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Abstract: Thyroid hormone influences brain maturation through interaction with nuclear receptors and regulation of gene expression. Their role on astrocyte maturation remains unclear. We have analyzed the role of thyroid hormone in rat cerebellar astrocyte maturation by comparing the sequential patterns of intermediate filament expression in normal and hypothyroid animals. During normal development astroglial cells sequentially express nestin, vimentin, and glial fibrillary acidic protein. Differentiated astrocytes appeared in the superior medullary vellum by postnatal day 2 and reached the white mater and internal granular layer by postnatal day 4. Intermediate filament marker expression was transiently lost from postnatal day 6 to 8 in anterior lobes, without an increased apoptosis. Vimentin expression was replaced by glial fibrillary acidic protein between postnatal days 10 and 32. The differentiated astrocytes were evenly distributed throughout the cerebellar slices, including the internal granular layer. Differences between normal and hypothyroid rats were observed starting from postnatal day 4, with lack of differentiated astrocytes in the

internal granular layer. The transient decrease of astrocyte markers immunoreactivity in the anterior lobe did not take place in hypothyroid rats. The vimentin-glial fibrillary acidic protein transition was delayed and most differentiated astrocytes remained confined to the white matter. The results indicate that thyroid hormone deficiency induces a delay and a partial arrest of astrocyte differentiation. Astrocytes express thyroid hormone receptor alpha and beta subtypes suggesting that astrocytes are direct target cells of thyroid hormones.

*** Response to Reviewers**

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Answer to reviewers:

Reviewer #1
We thank the reviewer for his/her positive and supportive coments

We included the data of Notch1 expression in hypothyroid rats, as requested, in Figure 3: there are no changes in Notch expression in the anterior lobes at P6, as occurs in normal rats, supporting the hypothesis that the Notch pathway may be involved in the extensive reprogramming taking place at this age.
We have also corrected all the spelling and writing mistakes.

Reviewer #2:

We thank the reviewer for his/her comments. We are hesitant to shorten the manuscript, if it is not a major requirement, because we are afraid of losing clarity.

Influence of thyroid hormones on maturation of rat cerebellar astrocytes

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Running title: Astrocytes in cerebellar development

Abstract

Thyroid hormone influences brain maturation through interaction with nuclear receptors and regulation of gene expression. Their role on astrocyte maturation remains unclear. We have analyzed the role of thyroid hormone in rat cerebellar astrocyte maturation by comparing the sequential patterns of intermediate filament expression in normal and hypothyroid animals. During normal development astroglial cells sequentially express nestin, vimentin, and glial fibrillary acidic protein. Differentiated astrocytes appeared in the superior medullary vellum by postnatal day 2 and reached the white mater and internal granular layer by postnatal day 4. Intermediate filament marker expression was transiently lost from postnatal day 6 to 8 in anterior lobes, without an increased apoptosis. Vimentin expression was replaced by glial fibrillary acidic protein between postnatal days 10 and 32. The differentiated astrocytes were evenly distributed throughout the cerebellar slices, including the internal granular layer. Differences between normal and hypothyroid rats were observed starting from postnatal day 4, with lack of differentiated astrocytes in the internal granular layer. The transient decrease of astrocyte markers immunoreactivity in the anterior lobe did not take place in hypothyroid rats. The vimentin-glial fibrillary acidic protein transition was delayed and most differentiated astrocytes remained confined to the white matter. The results indicate that thyroid hormone deficiency induces a delay and a partial arrest of astrocyte differentiation. Astrocytes express thyroid hormone receptor α and β subtypes suggesting that astrocytes are direct target cells of thyroid hormones.

Introduction

Normal brain development depends upon an adequate supply of thyroid hormones. Thyroid hormones have multiple actions on the developing brain, including effects on cell migration and differentiation (Bernal, 2005). For example some neurons in the neocortex do not reach their normal positions in the absence of thyroid hormone (Lavado-Autric *et al.*, 2003). Also, hypothyroidism delays migration of cerebellar granule cells from the external germinal layer to the internal granular layer (igl). The effects of thyroid hormone on cell differentiation are of great importance during myelination. Proper oligodendrocyte differentiation requires thyroid hormone in addition to other factors, and in hypothyroidism there are delays in myelination which result in a permanent lower number of myelinated axons (Berbel *et al.*, 1994; Schoonover *et al.*, 2004). Specific populations of neurons are very sensitive to the lack of thyroid hormone with strong deficits of maturation. For example, the Purkinje cells of the cerebellum, in which the lack of thyroid hormone results in arrested maturation with underdeveloped dendritic tree (Legrand, 1984). Not only neurons and oligodendrocytes are sensitive to thyroid hormones, but also astroglia and microglia are altered when hypothyroidism is induced early in development (Lima *et al.*, 1998; Lima *et al.*, 2001).

