Neuropeptides 45 (2011) 69–76

Contents lists available at ScienceDirect

Neuropeptides

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

Purkinje cells express Angiotensin II AT_2 receptors at different developmental stages

María E. Arce [a], Susana I. Sánchez [a], Francisco López Aguilera [b], Leonardo R. Seguin [a], Alicia M. Seltzer [b], Gladys M. Ciuffo [a,*]

[a] IMIBIO-SL CONICET, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Ejército de los Andes 950, San Luis, Argentina
[b] Laboratorio de Neurobiología, Instituto de Histología y Embriología IHEM-CONICET, FCM Universidad Nacional de Cuyo, Mendoza, Argentina

ARTICLE INFO

Article history:
Received 7 August 2010
Accepted 15 November 2010
Available online 10 December 2010

Keywords:
Purkinje cells
AT_2 receptors
Cerebellum development
Zebrin II
Molecular markers

ABSTRACT

Angiotensin II (Ang II) binds and activates two major receptors subtypes, namely AT_1 and AT_2. In the fetus, AT_2 receptors predominate in all tissues and decline shortly after birth, being restricted to a few organs including brain. Interpretation of the function of Ang II in the cerebellum requires a thorough understanding of the localization of Ang II receptors. The aim of the present paper is to evaluate the localization of Ang II AT_2 receptors in the Purkinje cell (PC) layer during development. By binding autoradiography, a clear complementary pattern of AT_1 and AT_2 binding labeled by [^{125}I] Ang II was observed in young rats within the cerebellar cortex. This pattern was present at the stages P8 and P15, but not at P30 and P60, where AT_2 binding appears low and superimposed with AT_1 binding. We demonstrate that AT_2 antibodies recognized postmitotic Purkinje cells, labeling the somata of these cells at all the stages studied, from P8 to P60, suggesting that PCs express these receptors from early stages of development until adulthood. In P8 and P15 animals, we observed a clear correspondence between immunolabeling and the well-defined layer observed by binding autoradiography. Confocal analysis allowed us to discard the co-localization of AT_2 receptors with glial fibrillary acidic protein (GFAP), a glial marker. Double immunolabeling allowed us to demonstrate the co-localization of Ang II AT_2 receptors with zebrin II, a specific PC marker. Since PCs are the sole output signal from the cerebellar cortex and considering the role of cerebellum in movement control, the specific receptor localization suggests a potential role for Ang II AT_2 receptors in the cerebellar function.

1. Introduction

Angiotensin II (Ang II), the primary effector of the renin–angiotensin system (RAS), plays a key role in the regulation of blood pressure, body fluid and electrolyte homeostasis. Ang II acts on two pharmacologically distinct receptors, AT_1 and AT_2, identified with selective antagonists (de Gasparo et al., 2000; Paul et al., 2006). Most of the known central effects of Ang II are mediated by AT_1 receptors. In the fetus, AT_2 receptors predominate in all tissues and decline shortly after birth, being restricted to a few organs including brain (Saavedra, 2005; Gallinat et al., 2000).

Mice with targeted disruption of AT_2 receptors showed attenuation of exploratory behavior and movement, impaired drinking responses and hypersensitivity to Ang II on blood pressure and sodium excretion (Ichiki et al., 1995; Hein et al., 1995; Okuyama et al., 1999). These mice also exhibited impaired spatial memory and altered dendritic morphology (Maul et al., 2008). In humans, X-linked mental retardation has been associated with Agtr2 gene mutations (Vervoort et al., 2002).

The cerebellum consists of a highly organized set of folia that, in rodents, are largely generated postnatally, providing a convenient model to study ongoing developmental processes (Altman, 1972; Swinny et al., 2005). Purkinje cells (PCs), one of the major types of neurons of the cerebellum, exhibit considerable outgrowth and dendrite differentiation during the first 3 postnatal weeks (Sotelo and Dusart, 2009). A combination of cell markers and transcription factors, allowed to establish a differential origin for precursors of the different cell types that populate the cerebellum (Morales and Hatten, 2006).

In addition, the mammalian cerebellar cortex is highly compartmentalized into a complex array of transverse zones and parasagittal stripes revealed by molecular markers such as the sphingosine kinase (SPHK) 1a isoform (Terada et al., 2004) and phospholipase C (PLC) β3 and β4 (Sarna et al., 2006). The most extensively studied compartmentation antigen is zebrin II (Brochu et al., 1990; Hawkes, 1997) expressed by PCs.

