Regulation of differentiation flux by Notch signalling influences the number of dopaminergic neurons in the adult brain

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ABSTRACT

Notch signalling is a well-established pathway that regulates neurogenesis. However, little is known about the role of Notch signalling in specific neuronal differentiation. Using Dll1 null mice, we found that Notch signalling has no function in the specification of mesencephalic dopaminergic neural precursor cells (NPCs), but plays an important role in regulating their expansion and differentiation into neurons. Premature neuronal differentiation was observed in mesencephalon of Dll1-deficient mice or after treatment with a Notch signalling inhibitor. Coupling between neurogenesis and dopaminergic differentiation was indicated from the coincident emergence of neuronal and dopaminergic markers. Early in differentiation, decreasing Notch signalling caused a reduction in NPCs and an increase in dopaminergic neurons in association with dynamic changes in the proportion of sequentially-linked dopaminergic NPCs (Msx1/2+, Ngn2+, Nurr1+) that were particularly evident in the substantia nigra pars compacta. Our results are in agreement with a mathematical model based on a Dll1-mediated regulatory feedback loop between early progenitors and their dividing precursors that controls the emergence and number of dopaminergic neurons.

KEY WORDS: Delta-like 1, Notch, Neurogenesis, Dopaminergic neurons

INTRODUCTION

At the cellular level, early embryogenesis involves stem and progenitor cell proliferation followed by their exit from the cell cycle and concurrent differentiation into specific cell types. In this context, the size and shape of the nervous system largely depend on the number of times that a neural stem cell (NSC) or its progeny re-enters the cell cycle. Importantly, timing of cell differentiation may also influence cell fate decision, given the fact that NSCs appear to change their potential over time throughout development (Bassett and Wallace, 2012; Okano and Temple, 2009). In particular, it has been shown that specific cortical neurons arise at a different developmental time, suggesting that specification is associated with the time of birth (Okano and Temple, 2009; Shen et al., 2006). Nonetheless, it is still unclear how cell differentiation timing influences cell fate choice and the histogenesis of specific brain regions.

The evolutionarily conserved Notch signalling pathway mediates cell-cell interactions that regulate the process of differentiation of neighbouring cells, providing a mechanism for consistent cell fate determination and patterning in time and space of highly organized tissues (Loui and Artavanis-Takas, 2006; Pierfelice et al., 2011). Notch is a family of transmembrane receptors that are activated by transmembrane ligands such as Delta-like (Dll1, Dll3 and Dll4) and Jagged (Jag1 and Jag2) in mammals. Upon activation, the Notch intracellular domain (NICD) is released and translocated to the nucleus, where it forms a complex with the DNA-binding protein RBPj (Pierfelice et al., 2011). In the CNS of mammals, the NICD-RBPj complex induces the expression of Hes1 and Hes5, genes encoding basic helix-loop-helix transcription factors that, in turn, can repress the expression of pro-neural genes (including Notch ligand genes), thereby inhibiting neuronal differentiation and maintaining the pool of neural precursor cells (NPC) (Ohtsuka et al., 1999). Blocking this pathway at different levels causes premature differentiation of NPC resulting in reduction in the number and spectrum of neuron types (Hatakeyama et al., 2004; Louvi and Artavanis-Takas, 2006; Ohtsuka et al., 1999). Thus, Notch signalling appears to be an essential component of the mechanisms that lead to the production of the neuronal diversity characteristic of the brain starting from apparently equivalent NSCs.

Despite the above, very little is known about the role of Notch signalling in the generation of specific brain regions and/or neuron types. In the developing midbrain, Notch1, 2, 3, Dll1 and Jag1 are expressed in the ventricular zone (Lindsell et al., 1996). Notch1 and Notch2 in rodents are essential genes (Conlon et al., 1995; Swiatek et al., 1994) but, while Notch2 appears mainly involved in diencephalon and mesencephalon roof plate development (Kadokawa and Marunouchi, 2002), conditional Notch1 deletion along the midbrain-hindbrain region results in the premature onset of neurogenesis (Lütolf et al., 2002). Dll1 and Dll4 are also essential genes in early mouse development (Duarte et al., 2004; Habé de Angelis et al., 1997); Dll1, in addition to be transiently expressed during gastrulation and early organogenesis, during CNS development is expressed in most of the neural tube (Betterhausen et al., 1995). Interestingly, in contrast with other brain regions, the mesencephalic floor plate (FP) expresses Dll1 (Ono et al., 2007), which associates with the peculiar neurogenic activity of this region (Joksimovic et al., 2009; Ono et al., 2007). Gene expression patterns and NPC differentiation potential of cells in the mesencephalic ventral midline (Lin et al., 2009; Ono et al., 2007) as well as fate mapping...
experiments (Kittappa et al., 2007) indicate that mesencephalic dopaminergic neurons originate from precursors within the FP. Therefore, Dll1 may play a role in the positioning, maintenance, and patterning of dopaminergic neurons and their NPCs.