The specific effects of thyroid hormone on astrocyte maturation *in vivo* are not well known. Early hypo or hyperthyroidism affect the astroglial cell population of the cerebellum (Clos *et al.*, 1980), thyroid hormones influence the expression of astroglial genes (Farwell and Dubord-Tomasetti, 1999; Alvarez-Dolado et al., 2000), and in cultured astrocytes thyroxine influences actin polymerization and integrin–laminin

interactions (Farwell *et al.*, 1995). The active thyroid hormone, T3 acts through nuclear receptors regulating gene expression. However the role of T3 receptors as mediators of thyroid hormone actions in astrocytes is unclear because whether or not astrocytes express thyroid hormone receptors has not been established (Leonard *et al.*, 1994; Carlson *et al.*, 1996). In a previous work we showed that null mutant mice for the thyroid hormone receptor α1 (TRα1) isoform display an aberrant pattern of astrocyte maturation which was normalized after induction of hypothyroidism (Morte *et al.*, 2004). Thyroid hormone was detrimental for astrocyte maturation in the absence of TRα1, indicating that proper astrocyte maturation requires a balance of opposing effects from both T3 receptor genes, TRα and TRβ. To provide a framework that could enable the interpretation of these data, and to clarify the role of thyroid hormone receptors on astrocyte maturation *in vivo*, we have compared the pattern of maturation of cerebellar astrocytes during postnatal development in normal rats and in rats made hypothyroid from embryonic stages. The results indicate that thyroid hormone deficiency induces a delay and a partial arrest of astrocyte differentiation, and that the actions of thyroid hormone are likely exerted through T3 receptors present in astrocytes.

Materials and Methods

Animals and treatment

Rats from the Wistar strain grown in our animal facilities were used. The animals were handled according to the European Communities Council Directive of 24 November 1986 (86/609/EEC), and the studies were approved by the Ethical Committee of the Spanish Council for Scientific Research (CSIC). Animals were under temperature (22 ± 2 ºC) and light (12:12 light-dark cycle; lights on at 7 a.m.) controlled conditions and had free access to food and water. To induce fetal and neonatal hypothyroidism, the drinking water of the dams was made 0.02% methyl-mercapto-imidazol (Sigma Chemical Co, St Louis Mo) and 1% $KClO_4$, which was given from the 9th day after conception and throughout the experimental period. At least four male rats, from different litters and for each condition were examined. The animals were sacrificed as indicated for each experiment.

Tissue processing.

Animals were perfused transcardially with buffered 4% paraformaldehyde, the brains were cryoprotected, and 25-µm sagital sections were obtained in a cryostat and processed as described in detail (Bernal and Guadaño-Ferraz, 2002).

Immunohistochemistry.

Immunohistochemistry was performed on free floating sections following the "ABC" immunoperoxidase procedure (Vector Laboratories, Burlingame, CA). The primary antibodies were monoclonal antibodies against nestin (diluted

1:7500,(McManus *et al.*, 1999)), vimentin (diluted 1:200, Dako, Glostrup, Denmark) and notch1 (diluted 1:200, Santa Cruz Biotechnology, Inc. Santa Cruz, CA.), and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, diluted 1:2000, Dako, Glostrup, Denmark). The secondary antibodies were a biotinylated horse anti-mouse or goat anti-rabbit (Vector laboratories) used at a 1:200 dilution. Peroxidase was visualized with diaminobenzidine (0.05%) and H_2O_2. For microscopy we used a Nikon Eclipse E400 optical microscope equipped with a Nikon DN100 digital camera.

For immunofluorescence, the slices were incubated with monoclonal anti-vimentin (diluted 1:200) and with rabbit anti-GFAP (diluted1:2000), followed by incubation with biotinylated horse anti-mouse. The slices were then incubated with Alexa Fluor® 594 goat anti-rabbit and streptavidin Alexa Fluor® 488 (Molecular probes, Invitrogen, Carlsbad, CA, USA). For microscopy we used a Leica TCS SP2 confocal microscope (Leica Microsystems GmBH, Wetzlar , Germany).

Double immunofluorescence and *in situ* hybridization.

In situ hybridization on free floating sections was performed as described in detail (Bernal and Guadaño-Ferraz, 2002). The riboprobes were made from templates encoding 1161-1428 nucleotides of mouse TRα1 cDNA and 21-329 nucleotides of mouse TRβ1 cDNA. Hybridization was performed with digoxigenin-labeled riboprobes at a final concentration of 200 ng/ml.

For cellular colocalization studies the slices were incubated overnight with an anti-digoxigenin (1:400) (Roche Diagnostics, Mannheim, Germany) and monoclonal anti-GFAP (1:2000) antibodies. The sections were then incubated with a biotinylated horse anti-mouse at a 1:200 dilution (Vector Laboratories, Burlingame, CA) followed by a

incubation with Alexa Fluor® 594 goat anti-rabbit and streptavidin Alexa Fluor® 488 (Molecular probes, Invitrogen, Carlsbad, CA, USA).

