. *J. Comp. Neurol.* **429**, 22-44.

Wilkinson, D. G. and Green, J. (1990). In situ hybridization and the three-dimensional construction of serial sections. In *Postimplantation Mammalian Embryos* (ed. A. Copp and D. Cockroft), pp. 155-171. Oxford, UK: Oxford University Press.

Zervas, M., Millet, S., Ahn, S. and Joyner, A. L. (2004). Cell behaviors and genetic lineages of the mesencephalon and rhombomere 1. *Neuron* **43**, 345-357.

DEVELOPMENT