Notch signaling coordinates the patterning of striatal compartments

Heather A. Mason[1], Staci M. Rakowiecki[1], Myrto Raftopoulou[1], Susana Nery[1], Yuanyuan Huang[1], Thomas Gridley[2] and Gord Fishell[1,*]

[1]Developmental Genetics Program and the Department of Cell Biology, The Skirball Institute of Biomolecular Medicine, New York University Medical Center, 540 First Avenue, New York, NY 10016, USA
[2]The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, USA
*Author for correspondence (e-mail: fishell@saturn.med.nyu.edu)

Accepted 21 July 2005

Development 132, 4247-4258
Published by The Company of Biologists 2005
doi:10.1242/dev.02008

Summary

Numerous lines of evidence suggest that Notch signaling plays a pivotal role in controlling the production of neurons from progenitor cells. However, most experiments have relied on gain-of-function approaches because perturbation of Notch signaling results in death prior to the onset of neurogenesis. Here, we examine the requirement for Notch signaling in the development of the striatum through the analysis of different single and compound *Notch1* conditional and *Notch3* null mutants. We find that normal development of the striatum depends on the presence of appropriate Notch signals in progenitors during a critical window of embryonic development. Early removal of *Notch1* prior to neurogenesis alters early-born patch neurons but not late-born matrix neurons in the striatum. We further show that the late-born striatal neurons in these mutants are spared as a result of functional compensation by *Notch3*. Notably, however, the removal of Notch signaling subsequent to cells leaving the germinal zone has no obvious effect on striatal organization and patterning. These results indicate that Notch signaling is required in neural progenitor cells to control cell fate in the striatum, but is dispensable during subsequent phases of neuronal migration and differentiation.

Key words: Notch1, Notch3, Striatum, Patch, Matrix, Neural progenitor

Introduction

Neural progenitor cells are responsible for producing the complex repertoire of diverse cell types that define the mature brain. Early during neurogenesis, the progenitor pool expands to generate additional neural progenitors through symmetric cell divisions. Once the progenitor pool has been established, neuronal production commences and sequential rounds of asymmetric cell divisions produce a postmitotic neuron as well as a progenitor cell. Neurogenesis ultimately draws to an end as neural progenitors undergo final symmetric divisions to give rise to postmitotic neurons, thus exhausting the progenitor cell population (Cai et al., 2002; Takahashi et al., 1996). Numerous studies have shown that the time at which a cell becomes postmitotic during forebrain development is a critical determinant of its ultimate cell fate (Frantz and McConnell, 1996; Livesey and Cepko, 2001; McConnell, 1995; McConnell and Kaznowski, 1991; van der Kooy and Fishell, 1987). Therefore, elucidating the signals that regulate the differentiation of progenitors within the developing brain is crucial to understanding how cell fate is regulated in the central nervous system (CNS).

The mature striatum is mosaically arranged into two distinct compartments, the patch (or striosome) compartment and the surrounding matrix. Patch and matrix neurons each display unique biochemical profiles and have different functional properties arising from separate corticostriatal and nigrostriatal afferent and efferent pathways (Gerfen, 1984; Gerfen, 1992; Ragsdale and Graybiel, 1990). Like the cerebral cortex (Angevine and Sidman, 1961; Luskin and Shatz, 1985; Rakic, 1974), neurons in the striatal patches and matrix are generated in a precise developmental sequence, with the majority of patch neurons being produced prior to those in the matrix (van der Kooy and Fishell, 1987). The earliest born patch neurons reside in the ventrolateral-most region of the striatum, called the subcallosal streak (SCS) (Song and Harlan, 1994). Little is known about the signals that regulate populations of neural progenitor cells in the basal forebrain and their subsequent differentiation into early- and late-born cell types in the striatum (Halliday and Cepko, 1992).

Notch signaling has been proposed to be a key regulator of the orderly progression of cell types during forebrain development (Schuurmans and Guillemot, 2002). Both Notch receptors and their Delta-Serrate-Lag2 (DSL) ligands are expressed within the proliferative ventricular and subventricular zones (VZ and SVZ, respectively) during neurogenesis (Lindsell et al., 1996). Gain-of-function studies have revealed that constitutive Notch signaling leads to cells remaining as progenitors (Henrique et al., 1997; Mizutani and Saito, 2005; Ohtsuka et al., 2001), whereas decreased Notch activity is correlated with a reduction in neural progenitors (Hitoshi et al., 2002; Yoon et al., 2004; Yoon and Gaiano, 2005) and increased neuronal differentiation (de la Pompa et al., 1997; Ishibashi et al., 1995). In addition, Notch signaling is thought to regulate glial versus neuronal identity (Furukawa et al., 2000; Gaiano et al., 2000; Morrison et al., 2000; Wang et al., 1998). Radial glia are stem cells in the nervous system

Development

Development

(Anthony et al., 2004; Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2004), and brain lipid-binding protein (BLBP), a marker of radial glia, has recently been shown to be a direct target of the Notch signaling pathway (Anthony et al., 2005). Although the majority of previous experiments have focused on the role of Notch activity in early developmental events, such as neurogenesis and cell fate determination, several studies have suggested that the Notch pathway may also play important roles in postmitotic neurons. In particular, in vitro experiments have implicated Notch signaling in regulating the growth of neurites (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999).

Because *Notch1* null mutants die at embryonic day 9.5 (E9.5) (Conlon et al., 1995; Swiatek et al., 1994), a time prior to formation of the nervous system, it has been impossible to examine the role of Notch signaling in neurogenesis and in subsequent stages of neuronal maturation in vivo. Neural progenitor cells sequentially give rise to different types of neurons, from which it can be predicted that the loss of Notch signaling would result in the production of early cell fates at the expense of later-born cell types in the striatum, because the progenitor population would become prematurely depleted in the absence of Notch activity. However, at least one Notch receptor, *Notch3*, has been reported to antagonize *Notch1* activity on the basis of gain-of-function experiments (Apelqvist et al., 1999; Beatus et al., 1999; Beatus et al., 2001). *Notch3* null mutants are viable (Krebs et al., 2003) and display some defects in vasculogenesis (Domenga et al., 2004), but the function of *Notch3* in striatal progenitor cells is at present unclear. Moreover, the requirement for Notch signaling once cells exit the VZ is unknown. Both *Notch1* and *RBP-Jκ* (an intracellular mediator of signaling through all Notch receptors) null mutants show signs of precocious neuronal differentiation, although *RBP-Jκ* mutants display more severe defects than *Notch1* null mutants, suggesting that another Notch family member may also play a role in forebrain neurogenesis (de la Pompa et al., 1997).

Like *Notch1*, *Notch3* is expressed by progenitor cells within the forebrain (Lindsell et al., 1996). To test the role of *Notch1* and *Notch3* receptors in regulating neurogenesis in the striatum, we have investigated the phenotypes occurring in single and compound *Notch1* conditional and *Notch3* null mutant animals. We used the Cre-LoxP system (Sauer and Henderson, 1988) and two different Cre-driver lines to produce two distinct conditional deletions of the *Notch1* receptor. In one case, *Notch1* is removed throughout the telencephalon from the beginning of neurogenesis onwards. In the second case, *Notch1* is deleted only after cells have exited the VZ in the ventral telencephalon. We have assessed striatal development in *Notch1* conditional; *Notch3* null double mutant mice in the context of both of these Cre-driver lines.

We show here that removing *Notch1* in the forebrain prior to neurogenesis preferentially affects early-born neurons in the striatum, whereas later born cell types are generated normally. In addition, we demonstrate that *Notch3* functionally compensates for the loss of *Notch1* in the nervous system and mediates the conservation of late-born neurons in *Notch1* conditional mutants. Notably, removal of *Notch1* and *Notch3* in cells after they have left the ventricular zone has no effect on striatal development. These experiments reveal that Notch signaling is not required in postmitotic neurons for their migration or the subsequent patterning of the striatum.

Materials and methods

Mice and mouse embryos

Floxed *Notch1* mice were a gift of Freddy Radtke and were genotyped as previously described (Radtke et al., 1999). Mutant mouse embryos were obtained by crossing homozygous floxed *Notch1* mice with mice heterozygous for floxed *Notch1* and $Foxg1^{Cre/+}$. The generation of $Foxg1^{Cre/+}$ mice was previously published (Hebert and McConnell, 2000) and $Foxg1^{Cre/+}$ heterozygous mice were maintained on a Swiss Webster background. *Dlx5/6-Cre-IRES-EFGP* mice were previously described (Stenman et al., 2003). *Notch3* null mutant mice are viable and fertile and were maintained as homozygous nulls (Krebs et al., 2003). Conditional *Notch1; Notch3* double mutant mice were acquired by breeding double homozygous floxed *Notch1; Notch3* null mutant mice with mice heterozygous for floxed *Notch1* and $Foxg1^{Cre/+}$ (or $Dlx5/6^{Cre}$) on a *Notch3* null mutant background. Two Cre recombination reporter lines were used, ROSA26 floxed stop *lacZ* (Soriano, 1999) and Z/EG (Novak et al., 2000). Plug date was defined as embryonic day 0.5 (E0.5).

BrdU birthdating

Pregnant mice were injected with intraperitoneally with 2 mg of bromodeoxyuridine (BrdU) (Sigma, St Louis, MO) in a solution of PBS with 7 mM NaOH. BrdU was administered at E10.5, E11.5, E12.5, E13.5, E14.5 and E15.5, and the embryos were subsequently allowed to develop until E18.5, at which point the dams were terminally anesthetized and the embryos were removed and perfused with 2% paraformaldehyde and postfixed for 2 hours at 4°C. At least three mutants and three wild-type littermates were analyzed for each time-point of BrdU administration.

Tissue preparation and in situ hybridization

Embryos were dissected in chilled PBS and fixed in either 2% or 4% paraformaldehyde for four hours at 4°C, cryoprotected in 30% sucrose, embedded in Tissue-Tek® OCT, and sectioned at a thickness of 14-16 μm on a Leica CM3050 S cryostat. RNA in situ hybridization was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993; Wilkinson and Nieto, 1993). RNA probes were labeled with digoxigenin and visualized with BM-Purple®, according the manufacturer's instruction (Roche Biosciences). The following cDNA probes were used: *Notch1*, *Notch2*, *Notch3*, *Hes1*, *Hes5*, *Mash1*, *Neurod* and *Ebf1*. Images were obtained using a Diagnostics 4.2 camera and Spot Advanced software, and processed using Adobe Photoshop.