Dopaminergic differentiation is characterized by the sequential expression of genes encoding certain transcription factors (e.g. En2, Otx2, Foxa2, Lmx1a, Msx1, Ngn2, Nurr1, Pitx3), which are downstream targets of extrinsic signals such as Shh, Fgf8 and Wnt1 (for a review see: Abeliovich and Hammond, 2007; Ang, 2006; Guerrero-Flores and Covarrubias, 2011; Hegarty et al., 2013). These transcription factors regulate the transition between different cell populations along the ventricular-alar axis of the developing ventral mesencephalon. Interestingly, Ngn2 and Mash1, recognized as proneural transcription factors, control the expression of Dll1 and, in consequence, also of some genes associated with Notch signalling, such as Hes5 (Castro et al., 2006; Kele et al., 2006).

Regulation of expression of Notch signalling genes has been studied in association with mesencephalic dopaminergic differentiation (Castro et al., 2006; Deng et al., 2011; Kele et al., 2006; Ono et al., 2007); however, in contrast, little is known about how Notch signalling regulates dopaminergic differentiation. In this study, we investigated the function of Notch signalling in the control of dopaminergic neurogenesis and the number of dopaminergic neurons produced.

RESULTS AND DISCUSSION

Dll1 and Hes5 are key mediators of Notch signalling in the mesencephalic dopaminergic niche

Dll1 transcript distribution in the developing mesencephalon has been previously determined by in situ hybridization; however, probably due to the quantitative limitations of this technique, the expression pattern has not been well defined showing scattered distribution with an apparent higher number of positive cells towards the subventricular area (Deng et al., 2011; Kele et al., 2006; Lahti et al., 2011). Here, we estimated Dll1 transcript distribution by in situ determination of lacZ activity in Dll1/lacZ mouse embryos, particularly at the initiation of dopaminergic differentiation. In the mesencephalon of embryonic day (E)10.5 and E11.5 embryos, Dll1 expression occurred mainly in the subventricular area with the highest levels found in the ventral half. Particularly in the dopaminergic niche at E10.5, the floor plate showed a thin layer of lacZ-stained cells just below the ventricular epithelium, which contrast with the pattern in the hindbrain (Fig. 1A). In E11.5 mesencephalons, a wider subventricular area of Dll1 expression was found around the ventral midline, corresponding to the location of intermediate progenitors, (Fig. 1A). At this latter stage, the ventral mesencephalon contained higher mRNA levels of Dll1 than of Dll3 or Dll4, whereas those of Notch1 and Notch2 were similar (Fig. 1B). In order to determine whether Dll1 is responsible for most Notch signalling occurring in the floor plate of mesencephalon in association with dopaminergic neuron differentiation, we compared the expression levels of two Notch effector genes, Hes1 and Hes5, in the ventral mesencephalon of E11.5 embryos lacking Dll1. Both Hes1 and Hes5 were expressed in wild-type samples, but the latter was apparently more than 100-fold more abundant than the former (Fig. 1B). Interestingly, the complete absence of Dll1 levels caused a corresponding near 30-fold reduction in Hes5 expression, whereas Hes1 expression was only partially (about half) affected (Fig. 1C). In agreement with this conclusion, developmental downregulation of Dll1 expression from E11.5 to E15.5 was best correlated with the expression level of Hes5 (Fig. 1B). Therefore, Dll1 and Hes5 are the major upstream and downstream mediators, respectively, of Notch signalling in the developing ventral mesencephalon.

