Developmental Biology 430 (2017) 129–141

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Retinoic acid controls early neurogenesis in the developing mouse cerebral cortex

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ARTICLE INFO

Keywords:
Retinoic acid
Cerebral cortex
Neurons
Neuronal progenitors

ABSTRACT

A tight regulation of neuron production is required to generate a functional cerebral cortex and is achieved by a proper balance between proliferation and differentiation of progenitor cells. Though the vitamin A (retinol) active derivative retinoic acid (RA) has been implicated as one of the signals acting during mammalian forebrain neurogenesis, its function at the onset of neurogenesis as well as during establishment of cortical layers and neuronal subtypes remains elusive. One limitation is that murine mutants for genes encoding key enzymes involved in RA synthesis die during early embryonic development. We analysed corticogenesis in *Rdh10* null mutants, in which an RA deficiency is generated as the intracellular retinol to retinaldehyde conversion is abolished. When analysed at the latest stage before lethality occurs (embryonic day [E]13.5), the mutants show smaller telencephalic vesicles and the thickness of their cortical plate is strongly reduced. The first progenitors formed in the cortical plate are radial glial (RG) cells which generate neurons either directly, or through an indirect mechanism involving the production of intermediate neuronal progenitors (INPs) which then give rise to neurons. We show that in absence of RA, the RG progenitors proliferate less and prematurely produce neurons, leading to their depletion at E11.5. Furthermore, we could demonstrate that lack of RA impairs the generation of INPs at E13.5 and affects the cell cycle exit of progenitor cells during corticogenesis, altogether leading to a deficit in projection neurons and to microcephaly.

1. Introduction

During neural development, several pathways including fibroblast growth factors (FGFs), transforming growth factors β (TGFβ), Sonic hedgehog (Shh) and retinoic acid (RA) are interacting in order to elaborate from a simple neuroepithelial sheet the most anterior neural structure, the telencephalon. Its dorsal part will generate the cerebral cortex, the site of higher cognitive functions. Before the start of neurogenesis, the neuroepithelial (NE) cells divide exclusively symmetrically to expand this cell pool, leading to a lateral expansion of the cortex (Chenn and McConnell, 1995). With the onset of neurogenesis (at embryonic day 10.5 [E10.5] in the mouse), the NE cells turn into radial glial (RG) cells, that undergo symmetric proliferative divisions to amplify their pool in the ventricular zone (VZ) of the neuroepithelium (Götz and Hüttner, 2005). RG cells further divide asymmetrically to self-renew and generate either a postmitotic neuron (direct neurogenesis), or an intermediate neuronal progenitor (INP) cell (Götz and Hüttner, 2005; Miyata et al., 2010; Noctor et al., 2004). INP cells localise in the subventricular zone (SVZ), and divide abventricularly in a symmetric differentiative mode generating two postmitotic neurons (indirect neurogenesis).

The timing of the transition of RG cells generating neurons directly or indirectly through INPs is critical for proper forebrain development. A premature or delayed transition will affect the number of neurons, and eventually the cortical size, leading to smaller or larger brains (i.e., microcephaly or macrocephaly) (Caviness et al., 1995; Rakic, 1995). The mechanisms that regulate this transition are still incompletely understood. Ablation of Wnt/β-catenin signalling leads to a premature differentiation of RG cells, but also to an increase of INPs (Draganova et al., 2015; Mutch et al., 2010; Wrobel et al., 2007). Attenuation of Notch signalling in RG leads to premature differentiation into nascent INPs (Mizutani et al., 2007). Another study showed that the zinc finger transcription factor Insm1 promotes basal progenitor formation, as Insm1 ablation reduces the generation of INPs whereas its over-