Antibodies and immunohistochemistry

Rabbit anti-DARPP-32 (Chemicon International, Temecula, CA) was used at 1:500, rabbit anti-tyrosine hydroxylase (Chemicon International) was used at 1:500, rabbit anti-glutamate receptor 1 (Chemicon International) was used at 1:50, mouse anti-BrdU (BD Biosciences, San Jose, CA) was used at 1:100, and rabbit anti-GFP (Molecular Probes, Eugene, OR) was used at 1:1000. Ephrin-A4/Fc (R&D Systems, Minneapolis, MN) was used at 2 μg/ml. Secondary antibodies conjugated with Cy3 or Alexa-488 were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and Molecular Probes, and raised in goats. α-human IgG-Alexa-488 (Molecular Probes) was used at 1:200. Fluorescent images were acquired using a cooled-CCD camera (Princeton Scientific Instruments, NJ) and Metamorph software (Universal Imaging, Downington, PA), and were processed using Adobe Photoshop.

Western blot

E12.5 mutant and wild-type brain lysates were prepared in 100 μl

RIPA buffer (10 mM Tris/HCl (pH 7.5), 140 mM NaCl, 1 mM orthovanadate, 1% Nonidet P-40, 2 mM PMSF, 5 mM EDTA, 20 μg/ml aprotinin, 20 μg/ml leupeptin), spun down and the supernatants were boiled in Laemli sample buffer. Proteins were resolved by 8% SDS-PAGE and transferred onto a PVDF membrane for western blot analysis. Rabbit anti-Notch1 (Upstate, Lake Placid, NY) followed by peroxidase-conjugated anti-Rabbit IgG (Jackson ImmunoResearch Laboratories) was used to detect the cleaved form of endogenous Notch1, and mouse anti-alpha tubulin (Sigma-Aldrich) followed by peroxidase-conjugated anti-Mouse IgG (Jackson ImmunoResearch Laboratories) was used to detect endogenous tubulin.

Striatal analysis

Coronal sections from E18.5 telencephalon were double immunostained with antibodies to Darpp32 and BrdU, and fluorescent images were obtained as described above. Using Metamorph software (Universal Imaging, Downington, PA), regions of Darpp32 immunoreactivity (which define the subcallosal streak and striatal patches) were outlined and the number of BrdU-positive cells in each compartment were counted. The matrix compartment of the striatum was defined as the region remaining around the clusters of Darpp32-positive cells. Six striatal sections were analyzed per animal and at least three mutant and three wild-type littermates were analyzed for each time-point of BrdU administration (E10.5-E15.5). Microsoft Excel was used to compute the data and perform the statistical analyses. Student's *t*-test (one-tailed) was used to compare the measurements of the mutant and wild-type animals at each time-point, and statistical significance was determined with *P*-values of less than 0.05.

Results

Targeted deletion of *Notch1* throughout the mouse forebrain

We used $Foxg1^{Cre/+}$ mice, in which Cre recombinase has been knocked into the *Foxg1* locus (Hebert and McConnell, 2000), to inactivate *Notch1* specifically in the embryonic mouse forebrain. As revealed by X-gal staining using the ROSA26 Cre reporter mouse (Soriano, 1999), $Foxg1^{Cre/+}$ induces recombination within the ventral telencephalon and anterior portion of the optic vesicles, beginning at embryonic day 8.5 (E8.5) (Fuccillo et al., 2004; Hebert and McConnell, 2000), and expanding throughout the entire telencephalon by E9.5 and E10.5 (Fig. 1A), encompassing all neuroepithelial cells. To obtain *Notch1* conditional mutants, we crossed homozygous mice in which exon 1 of the *Notch1* gene is flanked by loxP sites (floxed; $Notch1^{f/f}$) (Radtke et al., 1999) to $Notch1^{f/+}$;$Foxg1^{Cre/+}$ mice. Thus, Cre-mediated recombination of the floxed *Notch1* allele is expected to occur in all telencephalic cells, including the neural progenitor cells. We refer to $Foxg1^{Cre/+}$; $Notch1^{f/f}$ conditional knockout mice as $Foxg1^{Cre}$; *N1* cKOs for simplicity.

$Foxg1^{Cre}$; *N1* cKOs survive until birth. Neonates die within several hours and display a smaller forebrain than wild-type littermates do. To confirm that *Notch1* is completely removed as a result of this genetic cross, we examined the $Foxg1^{Cre}$; *N1* cKO telencephalon using in situ hybridization, immunoblotting and PCR. Although *Notch1* mRNA is observed throughout the VZ of the wild-type (WT) telencephalon at E10.5, *Notch1* transcripts are not detected in $Foxg1^{Cre}$; *N1* cKOs (Fig. 1B). In addition, Notch1 protein is abundant in forebrain lysates prepared from E12.5 wild-type embryos, but is absent from $Foxg1^{Cre}$; *N1* cKOs (Fig. 1C). PCR primers designed to recognize the recombined or wild-type allele show that only the recombined allele is present in telencephalic tissue at E12.5 (data not shown). *Hes5*, a downstream target of the Notch signaling pathway, is greatly diminished in conditional mutants when compared with wild-type embryos, and only persists in the most ventromedial region of the telencephalon, the medial ganglionic eminence (Fig. 1B, lower right panel), which does

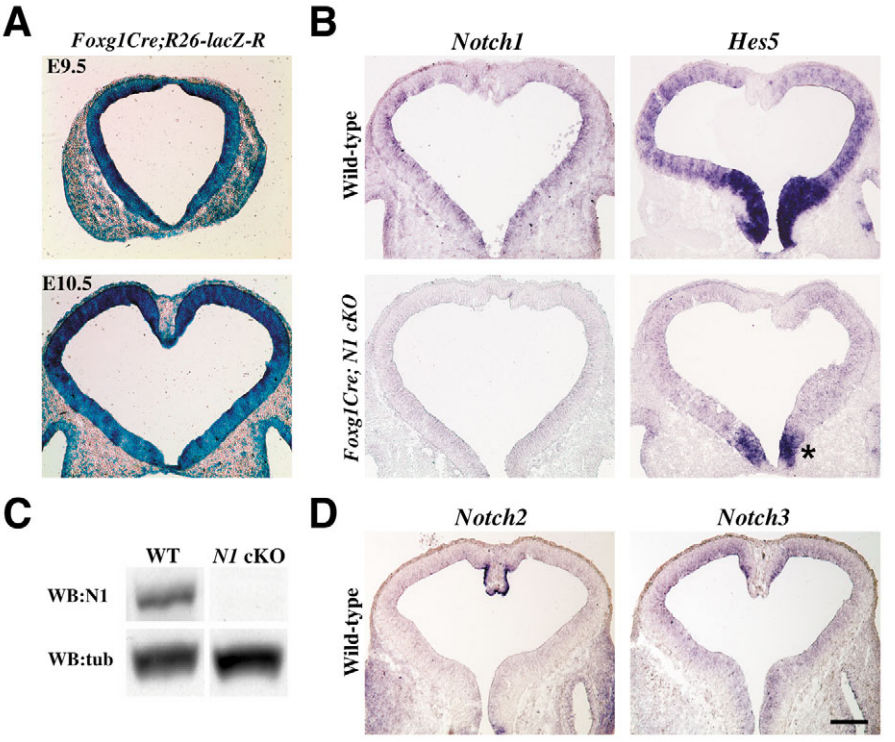

Fig. 1. Generation of a telencephalic-specific deletion of *Notch1*. (A) Coronal sections through the embryonic telencephalon of $Foxg1^{Cre/+}$; ROSA26-floxed-stop-*lacZ* reporter mice stained with X-gal to visualize Cre activity. Cre-mediated recombination is detected throughout the entire telencephalon by E9.5 and E10.5. (B) *Notch1* and *Hes5* are present in the telencephalic VZ in wild-type (WT) embryos, whereas *Notch1* mRNA is not detected and *Hes5* is substantially diminished in the telencephalon of *Foxg1Cre*; *N1* cKOs at E10.5. Notably, residual *Hes5* expression is detected only in the medial ganglionic eminence (asterisk), and not in the striatal anlage, the lateral ganglionic eminence, which is more laterally located. (C) At E12.5, Notch1 protein is not detected in telencephalic lysates from *Foxg1Cre*; *N1* cKOs (N1 cKO), whereas levels of α-tubulin are equivalent between N1 cKO and WT forebrain lysates, as assessed by immunoblotting for the cleaved portion of Notch1 and α-tubulin. Western blot (WB): anti-Notch1 (N1) and anti-α-tubulin (tub). (D) At E10.5, *Notch2* is expressed at high levels within the epithelium of the choroid plexus but is not detectable in the VZ, whereas *Notch3* is present at low levels throughout the VZ of wild-type embryos. Scale bar: 150 μm.

Development

not give rise to the striatum (Olsson et al., 1998; Wichterle et al., 2001). Thus, *Foxg1*^{Cre}; *N1* cKOs display an efficient recombination of the *Notch1* gene locus, which results in the removal of *Notch1* activity throughout the telencephalon by E9.5. To determine the potential contribution of other Notch family members to early neurogenesis within the telencephalon, we examined the expression of *Notch2* and *Notch3* at E10.5. *Notch2* is expressed at high levels within the epithelium of the choroid plexus but not within the VZ, whereas *Notch3* can be detected within the VZ, although at lower levels than *Notch1* is expressed in the ventral telencephalon at E10.5 (Fig. 1B,D).

Perturbation of early neurogenesis in *Notch1* conditional mutants

The telencephalon of *Foxg1*^{Cre}; *N1* cKOs exhibits striking morphological defects at E12.5, including a reduction in the overall size of the developing forebrain. The subcortical regions of the telencephalon, characterized by the lateral, medial and caudal ganglionic eminences (LGE, MGE and CGE, respectively), are particularly affected in the *Foxg1*^{Cre}; *N1* cKOs at E12.5. In *Foxg1*^{Cre}; *N1* cKOs, the MGE, LGE (Fig. 2A) and CGE (data not shown) are severely diminished in size. These ventral eminences comprise neural progenitors as well as their differentiating progeny. Both progenitor cells (visualized by *Hes1 and Hes5* expression, Fig. 2A) and newly differentiating neurons [identified by the expression of the neuron-specific marker TuJ1, as shown by Yoon et al. (Yoon et al., 2004)] are reduced in the *Foxg1*^{Cre}; *N1* cKOs when compared with wild-type littermates at E12.5.