Both Ang II AT_1 and AT_2 receptors have been described in cerebellum of young rats (Reagan et al., 1994; Jöhren and Saavedra, 1996; Arce et al., 2001). In 2-week-old animals, AT_2 receptors and its mRNA are discretely localized in neurons of the inferior olive (IO). Chemical lesion of the IO in 2-week-old rats decreased

* Corresponding author. Tel.: +54 2652 424027x180; fax: +54 2652 422644.
E-mail address: gciuffo@unsl.edu.ar (G.M. Ciuffo).

0143-4179/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.npep.2010.11.002

binding to AT_2 receptors and its mRNA levels in the IO and AT_2 receptor binding in the molecular layer of the cerebellar cortex (Jöhren et al., 1998). At present, data demonstrating the subtype receptor localization within the cerebellum remain controversial (Reagan et al., 1994; Jöhren et al., 1998; Arce et al., 2001). AT_2 mRNA expression during fetal and neonatal brain development was studied from 11 days of gestation to postnatal day 28 (Nuyt et al., 1999). Studies conducted in cell lines support the role of AT_2 receptor in neurite elongation, neuron migration, neuronal death and the establishment and maintenance of synaptic connections (Laflamme et al., 1996; Côté et al., 1999; Gallinat et al., 2000; Gendron et al., 2003; Kilian et al., 2008; Guimond et al., 2010). In adult cerebellar cortex, only recently it has been recognized the presence of Ang II AT_2 receptors (Fogarty and Matute, 2001).

In a previous study in 2-week-old rat hindbrain by autoradiography (Arce et al., 2001) we identified AT_2 binding in the cerebellar cortex, cerebellar nuclei, peduncles, and several brainstem nuclei related to either sensory or motor control activity. A clear complementary pattern of AT_1 and AT_2 binding was observed on adjacent layers within the cerebellar cortex, labeled by [^{125}I] Ang II. Interpretation of the function(s) of Ang II in cerebellum requires a thorough understanding about the localization of Ang II receptors. In the present paper, we demonstrate the co-localization of Ang II AT_2 receptors with specific PC marker zebrin II at the somata of PCs, during cerebellum development in correspondence with binding by autoradiography.

2. Materials and methods

2.1. Animals

Male Wistar rats were kept under controlled light–dark cycles (12:12 h), temperature (23 °C ± 1), and with free access to food and water. Animals were sacrificed and the brains removed at different developmental stages: postnatal day (P) 8, 15, 30 and 60. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. All efforts were made to minimize the number of animals used and their suffering.

2.2. Autoradiography of Ang II receptors

Binding by autoradiography was performed as described previously (Arce et al., 2001; Sánchez et al., 2008). Briefly, rats (n = 5–6 per age) were sacrificed by decapitation and the brains, carefully dissected, snap-frozen in dry ice-cooled 2-methylbutane and stored at −80 °C until use.

Consecutive coronal sections of hindbrain from rats at different ages (P8, P15, P30 and P60) were obtained at the selected level (Paxinos and Watson, 1986). Coronal sections (16 μm) were cut with a cryostat at −20 °C (Microm, Zeiss Inc.), thaw-mounted onto gelatine-coated glass slides, and desiccated at 4 °C overnight. Sections were preincubated in 10 mM sodium phosphate buffer pH 7.4, containing 120 mM NaCl, 5 mM disodium EDTA, 0.005% bacitracin (Sigma, St. Louis, CO), and 0.2% proteinase free bovine serum albumin (BSA) (Sigma) and incubated in fresh buffer with 0.2 nM [^{125}I] Ang II (DuPont-NEN, sp. act. 2200 Ci/mmol), a concentration below the Kd value, for 2 h. Non-specific binding was determined with an excess of Ang II (10^{-6} M). After incubation, slides were rinsed, in fresh ice-cold 50 mM Tris buffer, pH 7.6, followed by ice-cold water, and then dried under a stream of cool air.

For Ang II receptor subtype identification, consecutive sections were incubated with 0.2 nM [^{125}I] Ang II, in the presence of 10^{-6} M of AT_1 antagonist Losartan, to define AT_2 receptors, or 10^{-6} M PD123319, to define AT_1 subtype. The dried labeled sections were apposed to Kodak BioMax MR film in X-ray cassettes. Films were developed with D19 Kodak developer for 4 min at 4 °C, after 15–20 days exposition.