Reduced Notch signalling alters the number of dopaminergic precursor cells without affecting their specification

In order to determine whether Notch signalling has any role in maintaining the organization of the dopaminergic domain in the ventral mesencephalon, we compared the distribution pattern of Lmx1a and Foxa2 in Dll1+/+ and Dll1lacZ/lacZ embryos. We also
determined the distribution pattern of Nkx6.1; the gene encoding this transcription factor is expressed lateral to the Lmx1a expression domain and is repressed in dopaminergic NPCs after proper specification (Andersson et al., 2006b; Nakatani et al., 2010). Specification of dopaminergic NPC occurs between E9-E10 and neuronal differentiation markers start to be detected from E11 with a peak between E12 and E13 (Ang, 2006). Dll1 null mouse embryos die by E12 (Hrabe de Angelis et al., 1997, and our own observations), therefore, we limited the in vivo studies up to E11.5, stage at which most embryos are still alive though abnormalities were evident (Fig. S1). As shown in Fig. 2, the distribution pattern of Lmx1a, Foxa2 and Nkx6.1 is similar in midbrains of Dll1+/+ and Dll1lacZ/lacZ embryos at E10.5 and E11.5; however, at E11.5, although restricted distribution of each protein was still observed, the tissue seemed disorganized and fragile with fewer cells Lmx1a+ and Foxa2+ in mesencephalons of mutant than in those of wild-type embryos (Fig. 2B). Note, that within each specific expression domain, a high proportion of cells contained the corresponding marker (Fig. 2B), indicating that the decrease in number of presumably specified cells in mesencephalons of mice lacking Dll1 is not due to a failure in maintaining the mesencephalic dopaminergic fate. In agreement with these observations, Lmx1a and Foxa2 mRNA levels were similar in ventral mesencephalons of Dll1+/+ and Dll1lacZ/lacZ embryos at E11.5 (Fig. 2C). Interestingly, consistent Lmx1a up-regulation was observed in Dll1+/+ embryos between E13.5 and E15.5 (Fig. 2C). Since Lmx1a mRNA levels increased after the rapid decrease in association with specification and differentiation, this latter effect was likely related to a Lmx1a function in neuronal maturation. Although less conspicuous, Foxa2 expression at E13.5 showed a similar correlation (Fig. 2C; see also observations after Notch signalling inhibition in Fig. S2). Therefore, alterations in Notch signalling do not appear to affect dopaminergic niche specification.

**The poor expansion of dopaminergic NPCs lacking Dll1 correlates with the premature detection of neuronal markers**

There was no evident increase in cell death around the midline in mutant embryos at E10.5 and, at E11.5 (Fig. S3A, left panels). In contrast, the thinner neural tube of mesencephalons of E11.5 Dll1 null embryos correlated with a marked reduction in the number of cells that incorporated BrdU in comparison with wild-type mesencephalons at an equivalent developmental stage (Fig. 3A). Interestingly, consistent Lmx1a and Foxa2 mRNA levels were similar in ventral mesencephalons of Dll1+/+ and Dll1lacZ/lacZ embryos at E10.5 but was altered by E11.5 (Fig. 3A). Interestingly, the abundance and distribution of the immature neuronal marker βIII-tubulin suggest that neurogenesis is at a more advance stage in the mesencephalons of Dll1lacZ/lacZ embryos since E10.5, and became more evident by E11.5 in comparison with wild-type mesencephalons at an equivalent developmental stage (Fig. 3A). Marked reduction in Nestin+ cells and the extension of those βIII-tubulin+ to the ventricular zone was observed at E11.5 in the ventral region of mesencephalons lacking Dll1 (Fig. 3A). Of note was a pool of Nestin+ cells that were commonly detected around the midline in mutant mice; the identity of these cells remains to be determined (see Concluding remarks). Positive cells for NeuN, a mature neuronal marker, were not detected in the dopaminergic niche, even under the precocious differentiation observed in embryos lacking Dll1 (data not shown and see below). An expression analysis of neurogenic genes in the ventral mesencephalon of Dll1+/+, Dll1lacZ/lacZ and Dll1lacZ/lacZ embryos at E11.5 also supports premature neuronal differentiation with little or no marked alterations in the expression of genes associated with mature neurons (Fig. 3B). Of note was that, with the
exception of Nestin expression, the gene expression levels observed in ventral mesencephalons from Dll1lacZ/lacZ embryos were similar to those in samples from Dll1+/lacZ embryos.

Positive cells for Tyrosine hydroxylase (Th), a limiting enzyme in the synthesis of dopamine and one of the earliest markers of dopaminergic neurons, were detected at about the same time in wild-type and mutant tissues (E11.5), but their distribution in the latter samples resembled a more advanced developmental stage (i.e. E12.5) (Fig. 4A). In addition, cell quantification revealed a significant difference in the proportion of Th+ cells between mutant and control midbrain tissues at E11.5 (Fig. 4B). In agreement with the premature emergence of Th in mutant mice, Th mRNA levels were elevated in ventral mesencephalon of Dll1lacZ/lacZ embryos (Fig. 4C), in close similarity with the increase in βIII-tubulin; a marginal increase was detected in heterozygous embryos. The mRNA levels of Vmat and Dat, markers of mature dopaminergic neurons, did not markedly change between the different genotypes at E11.5, probably because the analysis was done at an early stage of differentiation.

