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A Novel Function of DELTA-NOTCH Signalling Mediates the Transition from Proliferation to Neurogenesis in Neural Progenitor Cells

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A complete account of the whole developmental process of neurogenesis involves understanding a number of complex underlying molecular processes. Among them, those that govern the crucial transition from proliferative (self-replicating) to neurogenic neural progenitor (NP) cells remain largely unknown. Due to its sequential rostro-caudal gradients of proliferation and neurogenesis, the prospective spinal cord of the chick embryo is a good experimental system to study this issue. We report that the NOTCH ligand DELTA-1 is expressed in scattered cycling NP cells in the prospective chick spinal cord preceding the onset of neurogenesis. These Delta-1-expressing progenitors are placed in between the proliferating caudal neural plate (stem zone) and the rostral neurogenic zone (NZ) where neurons are born. Thus, these Delta-1-expressing progenitors define a proliferation to neurogenesis transition zone (PNTZ). Gain and loss of function experiments carried by electroporation demonstrate that the expression of Delta-1 in individual progenitors of the PNTZ is necessary and sufficient to induce neuronal generation. The activation of NOTCH signalling by DELTA-1 in the adjacent progenitors inhibits neurogenesis and is required to maintain proliferation. However, rather than inducing cell cycle exit and neuronal differentiation by a typical lateral inhibition mechanism as in the NZ, DELTA-1/NOTCH signalling functions in a distinct manner in the PNTZ. Thus, the inhibition of NOTCH signalling arrests proliferation but it is not sufficient to elicit neuronal differentiation. Moreover, after the expression of Delta-1 PNTZ NP continue cycling and induce the expression of Tis21, a gene that is upregulated in neurogenic progenitors, before generating neurons. Together, these experiments unravel a novel function of DELTA–NOTCH signalling that regulates the transition from proliferation to neurogenesis in NP cells. We hypothesize that this novel function is evolutionary conserved.


INTRODUCTION

One of the greatest challenges in the field of neural development is to elucidate how developmental signals are integrated to generate the wide cellular diversity of the brain. In order to generate the correct number of cells in the proper places, the balance between cell proliferation and differentiation must be regulated in a very precise spatio-temporal manner during brain development.

The vertebrate CNS originates from a relatively small number of founder progenitor cells. At early developmental stages, the number of progenitors expands in an exponential manner through a series of proliferative divisions. Subsequently, NP cells begin to generate neurons through neurogenic divisions that give rise to a new progenitor and a neuron, [1–6]. Thus, the whole developmental process of neurogenesis comprises several cellular steps including the switch to neurogenic NP cells, the cell cycle exit after division of at least one of the daughter cells, and its differentiation into a neuron or glial cell [7–10]. Therefore, a complete account of neurogenesis involves understanding a number of complex underlying molecular processes. Numerous molecular mechanisms involved in the regulation of the asymmetric division of NP [11,12], cell cycle exit of neural cells [13,14] and neuronal differentiation [15] have been extensively studied. On the other hand, the genes and molecular processes that govern the switch from proliferative to neurogenic NP cells remain mostly unknown and, furthermore, little is known of how these sequential steps are coordinated.

Signalling through the NOTCH receptor is essential for correct cell-fate specification and differentiation throughout the animal kingdom [reviewed in 16,17]. The NOTCH proteins are cell-surface transmembrane receptors that upon binding to their ligands (DELTA, SERRATE, and JAGGED) located on the surface of adjacent cells, transduce a signal that influences cell fate choices. Several lines of evidence show that NOTCH signalling is involved in regulating neurogenesis in the vertebrate nervous system [reviewed in 18,19]. This takes place by the mechanism known as NOTCH mediated lateral inhibition. In brief, Delta-1 is expressed in single cells, which differentiate into neurons, and it impairs the differentiation of neighbouring cells by activating NOTCH signalling. Following DELTA binding to the NOTCH receptor in the neighbouring cells, NOTCH is cleared and its intracellular domain (NICD) translocates to the nucleus, where it stimulates the expression of the Hes family of bHLH transcription factors. In turn, these factors repress the expression of proneural bHLH transcription factors leading to the inhibition of neuronal differentiation. At the same time, the expression of Delta is inhibited in these cells and this decreases NOTCH activity in the Delta expressing cell by the feedback loop mechanism of lateral inhibition. Thus, the inhibited cells remain as progenitors and the Delta expressing cell differentiate as a neuron. This is the
mechanism that was originally proposed to happen in the rostral NZ of the developing chick spinal cord [20]. Nevertheless, DELTA-NOTCH signalling fulfils a different role in the growing caudal neural plate of the chick embryo. In this region, both Notch and Delta-1 are widely expressed in uncommitted progenitor cells. This leads to mutual NOTCH signalling which serves to maintain the proliferating progenitor pool necessary for the caudal extension of the body axis [21,22].

Here, we present compelling evidence for a new function of DELTA-NOTCH signalling, which regulates the transition from proliferation to neurogenesis in the prospective spinal cord of the chick embryo. This signalling takes place among NP cells which are located in between the caudal neural plate (stem zone) and the rostral NZ as the body axis extend caudally.

RESULTS

In the developing spinal cord, Delta1 is expressed in cycling neural progenitor cells preceding the onset of neurogenesis

The prospective spinal cord of the chick embryo presents a well defined rostro-caudal gradient of neurogenesis [23–25]. This yields a sequential separation of the cellular processes of proliferation and neurogenesis along the rostro-caudal axis [reviewed in 26]. Thus, this is a particularly suitable experimental system to investigate the molecular mechanisms underlying the transition from proliferation to neurogenesis. Although there are some small populations of neurons that appear very early in development [27], the main onset of neurogenesis in the chick embryo takes place after the closure of the neural tube [24–25].