The primary downstream effectors of the Notch signaling pathway are Hes genes (mammalian homologs of the *Hairy* and *Enhancer of split* genes in *Drosophila*) (Sasai et al., 1992; Takebayashi et al., 1995). Hes genes are expressed by neural progenitor cells, and encode transcriptional repressors that act to inhibit both the expression and the function of proneural basic helix-loop-helix (bHLH) genes (Ishibashi et al., 1995), such as *Mash1* and *Neurod*. As expected, both *Hes1* and *Hes5* are reduced in the forebrain of *Foxg1*^{Cre}; *N1* cKOs at E12.5 (Fig. 2A, left and center panels). *Foxg1* drives Cre expression in the developing eyes as well as in the forebrain (Hebert and McConnell, 2000), and therefore *Foxg1*^{Cre}; *N1* cKOs also lack *Notch1* activity in the nasal, temporal portion of the embryonic retina. Interestingly, levels of *Hes1* are decreased in the developing retina of *Foxg1*^{Cre}; *N1* cKOs, whereas *Hes5* is virtually undetectable (Fig. 2A, insets, left and middle panels). The persistence of *Hes5* expresssion in the telencephalon with its comparative absence in the embryonic retina indicated to us that another Notch receptor may function in the telecephalon. Although *Notch3* is present in the telencephalic VZ as early at E10.5 (Fig. 1D), we could only detect *Notch1* in the developing mouse retina (data not shown). Unlike *Hes5*, *Hes1* expression persists in the developing retina of *Foxg1*^{Cre}; *N1* cKOs, raising the possibility that *Hes1* could be regulated by Notch-

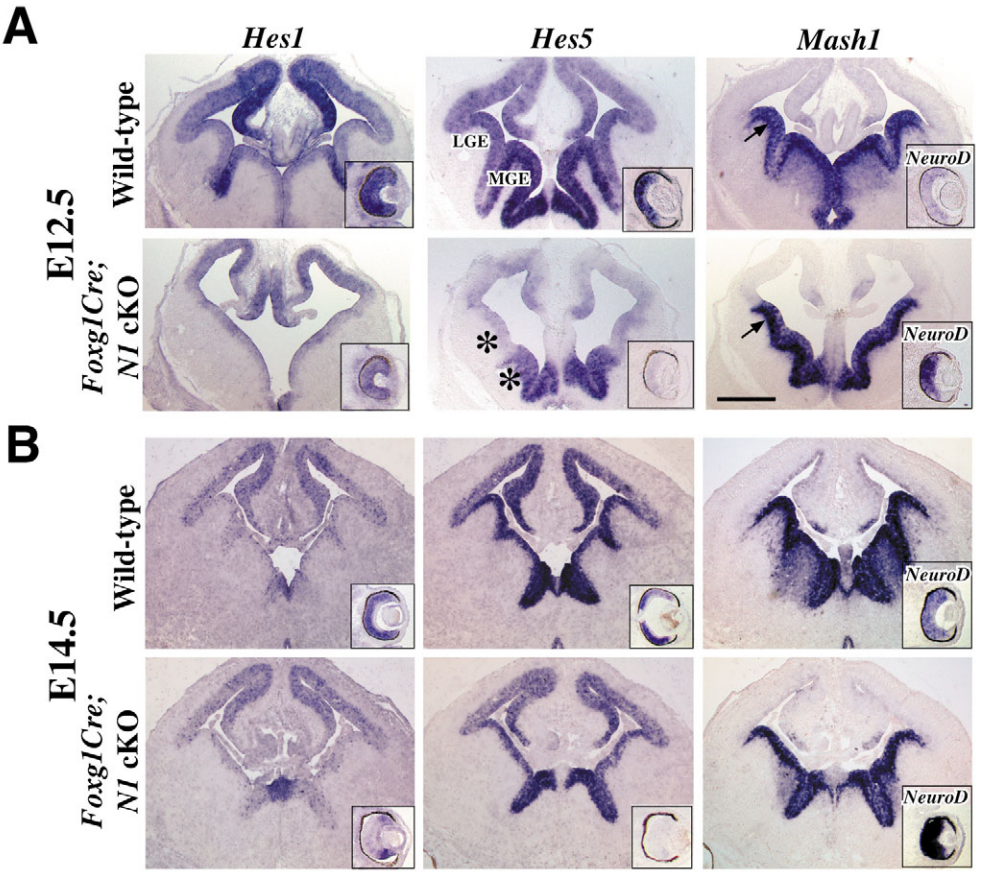

Fig. 2. Neurogenesis is perturbed in *Notch1* conditional mutants at E12.5 when compared with wild-type embryos, but largely recovers by E14.5. (A) The ganglionic eminences, the LGE and MGE, are drastically reduced in size in *Foxg1Cre; N1* cKOs (asterisks), as seen here in coronal sections through the forebrain at E12.5. *Hes1* and *Hes5*, genes that are activated by the Notch signaling pathway, are substantially decreased in the eye (insets) and telencephalon of *Foxg1Cre; N1* cKOs at E12.5. *Mash1*, a bHLH gene repressed by Hes activity, is increased in *Foxg1Cre; N1* cKOs at E12.5, particularly in the SVZ (arrows). In addition, the bHLH gene *Neurod* is upregulated in the developing retina (insets). Scale bar: 500 μm. (B) Overall brain morphology and neurogenesis is relatively normal at E14.5 in *Foxg1Cre; N1* cKOs. Expression of *Hes1* and *Hes5* appear equivalent in the VZ of *Foxg1Cre; N1* cKOs and wild-type embryos. Unlike at E12.5, *Mash1* is not noticeably upregulated in the SVZ of conditional knockouts when compared with wild-type embryos at E14.5. Neurogenesis is further impaired in the retina of *Notch1* conditional mutants, as *Hes1* and *Hes5* are virtually absent and *Neurod* is considerably elevated.

independent signaling pathways. In support of this idea, *RBP-Jκ* null mutants also display a lack of *Hes5* staining, whereas *Hes1* expression remains (de la Pompa et al., 1997).

Mash1 mRNA is expressed by differentiating cells in the ventral telencephalon and is upregulated in *Foxg1*^{Cre}; *N1* cKOs at E12.5 (Fig. 2A, right panels). The increase in *Mash1* expression is most apparent in cells residing within the SVZ. In addition, the mutant SVZ appears to be thicker than the wild-type SVZ, suggesting that cells precociously transit to the SVZ in the absence of *Notch1*. A similar observation was made in *Mash1* mutant mice in which VZ cells in the LGE expressed SVZ markers prematurely, a defect that the authors attributed to reduced Notch signaling (Casarosa et al., 1999). Like the forebrain, the developing retina of *Foxg1*^{Cre}; *N1* cKOs displays increased proneural gene expression, and *Neurod* is substantially elevated compared with wild-type littermates (Fig. 2A, insets, right panels). The upregulation of proneural genes in the eye and ventral forebrain, suggests that cells precociously initiate neuronal differentiation at E12.5 in the absence of *Notch1*. Although *Foxg1*^{Cre}; *N1* cKOs display obvious morphological defects at E12.5, we were surprised to note that the mutant brains appear relatively normal in their overall morphology at E14.5 (Fig. 2B). Furthermore, levels of *Hes1*, *Hes5* and *Mash1* in the forebrain of *Foxg1*^{Cre}; *N1* cKOs appear similar to those found in the forebrain of wild-type littermates at E14.5 (Fig. 2B), suggesting that neurogenesis occurs normally at this time and that telencephalic development recovers in *Foxg1*^{Cre}; *N1* cKOs. By contrast, removing *Notch1* in the embryonic mouse retina results in abnormalities that progressively worsen during development and fail to improve (Fig. 2A,B, insets, and H.A.M. and G.F., unpublished).

Aberrant patch and subcallosal streak development in the striatum of *Notch1* conditional mutants

To determine the consequences of abnormal neurogenesis at E12.5 in *Foxg1*^{Cre}; *N1* cKOs, we examined the specific cell types that are generated at E12.5 or earlier in the basal telencephalon. Patch neurons in the striatum are derived from the LGE (Olsson et al., 1998; Wichterle et al., 2001) and are produced within this temporal window (van der Kooy and Fishell, 1987). In addition, a ventrolateral to dorsomedial gradient of differentiation occurs such that the earliest born cells reside in a specialized region of the patch compartment known as the subcallosal streak (SCS), a crescent-shaped area along the ventrolateral edge of the striatum (Song and Harlan, 1994). Although SCS, patch and matrix neurons can be easily distinguished by a variety of molecular and histochemical markers in the adult striatum (Beckstead and Kersey, 1985; Graybiel and Chesselet, 1984; Graybiel et al., 1981), their segregation is only beginning to be apparent at E18.5. One indication that early-born striatal neurons are maturing and coalescing into characteristic SCS and patch compartments is the refinement of dopaminergic projections from the substania nigra (SN), forming what have been called islands of dopamine (Graybiel, 1984; Loizou, 1972; Murrin and Ferrer, 1984; Olson et al., 1972). In addition, these neurons express dopamine- and cyclic adenosine 3′:5′-monophosphate-regulated phosphoprotein (Darpp32; Ppp1r1b – Mouse Genome Informatics) (Foster et al., 1987) and glutamate receptor 1 (Glur1) (Snyder-Keller and Costantini, 1996) during embryonic development. Thus, we evaluated patch and SCS development in *Foxg1*^{Cre}; *N1* cKOs using antibodies to Darpp32, Glur1, and tyrosine hydroxylase (Th), all of which are abnormally expressed in mutants when compared with wild-type littermates at E18.5 (Fig. 3). The striatum, including the patch compartment, remains immature at E18.5, but this is the latest time-point that could be reliably examined because the conditional mutants die at birth. Glur1 and Darpp32 are expressed similarly in the SCS and some of the more laterally