Mesencephalic explant cultures recapitulate the effects of Notch signalling deficiency on dopaminergic neuronal differentiation

It was not unexpected to find that the premature neuronal differentiation described above at E11.5 was not reflected in the expression of genes encoding proteins associated with mature neurons (e.g. NeuN). Since mesencephalic dopaminergic differentiation was not completed before Dll1lacZ/lacZ embryos die, we analysed the differentiation potential of mesencephalic NPCs in culture.
Explant cultures embedded in collagen allow dopaminergic differentiation to a stage resembling the distribution and number of dopaminergic neurons present in the mesencephalon of E14-E15 embryos (Baizabal and Covarrubias, 2009). In concordance with the observations in E11.5 embryos, Dll1lacZ/lacZ mesencephalic explants cultured for 2 or 4 days showed a higher proportion of Th+ cells in comparison with equivalent samples from Dll1+/+ embryos. Most Th+ cells were NeuN+ in 2 days cultures of explants from embryos of either genotype, but fewer have this neuronal marker in 4 days cultures of Dll1lacZ/lacZ explants than of wild type (Fig. 5A). This is reminiscent to the process observed in newly born dopaminergic neurons of embryos at E13.5, which are NeuN+ and lost this marker by E15.5 (see Fig. 7). Therefore, late phases of dopaminergic neurogenesis in the absence of Dll1 can be observed in explant cultures, which showed more advanced differentiation/maturation with respect to that occurring in wild-type explants.

In order to get more insights into the direct role of Notch signalling in dopaminergic differentiation, we cultured
Therefore, Notch signalling inhibition reproduces the neurogenic mRNA levels in E9.5 explants, suggesting that NPC differentiation through Ngn2+ and Nurr1+ NPCs. Note that the increase in that are converted into dopaminergic neurons passing sequentially through the pathway, the mRNA levels determined are in agreement with a lagged that of Nurr1 (see below).

In general, the effect of Notch signalling inhibition on mRNA levels of neuronal and dopaminergic genes was in agreement with the observations in Dll1 deficient embryos (Fig. 5C). The proneurogenic effect of Notch inhibition was more pronounced in samples at E9.5 than at E10.5 (i.e. 4- vs 8-fold at either 2 days or 4 days treatment). Interestingly, there was no proportional decrease in the mRNA levels of Nestin (Fig. 5C), suggesting that a fraction of NPCs are dividing even under Notch signalling inhibition; nonetheless, note that Notch signalling inhibition reduced but did not affect the increasing rate of Nestin mRNA levels in E9.5 explants, suggesting that NPC differentiation but not proliferation is the target of the Notch signalling pathway. Therefore, Notch signalling inhibition reproduces the neurogenic effects of lacking Dll1, confirming that this ligand is the major mediator of Notch functions.

Absence of Notch signalling modifies the flux of dopaminergic differentiation

Msx1 and Ngn2 are transcription factors whose corresponding genes are transiently expressed during dopaminergic differentiation; the former is mostly present in ventricular NPCs and marks the initiation of dopaminergic differentiation (Andersson et al., 2006b), whereas the latter is the proneural factor more important for dopaminergic differentiation that shows higher levels in the intermediate progenitors before Nurr1 expression, a key transcription factor controlling Th expression (Kele et al., 2006). Because differentiating cells can spread to all directions from the site of birth, no direct relationship among the cells within a slice can be expected. Therefore, in order to estimate the transitions among the lineage-related dopaminergic precursors in the course of differentiation, we decided to determine the mRNA levels corresponding to those three transcription factors in ventral mesencephalons of embryos from E9.5 to E15.5 (Fig. 6A). The highest mRNA levels of Mxs1/2 were detected at E11.5, which represented a 30-fold increase in comparison with the level found at E9.5; this level value rapidly decreased to near the limit of detection since E12.5. Significant levels of Ngn2 mRNA were detected at E10.5, became highest at E11.5, and markedly dropped by E15.5. In contrast, Nurr1 mRNA levels increased gradually from E10.5 up to E12.5, at stage at which apparently became stable. Since Mxs1/2, Ngn2 and Nurr1 are expressed mostly in restricted non-overlapping NPCs that are linked along the dopaminergic differentiation pathway, the mRNA levels determined are in agreement with a differentiation flux starting with a definite number of Mxs1+ NPCs that are converted into dopaminergic neurons passing sequentially through Ngn2+ and Nurr1+ NPCs. Note that the increase in Th mRNA levels, representing the young dopaminergic neuroblasts, lagged that of Nurr1 (see below).