The neurogenic gene Delta-1 is expressed in an interesting rostro-caudal pattern along the prospective spinal cord of the chick embryo (Fig. 1A,B). In agreement to its role in neuronal differentiation [16,19,20], abundant Delta-1-expressing cells are present in the rostral NZ of the prospective spinal cord where neurons are born according to the expression of the early neuronal marker, class III β-tubulin (TUJ1) [28]. Intriguingly, we found numerous scattered Delta-1-expressing cells in the caudal region where neurons are practically absent (compare Fig. 1B and C). Therefore, it appears that Delta-1 expression in single cells of the caudal spinal cord precedes the onset of neurogenesis. Thus, we set out to determine the nature of these Delta-expressing cells. Interestingly, we observed that cells expressing Delta-1 were found at different apico-basal positions along the rostro-caudal axis. In the NZ (i.e. between somites 1–4 in a HH11 embryo), they were located preferentially in basal positions (Fig. 1D), in accordance with its expression in prospective neurons [20] that move to the mantle as they withdraw from the cell cycle. On the other hand, in the caudal region, they were found in all apico-basal positions of the neuroepithelium [Fig. 1E], suggesting that Delta-1 may be expressed by NP cells of the caudal spinal cord. Cycling NP cells are subject to interkinetic movements and hence, their apico-basal positions depend on the cell cycle phase [29]. To confirm this hypothesis, the coexpression of Delta1 with distinct cell cycle markers was analysed in the caudal spinal cord. The expression of Delta1 was assayed in mitosis (M phase), S phase, and interphase G2 using antibodies against phosphohistone-3 (PH3), BrdU, and cyclin B, respectively. As a result, we found that Delta-1 is expressed in the M, S, and G2 phases of the cell cycle in spinal cord NP cells (Fig. 2 and Table 1). Since there is no suitable antiserum available for immunocytochemistry against chick cyclin D that allowed to test the expression in interphase G1, the coexpression of cyclin D and Delta1 in NP cells was assayed in preneurogenic regions of the developing mouse brain where Delta1 expression has been also observed in scattered cells [30]. We indeed found a consistent proportion (32±5%, 3 embryos; 47/146 cells) of Delta-1/CYCLIN D double labelled cells in the forebrain of E10.5 mouse embryos. (Figure S1). Thus, it seems likely that Delta-1 is also expressed during the G1 phase in spinal cord NP cells. As expected, there were relatively very few Delta-1/TUJ1 double labelled cells in this region (Table 1 and Fig. 2G).
Together, these results show that the caudal spinal cord Delta-1-expressing cells are mostly cycling NP cells that define an intermediate domain between the rostral domain of Delta-1 expression in prospective neurons (approximately rostral to the 5 last formed caudal somites) and that of the proliferating uncommitted progenitor cells of the caudal neural plate (Fig. 1A–C). Accordingly, we will call this domain the “proliferation to neurogenesis transition zone” or just PNTZ.

DELTA/NOTCH signalling in neural progenitor cells of the prospective spinal cord of early chicken embryos

The scattered expression pattern of Delta-1 in the PNTZ suggests that these NP cells may be subject to DELTA-NOTCH lateral inhibition. In order to assess this idea, we tested whether the expression of Delta-1 was under the control of NOTCH signalling. At this end, we transfected a constitutively active truncated form of NOTCH (NICD) [31], along with a pEGFP reporter plasmid, into the PNTZ of HH10 embryos. As shown in Fig. 3A,B, this resulted in an almost complete suppression of Delta-1 expression (4/4 embryos).

In addition, we studied the relation of Delta-1 with the expression of Hes genes, the primary transducers of NOTCH signals in vertebrates [reviewed in 32]. Among them, Hes5.1 seemed to be the best candidate since it is abundantly expressed along the prospective spinal cord in a rostro-caudal distribution apparently overlapping with that of Delta-1 (compare Fig. 1A and Figure S2). Double FISH shows that Hes5.1 and Delta-1 are expressed in a mutually excluding cellular pattern with low Hes5.1 expression in Delta1-expressing cells and, conversely, high Hes5.1 expression in the adjacent ones. (Fig. 3C). Nevertheless, we observed that a few cells expressed neither Hes5.1 nor Delta-1. As shown in Fig. 3D,E, electroporation of the caudal spinal cord of HH110 chick embryos with pCIG-Delta1 in scattered cells reproduced the endogenous expression pattern of Hes5.1. These results strongly suggest that DELTA1-NOTCH lateral inhibition takes place through Hes5.1 in NP cells of the PNTZ.

Table 1. Quantitative analysis of Delta-1 expression with cell cycle markers.

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Marker</th>
<th>n</th>
<th>Nm/NDl1</th>
<th>% (Nm/NDl1) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Phosphohistone-3</td>
<td>3</td>
<td>31/241</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>G2* postmitotic</td>
<td>BrdU</td>
<td>3</td>
<td>54/232</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>G2*</td>
<td>Cyclin B</td>
<td>3</td>
<td>37/188</td>
<td>19 ± 3</td>
</tr>
<tr>
<td></td>
<td>TUJ1</td>
<td>3</td>
<td>10/202</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

Counting of double labelled cells was carried out on confocal images collected from transverse sections of HH10 chick embryos at the level of the last five caudal somites.

Counting of Delta-1/Cyclin B double labelled cells was restricted to the apical third of the neuroepithelium that does not incorporate BrdU (see dotted line in Fig. 2CE).

n: number of embryos analysed. Nm: number of Delta-1-expressing cells. Nm: number of cells co-expressing Delta-1 and the indicated marker. The error was calculated as the standard deviation (SD).

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doi:10.1371/journal.pone.0001169.g002
Activation of NOTCH signalling in caudal spinal cord progenitor cells represses neurogenesis

Since NOTCH lateral inhibition has been shown to repress neuronal differentiation in several vertebrate nervous systems [reviewed in 19, 33], we determined whether the activation of NOTCH signalling causes similar effects in the caudal spinal cord. As a read out of neuron production we used the pan-neuronal marker TUJ1, which we have previously shown to be expressed as early as 1–2 h after mitosis of early chicken spinal cord NP cells [34]. NICD was transfected into the PNTZ of HH10 embryos around prospective 12th–16th somite pairs where neurogenesis had not commenced yet. We found that 20 hours after transfection (16 h after the initial detection of GFP, n = 4), NICD completely impaired the co–expression of GFP and TUJ1 in that region (0/216 cells: Fig. 4A–A2,G). These results indicate that NOTCH activation in PNTZ NP cells inhibit neurogenesis.

For instance, DeltaDN has been previously shown to be an effective inhibitor of NOTCH signalling in chick nervous system [37,38]. Thus, we transfected DeltaDN into the PNTZ of HH10 embryos by electroporation and assayed proliferation by BrdU incorporation.

Inhibition of NOTCH signalling in caudal spinal cord neural progenitor cells arrests proliferation but does not elicit neuronal differentiation

The above results show that activation of NOTCH signalling in PNTZ NP cells represses neuronal production. Nevertheless, this does not seem to further stimulate the proliferation of these progenitors. Although this might be due to the fact that the expected increase (around a 10% of TUJ1 positive cells in the control, Fig. 4G) is in the range of the experimental error of the determination of BrdU labelled cells. In contrast, control pCIG transfected cells do not alter its expression level. In contrast, the pattern of Hes5.1 expression is not altered in an embryo transfected with the control morpholino(G–G').