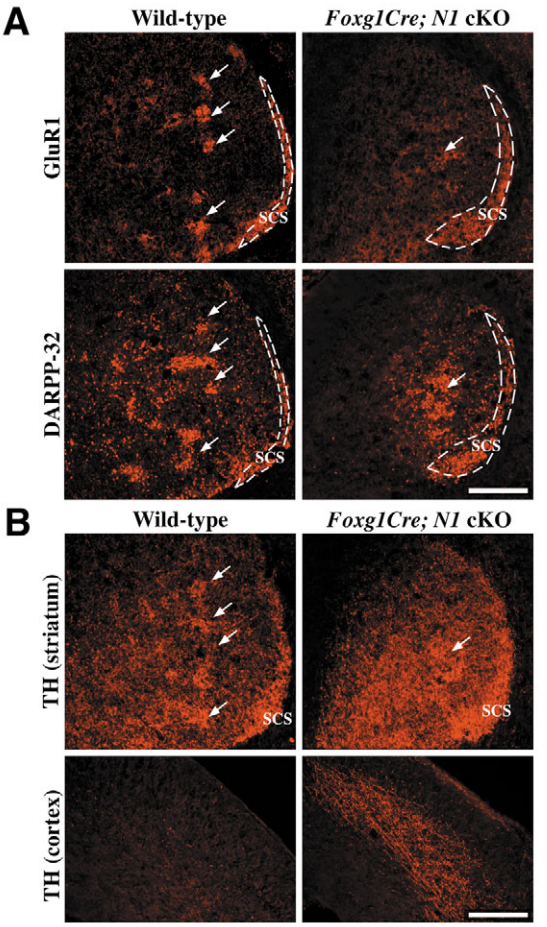

Fig. 3. The patch compartment, as well as its dopaminergic innervation from the midbrain, is significantly impaired in the absence of *Notch1*. (A) Patch neurons in the developing striatum begin to aggregate and display cell-specific markers at E18.5. Immunostained adjacent coronal forebrain sections show that glutamate receptor1 (Glur1) and Darpp32 are expressed by newly differentiating subcallosal (SCS) and patch neurons (arrows) in wild-type embryos but are abnormally expressed in *Foxg1Cre; N1* cKOs. The SCS is thicker in the *Foxg1Cre; N1* cKOs than wild-type embryos relative to the overall size of the striatum. Scale bar: 200 μm. (B) Incoming dopaminergic fibers from the substania nigra (SN) express tyrosine hydroxylase (Th) and become selectively localized to SCS and patch neurons in the wild-type striatum (arrows). *Foxg1Cre; N1* cKOs, which display an expanded SCS, show greater Th innervation than do wild-type mice at E18.5 (upper panels). In addition, fibers from the SN ectopically project to the cerebral cortex in the absence of *Notch1*. Excessive Th-positive fibers are present throughout the prefrontal cortex in *Foxg1Cre; N1* cKOs whereas Th-positive fibers are rarely observed in the wild-type cortex at E18.5 (lower panels). Scale bar: 100 μm.

located patches when these markers are examined in adjacent sections at E18.5 (Fig. 3A, left panels). α-Darpp32 labels additional cells compared with α-Glur1, including younger, more medially located neurons (Fig. 3A, left panels), suggesting that Darpp32 is expressed in less mature patch neurons than is Glur1.

There are several obvious defects in early-born striatal neurons in the absence of *Notch1*. First, the SCS appears thicker in *Foxg1*^{Cre}; *N1* cKOs than in controls, relative to the overall size of the striatum (Figs 3, 4, Fig. 5B). Second, the remaining patch clusters are fewer in number in these mutants, although the patches that do form tend to be larger in size than in wild-type embryos (Figs 3, 4, Fig. 5B). Finally, dopaminergic (Th-positive) fibers from the SN form aberrant projections in the forebrain of *Foxg1*^{Cre}; *N1* cKOs (Fig. 3B). Unlike wild-type littermates, which display an enrichment of Th-positive fibers in the SCS, as well as in numerous other patch compartments in the striatum, *Foxg1*^{Cre}; *N1* cKOs show expanded Th innervation of the SCS and fewer Th fibers forming characteristic clusters than in the wild-type striatum (Fig. 3B, upper panels). Because the dopaminergic fibers that innervate the striatum originate in the SN, an area of the midbrain that still retains *Notch1* activity, the aberrant Th immunostaining in the mutants reflects the functional consequences of defects in the patch targets. In addition, dopaminergic innervation of the developing striatum is delayed in *Foxg1*^{Cre}; *N1* cKOs by 2 days when compared with wild-type embryos (data not shown). Much to our surprise, *Foxg1*^{Cre}; *N1* cKOs also display numerous ectopic Th-positive fibers in the cerebral cortex compared with wild-type embryos, which display few, if any, Th projections to the cortex (Fig. 3B, lower panels). Because the size of the SN is indistinguishable between mutants and wild-type littermates (data not shown), one possible explanation is that afferent fibers from the SN fail to find normal or adequate patch targets in the mutant striatum and subsequently form aberrant projections to the frontal cortex.

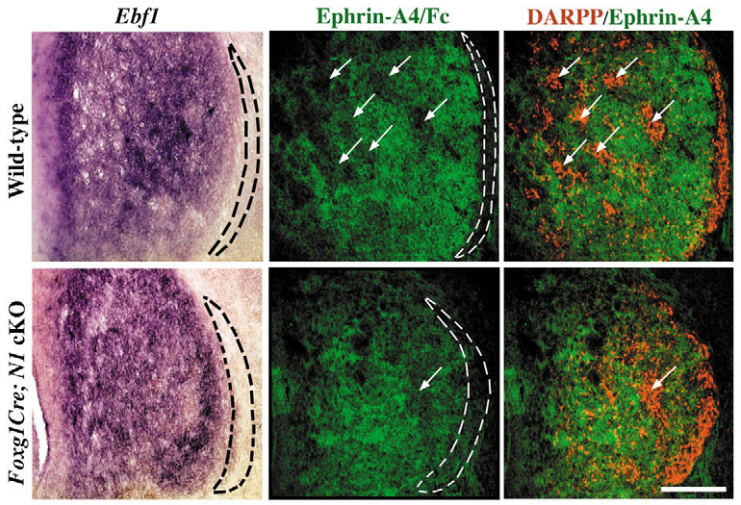

Fig. 4. Late-born matrix neurons develop normally in the absence of *Notch1*. *Ebf1* expression and ephrin A4/Fc binding is enriched in the matrix compartment of the striatum, and excluded from the SCS and patch regions. The patterns of *Ebf1* and ephrin A4/Fc staining appear to be equivalent in both wild-type embryos and *Foxg1Cre; N1* cKOs, as seen here in coronal sections through the striatum at E18.5. The SCS, which can be visualized by the lack of *Ebf1* and ephrin A4 staining (shown in green), is expanded in the absence of *Notch1* (dashed lines). The patch compartments, which also exclude *Ebf1* and ephrin A4, express Darpp32 (shown in red) and are reduced at E18.5 in the mutant (arrows). Scale bar: 200 μm.

The striatal matrix develops normally in the absence of *Notch1*

As morphological defects in the *Foxg1*^{Cre}; *N1* cKOs largely disappear by E14.5, we determined the phenotype of cells normally born at or after this time. In the ventral forebrain, these later-born neurons ultimately comprise the matrix compartment of the striatum (van der Kooy and Fishell, 1987). At E18.5, matrix neurons are still relatively immature and are in the initial stages of expressing their characteristic markers. One such marker is the helix-loop-helix transcription factor *Ebf1*, which is involved in the specification and compartmentalization of the striatal matrix (Garel et al., 1999). In addition, ephrin A4 ligands selectively bind to EphA receptors that are expressed by matrix neurons and are excluded from striatal patches (Janis et al., 1999). When we examine the expression of these matrix markers at E18.5, we find that they look relatively normal in *Foxg1*^{Cre}; *N1* cKOs compared with wild-type littermates. Both *Ebf1* and ephrin A4 binding is detected in the majority of cells in the striatum, and is excluded from the subcallosal streak and other patch compartments in conditional mutants and wild-type embryos (Fig. 4). Thus, in contrast to patch development *Foxg1*^{Cre}; *N1* cKOs, the matrix appears to develop normally in the absence of *Notch1*. Because the *Foxg1*$^{Cre/+}$ mice used to produce the *Notch1* conditional knockouts lack one copy of *Foxg1*, it is possible that the phenotype we observed in *Foxg1*^{Cre}; *N1* cKOs could be due to a decrease in *Foxg1* gene function rather than the selective loss of *Notch1*. However, when we examined patch and matrix compartments in *Foxg1*$^{Cre/+}$ mice, we found that the striatum forms normally (data not shown), confirming that the striatal phenotype in *Foxg1*^{Cre}; *N1* cKOs results from removing *Notch1* function.