Apoptosis assay

Apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labelling (TUNEL) assay using the Boehringer Mannheim cell death detection kit according to the manufacturer´s protocol.

Results

To assess the role of thyroid hormones during normal astrocyte maturation *in vivo*, we compared the maturational patterns of astrocytes from normal and hypothyroid rats during the postnatal period in the cerebellum. Maturing astrocytes sequentially express the intermediate filaments nestin, vimentin, and GFAP (Bovolenta et al., 1984; Kalman and Ajtai, 2001). Nestin is an intermediate filament expressed in immature precursor cells which may give rise to either neurons or glial cells. Once committed to the astroglial lineage, nestin is replaced in immature astrocytes by another intermediate filament, vimentin. Terminal differentiated astrocytes finally express GFAP. During maturation there is overlapping expression of these proteins, such that immature astrocytes may coexpress nestin and vimentin, and more mature astrocytes coexpress vimentin and GFAP. Therefore, we examined the effects of thyroid hormone deprivation on the sequential pattern of expression of vimentin and GFAP using confocal microcopy to assess the extent of coexpression of both differentiation markers.

A representative result of this kind of experiments is illustrated in figure 1. GFAP was detected using an antibody labelled with Alexa 594, which gives a red color, whereas vimentin was detected with Alexa 488, giving a green color. Slices were examined from rats at P10, P16, and P32. At P10 vimentin expression was present throughout the slice in normal animals (C), with many cells of astroglial morphology labelled in the white matter (wm) and in the igl. Bergmann processes (Bg) were also labelled. Most cells expressed GFAP, although there were also many cells that expressed only vimentin. In contrast, very few astrocytes expressed GFAP only. Expression of either vimentin or GFAP was greatly reduced in the hypothyroid animals

(H), and astrocytes were present mainly in the white matter (white arrows). The Bergmann processes were also stained as in control animals.

On P16, slices from normal animals showed a greatly reduced expression of vimentin and there were already many GFAP+ astrocytes, especially protoplasmic astrocytes of the igl. GFAP+ astrocytes were distributed homogeneously throughout the slice. In hypothyroid animals GFAP and vimentin expression increased in the white matter, and no GFAP+ cells were present in the igl. On P32, practically all astrocytes in the slices from normal rats were differentiated, according to expression of GFAP. Vimentin expression remained in Bergmann processes. In hypothyroid animals there were scattered astrocytes in the igl and in the white matter that still expressed vimentin. Vimentin expression was decreased in the white matter respect to P16. In the white matter there were also some GFAP+ cells that were not observed in the controls animals. Vimentin and GFAP coexpression was observed in the white matter and in some cells in the IGL. The results of this experiment clearly showed that hypothyroidism induced a strong delay, and a partial arrest of astrocyte differentiation. In hypothyroid rats the differentiated astrocytes remained confined to the white matter with few cells populating the igl.

Given that under hypothyroid conditions astrocytes were already altered at P10, we compared the pattern of expression of nestin, as a marker expressed in precursors, and the terminal differentiation marker, GFAP in normal and hypothyroid cerebella from P0 to P8. The result is shown in figure 2A. From P0 to P4 there were no apparent differences in the pattern of nestin staining between control and hypothyroid rats. Nestin was expressed in cells scattered throughout the igl, Golgi epithelial cells and their Bergmann processes (Fig 2B). Concerning GFAP expression, there were GFAP+

astrocytes in the posterior lobes of normal and hypothyroid animals as early as P0. At P2, differentiated astrocytes were clearly present in the superior medullary vellum from were they reach the white matter and the igl by P4 as shown in figure 2B. The Bergmann processes were also GFAP+. Differences between normal and hypothyroid animals were observed already at P4: GFAP+ cells were present in the igl from normal animals (2B), whereas in hypothyroid animals they were present mainly in the white matter.

From P6 onwards the differentiation wave reached the white matter in normal animals as could be observed for the increase staining of GFAP in this area. Surprisingly, at P6 there was a strong loss of immunoreactivity for nestin and GFAP taking place in the anterior lobes of normal animals. Immunostaining resumed again at P8. In contrast, this decreased staining was delayed in the sections from hypothyroid rats, and was not as strong as in the normal animals. Loss of antigen expression in the anterior lobes of normal rat sections was also observed by using a vimentin antibody and an alternative nestin monoclonal antibody (results not shown), which argues against an artefact of the immunohistochemistry.

As shown in panel 2B, at P6 nestin staining in controls animals appeared on the Bergman processes, whereas in the igl only blood vessels were stained. GFAP was absent throughout the slice, but not in the hypothyroid condition where the igl was populated by GFAP+ cells. At P8 nestin+ cells appeared in the igl, whereas GFAP+ cells were present in the white matter and in the igl. The hypothyroid rat presented a homogeneous staining for nestin at P6 and P8, but the cell staining was weaker than in the controls. At P8 the GFAP+ cells were localized in the white matter and there were less positive cells in the igl than in the controls.