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http://dx.doi.org/10.1016/j.ydbio.2017.08.006
Received 21 June 2017; Received in revised form 3 August 2017; Accepted 4 August 2017
Available online 05 August 2017
0012-1606/

expression increases their production (Farkas et al., 2008). Also, it has been shown that cortical neural progenitors express Robo1 and Robo2, receptors for Slit proteins that regulate axon guidance. The absence of these receptors or their ligands leads to an enhanced production of INPs in Robo1/2 and Slit1/2 mutants, suggesting that Slit/Robo signalling controls the transition to intermediate progenitors (Borrell et al., 2012). Furthermore, it has been shown recently that the loss of function of Elp3, an enzymatic subunit of the large macromolecular Elongator complex, favors direct neurogenesis at a time point where neurons should arise from INPs (Laguesse et al., 2015). On the other hand, while FGF (Kang et al., 2009) and Notch (Mizutani et al., 2007) signals inhibit the transition of RG cells to INPs, Shh (Komada et al., 2008) signalling induces INP amplification. Globally, the fate of progenitor cells is controlled by an interplay of numerous extrinsic and intrinsic signals (for review: Miyata et al., 2010).

In mice, the expansion phase holds until E10.5, when RG cells appear and the neurogenic phase can start. Among the various signalling molecules present from E8.5 until the beginning of neurogenesis, the active vitamin A (retinol) derivative retinoic acid (RA) directly promotes gene expression through binding and activation of nuclear receptors (RARs). This small lipophilic molecule is produced in a two-step oxidative reaction involving RDH10, the main retinol dehydrogenase present in the embryo (Cammas et al., 2007; Sandell et al., 2007), and retinaldehyde dehydrogenases (RALDH1, 2, 3) (for reviews: Duester, 2008; Rhinn and Dollé, 2012). During brain development, RALDH2 is the first RALDH to be expressed, starting at E8.5, followed by RALDH3 (Ribes et al., 2006). *Raldh2* $^{-/-}$ mutant mice are lethal at E9.5, preventing the generation of double *Raldh2*;*Raldh3* mutants and their analysis at the start of the neurogenic phase and later on. We have previously generated an *Rdh10* knockout allele that is lethal at midgestation, though some of the mutants escape early lethality and survive until E13.5 (Rhinn et al., 2011). This provides us with a model where we can ask about a potential role of RA during early corticogenesis, and analyse loss-of-function mutants until E13.5.

Using *Rdh10* $^{-/-}$ mutant mice, we have found that mutants have a reduced number of developing cortical projection neurons, resulting in a microcephalic phenotype. To understand the origin of the microcephaly, we analysed progenitor populations and their proliferating behaviour. We observed that at E11.5, RG cells proliferate less and undergo premature neurogenesis, suggesting that the absence of RA affects the size of the initial progenitor pool. Interestingly, we found that in absence of RA there is a strong depletion of INP cells at E13.5, suggesting a key function of RA during early cortical neurogenesis in regulating the transition phase of RG cells to INPs, and thus indirect neurogenesis. Globally, our findings provide evidence for a new role for RA in controlling early cortical neurogenesis.

2. Results

2.1. Rdh10 deletion leads to a reduction of radial glia progenitors

We previously generated an *Rdh10* loss of function allele by gene targeting, eliminating the catalytic domain (Rhinn et al., 2011). We showed that *Rdh10*-null (*Rdh10*$^{-/-}$) mutant embryos lack RA activity in the early forebrain, and that *Rdh10* loss of function is lethal shortly after midgestation (E10.5–11.5), with a small fraction of the mutants surviving until E13.5. The *Rdh10*$^{-/-}$ mutants obtained at E13.5 had severe external abnormalities including nasal/facial clefting, truncation of the nasal process and intracranial eye development (Rhinn et al., 2011). To determine the function of RA in early cortical development, we analysed *Rdh10*$^{-/-}$ mutants that survive at E11.5 (Fig. 1A, B). Remarkably, *Rdh10*$^{-/-}$ animals showed a reduced cortical thickness (Fig. 1C-D′, K, $p < 0.01$ rostrally and $p < 0.05$ more posteriorly). At this stage, the major populations of neural progenitor cells found in the developing cortex are radial glial (RG) cells localised in the ventricular zone (VZ) and which express Pax6 (Englund et al., 2005). Interestingly, the number of Pax6-positive RG progenitors was significantly decreased in *Rdh10*$^{-/-}$ mutants: it was diminished by 25% in the rostral cortex (Fig. 1E, F, L; $p < 0.01$) and more posteriorly by 20% (Fig. 1E′, F′, L; $p < 0.05$) when compared to controls. No abnormal apoptosis was detected in mutants at E11.5 (Supplementary material, Fig. S1C-D′, I-J′).