The increase in Th mRNA levels and relative number of dopaminergic neurons in the absence of Dll1 suggest that Notch signalling regulates the emergence of dopaminergic features. A marked increase in Ngn2, Nurr1 and Th mRNA levels in mesencephalon of E10.5 embryos lacking Dll1 (Fig. 6A) suggests that the cell populations expressing Ngn2, Nurr1 and Th are prematurely emerging. Interestingly, at E11.5, Mxs2 and Ngn2 mRNA levels in the ventral mesencephalon of Dll1+/lacZ embryos were lower than in the one of wild-type embryos; Mxs1 and Mash1 showed similar regulation as Mxs2 and Ngn2, respectively (Fig. S4). In agreement with the correlation between the proportion of NPCs and mRNA levels, the decrease in Ngn2 mRNA levels correlated with a decrease in the number of Ngn2+ NPCs (Fig. 6B,C). In contrast, Nurr1 and Pitx3, two genes downstream Ngn2 whose expression remains in the emerging Th+ neurons, showed slight differences in their mRNA levels in the presence or absence of Dll1 at E11.5; only Pitx3 mRNA levels were in line with the increase in Th expression but in much lower proportion (Fig. 4). Of note was that the reduction in Dll1 (i.e. to the amount present in Dll1+/lacZ embryos) caused a marked drop in Ngn2 mRNA levels only in embryos at E11.5 but produced similar mRNA levels and expression patterns as wild-type of all other genes tested (Fig. 6A). Therefore, reduction in Dll1 dose promotes dopaminergic differentiation in association with a decrement in the early specific NPCs (i.e. Mxs2+ and Ngn2+).

The above observations suggest that Dll1-Notch signalling is controlling the differentiation flux once dopaminergic NPCs are specified such that, during the process, the effect is mainly noted in the reduction of Ngn2+ transient progenitors but not evident in the proportion of differentiated dopaminergic neuroblasts. The dopaminergic differentiation flux can be observed in explants cultures treated with DAPT (Fig. 6D). During culture of mesencephalic explants active differentiation was indicated by the rise in mRNA levels of Mxs1, Ngn2 and Nurr1. The differentiation dynamics in E9.5 and E10.5 explants was similar but the increase from 2 days to 4 days of culture in mRNA levels of early markers (i.e. Mxs1/2, Ngn2) was less pronounced, whereas of late markers (i.e. Nurr1, Th) was more pronounced at the more advanced developmental stage, consistent with the differentiation trend occurring. Upon Notch signalling inhibition, consistent decrease in Mxs1 mRNA levels was observed in E9.5 explants despite their increasing phase at this developmental stage which was still observed; this observation is in agreement with a no relevant function of Notch in dopaminergic NPC proliferation. In contrast, Notch inhibition caused a consistent decrease in Ngn2 mRNA levels in E10.5 explants, whereas this effect was noted in E9.5 only after 4 days DAPT inhibitor treatment; apparent lack of effect on Ngn2 mRNA levels in 2 days-treated E9.5 explants supports a transitory role of Ngn2+ cells during differentiation. Nurr1 mRNA levels showed lower levels in E9.5 than in E10.5 explants, but a higher increment was triggered by Notch inhibition. Similar behaviour was observed for the Th mRNA but its levels did not rise in E10.5 explants after 4 days Notch inhibitor treatment possibly due to the depletion of progenitor cells. Together, these data are in agreement with a model in which the proliferation and size of the pool of NPCs (i.e. E9.5>E10.5) defines how the dopaminergic differentiation flux is affected by Notch inhibition such that, NPC depletion and neuron generation induced by the Notch inhibitor strengthen and weaken, respectively, at late phases of differentiation (possibly after E11.5).