The Notch pathway has been implicated in differentiation of astrocytes. Notch1 may play an instructive role in promoting glial development. Notch1 stained cells were present mainly in the Purkinje cell layer and in the IGL (Fig 3). Interestingly, Notch1 staining was also almost absent in anterior lobules of P6 cerebella from normal rats in parallel to the decreased staining of intermediate filaments described above. These changes were absent from the posterior lobes. In contrast to normal rats, no changes in Notch1 expression were observed at P6 in hypothyroid rats.

To check whether the loss of intermediate filament antigens was due to extensive cell death we performed a TUNEL assay for programmed cell death. Fig 4 shows the anterior and central lobe (C and D respectively) of a control P6 animal. In the anterior lobe few TUNEL positive cells were detected, with even less apoptotic cells than in the central lobe. Panels A and B represent the positive and negative controls, respectively, of the assay. We also performed the TUNEL assay at P4 and P5 with the same result. This experiment rules out the possibility that loss of antigens was due to massive astrocytic death. In addition, Nissl staining of the sections did not reveal any decrease in cell density from the anterior or the posterior lobules (not shown).

To check whether astrocytes express T3 receptors we performed a double fluorescence in situ hybridization-immunohistochemistry analysis using probes for the TRα1 and TRβ1 receptor subtypes. The probes were labelled with digoxigenin, and the in situ hybridization was followed by immunohistochemistry using anti-digoxigenin and GFAP antibodies to co-localize the receptor mRNA signal (green) on GFAP+ cells (red) in the granular cell layer. The results shown in fig 5 demonstrate that differentiated astrocytes in vivo express both, TRα1 and TRβ1. The receptor signal gave a granular appearance, in contrast to the GFAP stain which was diffuse in the cytoplasm. Therefore, within a single astroglial cell there was not an exact co-localization of both

markers. However, the receptor signal delimited cell shapes corresponding to the GFAP ones. The green colour observed in the background most likely corresponds to receptor present in the granular cells.

DISCUSION

In this paper we show that absence of thyroid hormones during the postnatal period in the rat delays astrocyte maturation as assessed by the sequential expression of intermediate filament proteins. The transition vimentin-GFAP was delayed in hypothyroid rat cerebella. In addition there was evidence for differential effects of hypothyroidism on the patterns of differentiation of the two major subpopulations of astrocytes. Fibrous astrocytes, which are present in the white matter, showed an increased vimentin and GFAP staining in hypothyroid rats compared to control rats. On the other hand, protoplasmic astrocytes, which are located mainly in the igl, showed a decreased staining at all ages. At P16, for example, the contrast between vimentin staining in normal and hypothyroid rats illustrates this observation. Other studies have also addressed the role of thyroid hormone on astrocyte maturation. Thyroid hormone regulates the number of astrocytes in vivo, and also the maturation of Golgi epithelial cells in the rat cerebellum (Nicholson and Altman, 1972). In culture, T3 stimulates astrocyte proliferation and affects GFAP expression and organization (Trentin *et al.*, 1995; Lima *et al.*, 1998).

The cellular basis for the actions of thyroid hormone on astrocytic maturation remains mostly unknown. A most basic question is whether thyroid hormone acts through nuclear receptors present in astrocytes, or whether its effects are mediated by indirect interactions with other cells, or even by non genomic actions of the hormone. The presence or absence of T3 receptors in astrocytes has been debated and the issue not entirely settled. Estimations of T3 binding to isolated nuclei showed that astrocytes contained about 200 sites per nucleus, whereas neurons have around 5000 sites / nucleus (Kolodny *et al.*, 1985a; Kolodny *et al.*, 1985b). Regarding expression of

individual receptor isoforms, astrocytes in primary cultures from cerebral hemispheres express TRα and TRβ (Lebel *et al.*, 1993; Carlson *et al.*, 1996) but other studies showed expression of TRα1 but not TRβ1 (Leonard *et al.*, 1994). We have previously analyzed astrocyte maturation in TRα1$^{-/-}$ mice and found an aberrant pattern of expression of intermediate filament antigens (Morte *et al.*, 2004). Surprisingly, thyroid hormone deficiency in these mice largely corrected the observed alterations in TRα1$^{-/-}$ euthyroid mice, but treatment with the TRβ agonist GC-1 greatly arrested astrocyte differentiation. The results suggested that thyroid hormone could act on astrocyte differentiation by opposing pathways through TRα1 and TRβ, and that proper differentiation requires a balance of the two receptors. This conclusion is strengthened by our finding that astrocytes express both, TRα1 and TRβ1 mRNAs.