One explanation for the defects observed in the patch and SCS neurons in the absence of *Notch1* is that Notch signaling acts in progenitors to preserve a sufficient number of progenitor cells to ensure the generation of all striatal cell types. Without adequate Notch signaling, progenitor cells may produce postmitotic neurons without replenishing themselves. However, the defects observed in the patch compartment may also reflect changes in cell proliferation, cell death, or neuronal differentiation and we have examined each of these possibilities. We do not observe any obvious differences in the proliferation of neural progenitors residing in the VZ (as assessed by BrdU incorporation or markers for cycling cells such as Ki67 or phosphohistone-H3) between wild-type and mutant mice (data not shown), indicating that the increase in the SCS is not likely to be due to enhanced proliferation of SCS neurons or their progenitors. Next, we examined whether cell death is increased in *Foxg1*^{Cre}; *N1* cKOs using antibodies that recognize cleaved caspase 3 and TUNEL labeling, both hallmarks of apoptosis. The number of cleaved caspase 3 immunoreactive cells is increased in *Foxg1*^{Cre}; *N1* cKOs when compared with wild-type littermates from E12.5 through E16.5, as is the number of TUNEL-

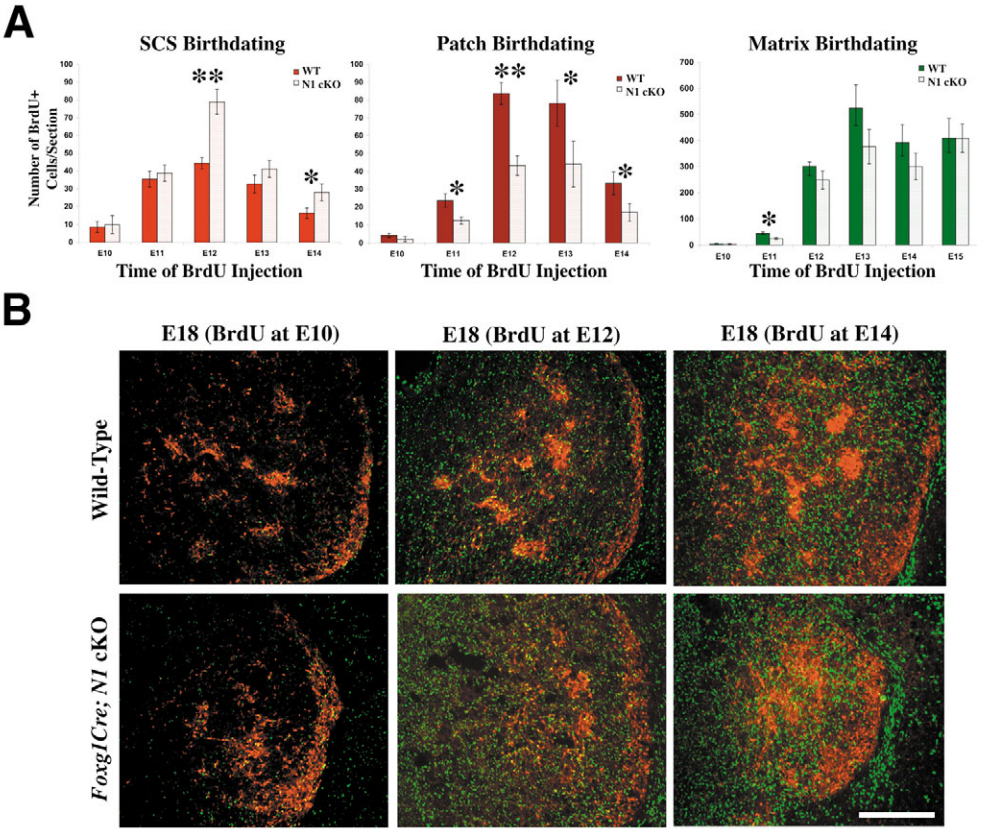

Fig. 5. Birthdating analysis of neurons in the striatum. (A) BrdU was administered at different embryonic time-points (E10.5-E15.5), and the striatum was subsequently analyzed at E18.5. BrdU-positive neurons were counted with respect to their localization within the striatal compartments (SCS, patch or matrix). The SCS neurons born at E12.5 are significantly increased in *Foxg1Cre; N1* cKOs, whereas neurons born at E12.5 in the patch compartment are significantly decreased in mutants compared when with wild-type littermates. Equivalent numbers of matrix neurons are born in the mutants and in wild-type littermates, except at E11.5, at which time there is a small but statistically significant decrease in the birth of matrix neurons in *Foxg1Cre; N1* cKOs. A single asterisk (*) denotes a *P*-value of <0.05, whereas two asterisks (**) signify a *P*-value of <0.005. Three *Foxg1Cre; N1* cKOs and three wild-type embryos were analyzed for each time-point. Error bars represent s.e.m. (B) Coronal sections of the striatum at E18.5 with three representative ages of BrdU administration in *Foxg1Cre; N1* cKOs and wild-type littermates. Tissue was immunostained with antibodies to Darpp32 (red) to visualize the SCS and patch compartments, and with antibodies to BrdU (green) to detect cells that become postmitotic shortly after the pulse of BrdU. Scale bar: 250 μm.

positive nuclei (Mason et al., 2005). However, even though cell death is increased in the absence of *Notch1*, we could not find a selective increase in cell death at the time when most patch neurons are generated (E12.5-E13.5) compared with later time-points, when the bulk of matrix neurons are produced (E13.5 and later) (Mason et al., 2005). These data suggest that there is a generalized increase in cell death in the absence of *Notch1* that equally affects patch and matrix neurons. Indeed, Notch has been reported to regulate cell survival via mechanisms distinct from its effects on neurogenesis (Oishi et al., 2004). Therefore, we favor a model in which *Notch1* acts in progenitors to control their differentiation as early-born cell types in the striatum, and in which the loss of *Notch1* activity results in a majority of cells differentiating as SCS neurons at the expense of remaining patch neurons.

To further test this hypothesis, we performed a series of birthdating experiments to quantify the effect of *Notch1* function on the generation of specific neuronal cell types (SCS, patch and matrix) during embryonic development. BrdU pulses were administered at different times of development, from E10.5 through E15.5, and the brains were subsequently analyzed at E18.5. Cells that were in S-phase at the time of BrdU administration and then subsequently exited the cell cycle will retain BrdU at E18.5, whereas cells that continued proliferating would have diluted the BrdU label. Darpp32 was used to distinguish the patch versus matrix compartments at E18.5 and the number of BrdU-positive cells was quantified in each compartment (Fig. 5). We find that significantly more SCS neurons are born at E12.5 in the *Foxg1*^{Cre}; *N1* cKOs compared with in wild-type embryos at this time (Fig. 5A, left panel). In addition, there are correspondingly fewer patch neurons in the mutants born on E12.5 and E13.5, than in wild-type embryos (Fig. 5A, middle panel). Interestingly, there is a small but statistically significant decrease in the number of matrix neurons in the mutants at E11.5, but after E14.5 the number of matrix neurons that are produced are equivalent in the *Foxg1*^{Cre}; *N1* cKOs and the wild-type embryos (Fig. 5A, right panel). These data suggest that *Notch1* acts in progenitor cells to control the time at which they differentiate and to influence what type of neuron is produced. Furthermore, there is a defect in early-born cells (more SCS neurons at the expense of patch neurons), whereas late-born matrix neurons develop normally. These results raise the question of why there is a selective defect in early-born cells followed by a period during which progenitors are able to produce a relatively normal cohort of matrix neurons.

The role of *Notch3* in the development of the striatum in the absence of *Notch1*

It seemed likely that the normal development of matrix neurons in the striatum of *Foxg1*^{Cre}; *N1* cKOs is mediated through the activity another member of the Notch family of receptors. We examined both *Notch2* and *Notch3* expression at E10.5 using in situ hybridization and found detectable levels of *Notch3*

mRNA in the VZ (Fig. 1D). By contrast, *Notch2* is present primarily within the epithelium of the choroid plexus and not within the VZ at E10.5 (Fig. 1D). Thus, *Notch3* seemed to be a more likely candidate than *Notch2* based on their expression patterns. However, previous gain-of-function studies on *Notch3* activity reported that *Notch3* is a weak activator of canonical Notch target genes and can even inhibit *Notch1* signaling (Apelqvist et al., 1999; Beatus et al., 1999; Beatus et al., 2001). *Notch3* alone is not essential for striatal development because *Notch3* null mutants display normal patch and matrix compartments (data not shown). However, to resolve whether or not *Notch3* (*N3*) can functionally compensate for the loss of *Notch1*, we examined the development of the striatum in conditional *N1; N3* null double mutants (*Foxg1*^{Cre}; *N1; N3* DKOs). These double knockouts were generated by crossing *N3* null mutants, which are viable and fertile (Krebs et al., 2003), onto the *Foxg1*^{Cre}; *N1* cKO background.

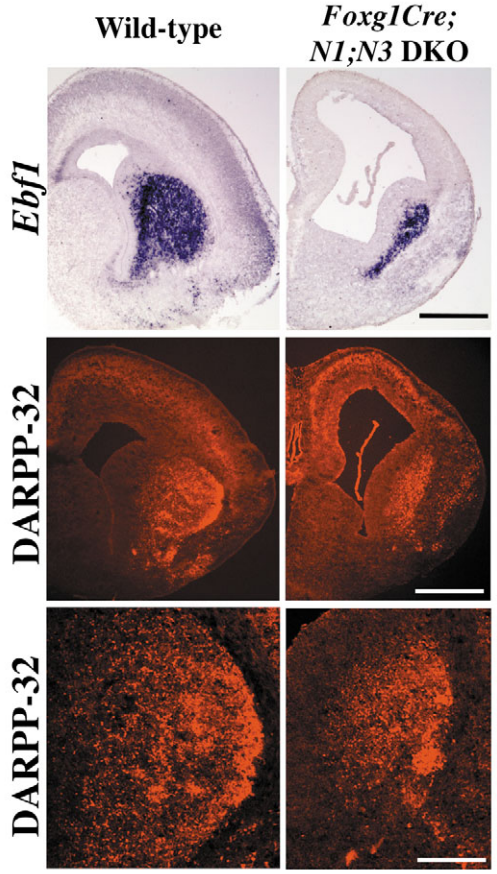

Fig. 6. The entire striatum is severely compromised in *Foxg1Cre; Notch1* conditional; *Notch3* null double mutants (*Foxg1Cre; N1; N3* DKOs). As visualized using in situ hybridization for *Ebf1* in E17.5 coronal sections of the striatum, only a small region of the matrix compartment develops in the absence of both *Notch1* and *Notch3*. Scale bar: 500 μm (upper right panel). Similarly, both the SCS and patch regions, immunostained here with antibodies to Darpp32, are substantially reduced in size and are severely disorganized in the double knockouts at E17.5. Scale bar: 500 μm. As seen in the lower panels, which show a higher magnification view than the middle panels, the SCS is disrupted and no characteristic clusters of Darpp32-positive cells are observed in *Foxg1Cre; N1; N3* DKOs. Scale bar: 250 μm.