DII1 haploinsufficiency causes a reduction in dopaminergic neurons of the adult brain substantia nigra

The alterations in mRNA levels of genes involved in neuronal dopaminergic differentiation as well as the mesencephalic phenotype observed in DII1+/lacZ embryos, both consistent with mild premature neuronal differentiation, prompted us to study the
consequences in adult mice. Although no differences in mRNA levels of neuronal or dopaminergic differentiation markers were detected at E13.5 or E15.5, lower density of dopaminergic neurons in the ventral mesencephalon of mutant in comparison with wild-type mice was not obvious at E13.5 but apparent at E15.5 when they are nearly to establish their final allocation (Fig. 7A). As mentioned above, NeuN downregulation correlated with dopaminergic neuron maturation such that some Th+/NeuN+ were detected in Dll1+/lacZ E13.5 embryos but almost none in E15.5 embryos of either genotype. Interestingly, this reduction in dopaminergic neurons
Fig. 7. Number of dopaminergic neurons in the adult brain under reducedDll1 levels. (A) Dopaminergic neurons were detected by the presence of Th (green). No apparent difference in the density of Th+ neurons was detected in embryonic samples. Note that some Th+ neurons in the mesencephalon of E13.5 embryos (arrowheads) but not in that of E15.5 embryos contained nuclear NeuN (red), a marker frequently found in mature neurons. Evident lower density of Th+ neurons, all lacking nuclear NeuN, was observed in the SNpc of Dll1lacZ mice in comparison with the density in this region of wild-type mice. Scale bars are 100 µm; arrows indicate Th−/NeuN+ cells within the SNpc. (B) Th+ neurons were counted in slices from brains of 14 month-old mice. Data represented as average±s.d.
became evident in adult mice, where it was readily visible in the substantia nigra pars compacta (SNpc) where most Th+ neurons remained NeuN negative (Fig. 7A,B). This is not related to a Dll1 role in neuron survival as similar phenotype was observed in young and old mice (see Fig. S5). Nonetheless, lower neuronal density was not restricted to dopaminergic neurons or the mesencephalic area but rather noted throughout the brain. The functional consequences of this reduction in neuronal density will be published elsewhere (manuscript in preparation). Therefore, a lower dose of Dll1 decreases the production of several neuronal types, possibly by disrupting a differentiation flux similar to the one described for mesencephalic dopaminergic neurons.

A mathematical model of dopaminergic differentiation flux

During neurogenesis, early ventricular neural precursors move to the subventricular zone as they differentiate (Fig. 8A). Dll1 expression was detected in the subventricular area of most mesencephalon at E10-E11 (Fig. 1) (see also Kele et al., 2006; Lahti et al., 2011; Ono et al., 2007), whereas Hes5, the main mediator of Dll1-Notch signalling found, has been reported to be mostly located in the ventricular area (Kele et al., 2006; Vernay et al., 2005). From these observations we inferred that cells targeted by Dll1 are located in the ventricular zone (Fig. 8A), though we cannot discard a partial overlapping between Dll1 and Hes5 expression domains. Analysis of cell death and proliferation and emergence of neuronal markers at different amounts of Dll1 (i.e. those in Dll1+/+, Dll1+/LacZ, Dll1lacZ/lacZ mice) suggest that as Dll1 decreases, the differentiation rate increases, meaning that fewer NPCs divide and more exit the cell cycle to become neuroblasts. This phenomenon has been observed in other brain regions and interpreted as premature neuronal differentiation due to the lack of the inhibitory differentiation effect of Notch signalling (Hatakeyama et al., 2004; de la Pompa et al., 1997; Louvi and Artavanis-Tsakonas, 2006). Premature differentiation during the expansion of dividing precursors could cause their rapid exhaustion and, consequently, a reduction in the number of neurons produced.

Increased rate of differentiation can also be observed following specifically the mesencephalic dopaminergic lineage (Fig. 8A). Our data show that initiation of neuronal differentiation, as detected by the emergence of βIII-tubulin, is closely coupled with the acquisition of markers of early dopaminergic neuroblasts such as Nurr1 and Th, and negatively regulated by Dll1. As expected, Msx1/2, a marker of early dividing dopaminergic precursors, concomitantly decreases. As inferred from mRNA levels, the number of Ngn2+ cells increases and decreases in coordination with that of Msx1/2+ cells, but under reduced Dll1 levels, early emergence of Ngn2+ cells occurs (Fig. 6A). Because the Ngn2+ NPCs population is a transient poorly dividing cell population (Andersson et al., 2006a; Kele et al., 2006; Thompson et al., 2006), these observations support a mechanism in which there is no additional restriction on differentiation from Ngn2+ progenitors into Nurr1+ neuroblasts.