Quantification: Autoradiographic images (short exposure) were quantified by densitometry with Scion software for Windows. For statistical comparisons, values from etarian groups were obtained within the same film. Results were expressed as optical density units from a 256 grey scale.

2.2.1. Statistics analysis

Means and standard errors were calculated for every data set. Differences between groups were evaluated using one-way Analysis of Variance (ANOVA) followed by Tukey–Kramer Multiple Comparisons test.

2.3. Immunohistochemistry

For immunohistochemistry, animals (n = 4–5 per age) were deeply anesthetized with chloral hydrate, perfused intracardially with washing solution (0.8% NaCl, 0.8% sucrose, 0.4% glucose for 10 min) and then with 4% paraformaldehyde in 0.01 M borate buffer plus 0.35 M Na_2SO_3. The whole heads were wrapped with aluminium foil and stored overnight at 4 °C. The following day, brains were removed and cryoprotected with 30% sucrose in paraformaldehyde buffer. After 2–3 days, brains were snap frozen at −30 °C in isopentane on dry ice, and stored at −80 °C until use.

Immunohistochemistry was performed as described previously (Ezquer et al., 2006). Coronal sections were obtained (14–16 μm) with a cryostat at −20 °C and mounted on 3-aminopropyltrietoxysilane-coated slides. Initially, sections were washed in 50 mM Tris–HCl, 0.9% NaCl, pH 7.4, reacted with 3% hydrogen peroxide for 20 min and then incubated for 45 min with 10% mouse serum in blocking solution (saline/Tris buffer with 0.1% Triton X-100, 1% mouse serum and 1% BSA). After additional washings, sections were incubated with goat anti-AT_2 antibody (Santa Cruz Biotechnology, AT_2 C-18: sc-7420) diluted 1:50, in buffer Tris 50 mM, Triton 0.1% for 48 h at 4 °C. Sections were washed and then incubated with a biotinylated-secondary anti-goat antibody (1:200, Goat ExtrAvidin Peroxidase kit, Sigma). Diaminobenzidine, glucose oxidase (Sigma) plus nickel ammonium sulphate (as colour enhancer) were used as chromagen. Slices were mounted, dehydrated and coverslipped with Permount (Fisher). Immunopositive PCs were quantified in coronal sections of P30 and P60 animals, between Bregma levels −9.80 and −10.80 mm by using the Image J software.

Sections were examined and photographed with a light microscope (Nikon Eclipse 50i). As a negative control, the primary antibodies were omitted and no specific labeling was identified in these sections.

2.4. Immunofluorescence staining

For single immunofluorescence labeling, rats (n = 4–5 per age) were sacrificed by decapitation, brains carefully dissected, snap frozen under dry ice-cooled 2-methylbutane and stored at −80 °C until use.

Sagittal sections (12 μm) were obtained in a cryostat and mounted on glass slides optimized for immunohistochemistry (HiFix, NH). Sections were fixed for 10 min with cold acetone, air dried and rinsed in PBS for 10 min. Following blockade (Triton X-100, 0.5% BSA in PBS) for 45 min at room temperature, sections were reacted overnight at 4 °C with rabbit anti-AT_2 antibody (diluted 1:100, Santa Cruz Biotechnology, AT_2 H-143: sc-9040) or a mouse anti-zebrin II monoclonal antibody (a gift from Dr. R. Hawkes, University of Calgary, Canada). The slides were rinsed with PBS and then incubated with fluorescein isothiocyanate

(FITC)-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology, diluted 1:200) for 2 h at room temperature. Images of immunostained sections were acquired with an epifluorescence microscope (Nikon Eclipse 50i).

To verify the specificity of the fluorescent staining, sections were processed as before, without primary antibody. The resolution, brightness, and contrast of the images were optimized using the Adobe Photoshop CS software (Adobe Systems Inc., San Jose, CA, USA) but not otherwise manipulated. To test the specificity of the rabbit anti-AT_2 antibody (Santa Cruz Biotechnology, sc-9040) dot blot analysis was performed (1:2500, 3 h) on negative (rat liver) and positive (E20 fetus and P15 cerebellum) tissues.