The reduced number of Pax6-expressing cells could be due to decreased cell proliferation, and/or premature differentiation. To determine whether impaired RA synthesis affects cell proliferation, we performed 1 h bromodeoxyuridine (BrdU) pulse labeling at E11.5. We quantified BrdU+ cells, corresponding to cells that were undergoing DNA replication at the time of BrdU injection. An abnormal proliferation rate was observed in *Rdh10*$^{-/-}$ mutants: quantification of BrdU+ cells over the total number of DAPI-positive cells showed a decrease by 20% (Fig. 1G-H′, M; $p < 0.01$ rostrally, $p < 0.001$ posteriorly) in comparison with controls. We quantified the fraction of Pax6-labelled BrdU+ cells (RG progenitors in S phase), and found a decrease by 20% (Fig. 1I-J′, N; $p < 0.001$ rostrally, $p < 0.01$ posteriorly) in *Rdh10*$^{-/-}$ embryos. These observations indicate an early role for RA in controlling the proliferation rate of cortical neural progenitors, more precisely in Pax6-positive RG progenitors.

2.2. Premature neurogenesis in Rdh10 mutants at E11.5

Next, we addressed if the loss of proliferating progenitors could be due to a change in the mode of cell cycle exit. For this purpose, we examined the ratio of cells that exit and reenter the cell cycle, by exposing control and *Rdh10*$^{-/-}$ embryos to a pulse of BrdU 18 h prior to analysis, which was performed at E10.5. Double immunolabeling for BrdU and Ki67, a marker for proliferating progenitors in all cell cycle phases except G2, allowed us to quantify BrdU+/Ki67- cells, corresponding to the cells that were dividing at the time of BrdU injection, but had presumably exited the cell cycle by the time of analysis. Altogether, this analysis revealed a significantly increased cell cycle exit rate: in controls 12% of cells presumably left the cell cycle, whereas in *Rdh10*$^{-/-}$ mutants 25% of the cells did so (Fig. 2A-D′, K; $p < 0.001$).

We further examined expression of the neuronal differentiation marker Tuj1 at E11.5, and found a markedly increased number of newborn neurons rostrally by 15% (Fig. 2E, F, L; $p < 0.05$) and more posteriorly by 35% (Fig. 2E′, F′, L; $p < 0.01$) in *Rdh10*$^{-/-}$ mutants compared to controls. Tbr1 immunostaining showed a co-labeling of the Tuj1+ supernumerary neurons, confirming the post-mitotic character of those cells (Fig. 2G-J′). Thus, premature differentiation occurring at this stage in the developing cortex contributes to deplete the pool of RG progenitors. Altogether, these data indicate a role of RA at E11.5 in the establishment of the pool of RG progenitors.

2.3. Rdh10 deletion affects neurogenesis, leading to microcephaly

To further dissect the effect of RA on cortical development, we focused our analysis on the cortex of *Rdh10*$^{-/-}$ mutants that survive until E13.5. The mutants obtained at this stage had severe external abnormalities, as described above at E11.5 (Rhinn et al., 2011). Remarkably, when dissected, the brains of *Rdh10*$^{-/-}$ mutants displayed smaller telencephalic vesicles compared to controls (Fig. 3A-D). Interestingly, by combining a loss of function of *Raldh3* and *Raldh2* using a conditional approach (see Section 4, and Haushalter et al., 2017), we observed a craniofacial and brain phenotype comparable to *Rdh10*$^{-/-}$ mutants at E13.5 (Supplementary material, Fig. S2A-E), indicating that the severe craniofacial abnormalities accompanied with smaller brains are due to a lack of RA production.

The observation of smaller neocortices in *Rdh10*$^{-/-}$ mutants suggests a possible role of RA in controlling neurogenesis. Remarkably, *Rdh10*$^{-/-}$ animals showed a reduced cortical thickness (Fig. 3E-F′, I; $p < 0.05$ rostrally, $p < 0.01$ posteriorly). Interestingly, immunostaining using Tuj1 showed a markedly reduced thickness of the intermediate