Figure 3. DELTA-1/NOTCH signaling in the caudal spinal cord of chick embryos. A–A’ Confoal projection over 50 μm from a dorsal aspect of the prospective spinal cord at the level of the 12th and 13th somites counted from rostral of a HH13 chick embryo that was electroporated with NICD at stage HH10 when that region was part of the PNTZ. Transfected cells express the c-myc reporter. Note the clear decrease in the number of Delta-1 expressing cells within the electroporated area compared to the contralateral side and to the control transfected embryo (B–B’). C–C’. Transversal optical section of a double FISH of Delta1 and Hes5.1 taken at the level of the 12th and 13th somite. Notice that Delta-1 and Hes5.1-expressing cells are mutually exclusive and that a few cells lack expression of both genes. D–D’. Confoal projection over 50 μm taken from a dorsal view point of the prospective spinal cord at the level of the 12th–14th somites counted from rostral of an embryo that was electroporated with pCIG-Delta1 at HH10 stage and allowed to develop until HH12/13 stage. Transfected cells express the reporter GFP protein. Notice the clear decrease in Hes5.1 expression in Delta-1 transfected cells while Hes5.1 expression is maintained in most non transfected cells. In control, electroporated embryos do not alter Hes5.1 expression (E–E’). F–F’. Confoal projection over 50 μm taken from a dorsal view point of the prospective spinal cord at the level of the 12th–14th somites counted from rostral of an embryo which was electroporated with the antisense morpholino oligo Mo2-cDelta1 at HH10 stage and allowed to develop until HH12/13. Notice the decrease in the expression level of Hes5.1 in the transfected side, as compared to the non-transfected contralateral side. doi:10.1371/journal.pone.0001169.g003
In order to make sure of the cell autonomous effect of \( \text{Delta}^{DN} \) we transfect few scattered cells. As expected, \( \text{Delta}^{DN} \) induced a strong arrest of proliferation 12 h posttransfection (Fig. 4C–C').

Thus, we conclude that NOTCH signalling is required to maintain proliferation of caudal spinal cord NP cells. Interestingly, the arrest in proliferation induced by \( \text{Delta}^{DN} \) was not accompanied by a significant increase in the proportion of cells co-expressing TUJ1 18 h (4 embryos, 20/194 cells; 10.3%; Fig. 4D,D',G) and 26 h (3 embryos, 14/149 cells; 9.4%, not shown): after transfection as compared to controls (4 embryos, 8/113 cells, 7.1%). The fact that a large part of the transfected cells were located on basal positions (Fig. 4D) may suggest that they were driven to exit the cell cycle by the expression of \( \text{Delta}^{DN} \). This possibility was assessed by analysing the expression of the cyclin-dependent kinase inhibitor \( \text{p27}^{kip1} \), which is a major regulator of cell cycle exit [14] and whose expression has been associated to the birth of neurons in the mouse forebrain [39] and the chick spinal cord [40]. We found...
that most $\text{Delta}^{DN}$ transfected cells lacked or exhibited very low $p27^{kip1}$ expression (Fig. 4E). This makes very unlikely that NP cells were removed from the cell cycle. An alternative explanation for the basal localization of the cells is that they were arrested in G1 by the decrease of NOTCH signalling as it happens when NOTCH signalling is inhibited by $\gamma$-secretase inhibitors [41]. The lack of suitable cyclin D antisera precluded to assess this possibility. Together, these experiments indicate that suppression of NOTCH signalling in caudal spinal cord NP cells arrests proliferation but it is not sufficient to elicit cell cycle exit and neuronal differentiation. This is in clear contrast to the induction of $p27^{kip1}$ (not shown) and TUJ1 expression (4 embryos, 37.5% of double labelled cells vs. the basal localization of the cells) by $\text{Delta}^{DN}$ in the NZ (around prospective 3rd–7th somite pairs of HH12 embryos) 18 h after transfection.

**Delta-1 expression in cycling neural progenitor cells is necessary and sufficient to induce neuronal generation**

So far, we have shown that DELTA-NOTCH signalling in cycling NP cells precedes the onset of neuronal generation at the caudal spinal cord. Although suppression of NOTCH signalling induced proliferation arrest of these cells, it was not sufficient to elicit neuronal differentiation. Thus, it remains unclear how the expression of $\text{Delta-1}$ in cycling NP cells is related to the process of neurogenesis and in what context of the different cellular steps along the rostro-caudal axis may be acting. To address these questions, we performed gain and loss of function experiments by focal electroporation at different rostro-caudal positions of the prospective spinal cord of stage HH10-HH12 chicken embryos. Experiments are summarised in Table 2. Loss of function was carried out by posttranscriptional gene silencing of $\text{Delta-1}$ with two anti-sense and one control morpholino oligos (see Material and Methods for details). Since we have no available anti-DELTA-1 antisera to test the decrease of protein expression on the tissue, the efficiency of the $\text{Delta-1}$ antisense morpholino oligos was assessed by analysing their effect on the expression of $\text{Hes5.1}$. As exemplified in Fig. 3F,G, the expression level of $\text{Hes5.1}$ in the PNTZ was substantially reduced by electroporation of anti-sense morpholinos (4/5 embryos) while the control morpholino oligo did not modify the $\text{Hes5.1}$ expression pattern (3/3 embryos). Accordingly, we tested the effect on neuronal generation. As shown in Fig. 5A-C, electroporation of morpholino anti-sense oligos induced an extensive decrease of TUJ1 immunolabelling whereas the control morpholino did not.

In order to test the effects of $\text{Delta-1}$ gain of function, we electroporated the pCIG-$\text{Delta}$ vector in the PNTZ. Nevertheless, as previously found in other chick neural tissues [37,38], we observed that widespread transfection of cells with $\text{Delta-1}$ inhibited neurogenesis in the PNTZ (Figure S3). This predictable inhibitory effect is explained by the mutual lateral induction of NOTCH signalling when many neighbour cells express both DELTA and NOTCH. To overcome this problem we used electroporation conditions for transfecting scattered cells with high levels of $\text{Delta-1}$ expression, emulating the endogenous pattern of expression. Embryos were incubated for 10, 18 and 26 h, and TUJ1 labelling was analysed in the transfected cells (Fig. 5D–G,L). The percentage of $\text{Delta-1}$ transfected cells expressing TUJ1 10 h after transfection was less than 2% (not shown). 8 hrs later, there was no significant increase in the percentage of $\text{Delta-1}$ transfected TUJ1 labelled cells (8.5±4% vs. 7±2%). However, this effect increased greatly at 26 h after transfection (45±7%, vs. 7±1% in control embryos). Together with the electroporation of antisense