A simplified model to describe the transition from specified dopaminergic precursor (Mx1/2⁺; P), going through intermediate
progenitors (Ngn2+, Nurr1+; I) up to becoming a young dopaminergic neuron (Nurr1+/Th+; N) (Fig. 8A) is:

\[ \mathbb{O}P \to I_1 \to I_2 \to N. \]

Given that Ngn2 controls Dll1 expression, effects of Dll1 on P are proportional to the amount of I adjacent to P (I1; I2 refers to the I population moving away from P possibly Nurr1+), thus, this effect varies according with the equation

\[ \partial = \frac{I_1}{P + I_1 + I_2 + N}. \]

Considering that there is no restriction on differentiation from I to N, as data suggested, the dynamic change in the number of P, I1, I2 and N during differentiation can be modelled by the following set of differential equations,

\[
\begin{align*}
\frac{dP}{dt} &= \frac{P}{1+I_1+I_2+N} + Pr_d \partial - Pr_d \\
\frac{dI_1}{dt} &= Pr_d - I_1 r_d \\
\frac{dI_2}{dt} &= I_1 r_d - N r_d \\
\frac{dN}{dt} &= N r_d,
\end{align*}
\]

which develop over time from an initial population P0 (P when I1+I2+N=0) and where the term P/(1+I1+I2+N) represents the fraction of P with proliferation independent of Dll1-Notch signalling. \(r_p\) and \(r_d\) denote the probability that one P cell self-divides or differentiates, respectively. Thus,

\[ r_p + r_d = 1. \]

To estimate the value of \(r_p\) and \(r_d\), we consider our experimental observations showing that the population Msx1/2+ (P) initially grows and extinguishes during the 6 days of differentiation (E9-E15) with a maximum around E11.5, and that the plateau of dopaminergic neurons (Th+; N) is reached between E13.5-E14.5. Using Eqs 1-4 and \(P_0=10\) to describe the differentiation dynamics, it can be determined that P grows when the \(r_p\) value is above 0.70, and gets restricted to the short range of 0.75-0.80 when considering the time at which N reaches a plateau (Fig. 8B). The \(r_p/r_d\) value might correlate with a short window of the cell cycle at which the neurogenic process can initiate (Latasa et al., 2009).

Fig. 8C shows the dynamics of dopaminergic differentiation by comparing the growth pattern of the major distinct cell populations considered in this work (\(P_0=10, r_p=0.78\)). Interestingly, the pattern shown resembles the one experimentally determined using the gene expression levels of the specific markers for P, I1, I2 and N (Fig. 6). A major conclusion derived from this dynamic model is that, the initial P0 population produces a 15-20-fold larger N population, which implies it can be determined that P grows when the \(r_p\) value is above 0.70, thus, this effect varies according with the equation

\[ \partial = \frac{I_1}{P + I_1 + I_2 + N}. \]

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The production of specific neurons during development should comply with the number needed for their functions in the adult brain. Moreover, it is expected that neuronal differentiation is coordinated in time with the processes that control migration of neuroblasts or young neurons to the definitive residence site, such that successful connections between neurons occur. It is generally thought that more neurons than needed are produced and that many die as they move through the path to their home and their axons compete for reaching the target cells. However, only in few instances have been demonstrated the role of natural neurodegeneration in the establishment of the final number of neurons, therefore, it is still relevant to determine the contribution of neurons produced. Here we found that the Notch-regulated differentiation flux from mesencephalic dopaminergic precursors up to becoming dopaminergic neurons is critical for determining the number of dopaminergic neurons present in the adult brain.

MATERIALS AND METHODS

Maintenance of mutant mouse line Dll1<sup>lacZ/+</sup>

The Dll1<sup>lacZ/+</sup> mutant mouse line in the CD1 strain genetic background was kindly provided by Dr Olivier Pourquié at the Stowers Institute for Medical Research. Because CD1 is an outbred strain, phenotypes resulting from the Dll1 mutation or its penetrance for certain phenotypes might vary among

\[
\begin{align*}
\text{dP} \quad &\text{dI}_1 \\
\text{dI}_2 \quad &\text{dN}
\end{align*}
\]
homozygous embryos; nonetheless, the particular phenotype studied here was highly reproducible suggesting low influence of potential modifiers present in this strain. The mutation resulted from the in-frame replacement of exons1 by the lacZ gene, as described by Hrabé de Angelis et al. (1997). The null allele was maintained in the heterozygous state, since homozygous DII1lacZ/lacZ embryos die at around embryonic day E12.5, similar as previously reported (Hrabé de Angelis et al., 1997). With little variation, the genotype determined by PCR (forward primer: 5'-GGTCAAGCGAAGATGAAACGC-3'; reverse primer: 5'-AAGCCGACGAAAGGAAACCG-3') was coincident with lacZ expression level and with E10-E12 embryo phenotype (e.g. head morphology, haemorrhagic spots). All animal manipulations required for the present work were in compliance with the ‘Guide for the Care and Use of Laboratory Animals’ (National Research Council) and approved by our Bioethical Committee.