Examination using *Ebf1*, a striatal matrix marker, revealed that this region is severely compromised in the double knockouts when compared with wild-type littermates at E17.5 (Fig. 6, upper panels). This data suggests that *Notch3* compensates for the loss of *Notch1* in our previous single conditional knockout analysis. Furthermore, these results strongly suggest that *Notch3* activity can functionally replace *Notch1* activity to regulate proper matrix development. Because *Notch3* compensates for the loss of *Notch1* in the matrix neurons of the striatum, we tested whether *Notch3* also plays a role in regulating the development of the SCS and patch neurons. Indeed, the striatal SCS and patches display severe defects in the *Foxg1*^{Cre}; *N1; N3* DKOs as visualized by Darpp32 immunostaining (Fig. 6, middle and lower panels). The SCS of *Foxg1*^{Cre}; *N1; N3* DKOs shows abnormal aggregates and is not a smooth crescent shape as it is in wild-type mice or even in *Notch1* single conditional mutants (Fig. 6, middle and lower panels, and Figs 3-5). In addition, no clusters of Darpp32 immunoreactivity, characterizing the patch compartment, are observed within the striatum of the double mutants, and only scattered Darpp32-positive cells are found within this region (Fig. 6, lower panels). Thus, the striatal development of *Foxg1*^{Cre}; *N1; N3* DKOs is significantly impaired and all compartments (SCS, patch and matrix) are disrupted. Unfortunately, at present, no markers exist to distinguish between patch and SCS cells. It is therefore unclear whether the loss of striatal patches in *Foxg1*^{Cre}; *N1; N3* DKOs is a result of a change in cell fate or simply of a severe disorganization of the patch, SCS and matrix compartments.

Notch1 and *Notch3* act within the VZ to regulate the distinct cell types that form the compartments of the striatum

The defects observed in the *Foxg1*^{Cre}; *N1* cKOs and the *Foxg1*^{Cre}; *N1; N3* DKOs could result either from the lack of Notch signals in progenitor cells in the VZ, or during a later developmental stage, such as migration and differentiation, as *Foxg1*^{Cre} results in the permanent removal of *Notch1* throughout the telencephalon. Notch signaling has been reported to function in postmitotic neurons, such as in controlling neurite morphology (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). In addition, Presenilin 1, a membrane protein responsible for the cleavage activation of the Notch receptor (De Strooper et al., 1999; Struhl and Greenwald, 1999) has been shown to play important roles in neuronal migration (Louvi et al., 2004). These findings raise the possibility that the striatal disorganization observed in our *Foxg1*^{Cre} single and double mutants results from the loss of Notch signaling during the migration and differentiation of neurons in the striatum.

To test this idea, we selectively removed Notch function after cells exit the VZ using *Dlx5/6-Cre-IRES-EGFP* transgenic mice (*Dlx5/6*^{Cre}), in which Cre recombinase is absent from the striatal VZ and is expressed only when cells transit into the subventricular zone (SVZ) and underlying mantle (Stenman et al., 2003) (Fig. 7A). When the *Dlx5/6*^{Cre} line is crossed to the Z/EG recombination reporter line (Novak et al., 2000), EGFP permanently marks cells that

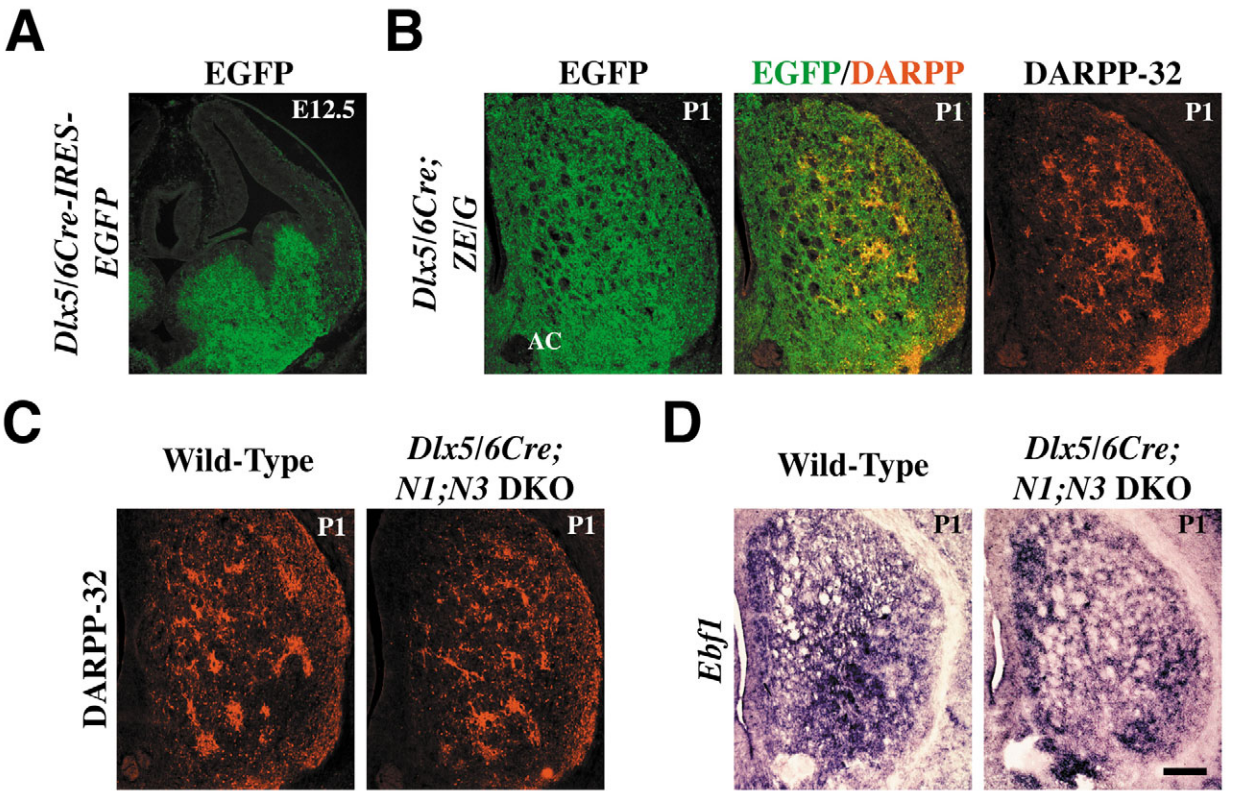

Fig. 7. *Notch1* and *Notch3* are not required for normal striatal development after cells exit the VZ. (A) A coronal section of an E12.5 *Dlx5/6Cre-IRES-EGFP* forebrain indicates that the *Dlx5/6* enhancer directs the expression of EGFP throughout the ventral SVZ and mantle, but not in the VZ. (B*) Dlx5/6Cre* induces the recombination of floxed alleles throughout the striatum, including the SCS, patch and matrix compartments. Shown here is in a coromal section of a P1 striatum from a cross between the *Dlx5/6Cre-IRES-EGFP* transgenic and the Z/EG reporter mouse. Cells that have undergone Cre-mediated recombination permanently express EGFP (shown here in green). EGFP is expressed in all regions of the striatum at P1 in progeny from *Dlx5/6Cre* and Z/EG reporter mice. Antibodies to Darpp32 mark the SCS and patches (shown here in red). The EGFP-negative regions are fiber tracts passing through the striatum, with the anterior commissure (AC) visible in the lower left corner. (C) The SCS and patch compartments develop normally in *Dlx5/6Cre; N1; N3* DKOs, as seen here with antibodies to Darpp32 at P1. (D) The matrix compartment is equivalent in both *Dlx5/6Cre; N1; N3* DKOs and wild-type littermates at P1. Scale bar: 250 μm.

have undergone Cre-mediated recombination during their development. This fate-mapping experiment reveals that the entire striatum, including both patch and matrix compartments, has undergone recombination by postnatal day 1 (P1) in Z/EG; *Dlx5/6*^{Cre} mice (Fig. 7B). Therefore, the *Dlx5/6*^{Cre} transgenic mice will facilitate the removal of floxed *Notch1* in all neurons in the striatum but only after they exit the VZ. Because we have shown that *Notch3* functionally compensates for the loss of *Notch1* (Fig. 6), we generated *Dlx5/6*^{Cre} conditional mutants on the *Notch3* null background (*Dlx5/6*^{Cre}; *N1; N3* DKOs). Unlike *Foxg1*^{Cre}; *N1* mutants, *Dlx5/6*^{Cre}; *N1; N3* DKOs survive into adulthood. When we examined patch and matrix development in *Dlx5/6*^{Cre}; *N1; N3* DKOs at P1 (Fig. 7C-D) or in adults (data not shown), the mutant striatum appeared to be indistinguishable from the wild-type striatum. The patch marker Darpp32 (Fig. 7C) and the matrix marker *Ebf1* (Fig. 7D) both show normal patterns of expression in the *Dlx5/6*^{Cre} double knockouts at P1. Thus, activity through Notch receptors 1 and 3 is not critical once cells are in the SVZ and mantle. Taken together, these results suggest that Notch signaling is essential when cells are in the VZ to regulate the distinct neuronal cell types found in the striatum. Once cells progress to the SVZ and mantle, Notch signaling is not required for the subsequent stages of maturation and morphogenesis that will ultimately form the mature striatum.

Discussion

To test the role of Notch in regulating striatal development, we used genetic strategies to study the consequences of the loss of Notch signaling at different stages of the development of this structure. Surprisingly, we found that only early-born, not late-born, neurons are affected in the striatum of *Foxg1*^{Cre}; *N1* cKOs. More specifically, the earliest born subcallosal streak cells are increased, whereas the remaining patch neurons are correspondingly decreased. The late-born matrix neurons in the striatum of *Foxg1*^{Cre}; *N1* cKOs develop normally. Thus, *Notch1* is essential for the proper development of the early-born neurons in the striatum and is dispensable for the formation of the late-born populations.

We show that *Notch3* gene function underlies the production of late-born matrix neurons in *Foxg1*^{Cre}; *N1* cKOs, as these cell types are severely impaired in *Foxg1*^{Cre}; *N1; N3* DKOs. In addition, the generation of patch neurons is also further compromised, revealing that *Notch3* also plays a role early in neurogenesis in the absence of *Notch1*. These results raise two

Development

interesting points. First, they show that *Notch3* is capable of functioning in place of *Notch1* to regulate stritatal neurogenesis. Second, the defects observed in the early-born cell types in the *Foxg1^{Cre}*; *N1* cKOs suggest that *Notch3* cannot perfectly replace the activity of *Notch1*, a point that will be discussed in more detail below. We interpret these results as indicating that the loss of *Notch1* alone results in an early temporal window of severely compromised Notch signaling that in turn leads to specific defects in the patch compartment. However, by E14.5 the overall forebrain morphology and birthdating data suggests that neurogenesis is occurring normally in the absence of *Notch1*. This contrasts sharply with other regions of the developing CNS, such as the cerebellum and the eye, in which *Notch1* removal alone results in severe, progressive and permanent defects (Lutolf et al., 2002) (Fig. 2).