### Table 2. Phenotypic analysis in gain and loss of function experiments of $\text{Delta-1}$.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Region</th>
<th>Phenotype or Test</th>
<th>Exp. $N_{\text{Ph}}/N_T$</th>
<th>Control $N_{\text{Ph}}/N_T$</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO-Delta HH10+24 h</td>
<td>PNTZ</td>
<td>Decreased TUJ1 labelling</td>
<td>9/9</td>
<td>0/3</td>
<td>5A,B,C</td>
</tr>
<tr>
<td>Delta HH10+18 h</td>
<td>PNTZ</td>
<td>Normal TU1 labelling</td>
<td>13/14</td>
<td>4/4</td>
<td>5D,EL</td>
</tr>
<tr>
<td>Delta HH10+26 h</td>
<td>PNTZ</td>
<td>Increased TUJ1 labelling</td>
<td>9/9</td>
<td>0/4</td>
<td>5FG,L</td>
</tr>
<tr>
<td>Delta HH12+18 h</td>
<td>PNTZ</td>
<td>Normal TU1 labelling</td>
<td>7/8</td>
<td>4/4</td>
<td>5H,L</td>
</tr>
<tr>
<td>Delta HH12+26 h</td>
<td>PNTZ</td>
<td>Increased TUJ1 labelling</td>
<td>3/3</td>
<td>0/4</td>
<td>5L</td>
</tr>
<tr>
<td>Delta HH10+18 h</td>
<td>PNTZ</td>
<td>Normal BrdU incorporation</td>
<td>5/5</td>
<td>0/6</td>
<td>6A,B,E</td>
</tr>
<tr>
<td>Delta HH10+26 h</td>
<td>PNTZ</td>
<td>Decreased BrdU incorp.</td>
<td>5/5</td>
<td>0/6</td>
<td>6C,D,E</td>
</tr>
<tr>
<td>Delta HH10+18 h</td>
<td>PNTZ</td>
<td>Co-expression of PH3</td>
<td>3/3</td>
<td>3/3</td>
<td>6F</td>
</tr>
<tr>
<td>Delta HH10+18 h</td>
<td>PNTZ</td>
<td>Co-expression of Cyclin B</td>
<td>3/3</td>
<td>3/3</td>
<td>6G</td>
</tr>
<tr>
<td>Delta HH10+18 h</td>
<td>PNTZ</td>
<td>Normal TU1 labelling</td>
<td>4/4</td>
<td>4/4</td>
<td>6G</td>
</tr>
<tr>
<td>Delta HH10+18 h</td>
<td>PNTZ</td>
<td>Normal TU1 labelling</td>
<td>3/3</td>
<td>3/3</td>
<td>N.S</td>
</tr>
<tr>
<td>Delta HH10+26 h</td>
<td>PNTZ</td>
<td>Normal TU1 labelling</td>
<td>3/3</td>
<td>3/3</td>
<td>5L</td>
</tr>
<tr>
<td>Delta HH10+18 h</td>
<td>PNTZ</td>
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<td>3/3</td>
<td>3/3</td>
<td>N.S</td>
</tr>
<tr>
<td>Delta HH10+26 h</td>
<td>PNTZ</td>
<td>Decreased BrdU incorporation</td>
<td>3/3</td>
<td>3/3</td>
<td>6E</td>
</tr>
<tr>
<td>Delta HH10+18 h</td>
<td>PNTZ</td>
<td>Normal p27KIP1 labelling</td>
<td>3/3</td>
<td>3/3</td>
<td>4E</td>
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<tr>
<td>Delta HH10+26 h</td>
<td>NZ</td>
<td>Increased p27KIP1 labelling</td>
<td>3/3</td>
<td>3/3</td>
<td>N.S.</td>
</tr>
<tr>
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<td>NZ</td>
<td>Increased TU1 labelling</td>
<td>4/4</td>
<td>4/4</td>
<td>5J</td>
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<tr>
<td>Delta HH12+26 h</td>
<td>NZ</td>
<td>Increased TU1 labelling</td>
<td>3/3</td>
<td>3/3</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

The phenotypic analysis was carried out by Confocal Microscopy of whole mount embryos. $N_{\text{Ph}}$: Number of embryos with clear phenotype. $N_T$: Total number of analysed embryos.

N.S. = not shown.

1 doi:10.1371/journal.pone.0001169.t002
morpholinos, these experiments demonstrate that the expression of Delta-1 in NP cells of the PNTZ is necessary and sufficient to induce the generation of neurons. Interestingly, the onset of neuronal generation after Delta-1 expression needs a longer period in the PNTZ than in the NZ where we measured a consistent increase in TUJ1 labelled cells 18 h after transfection of Delta-1 (35.2±3.5%, vs. 9.8±0.6% in control embryos; Fig. 5J, K, L). Thus, neurons need approximately 8 and 16 h to arise in the NZ and PNTZ, respectively, after Delta-1 expression if we consider that there was a good correlation between GFP and Delta-1 mRNA expression at 8 h post-transfection (Figure S3), and TUJ1 can be detected as early as 1–2 h after mitosis [34]. These

Figure 5. Spatio-temporal analysis of neuronal generation induced by Delta-1 expression. A–I Confocal projections (40–50 μm) from a dorsal point of view of the prospective spinal cord of embryos transfected with pCIG-Delta-1, pCIG-Delta-1/pCIG-DeltaDN, pCIG (controls), Delta-1 anti-sense (MO1-cDelta1 and MO2-cDelta1) and control morpholinos at the stages and times indicated. Arrows point to double labelled cells. J and K. Confocal transverse projections (50 μm) of two embryos electroporated at the NZ with pCIG-Delta-1 and pCIG, respectively, and allowed to develop for 18 h. J' and J'', higher magnification of a single optical section (5 μm) of the boxed area in J showing two GFP/TUJ1 labelled cells. L. Statistical analysis of the GFP/TUJ1 double labelled cells at the indicated stages and times after transfection with pCIG-Delta-1, pCIG-Delta-1/pCIG-DeltaDN and pCIG (controls). Notice that a consistent increment in the proportion of double labelled cells are only obtained at 26 h postransfection with pCIG-Delta-1 in PNTZ independently of the transfected embryonic stage.

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additional 8 h between the NZ and the PNTZ could reflect the time required by a rostro-caudal wave of differentiation to reach the transfected cells of the PNTZ. To assess this possibility, we transfected the same region (prospective somites 12–16) of older embryos (HH12) and allowed them to develop for 18 h, the time required to reach the same developmental stage (HH16) as HH10 embryos incubated for 26 hours. However, no increase in the percentage of TUJ1-positive cells was observed under these experimental conditions (Fig. 5H,L). On the other hand, a similar percentage of TUJ1-positive cells was obtained when both HH10 and HH12 Delta-1 transfected embryos were allowed to develop for 26 h (Fig. 5L). These results rule out the possibility that a rostro-caudal gradient of differentiation might explain the delayed generation of neurons after Delta-1 expression in the PNTZ.