Collagen explant cultures

The midbrain explants were prepared as described by Baizabal and Covarrubias (2009). When explants (E9.5 or E10.5) were treated with the γ-secretase inhibitor N-[3,5-Difluorophenyl]acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT) (5 μM, Tocris), control medium had same volume (0.5 μl) of dimethylsulfoxide (DMSO); under these conditions, explants were not cultured for longer than 4 days.

Tissue processing and immunofluorescence

Embryonic tissue and midbrain explants sections were processed as described by Baizabal and Covarrubias (2009). All coronal sections analysed were perpendicular to the tangent near the midportion of the mesencephalic flexure and those showing the complete ventral neuroepithelium (Fig. S1); thus, the extreme anterior and posterior mesencephalic areas were excluded. Tissue sections were incubated with the appropriate mixture of antibodies (Table S1). To determine active cell proliferation, pregnant mice were injected with 50 μl of BrdU (50 μg/ml; Sigma-Aldrich) 1 h before sacrifice; BrdU incorporation was determined by immunofluorescence. On the other hand, the TUNEL assay (Roche) was used to detect apoptotic cells in embryo brain cryosections. Preparations were counterstained with DAPI (1:10,000; Invitrogen), and analysed for immunofluorescence using a Zeiss LSM 510 confocal microscope and Zeiss Apotome microscope Axios Observer Z1.

Real-time quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen) following the manufacturer’s instructions. The ventral midbrain (defined by the one-forth of midbrain around the midline) of embryos was dissected and pooled for RNA extraction from no less than two litters at each developmental stage tested (i.e. E9.5-E15.5 dpc). Single ventral midbranes were not used for this analysis because rapid changes in gene expression occur in this region within the relevant developmental window, and because the low RNA yield would prevent from using the same sample for comparing the expression pattern of all genes. For explants, 3 midbrain explants (E9.5 or E10.5) of each condition and/or culture stage were collected per experiment; at least two experiments were performed per condition and/or culture stage. First strand cDNA was synthesized using AMV Reverse Transcriptase (Invitrogen) and oligo-dT16 primer. Quantitative RT-PCR was performed using KAPA SYBR FAST mix (KAPA Biosystems) in the presence of the specific primers (Table S2) and the Rotor-Gene 3000 thermocycler (Corbett Research). Gene expression was evaluated using 2ΔΔCt method. The housekeeping gene Rplp0 was used to normalize gene expression levels.

Data analysis and mathematical modelling

For cell quantification in tissue sections of embryos and explants cultures, single optical planes were analysed independently within each confocal stack. Total number of cells positive for the specific marker was determined within the stack. Unless indicated, cells counted were within the marked area, usually corresponding to the Lmx1a expression domain (determined in a parallel section). For adult brains, mice of 5, 8, 12 and 14 months of age were used. Although difference between DII1+/− and DII1lacZ/lacZ in the number of Th+ neurons was similar at all ages, robust analysis was done for one-year-old animals. We counted the number of Th+ cells in the SNpc of five different slices along the antero-posterior axis for each DII1+/− and DII1lacZ/lacZ mouse (n=4). The proportion of positive cells for a given marker is expressed as a percentage of the total cell number (DAPI+ cells)=s.d. (standard deviation) calculated from at least 3 different tissue samples. Asterisks in graphs indicate that the experimental groups were significantly different from control groups (P<0.05 as determined by the t-test). Mathematical modelling was performed in Matlab software (MathWorks, Natick, Massachusetts).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

N.T.-P., C.V., G.G.-F., D.-M.A., M.G.-C., A.F.-H., and I.Z.-A. performed the experiments and data analysis; J.-M.B. and L.C. conceived the approaches; L.C. developed the concepts; N.T.-P., G.G.-F and L.C. wrote the manuscript.

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Supplementary information

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