One of the major problems to interpret the actions of thyroid hormone on astrocyte maturation is that relatively little is known on this process *in vivo*. In the process of generation of mature myelinating oligodendrocytes, glial precursors arise in the subependymal layers of the IV ventricle and migrate to their final positions in the cerebellum mainly through the superior medullary vellum. Then cells migrate from the base of the cerebellum up to the white matter and then from this central position into the granular cell layer (Reynolds and Wilkin, 1988). A similar pattern of astroglial differentiation was observed in this study. Most likely, the lack of thyroid hormone interferes with the progression of astroglial differentiation from the central position in the lobule to the internal granular layer. Therefore, hypothyroid animals show an increased microfilament expression in the white matter and a decreased in the granular layer.

A previously undescribed phenomenon is the disappearance of filament staining around P6 in anterior lobules of normal animals. We do not know the reasons for this

phenomenon, but apparently massive cell death is not an explanation. Intensive cell death has been previously described in normal cerebellum (Krueger *et al.*, 1995), but we did not observed an increased apoptosis, or a decreased total cellularity in Nissl-stained sections. Therefore our provisional interpretation of this phenomenon is that there is extensive reprogramming of cell fate at this age, which may be related to the appearance of differentiated oligodendrocytes.

Notch and its ligands Delta and Jagged are involved in neuronal and glial cell differentiation through complex processes involving cell to cell contact (Gaiano and Fishell, 2002). It is thought that Notch actively promotes differentiation of astroglial cells in the forebrain and cerebellum. Conditional ablation of Notch1 in cells of the midbrain-hindbrain region results in premature onset of neurogenesis and a decrease in glial cell number (Lutolf *et al.*, 2002). As previously reported, we found that in the cerebellum Notch is expressed in Purkinje cells and in cells of the internal granular layer, presumably immature astrocytes and oligodendrocytes (Stump *et al.*, 2002). Moreover primary cultures of astrocytes show that GFAP and nestin+ cells express Notch1 (Irvin *et al.*, 2001). The concordance in the pattern of expression of intermediates filament of astrocytes and Notch1, and the essential action of Notch in promoting glial linage, suggests that the Notch pathway is involved in the disappearance of astrocyte microfilament markers at P6. This is also supported by the lack of changes of Notch1 expression in the anterior lobes of hypothyroid rats.

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Abbreviations: GFAP: glial fibrillary acidic protein; igl: internal granular layer**;** TR: thyroid hormone receptor

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Figure legends

Fig 1:

Colocalization of GFAP (red) and vimentin (green) in lobe VI of the cerebellum from normal and hypothyroid rats on P10, P16 and P32. Arrows point to the white matter; igl, internal granular layer; Bg, Bergmann processes; wm, white matter; C, normal rats; H, hypothyroid rats. Scale bar: 40µm.

Fig 2:

GFAP and nestin staining of cerebellar slices on P0, P2, P4, P6 and P8 of normal and hypothyroid rats. Panel 2A shows the entire slice and panel 2B shows a higher magnification of the square area shown on the lobule. C, normal rats; H, hypothyroid rats; smv, superior medullary vellum; Bg, Bergmann processes; igl, internal granular layer; ant, anterior lobules; post, posterior lobules. Scale bars: Panel 2A: 1,5mm; panel 2B: 20µm.

Fig 3:

Notch staining of normal and hypothyroid cerebellar slices on P4, P6 and P8. The figure shows the anterior (C-Ant) and posterior (C-Post) lobules of normal control rats and the anterior lobules of hypothyroid rats (H-Ant). egl, external granular layer; Pl, Purkinje layer; igl, internal granular layer. Scale bar: 40µm.

Fig 4:

TUNEL assay of cerebellar slices of P6 normal rats. The positive controls (A) correspond to a slice previously treated with DNAse. The negative controls (B)

correspond to a slice in which the assay was performed without terminal transferase. (C) anterior lobule; (D) central lobule. Scale bars: upper panels 40µm, lower panels 20µm.

Fig 5:

Colocalization assay of GFAP and TRα1 and TRβ1 mRNAs. The receptors were detected by in situ hybridization using digoxygenin-labelled riboprobes followed by immunohistochemistry with anti digoxygenin antibodies. The TRs present a punctuate pattern in green. GFAP was detected by immunohistochemistry (red). Scale bar: 8µm.