It has been proposed that the upregulation of DeltaA in proliferating neural progenitor cells of the zebrafish neural tube induced cell cycle exit and differentiation [42]. It has been also found that the expression of the intracellular domain of DELTA-1 resulted in a non proliferating senescent-like cell phenotype [43]. This made us wonder whether the delayed effect of Delta-1 could be due to a long delay between cell cycle exit and neuronal differentiation. In order to test this possibility, the caudal spinal cord of HH10 chicken embryos was transfected with Delta-1 and BrdU incorporation was analyzed at 12, 18 and 26 h post-transfection. The percentage of Delta-1 transfected cells that incorporated BrdU was very similar to that of control transfected embryos at 12 h (56±5% vs. 48±4%; not shown) and 18 h post-transfection (46±6% vs. 45±2%, Fig. 6A,B,E). Moreover, the proportion of Delta-1 transfected cells coexpressing the mitotic marker PH3 (12±3%, Fig. 6F) and the G2 phase marker cyclin B (19±5%, Fig. 6G) 18 h after transfection were similar to those of NP cells endogenously expressing Delta-1 (Table 1). Therefore, in contrast to the immediate cell cycle arrest caused by Delta<sub>DN</sub> (Fig. 4), Delta-1 transfected PNTZ NP cells do not stop cell cycling. Interestingly, the percentage of Delta-1 transfected cells in the PNTZ that incorporated BrdU diminished substantially after 26 h (23±3% vs. 53±1%, Fig. 6C,D,E). Remarkably, the approximate 50% decrease in the number of cells that incorporate BrdU between 18 and 26 h post-transfection (Fig. 6E) coincides with the increase of TUJ1 labelled cells for the same period (Fig. 5L). These results can be interpreted as if Delta-1 expressing NP cells of the PNTZ generate neurons by undergoing neurogenic cell cycles, those that give rise to a new progenitor and a neuron. This also implies that in order to generate neurons, Delta-1 transfected NP cells of the PNTZ need to continue cycling. The fact that the inhibition of NOTCH signalling induced a cell cycle arrest of PNTZ NP cells (Fig. 4C,H) suggest that NOTCH signalling is required for Delta-1 expressing NP to continue cycling. To assess this idea, we co-transfected the PNTZ with Delta-1 together with Delta<sub>DN</sub>. As predicted, the cotransfection resulted in a proliferation arrest (18±3±4%, vs. 45±2% in control embryos; Fig. 6E) and a strong decrease in the production of neurons (16±3%; Fig. 5L) 26 h post-transfection as compared with the effect of Delta-1 alone (Fig. 5L).

Together, these experiments show that Delta-1 expression in PNTZ NP cells is necessary and sufficient to induce neuronal generation. In addition, these results suggest that neuronal production takes place through neurogenic cell cycles rather than by inducing neuronal differentiation of the NP cells.

**DELTA-NOTCH signalling regulates the expression of Tis21**

In order to assess a possible switch to neurogenic NP cells in response to Delta-1, we examined the expression of BTG2/PC3/
**DISCUSSION**

**DELTA/NOTCH signalling at the transition from proliferation to neurogenesis of neural progenitor cells**

The regulation of the balance between cell proliferation and differentiation is essential for the correct growth, shaping, and evolutionary diversification of the nervous system [2–5,49,50]. Thus, the switch from proliferative to neurogenic divisions of NP cells appears as a key regulatory point. Despite its biological relevance, the molecular processes that govern this switch have remained elusive. We have taken advantage of the sequential separation of the cellular processes of proliferation and neurogenesis in the prospective spinal cord of chick embryo to study the mechanisms that regulate this transition.

The chick spinal cord is generated in a rostrocaudal sequence as the body axis extends during embryonic development. This growth relies on the generation of NP cells from a region known as the caudal neural plate or stem zone, which moves caudally by regressing alongside the primitive streak. NP cells are generated in the stem zone and are left behind to form the spinal cord [22]. This process is promoted by a caudal FGF signalling gradient while an opposing rostral gradient of retinoic acid is required for neuronal differentiation [reviewed in 26]. It has been shown that FGF dependent NOTCH signalling gradient is increased at the transition zone of the stem zone [21]. In this region, all cells express high levels of Delta-1 and Notch. As a consequence, there is mutual activation of NOTCH signalling which maintains proliferation of this pool of cells.

A molecular marker of neurogenically dividing NP cells [44, 45], which we have previously found to be expressed in the developing spinal cord preceding the appearance of neurons [46]. Double ISH of chick embryos indicated that Tis21 and Delta-1 are indeed co-expressed in caudal spinal cord NP cells (Fig. 7A). We found that among the labelled cells located within the PNTZ of 3 embryos, 71% co-expressed both genes, while 23% expressed only Delta-1 and 6% expressed Tis21 alone. Accordingly, we next assessed whether Tis21 expression could be regulated by DELTA-1-NOTCH signalling. As shown in Fig. 7BC, Tis21 expression was extensively suppressed by electroporation with NICD (6/6 embryos). Conversely, the electroporation with pCIG-Delta-1 of the caudal spinal cord of HH10 embryos, around prospective somites 16–20 that practically lack Tis21 expression [46] induced ectopic Tis21 expression beginning at 11 h (2/3 embryos, 12% of transfected cells, not shown) and highly increasing at 15 h post-transfection (10/11 embryos, 51% of transfected cells; Fig. 7D,E). Altogether, these experiments demonstrate that DELTA1-NOTCH signalling in the PNTZ regulates the expression of Tis21.

It is known that in the mammalian CNS neuroepithelium there is an increase in the length of the cell cycle concomitant with the switch from proliferative to neurogenic divisions [47]. Since former studies have estimated that the duration of the cell cycle in the developing chick spinal cord is in the range of 6–8 h [1,48], the generation of neurons 16 h after Delta-1 expression could be explained if Delta-1 were to drive the PNTZ NP cell into a long neurogenic cycle. However, we found that the expression of Delta-1 in NP cells of the prospective spinal cord does not increase the duration of the cell cycle as measured by in vivo monitorisation of GFP transfected cells (Fig. 8).

![Figure 7. Relation of DELTA-1/NOTCH signalling and Tis21 expression in the prospective spinal cord. A–A”. Confocal projection (25 μm) of double ISH for Delta-1 and Tis21 in the transition zone of a HH10 embryo showing several double labelled cells (white arrows) and cells expressing either Tis21 (magenta arrows) or Delta-1 (green arrows). B,C. Confocal projection (50 μm) over the PNTZ of embryos electroporated with pCIG-Delta-1 and pCIG (controls) and pCIG-Delta1m at HH10 and allowed to develop until HH12/13. D–F. Confocal projection (25 μm) around the 6 most caudal somites of embryos electroporated with pCIG-Delta-1, pCIG (controls) and pCIG-Delta1m at HH10 and allowed to develop until HH12/13. G,H. Confocal projection (50 μm) around the PNTZ of embryos electroporated with MO2-cDelta1 anti-sense and control morpholinos. Notice the decrease in the number of cells expressing Tis21 and their expression level in the side transfected with MO2-cDelta1 as compared to the contralateral one. doi:10.1371/journal.pone.0001169.g007](image-url)
DELTA-1-NOTCH signalling on this transition. We have found that DELTA-1 is expressed in cycling NP cells located between the intermediate region (PNTZ) that expands more rostrally than the NZ of the chick spinal cord (this paper). Together, these results support the role of DELTA-NOTCH signalling in neuronal differentiation.