Double immunofluorescence labeling was performed in coronal sections (20 μm) from perfused P15 rat brains, as described previously. Brains were placed in 10 mM Sodium Citrate buffer, pH 6 at 95 °C, during 5 min and placed in the same buffer during 15 min at room temperature, for antigen retrieval. Then the slices were washed in 10 mM phosphate buffer, 0.9% NaCl, pH 7.4, and then incubated for 45 min in 10% goat serum in blocking solution (10 mM phosphate buffer, 0.9% NaCl, pH 7.4 with 0.1% Triton X-100, 10% goat serum and 1% BSA). After additional washings, sections were incubated overnight at 4 °C in a mixture containing rabbit anti-AT_2 antibody (1:40, sc-9040, Santa Cruz Biotechnology) and mouse monoclonal anti-zebrin II antibody (1:100) or mouse anti-GFAP antibody (1:500, Sigma). After rinsing in PBS, sections were incubated in a mixture containing FITC-conjugated anti-rabbit IgG (1:400, Sigma) and Cy5-conjugated anti-mouse IgG (1:200; Jackson ImmunoResearch Lab.) for 2 h at room temperature. For the labeling of the nuclei, the blue dye Hoechst (1:2000 Sigma) was used together with fluorescent mounting (Dako) to enhance the fluorophore's life. Sections were examined by confocal laser scanning microscope (Olympus FV1000 Microsystem).

3. Results

In a previous paper, we observed by binding autoradiography the presence of both receptor subtypes of Ang II in the cerebellar cortex of 2-week-old animals (Arce et al., 2001). Localization of Ang II receptor subtypes AT_1 and AT_2 exhibited a complementary pattern in all cerebellar cortex areas at different Bregma levels. To further characterize the cellular layer that expresses AT_2 receptors, we designed this study by using immunostaining with specific molecular markers of Purkinje cells to compare with binding