Unlike later phases of neurogenesis, *Notch3* alone is insufficient for the normal development of patch neurons in the absence of *Notch1*. One simple explanation may be that the levels of *Notch3* are too low early in neurogenesis to provide effective Notch signaling. Although it remains a possibility that the selective expression of *Notch3* in matrix progenitors and not in patch progenitors underlies this difference, the expression of *Notch3* appears uniform in VZ progenitors. We therefore favor a model in which progenitor cells giving rise to early- and late-born neurons arise from distinct progenitor pools that require Notch signaling at sequential times during development. One intriguing possibility is that the mode of cell division is linked to the requirement of a progenitor cell for Notch signaling. According to our data, the progenitors that are likely to be dependent on Notch signaling are the ones in a neurogenic mode of division at the time *Notch1* is removed in *Foxg1^{Cre}*; *N1* cKOs, which are the progenitors that are producing patch neurons. Progenitors that give rise to late-born neurons appear to be in a Notch-independent mode of division, most likely undergoing self-renewing divisions that produce additional progenitor cells rather than post-mitotic neurons. The progenitors that give rise to late-born neurons ultimately become dependent on Notch signaling to regulate their differentiation (most likely when they initiate neurogenic divisions) because matrix development is severely impaired in *Foxg1^{Cre}*; *N1; N3* DKOs (Fig. 6). This model is consistent with the evidence that *Foxg1^{Cre}*; *N1* cKOs do not display any obvious defects during the initial phases of neural development (between E9.5 and E10.5; Fig. 1B), a period characterized primarily by symmetric cell divisions that amplify the progenitor population rather than neurogenic divisions. A growing number of genes, including *Numb* and *lethal giant larvae 1* (*Lgl1*) also appear to be required at the onset of neurogenesis (Klezovitch et al., 2004; Li et al., 2003; Petersen et al., 2002; Petersen et al., 2004; Shen et al., 2002). These genes may function to promote asymmetric divisions through interactions with the Notch pathway.

The selective effect of *Notch1* on early-born cells in the striatum in *Foxg1^{Cre}*; *N1* cKOs, in conjunction with previous studies that demonstrated that Notch activity prevents differentiation and maintains a progenitor state (Hitoshi et al., 2002; Ohtsuka et al., 2001), suggested to us that Notch signaling is critical in neural progenitors that reside in the VZ. However, *Notch1* and *Notch3* gene function in *Foxg1^{Cre}*; *N1; N3* DKOs is also absent during all subsequent development stages, including neuronal migration and differentiation. It is therefore impossible to know from this analysis whether Notch signaling is used iteratively for a variety of developmental steps. To address the potential role of Notch signaling in later stages of neuronal development, we used the *Dlx5/6^{Cre}* driver line to remove Notch signaling after cells have exited the VZ. In *Dlx5/6^{Cre}*; *N1; N3* DKOs, both the patch and matrix compartments develop normally (Fig. 7). These results suggest that Notch signaling is not required for proper striatal patterning once cells have exited the VZ. Recent reports have suggested that Notch activity is important for regulating neurite morphology in postmitic neurons in the cortex (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). The present study did not examine axonal or dendrite morphology although it will be interesting to address this question in future studies. The neuronal migration defects in *presenilin 1* mutants raised the possibility that Notch signaling might be involved in migration, as Notch receptors require cleavage by presenilins to be activated. However, our data supports the idea the presenilin 1 exerts its effects on migration by acting on other proteins, such as cytoskeletal proteins (Louvi et al., 2004). Therefore, *Notch1* and *Notch3* are not necessary for the subsequent phases of neuronal development that ultimately form the characteristic striatal mosaic, such as neuronal migration, the segregating of SCS, patch and matrix neurons, and their ultimate differentiation and expression of specific cellular and molecular markers. In these mutants, it remains possible that *Notch2* could be compensating for the absence of *Notch1* and *Notch3*. However, several observations do not support this possibility. First, the fact that the morphology of the *Foxg1^{Cre}*; *N1; N3* DKOs is severely compromised (Fig. 6) suggests that *Notch2* activity is not sufficient to mediate normal striatal development. Second, we do not observe *Notch2* upregulation in either *Foxg1^{Cre}* or *Dlx5/6^{Cre}* double knockouts (data not shown). Third, we see no indication of *Notch2* expression outside of the VZ at any time-point (data not shown).

Apart from the role of Notch in regulating neurogenesis in the VZ, the only other developmental process we found to be affected when Notch signaling was removed later (in neurons after they exited the ventricular zone) was for cell survival. Specifically, we observed elevated levels of cell death in both *Foxg1^{Cre}* (Mason et al., 2005) and *Dlx5/6^{Cre}* double knockouts (data not shown). These results suggest that Notch signaling plays a generalized role in cell survival, and that in the absence of *Notch1* and *Notch3*, cells have a higher probability of undergoing programmed cell death during embryonic development. However, this increased apoptosis appears to affect all types of neurons equally, as we could not find a selective effect on either the patch or the matrix neurons in either of our conditional knockouts.

In conclusion, our data indicates that early-born neuronal fates are selectively altered in the striatum of *Foxg1^{Cre}*; *N1* cKOs, whereas later born cell types are generated normally. Ectopic innervation of the cortex from midbrain dopaminergic fibers is observed in these mutants, most likely as a consequence of this defect. We further show that *Notch3* can compensate for the loss of *Notch1* in the generation of late-born matrix neurons in the striatum. Finally, we demonstrate that both the patch and matrix compartments develop normally when *Notch1* and *Notch3* are removed after cells have exited

the VZ. The results pinpoint the critical window of Notch activity in progenitor cells in the VZ, and suggest that neurons can migrate and differentiate in the absence of additional Notch signaling.

We thank the Fishell lab members for invaluable discussion and the critical reading of this manuscript; Richard Christie for assistance with the data analyses; Freddy Radtke for the gift of the floxed *Notch1* mice; Jean Hebert and Susan McConnell for the gift of the *Foxg1*$^{Cre/+}$ mice; Kenny Campbell for generously providing the *Dlx5/6Cre-IRES-EGFP* mice; Janet Rossant for the *Notch1* RNA in situ probe; Domingos Henrique for the *Notch2* and *Notch3* RNA in situ probes; Ryoichiro Kageyama for the *Hes1, 5* RNA in situ probes; Sonia Garel for the *Ebf1* RNA in situ probe; Jane Johnson for the *Mash1* RNA in situ probe; David Anderson for the *Neurod* RNA in situ probe; and Larry Kromer for advice on ephrin A4/Fc labeling. H.A.M. is supported by an institutional postdoctoral training grant from the NIH (NS 07457-03). T.G. is supported by NINDS (NS036437). G.F. is supported by NIMH (5 R01 MH068469-02) and NYS Spinal Cord Injury Research Program (C017684).

References

Angevine, J. B. J. and Sidman, R. L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* **192**, 766-768.

Anthony, T. E., Klein, C., Fishell, G. and Heintz, N. (2004). Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron* **41**, 881-890.

Anthony, T. E., Mason, H. A., Fishell, G. and Heintz, N. (2005). Brain lipid-binding protein is a direct target of Notch signaling in radial glial cells. *Genes Dev.* **19**, 1028-1033.

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendahl, U. and Edlund, H. (1999). Notch signalling controls pancreatic cell differentiation. *Nature* **400**, 877-881.

Beatus, P., Lundkvist, J., Oberg, C. and Lendahl, U. (1999). The notch 3 intracellular domain represses notch 1-mediated activation through Hairy/Enhancer of split (HES) promoters. *Development* **126**, 3925-3935.

Beatus, P., Lundkvist, J., Oberg, C., Pedersen, K. and Lendahl, U. (2001). The origin of the ankyrin repeat region in Notch intracellular domains is critical for regulation of HES promoter activity. *Mech. Dev.* **104**, 3-20.

Beckstead, R. M. and Kersey, K. S. (1985). Immunohistochemical demonstration of differential substance P-, met-enkephalin-, and glutamic-acid-decarboxylase-containing cell body and axon distributions in the corpus striatum of the cat. *J. Comp. Neurol.* **232**, 481-498.

Berezovska, O., McLean, P., Knowles, R., Frosh, M., Lu, F. M., Lux, S. E. and Hyman, B. T. (1999). Notch1 inhibits neurite outgrowth in postmitotic primary neurons. *Neuroscience* **93**, 433-439.

Cai, L., Hayes, N. L., Takahashi, T., Caviness, V. S., Jr and Nowakowski, R. S. (2002). Size distribution of retrovirally marked lineages matches prediction from population measurements of cell cycle behavior. *J. Neurosci. Res.* **69**, 731-744.

Casarosa, S., Fode, C. and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. *Development* **126**, 525-534.

Conlon, R. A., Reaume, A. G. and Rossant, J. (1995). Notch1 is required for the coordinate segmentation of somites. *Development* **121**, 1533-1545.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. et al. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-1148.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J. et al. (1999). A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518-522.

Domenga, V., Fardoux, P., Lacombe, P., Monet, M., Maciazek, J., Krebs, L. T., Klonjkowski, B., Berrou, E., Mericskay, M., Li, Z. et al. (2004). Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Genes Dev.* **18**, 2730-2735.

Foster, G. A., Schultzberg, M., Hokfelt, T., Goldstein, M., Hemmings, H. C., Jr, Ouimet, C. C., Walaas, S. I. and Greengard, P. (1987). Development of a dopamine- and cyclic adenosine 3′:5′-monophosphate-regulated phosphoprotein (DARPP-32) in the prenatal rat central nervous system, and its relationship to the arrival of presumptive dopaminergic innervation. *J. Neurosci.* **7**, 1994-2018.