Figure 1

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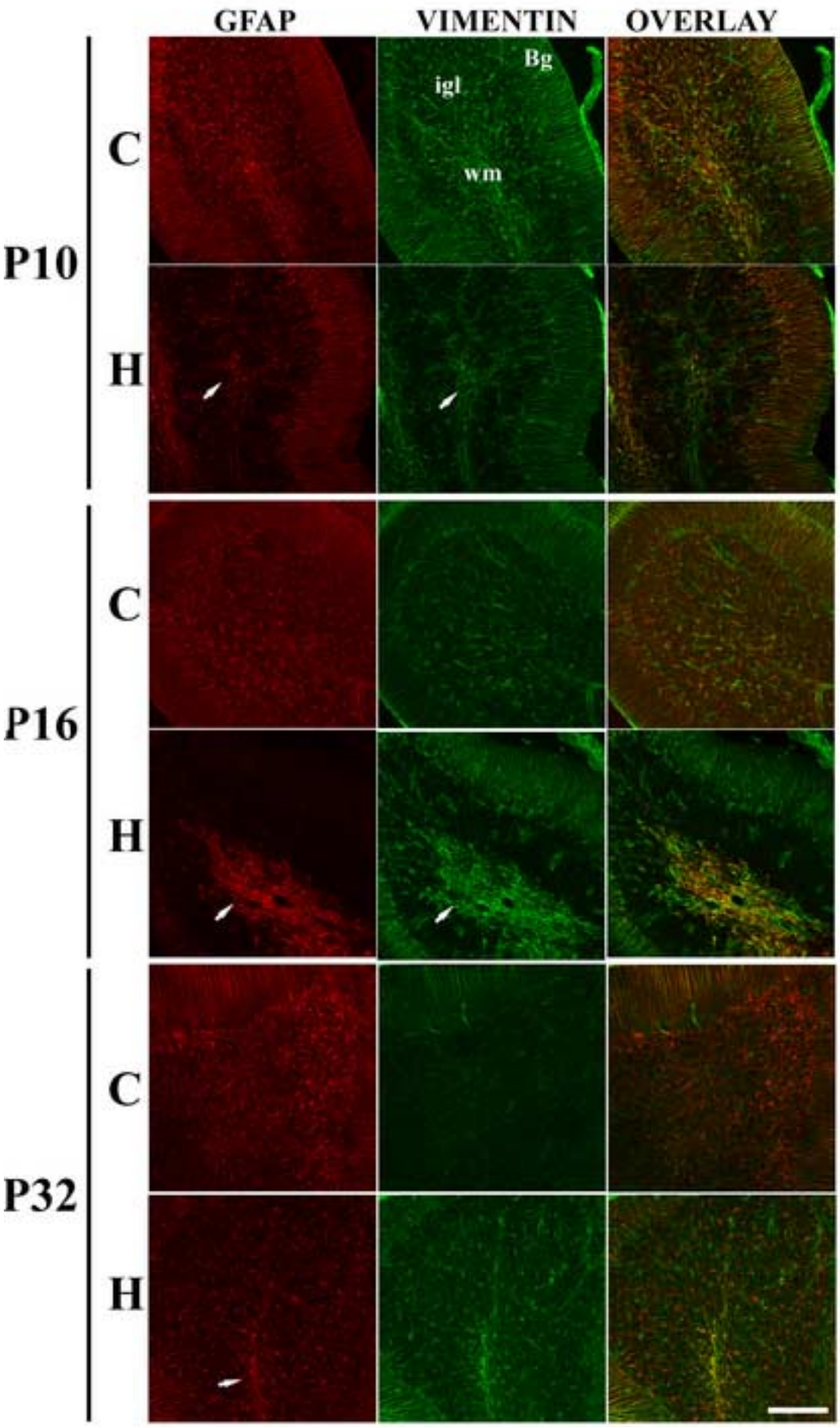