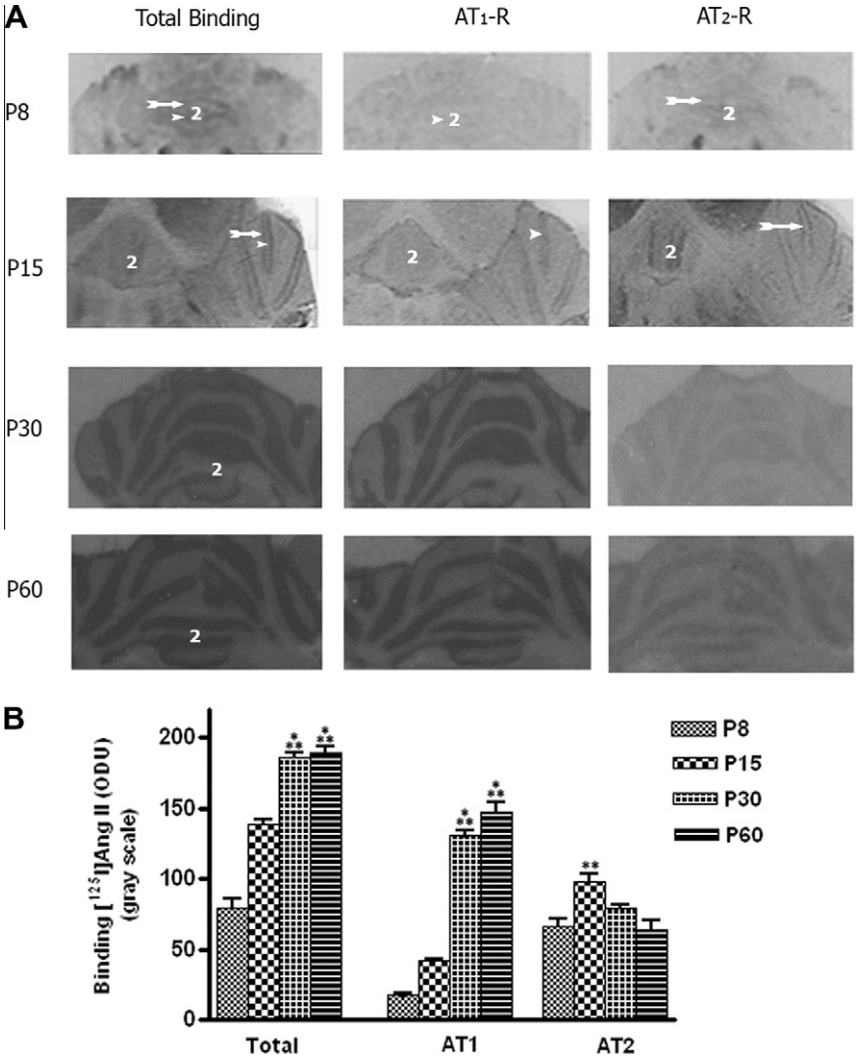

Fig. 1. Autoradiographic localization of the Ang II receptors in rat cerebellum at different postnatal ages. (A) Consecutive coronal sections of P8, P15, P30 and P60 cerebellum were incubated with [^{125}I] Ang II (0.2 nM) in the absence (Total) or in the presence of PD123319 (AT_1 receptors, AT_1-R 10^{-6} M) or Losartan (AT_2 receptors, AT_2-R 10^{-6} M). The differential localization of Ang II receptors is indicated at the cerebellar cortex: AT_1 (arrowhead) and AT_2 (arrow). 2: Area 2 of the cerebellar cortex. Representative image from $n = 5$–6 animals. (B) [^{125}I] Ang II binding densities obtained by autoradiography in rat cerebellum at different postnatal ages. Data are mean ± SEM of total specific binding, Total ($\overset{*}{**}P < 0.001$ vs. P8), AT_1 subtype (AT1), $\overset{*}{**}P < 0.001$ vs. P15 and P8 and AT_2 subtype (AT2), $^{**}P < 0.01$ vs. P60 and P8.

autoradiography. Taking into account the postnatal maturation process of the cerebellar cortex, the study was performed at different developmental stages: P8, P15, P30 and P60.

3.1. Binding autoradiography

Coronal sections from rat cerebella at ages P8, P15, P30 and P60 (area 2, cerebellar cortex) were used for binding autoradiography with [^{125}I] Ang II (0.2 nM). Fig. 1A shows the total binding, AT_2 and AT_1 binding on different columns. At P8 and P15, the neuronal layers of the cerebellar cortex exhibited differential AT_1 (arrowhead) or AT_2 (arrows) labeling in a complementary pattern when compared to the total binding (see Fig. 1A). This pattern was not observed at P30 and P60, where AT_2 binding appears superimposed with AT_1 binding (see Fig. 1A). The increase with age of the total binding is due to the increase in AT_1 binding (Fig. 1B). While AT_1 binding increases with age, AT_2 binding was higher at early stages being maximal at P15 ($p < 0.001$) and decreased at P60 (Fig. 1B).

Fig. 2 shows cerebellar cortex at the level Bregma −10.52 mm. A comparison with the histology shows that the total binding comprises both the molecular and PC layers. A clear correspondence of AT_1 binding with the molecular layer of the cerebellar cortex can be observed (Fig 2). The thin layer showing AT_2 binding seems localized at the Purkinje cell layer while no binding was observed at the granular layer which exhibits intensively stained nuclei (Fig. 2).

3.2. Immunolabeling of AT_2 receptors in developing cerebella

In order to confirm our assumption that AT_2 receptors labeled the PC layer, we performed immunohistochemistry and immunofluorescence staining of cerebellar cortex form P8, P15, P30 and P60 rats.

To establish the antibody specificity we performed assays in the presence or absence of AT_2 antibodies. Fig. 3 shows the specificity of the antibodies used, since no immunoreactivity was observed in the absence of the primary antibody (control sections, Fig. 3B and D). AT_2 antibodies selectively labeled the PC layer (Fig. 3A and C). The molecular layer, which exhibits AT_1 receptors by binding autoradiography, was not immunostained by the anti-AT_2 antibody. The specificity of the antibody was also assayed by dot blots of tissues expressing or not AT_2 receptors (Fig. 3 E).

Sagittal or coronal sections from cerebella at the different developmental stages were assayed by immunofluorescence (Fig. 4, left panels) or immunohistochemistry (Fig. 4, right panels) with anti-AT_2 receptor antibodies ($n = 4, 5$).

P8: By using two different antibodies and experimental conditions, we observed labeling of a cellular layer, corresponding to the Purkinje cells (Fig. 4A and B). At P8 the Purkinje cells have already migrated to their final localization and constitute a monolayer. At this developmental stage the pyramidal somata appeared very well defined (Fig. 4A) and the efferent processes were weakly immunostained (Fig. 4A, inset). Fig. 4B shows a lower resolution view where PC layer appeared very well immunolabeled in a cerebellar lobule.

P15: Sagittal sections of P15 animals were stained with anti-AT_2 antibodies either for immunofluorescence or immunohistochemistry (Fig. 4C and D), revealing a monolayer with the typical disposition of Purkinje cells. Binding by autoradiography shows a narrow cellular band exhibiting AT_2 labeling, in correspondence with the immunolabeling of PCs at this stage. Fig. 4C and D shows the somata of Purkinje cells immunolabeled by AT_2 antibody, while the cellular processes where not labeled. At this stage, the dendritic trees are not fully developed.