It has been proposed that as new neuroepithelium is generated immediately rostral to the caudal stem zone, there is a “transition zone” with a gradual change from mutual inhibition between all Delta-1/Notch-expressing cells of the caudal neural plate to lateral inhibition between single Delta-1 expressing prospective neurons and adjacent progenitors [21].

We have taken advantage of the sequential separation of the cellular processes of proliferation and neurogenesis along the rostrocaudal axis to study the possible role of DELTA1-NOTCH signalling on this transition. We have found that Delta-1 is expressed in cycling NP cells located between the DELTA-1 expressing prospective neurons of the rostral neurogenic region [20] and the DELTA-1 expressing uncommitted progenitors of the caudal neural plate [21]. Thus, the expression of Delta-1 in these single NP cells defines an intermediate region (PNTZ) that expands more rostrally than the previously described “transition zone” [21]. Most importantly, we have found that lateral inhibition in the PNTZ occurs between cycling NP cells rather than between a NP and a prospective neuron as in the rostral NZ (see Fig. 9 for a schematic representation). It must be also emphasized that the suppression of NOTCH signalling does not elicit neuronal differentiation of PNTZ NP cells in spite of inducing a proliferation arrest. These results contrast with the overproduction of neurons observed in other vertebrate nervous tissues after Delta-1 transfection [37,38,42,54], including the rostral NZ of the chick spinal cord (this paper). Together, these results indicate that neuronal differentiation is not an automatic consequence of reducing NOTCH signalling in CNS progenitors but it depends on the cellular context.

The expression of delta genes in proliferating NP cells has been previously reported in the embryonic zebrafish neural tube [42]. This was interpreted to mean that those cells that upregulate delta expression, decreased NOTCH activity by the feedback loop mechanism of lateral inhibition, and were driven to exit the cell cycle and to differentiate. However this does not happen in the PNTZ because, as we have here shown, after the expression of Delta-1, PNTZ NP continue cycling and the suppression of NOTCH signalling did not induce cell cycle exit and neuronal differentiation. Another possibility is that Delta-1 could function to keep selected neuronal progenitors in a pre-differentiated state until rostral differentiating gradients (i.e. retinoic acid) reach them. However, two set of results rule out this possibility. First, if this would be the case, one should expect that removing Delta-1 function will result in precocious neuronal differentiation. However, gene silencing with Delta-1 antisense morpholinos gave the opposite result. Secondly, the transfection with Delta-1 in the same region of older embryos in which the rostral gradient of differentiation has advanced more caudally did not result in an earlier generation of neurons. Thus, the expression of Delta-1 in PNTZ NP cells reveals a novel function.

We have here presented compelling results showing that DELTA–NOTCH signalling in the PNTZ regulates the transition from proliferation to neurogenesis in NP cells and that this signalling is different from that of classical lateral inhibition as it happens at the rostral NZ of the spinal cord. These major conclusions are based on the following evidences: I, Activation of NOTCH signalling in the NZ of the chick spinal cord is necessary for maintaining proliferation of NP cells. II, However, in contrast to what happens in the NZ, the suppression of NOTCH signalling in the PNTZ is not sufficient to elicit neuronal differentiation. III, The expression of Delta-1 in cycling NP cells of PNTZ is necessary and sufficient to induce neuronal generation and maintain proliferation of NP cells. IV, After cycling, approximately half of the Delta-1 expressing cells incorporate BrdU while the other half become neurons. This fits with the switch to neuron-generating divisions, those yielding one NP cell and one neuron. V, This idea is further supported by the fact that Delta-1 expression in cycling NP cells of PNTZ is necessary and sufficient to induce the expression of Tis21, a molecular marker that identifies in the mouse CNS those NP cells that have switched from proliferative to neurogenic divisions [44,62]. This suggest that the transition from proliferation to neurogenesis is regulated in the PNTZ by turning proliferating progenitors (i.e. self-renewing progenitors) into neurogenic progenitors.

A common feature of lateral inhibition by NOTCH signalling throughout the animal kingdom is that mediates the binary decision of adjacent cells between two alternative fates, which depend on the developmental context [9,17]. Accordingly, one would expect that DELTA-NOTCH signalling in different organisms under similar developmental contexts should result in equivalent fate decisions. The expression of Delta-1 that we have found in cycling NP cells of preneurogenic regions of the mouse CNS (Figure S1) supports this idea. The increase in the ratio of symmetric/asymmetric divisions of progenitor cells found in Numb and Numblike mouse mutants 60,63) and the regulation of asymmetric divisions by Mash1 in certain spinal cord lineages [64] also fit with this hypothesis. Interestingly, NOTCH signalling [65,66] and Mash1 [67] seem to promote the neuronal commitment of pluripotent stem cells.

In the embryonic CNS of Drosophila, neuronal progenitors (neuroblasts) arise from a neuroectoderm in which all cells initially express Delta and Notch, and have the potential to become neuroblasts or epidermolasts. Pioneural genes are expressed in clusters of cells and predispose them to a neural fate. Within each
cluster Delta is only upregulated in a single cell that becomes a neuroblast and, through NOTCH-signalling, inhibits the neighbouring cells that remain as epidermoblasts [reviewed in 7,8]. Cells in the neuroectoderm divide symmetrically but after delamination, neuroblasts undergo repeated rounds of asymmetric divisions. Therefore, one may interpret that after DELTA/NOTCH lateral inhibition, the neuroblast changes from proliferative to neurogenic divisions. Thus, the regulation of this switch might be an evolutionarily conserved function of DELTA-NOTCH signalling. Nevertheless, since we have not directly studied the pattern of division of the PNTZ NP cells, it is unclear whether DELTA–NOTCH signalling changes PNTZ progenitors to intrinsic asymmetrically dividing NP cells or it makes the daughter cells competent to respond to other signals that determine their different fates (i.e. DELTA1-NOTCH lateral inhibition at the NZ).

It must be stressed that the switch to neurogenic NP cells does not happen immediately after Delta-1 expression since the expression of Tis21 was induced 8 h after Delta-1 expression and the generation of neurons after 16 h. The duration of the cell cycle in the prospective spinal cord has been previously determined to be in the range of 6–8 h by double labelling experiments with BrdU and [3H]-Tymidine [1,48]. This fits very well with our in ovo measurements of the time required to duplicate the number of GFP-expressing cells in the PNTZ (Fig. 8) and contrast to the results of a recent report that found cell cycles to last 12–24 h based on time lapse determinations in the spinal cord [68]. The fact that this last study was carried out in cultured transversal slices of spinal cord that are deprived of the rostro-caudal signalling factors, which are known to stimulate proliferation, might explain the timing discrepancies.