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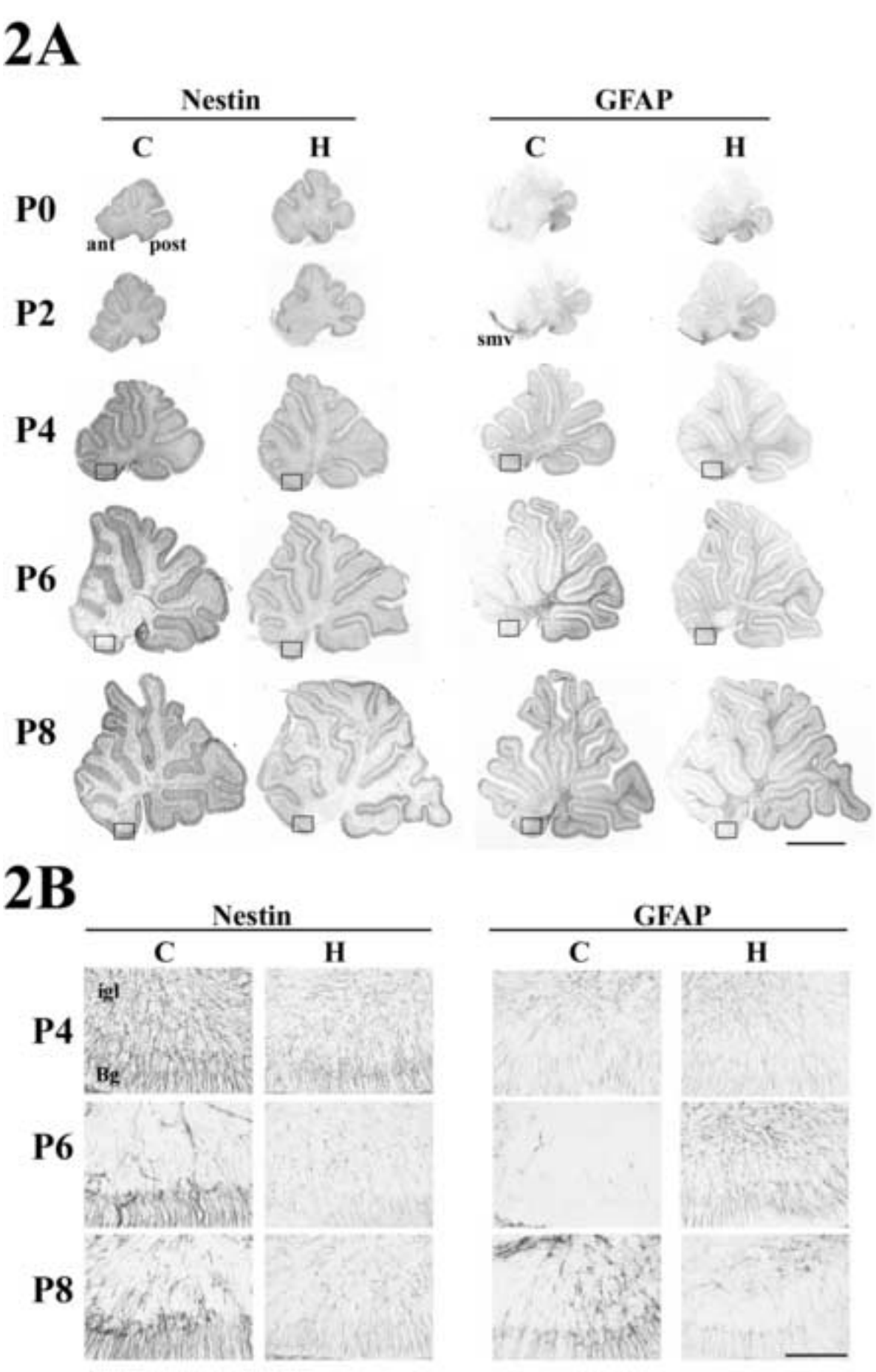

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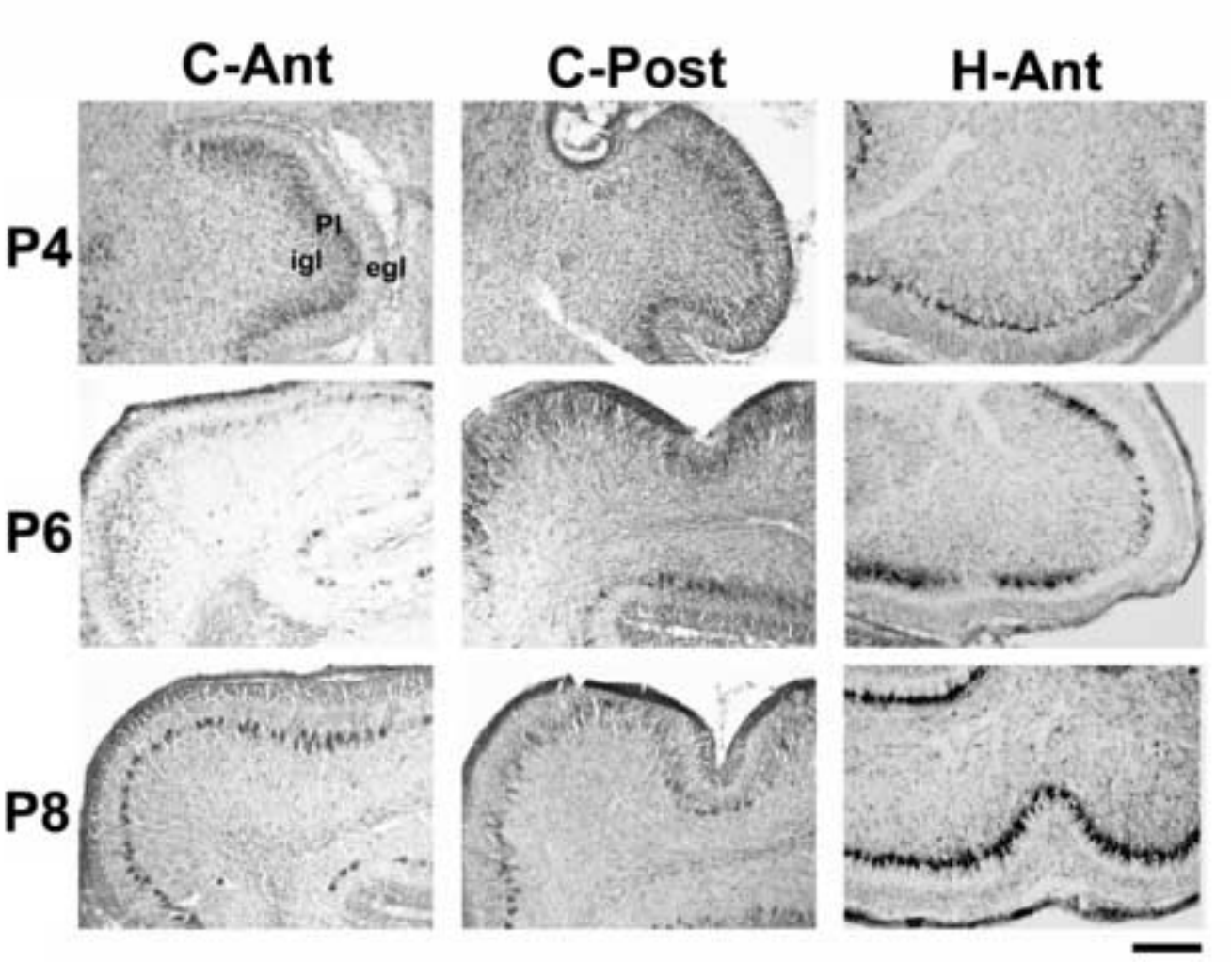

