Notch signaling coordinates the patterning of striatal compartments

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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 corticostral 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...
(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 (Domena 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-Jk (an intracellular mediator of signaling through all Notch receptors) null mutants show signs of precocious neuronal differentiation, although RBP-Jk 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 Foxg1Cre/+. The generation of Foxg1Cre/+ mice was previously published (Hebert and McConnell, 2000) and Foxg1Cre/+ heterozygous mice were maintained on a Swiss Webster background. Dlx5/6-Cre-IRE-EGFP mice were previously described (Steenman 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 Foxg1Cre/+ (or Dlx5/6Cre) 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 labeling
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 littersmates 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 (Schaeper-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. Antibodies and immunohistochemistry

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

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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 Laemmli 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, Downingtown, 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 wild-type littersmates were analyzed for each time-point of BrdU administration (E10.5-E15.5). Microsoft Excel was used to analyze the data 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 wild-type littersmates 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 Foxg1Cre/+ 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), Foxg1Cre/+ 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; Notch1loxPloxP (Radtke et al., 1999)) to Notch1Cre/+;Foxg1Cre/+ 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 Foxg1Cre/+; Notch1floxed conditional knockout mice as Foxg1Cre/+; Ni cKOs for simplicity.

Foxg1Cre/+; Ni cKOs survive until birth. Neonates die within several hours and display a smaller forebrain than wild-type littersmates do. To confirm that Notch1 is completely removed as a result of this genetic cross, we examined the Foxg1Cre/+; Ni 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 Foxg1Cre/+; Ni cKOs (Fig. 1B). In addition, Notch1 protein is abundant in forebrain lysates prepared from E12.5 wild-type embryos, but is absent from Foxg1Cre/+; Ni 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 not.

**Fig. 1.** Generation of a telencephalic-specific deletion of Notch1. (A) Coronal sections through the embryonic telencephalon of Foxg1Cre/+; 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/+; Ni 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/+; Ni cKOs (Ni cKO), whereas levels of α-tubulin are equivalent between Ni 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.
not give rise to the striatum (Olsson et al., 1998; Wichterle et al., 2001). Thus, Foxg1Cre; 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 Foxg1Cre; 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 Foxg1Cre; N1 cKOs at E12.5. In Foxg1Cre; N1 cKOs, the MGE and CGE (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 Foxg1Cre; 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 Foxg1Cre; 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 Foxg1Cre; 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 Foxg1Cre; N1 cKOs, whereas Hes5 is virtually undetectable (Fig. 2A, insets, left and middle panels). The persistence of Hes5 expression 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 as 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 Foxg1Cre; N1 cKOs, raising the possibility that Hes1 could be regulated by Notch-

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

To determine the consequences of abnormal neurogenesis at E12.5 in Foxg1Cre; 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 substantia 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 Foxg1Cre; 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 receptor 1 (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 substantia 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 Foxg1Cre; 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 Foxg1Cre; 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, Foxg1Cre; 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 Foxg1Cre; N1 cKOs by 2 days when compared with wild-type embryos (data not shown). Much to our surprise, Foxg1Cre; 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.

The striatal matrix develops normally in the absence of Notch1

As morphological defects in the Foxg1Cre; 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 Foxg1Cre; 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 Foxg1Cre; N1 cKOs, the matrix appears to develop normally in the absence of Notch1. Because the Foxg1Cre; N1 cKOs used to produce the Notch1 conditional knockouts lack one copy of Foxg1, it is possible that the phenotype we observed in Foxg1Cre; 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 Foxg1Cre; N1 cKOs mice, we found that the striatum forms normally (data not shown), confirming that the striatal phenotype in Foxg1Cre; 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 Foxg1Cre; 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 Foxg1Cre; N1 cKOs when compared with wild-type littermates from E12.5 through E16.5, as is the number of TUNEL-

![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.](image-url)
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 progenitor cells 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 Foxg1Cre; N1 cKOs compared with in wild-type littersates. Three Foxg1Cre; N1 cKOs and three wild-type embryos were analyzed for each time-point. 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.

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 Foxg1Cre; 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 (Foxg1Cre; N1; N3 DKO). These double knockouts were generated by crossing N3 null mutants, which are viable and fertile (Krebs et al., 2003), onto the Foxg1Cre; N1 cKO background.

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 Foxg1Cre; N1; N3 DKO as visualized by Darp32 immunostaining (Fig. 6, middle and lower panels). The SCS of Foxg1Cre; N1; N3 DKO 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 Darp32 immunostaining, characterizing the patch compartment, are observed within the striatum of the double mutants, and only scattered Darp32-positive cells are found within this region (Fig. 6, lower panels). Thus, the striatal development of Foxg1Cre; N1; N3 DKO 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 Foxg1Cre; N1; N3 DKO 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 Foxg1Cre; N1 cKO and the Foxg1Cre; N1; N3 DKO 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 Foxg1Cre 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 Foxg1Cre 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 (Dlx5/6Cre), 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 Dlx5/6Cre line is crossed to the Z/EGER recombinator line (Novak et al., 2000), EGFP permanently marks cells that...
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; Dlx5/6Cre transgenic mice (Fig. 7B). Therefore, the Dlx5/6Cre 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 Dlx5/6Cre conditional mutants on the Notch3 null background (Dlx5/6Cre; N1; N3 DKO). Unlike Foxg1Cre; N1 mutants, Dlx5/6Cre; N1; N3 DKO survive into adulthood. When we examined patch and matrix development in Dlx5/6Cre; N1; N3 DKO 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 Darp32 (Fig. 7C) and the matrix marker Ebf1 (Fig. 7D) both show normal patterns of expression in the Dlx5/6Cre 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 Foxg1Cre; 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 Foxg1Cre; 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 Foxg1Cre; N1 cKOS, as these cell types are severely impaired in Foxg1Cre; N1; N3 DKO. 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
interesting points. First, they show that Notch3 is capable of functioning in place of Notch1 to regulate striatal neurogenesis. Second, the defects observed in the early-born cell types in the Foxg1Cre; 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 Foxg1Cre; 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 Foxg1Cre; N1; N3 DKOs (Fig. 6). This model is consistent with the evidence that Foxg1Cre; 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 Foxg1Cre; 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 Foxg1Cre; 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 Dlx5/6Cre driver line to remove Notch signaling after cells have exited the VZ. In Dlx5/6Cre; 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 postmitotic 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 Foxg1Cre; 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 Foxg1Cre or Dlx5/6Cre 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 Foxg1Cre (Mason et al., 2005) and Dlx5/6Cre 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 Foxg1Cre; 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
Notch activity regulates striatal patterning

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; Freedly 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-IRESF/EGFP mice; Domingos Henrique for the *Notch2* and *Notch3* RNA in situ probes; Ryoichiro Kageyama for the *Hexl* 5 RNA in situ probe; Sonia Garel for the *Ebf1* RNA in situ probe; Jane Johnson for the *Ebf1* RNA in situ probe; Sonia Garel for the *Ebf1* RNA in situ probe; and Larry Kromer for advice on ephrin A4Fc 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 an institutional postdoctoral training grant from the NIH (NS 07457-03) and NYS Spinal Cord Injury Research Program (CO17684).

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