Since Delta-1 does not seem to increase the length of the cell cycle (Fig. 8), 16 h allow two cells cycles to pass from Delta-1 expression to neuronal birth. Thus, the delay in the production of neurons could be explained if Delta-1 were to drive NP cells of the PNTZ into neurogenic cell cycles after an intermediate cycle. This intermediate cell cycle might be required to rearrange the cell machinery of proliferating NP cells through the activity of possible mediators of neurogenic competence induced in response to DELTA1-NOTCH signalling.

Thus, our data fits with a model (Fig. 9) in which the single Delta-1 expressing NP cell divides into two new NP cells, which in turn divide in a neurogenic manner. Concomitantly, DELTA-1 activates lateral inhibition NOTCH signalling (as indicated by Hes5 expression) in the neighbouring NP cells of the PNTZ, which remain proliferating (self-replicating). Tis21 transcription has been
reported to begin in G1 and stops at the beginning of S-phase of mouse NP [44]. Our former results in the chick spinal cord are in agreement with this [46]. Thus, our observation that Tis21 is strongly induced 8 h (the approximate duration of one cell cycle) after Delta-1 expression, suggest that Tis21 expression begins after the Delta-1 NP divide. Thus, the resulting daughter NP cells will coexpress Delta-1 and Tis21. This fits with the high proportion of Delta-1/Tis21 double labelled cells in the PNTZ. Nevertheless, Delta-1 expression probably needs to be down regulated in these NP before they reach the NZ since in this region high levels of Delta-1 are detected in prospective neurons rather than in neurogenic progenitors [20]. Identifying the diverse cell populations that are involved in the sequential steps of the neurogenesis process is crucial to understanding the underlying molecular mechanisms. This goal has remained elusive by the intermingling of the diverse cell types in the neuroepithelium and the shortage of specific markers. Our results can provide some molecular markers that might help to discriminate among different progenitor pools in the developing CNS. For instance, it has been shown that some HES proteins are required for maintenance of the undifferentiated state of NP cells [59,65]. Thus, Hes5 expression could label self-replicating NP cells in this context. Similarly, Hes5 expression seems to identify self-replicating multipotent progenitors in the embryonic mouse nervous system [69]. Additionally, we propose that the expression of Delta-1 in single progenitors of preneurogenic neuroepithelium may identify NP cells that are switching from a proliferative to neurogenic state while the co-expression of Delta-1 and Tis21 may label those NP that are ready to begin to generate neurons.

It must be highlighted that the way in which DELTA1-Delta-1 NOTCH signalling regulates the switch from proliferative to neurogenic NP does not seem to occur through a standard lateral inhibition as demonstrated by the fact that the suppression of NOTCH signalling by DeltaDN induces neither the expression of Tis21 nor the production of neurons. Thus, in addition to its role as NOTCH ligand in maintaining the self-replicating state of the adjacent NP cells, our results indicate that Delta-1 may have a cell autonomous contribution to the switch to neurogenic NP cells as indicated by the cell autonomous induction of Tis21. Nevertheless, this process can not be regulated exclusively by cell autonomous effects of Delta-1 since inhibition of NOTCH signalling blocks the transition to neurogenic NP cells as indicated by the reversion of the neurogenic effect of Delta-1 by co-transfection with Delta-DN. Thus, NOTCH signalling is also required for this transition. Together, our results indicate that cell autonomous effects of Delta-1 act concomitantly with NOTCH signalling to regulate this transition.

The way how Delta-1 may act cell-autonomously in this context remains to be studied. Nevertheless, it is known that high level expression of NOTCH ligands can produce cell-autonomous inhibition of NOTCH signalling [36,70,71]. Interestingly, it has been found that Delta-like-3 promotes primary neurogenesis in Xenopus laevis by suppressing NOTCH signalling in a cell autonomous manner [72]. However, the effects of Delta-1 in the PNTZ can not be explained by a cell autonomous reduction of NOTCH signalling since we have found that the inhibition of NOTCH signalling in a cell-autonomous manner by DeltaDN does not yield neurons.

NOTCH ligands have been for long time considered unable to transmit signals in the cells where they are expressed. However, evidences supporting a signalling role of these ligands have recently been accumulating. For instance, it has been shown that ADAM protease and γ-secretase can release an intracellular domain of Delta, which can be localized in the nucleus [73–75]. Furthermore, the over-expression of this intracellular domain in cultured neural stem cells induced neurons [75]. Thus, these observations strongly suggest the involvement of DELTA-1 mediated signalling on neurogenesis and help to build a hypothesis for its possible implication on the transition from proliferative to neurogenic NP cells. Although, we have not approached here the molecular mechanisms underlying this signalling, we have identified Tis21 as a possible downstream mediator. The possibility that DELTA-1/NOTCH signalling triggers the switch from proliferative to neurogenic NP through activation of Tis21 is an attractive working hypothesis that is supported by the precocious increase in the production of neurons in transgenic mice overexpressing Tis21 [76].

In addition to unravelling this novel function of DELTA-1 NOTCH signalling in the PNTZ, our data suggest that the balance between neural proliferation and differentiation in the developing spinal cord is regulated by the sequential use of NOTCH signalling in three consecutive cellular contexts: proliferation of uncommitted progenitors, switch from proliferative to neurogenic NP cells, and neuronal differentiation (Fig. 9). It will be very interesting to uncover the molecular mechanisms that regulate DELTA/NOTCH signalling in these three sequential cellular domains and how they are coordinated within the overall process of neurogenesis.

**MATERIALS AND METHODS**

**Embryos**

Normal fertilized chicken eggs (Gallus domesticus) were incubated at 38°C until they had reached the desired stage [77]. In some experiments, mouse embryos of the ICR strain were used.

**In ovo electroporation**

Plasmid DNA (1–2 μg/ml) was injected into the neural tube of HH 10-12 chicken embryos. Two platinum electrodes were placed in parallel on either side of the neural tube, at a distance of 5mm from one another, and the embryos were pulsed 5 times (30–40 V/50 ms) using an Intrasept TSS10 pulse stimulator (Intracell). The DNA concentration and pulse voltage were adjusted depending on the desired transfection efficiency. After electroporation, the embryos were incubated at 38°C. Transfection efficiency was tested by in vivo observation of GFP or Fluorescein fluorescence under a microscope 4–12 h post-transfection. After further incubation, the embryos were either BrdU labelled or immediately fixed and processed for immunocytochemistry or FISH as described below.