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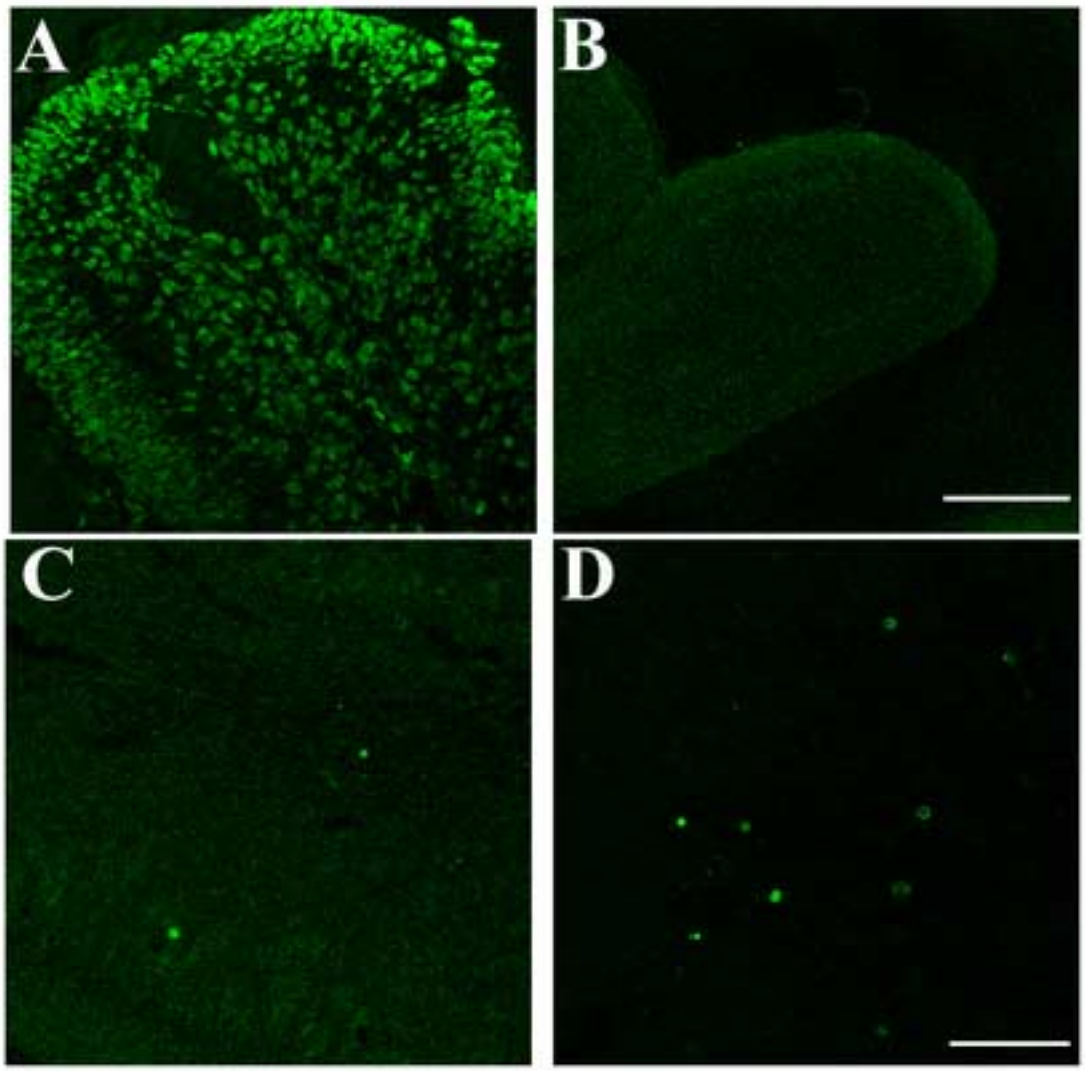

Figure 5

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