Franklin, J. L., Berechid, B. E., Cutting, F. B., Presente, A., Chambers, C. B., Foltz, D. R., Ferreira, A. and Nye, J. S. (1999). Autonomous and non-autonomous regulation of mammalian neurite development by Notch1 and Delta1. *Curr. Biol.* **9**, 1448-1457.

Frantz, G. D. and McConnell, S. K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. *Neuron* **17**, 55-61.

Fuccillo, M., Rallu, M., McMahon, A. P. and Fishell, G. (2004). Temporal requirement for hedgehog signaling in ventral telencephalic patterning. *Development* **131**, 5031-5040.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M. and Cepko, C. L. (2000). rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. *Neuron* **26**, 383-394.

Gaiano, N., Nye, J. S. and Fishell, G. (2000). Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* **26**, 395-404.

Garel, S., Marin, F., Grosschedl, R. and Charnay, P. (1999). Ebf1 controls early cell differentiation in the embryonic striatum. *Development* **126**, 5285-5294.

Gerfen, C. R. (1984). The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems. *Nature* **311**, 461-464.

Gerfen, C. R. (1992). The neostriatal mosaic: multiple levels of compartmental organization. *Trends Neurosci.* **15**, 133-139.

Graybiel, A. M. (1984). Correspondence between the dopamine islands and striosomes of the mammalian striatum. *Neuroscience* **13**, 1157-1187.

Graybiel, A. M. and Chesselet, M. F. (1984). Compartmental distribution of striatal cell bodies expressing [Met]enkephalin-like immunoreactivity. *Proc. Natl. Acad. Sci. USA* **81**, 7980-7984.

Graybiel, A. M., Ragsdale, C. W., Jr, Yoneoka, E. S. and Elde, R. P. (1981). An immunohistochemical study of enkephalins and other neuropeptides in the striatum of the cat with evidence that the opiate peptides are arranged to form mosaic patterns in register with the striosomal compartments visible by acetylcholinesterase staining. *Neuroscience* **6**, 377-397.

Halliday, A. L. and Cepko, C. L. (1992). Generation and migration of cells in the developing striatum. *Neuron* **9**, 15-26.

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.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D. and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. *Curr. Biol.* **7**, 661-670.

Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A. J., Nye, J. S., Conlon, R. A., Mak, T. W., Bernstein, A. and van der Kooy, D. (2002). Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes Dev.* **16**, 846-858.

Ishibashi, M., Ang, S. L., Shiota, K., Nakanishi, S., Kageyama, R. and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev.* **9**, 3136-3148.

Janis, L. S., Cassidy, R. M. and Kromer, L. F. (1999). Ephrin-A binding and EphA receptor expression delineate the matrix compartment of the striatum. *J. Neurosci.* **19**, 4962-4971.

Klezovitch, O., Fernandez, T. E., Tapscott, S. J. and Vasioukhin, V. (2004). Loss of cell polarity causes severe brain dysplasia in Lgl1 knockout mice. *Genes Dev.* **18**, 559-571.

Krebs, L. T., Xue, Y., Norton, C. R., Sundberg, J. P., Beatus, P., Lendahl, U., Joutel, A. and Gridley, T. (2003). Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genesis* **37**, 139-143.

Li, H. S., Wang, D., Shen, Q., Schonemann, M. D., Gorski, J. A., Jones, K. R., Temple, S., Jan, L. Y. and Jan, Y. N. (2003). Inactivation of Numb and Numblike in embryonic dorsal forebrain impairs neurogenesis and disrupts cortical morphogenesis. *Neuron* **40**, 1105-1118.

Lindsell, C. E., Boulter, J., diSibio, G., Gossler, A. and Weinmaster, G. (1996). Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. *Mol. Cell Neurosci.* **8**, 14-27.

Livesey, F. J. and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. *Nat. Rev. Neurosci.* **2**, 109-118.

Loizou, L. A. (1972). The postnatal ontogeny of monoamine-containing

neurones in the central nervous system of the albino rat. *Brain Res.*. **40**, 395-418.
Louvi, A., Sisodia, S. S. and Grove, E. A. (2004). Presenilin 1 in migration and morphogenesis in the central nervous system. *Development* **131**, 3093-3105.
Luskin, M. B. and Shatz, C. J. (1985). Neurogenesis of the cat's primary visual cortex. *J. Comp. Neurol.* **242**, 611-631.
Lutolf, S., Radtke, F., Aguet, M., Suter, U. and Taylor, V. (2002). Notch1 is required for neuronal and glial differentiation in the cerebellum. *Development* **129**, 373-385.
Malatesta, P., Hartfuss, E. and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* **127**, 5253-5263.
Mason, H. A., Rakowiecki, S. M., Gridley, T. and Fishell, G. (2005). Loss of Notch activity in the developing central nervous system leads to increased cell death. *Dev. Neurosci.* (in press).
McConnell, S. K. (1995). Strategies for the generation of neuronal diversity in the developing central nervous system. *J. Neurosci.* **15**, 6987-6998.
McConnell, S. K. and Kaznowski, C. E. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* **254**, 282-285.
Mizutani, K. and Saito, T. (2005). Progenitors resume generating neurons after temporary inhibition of neurogenesis by Notch activation in the mammalian cerebral cortex. *Development* **132**, 1295-1304.
Morrison, S. J., Perez, S. E., Qiao, Z., Verdi, J. M., Hicks, C., Weinmaster, G. and Anderson, D. J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. *Cell* **101**, 499-510.
Murrin, L. C. and Ferrer, J. R. (1984). Ontogeny of the rat striatum: correspondence of dopamine terminals, opiate receptors and acetylcholinesterase. *Neurosci. Lett.* **47**, 155-160.
Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S. and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714-720.
Noctor, S. C., Martinez-Cerdeno, V., Ivic, L. and Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* **7**, 136-144.
Novak, A., Guo, C., Yang, W., Nagy, A. and Lobe, C. G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* **28**, 147-155.
Ohtsuka, T., Sakamoto, M., Guillemot, F. and Kageyama, R. (2001). Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain. *J. Biol. Chem.* **276**, 30467-30474.
Oishi, K., Kamakura, S., Isazawa, Y., Yoshimatsu, T., Kuida, K., Nakafuku, M., Masuyama, N. and Gotoh, Y. (2004). Notch promotes survival of neural precursor cells via mechanisms distinct from those regulating neurogenesis. *Dev. Biol.* **276**, 172-184.
Olson, L., Seiger, A. and Fuxe, K. (1972). Heterogeneity of striatal and limbic dopamine innervation: highly fluorescent islands in developing and adult rats. *Brain Res.* **44**, 283-288.
Olsson, M., Bjorklund, A. and Campbell, K. (1998). Early specification of striatal projection neurons and interneuronal subtypes in the lateral and medial ganglionic eminence. *Neuroscience* **84**, 867-876.
Petersen, P. H., Zou, K., Hwang, J. K., Jan, Y. N. and Zhong, W. (2002). Progenitor cell maintenance requires numb and numblike during mouse neurogenesis. *Nature* **419**, 929-934.
Petersen, P. H., Zou, K., Krauss, S. and Zhong, W. (2004). Continuing role for mouse Numb and Numbl in maintaining progenitor cells during cortical neurogenesis. *Nat. Neurosci.* **25**, 25.
Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R. and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547-558.
Ragsdale, C. W., Jr and Graybiel, A. M. (1990). A simple ordering of neocortical areas established by the compartmental organization of their striatal projections. *Proc. Natl. Acad. Sci. USA* **87**, 6196-6199.
Rakic, P. (1974). Neurons in rhesus monkey visual cortex: systematic relation between time of origin and eventual disposition. *Science* **183**, 425-427.
Redmond, L., Oh, S. R., Hicks, C., Weinmaster, G. and Ghosh, A. (2000). Nuclear Notch1 signaling and the regulation of dendritic development. *Nat. Neurosci.* **3**, 30-40.
Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev.* **6**, 2620-2634.
Sauer, B. and Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. USA* **85**, 5166-5170.
Schaeren-Wiemers, N. and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* **100**, 431-440.
Schuurmans, C. and Guillemot, F. (2002). Molecular mechanisms underlying cell fate specification in the developing telencephalon. *Curr. Opin. Neurobiol.* **12**, 26-34.
Sestan, N., Artavanis-Tsakonas, S. and Rakic, P. (1999). Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science* **286**, 741-746.
Shen, Q., Zhong, W., Jan, Y. N. and Temple, S. (2002). Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. *Development* **129**, 4843-4853.
Snyder-Keller, A. and Costantini, L. C. (1996). Glutamate receptor subtypes localize to patches in the developing striatum. *Brain Res. Dev. Brain Res.* **94**, 246-250.
Song, D. D. and Harlan, R. E. (1994). Genesis and migration patterns of neurons forming the patch and matrix compartments of the rat striatum. *Brain Res. Dev. Brain Res.* **83**, 233-245.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Stenman, J., Toresson, H. and Campbell, K. (2003). Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. *J. Neurosci.* **23**, 167-174.
Struhl, G. and Greenwald, I. (1999). Presenilin is required for activity and nuclear access of Notch in Drosophila. *Nature* **398**, 522-525.
Swiatek, P. J., Lindsell, C. E., del Amo, F. F., Weinmaster, G. and Gridley, T. (1994). Notch1 is essential for postimplantation development in mice. *Genes Dev.* **8**, 707-719.
Takahashi, T., Nowakowski, R. S. and Caviness, V. S., Jr (1996). The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neuronogenesis. *J. Neurosci.* **16**, 6183-6196.
Takebayashi, K., Akazawa, C., Nakanishi, S. and Kageyama, R. (1995). Structure and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-5. Identification of the neural precursor cell-specific promoter element. *J. Biol. Chem.* **270**, 1342-1349.
van der Kooy, D. and Fishell, G. (1987). Neuronal birthdate underlies the development of striatal compartments. *Brain Res.* **401**, 155-161.
Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G. and Barres, B. A. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* **21**, 63-75.
Wichterle, H., Turnbull, D. H., Nery, S., Fishell, G. and Alvarez-Buylla, A. (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development* **128**, 3759-3771.
Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**, 361-373.
Yoon, K. and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat. Neurosci.* **8**, 709-715.
Yoon, K., Nery, S., Rutlin, M. L., Radtke, F., Fishell, G. and Gaiano, N. (2004). Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. *J. Neurosci.* **24**, 9497-9506.

Development