We transfected a full coding sequence c-Delta-1 cDNA and a truncated version (DeltaDN) lacking all but 13 of the amino acids in the intracellular region [37,55] cloned into pCIG, a bicistronic vector that coexpresses nuclear GFP [78]. The intracellular domain of NOTCH (NICD) was cloned into the pEVRF vector [79]. In order to control the transfection efficiency, the GFP containing EGFPN1 vector (Clontech) was co-transfected together with pEVR-NICD.

**Posttranscriptional gene silencing of Delta-1**

We used two 23-mers fluorescein-labelled morpholino anti-sense oligos (Mo1-cDelta1, GGCTGACGAGGAGGAGCTCCTCCCAT and Mo2-cDelta1, GCGTTCTGACCCCCGTGTCTTCTCGTG). These are complementary to two non-overlapping segments of sequence located around the translation start site (NCBI, Gallus gallus genome, Chromosome 3, GeneID: 395820, NW_001471668, positions 1153691–1153811). Microinjection and focal electroporation of the morpholinos in HH10 chick embryos were carried out according to experimental conditions previously described in detail.

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DI1/N Switch to Neurogenesis

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by others [80]. Both anti-sense morpholinos interfered with Delta-1 function although Mo2-c-Delta1 was more efficient (not shown). As a control morpholino we used a 25-mer fluorescein-labeled morpholino having a sequence (CCT TCTATTGTTTGGAGTTTAA) of the mutated β-globin of human thalassemia patients that is the standard control morpholino used in chicken [80]. All morpholinos were purchased from GeneTools, LLC.

In situ Hybridisation and Immunocytochemistry

Chicken embryos were fixed with 4% paraformaldehyde for 3 hrs at RT. Whole mount in situ hybridization (ISH) with RNA probes for cDelta1, cHairy1 and cHairy2 was performed essentially as previously described [20,81]. The cHairy1 mRNA probe was prepared from a cDNA clone (C.chicken Hairy1 EST clone) that contains the Hairy1 sequence between 178–2179 bp (Accession number XM_417554). For the Ti21 RNA probe, a 709 bp fragment of an EST clone (ptr1.pk001.n8, University of Delaware Chick EST project) corresponding to bp 1–709 of the predicted Gallus gallus Ti21 sequence (Accession number XM_418053) was subcloned into plasmid SK+

Double fluorescent in situ hybridization (FISH) was carried with DIG- and fluorescein-labeled RNA, which were detected with the TSA Plus Fluorescence System (Perkin Elmer) following standard protocols.ISH was performed on whole mount embryos and 50 μm vibratome sections were then obtained to facilitate the immunocytochemical analysis. When combined with immunocytochemical detection of proteins, ISH was performed at lower temperature (52°C), lower salt concentration (SSC 1.3x), and pH = 5.0 or alternatively, using DNA probes that were generated by PCR using two templates of the cDelta1 cDNA (678–914 bp and 1085–1425 bp: Accession number XM_418053) was subcloned into plasmid SK+

Conditions for the use of antisera against GFP (Invitrogen), phosphorylated-histone H3 (PH3) and cyclin D (Upstate Biotechnology), cyclin B1 (clone V152, Abcam), p27KIP1 (clone 57, BD Transduction Laboratories), neuronal class III β-tubulin (TUJ1, Covance) were optimized. Cy2, Cy3, and Cy5-conjugated secondary antibodies were used as recommended by the supplier (Jackson Immunnochemicals Ltd). Images were acquired on a Leica TCS-SL spectral confocal microscope.

BrdU labelling and cell cycle analysis

Proliferating cells were detected in chick embryos by in ovo incorporation of BrdU. Thus, 50 μl of a 5 mg/ml solution of BrdU in PBS was applied to the top of the embryo after opening a window in the eggshell. After incubation (20 min–4 h), the embryos were fixed as described above. Immunodetection of BrdU labelled cells was carried out on 4 μm vibratome sections with anti-BrdU (Becton Dickinson) and detected with Cy3-conjugated secondary antibody. When ISH was combined with BrdU labelling, denaturation of DNA by treatment with 2 N HCl for 30 min was carried out after, DIG immunolabelling and before the subsequent BrdU immunodetection.

The analysis of expression of Delta-1 at the different stages of the cell cycle was based on the use of the appropriate markers and the differential apical-basal position of the nuclei. Thus, replication of DNA during S-phase takes place in the basal half of the neuroepithelium; during G2, the nuclei move towards the ventricular surface where mitosis takes place; during G1 nuclei move towards the basal region [29]. Mitotic cells were labelled with anti-phosphohistone-H3. Anti-cyclin D was used to detect cells in G1 phase. To assess the expression of Delta1 during the S-phase, embryos were exposed to a pulse of BrdU for a very short period of time (20 min) in order to avoid labelled cells moving to G2 by the end of the pulse. Accordingly, only cells in the basal half of the neuroepithelium were labelled under these conditions. Anti-cyclin B was used to label cells in G2 phase. Since Cyclin B is expressed in the cytoplasm during the S and G2-phases and it translocates into the nucleus during mitosis [82], statistical counts of double labelled cells focused only on cells containing cytoplasmic Cyclin B and being located within the apical third of the neuroepithelium where practically no BrdU-labelled cells were found after a short pulse. Cell counting was carried out over single optical confocal sections. The mean proportion of cells co-expressing specific genes was obtained for each embryo and the error calculated as the standard deviation. The statistical significance (P value) between experimental and control samples was determined using the Student’s t-test.

In vivo analysis of cell cycle duration was carried on HH10 chick embryos electroporated with either pCIG-Delta-1 or pCIG-Dl1/N Switch to Neurogenesis

The time required for GFP expressing cells to double their number was determined by counting labelled cells in the neural tube region between somites 12–13th with a Leica MZFLIII stereo microscope using a ×2 magnification objective. To make sure that our estimation of the increase in the number of GFP labelled cells with time was due to cell division and not to upregulation of GFP expression, the determination was started at 12 h post-transfection (i.e. 5 h from beginning of GFP expression). Also, in order to avoid dilution of GFP signal by cell division, cell counting was not carried after 24 h post-transfection.

SUPPORTING INFORMATION

Figure S1 Coexpression of Delta1 and cyclin D in early mouse neuroepithelium.

Found at: doi:10.1371/journal.pone.0001169.s001 (0.13 MB PDF)

Figure S2 Expression pattern of Hes genes in the developing caudal spinal cord of chick embryos.

Found at: doi:10.1371/journal.pone.0001169.s002 (0.07 MB PDF)

Figure S3 Control electroporation experiments.

Found at: doi:10.1371/journal.pone.0001169.s003 (0.52 MB PDF)

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Author Contributions

Conceived and designed the experiments: FT. Performed the experiments: FT. Analyzed the data: FT BH. Wrote the paper: FT.
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