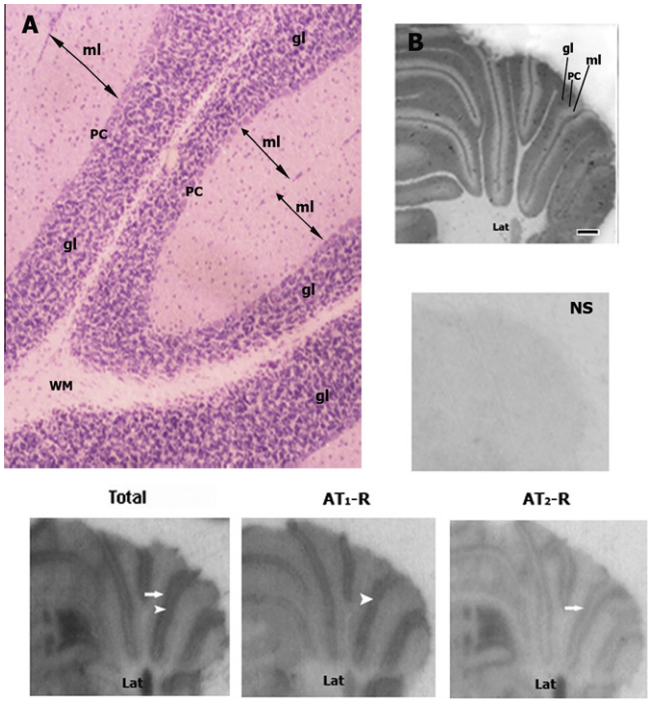

Fig. 2. Binding autoradiography at the cerebellar cortex (level Bregma −10.52 mm) of P15 rats. (A) and (B) Hematoxylin–eosin staining at high (A) and low (B) resolution. Consecutive coronal sections of P15 cerebellum were incubated with [^{125}I] Ang II (0.2 nM) in the absence (Total) or in the presence of PD123319 (AT_1 receptors, AT_1-R 10^{-6} M) or Losartan (AT_2 receptors, AT_2-R 10^{-6} M). AT_2 (arrow) and AT_1 (arrowhead) binding localized on adjacent neuronal layers, in correspondence with the Purkinje cell layer (PC) and the molecular layer (ml), respectively. No Ang II binding was observed at the granular (gl) layer. NS, non-specific binding; Lat, lateral nucleus; WM, white matter.

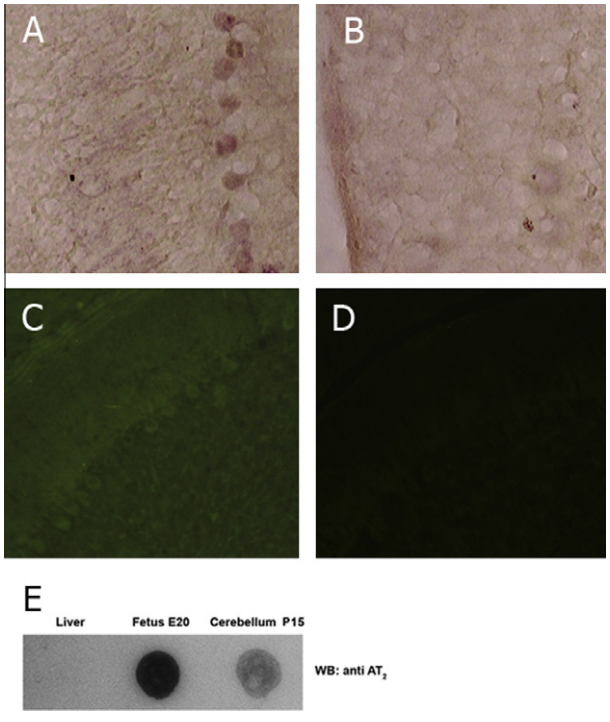

Fig. 3. AT_2 antibody specificity. Cerebellar sections were stained with (A and C) or without (B and D) anti-AT_2 primary antibody and processed by immunohistochemistry (AT_2 C-18, sc-7420) (A and B) or immunofluorescence (AT_2 H-143, sc-9040) (C and D). (B) and (D) are negative controls, with omission of the primary antibody. (E) Dot blot developed with anti-AT_2 antibody from liver, fetus E20, and cerebellum P15.

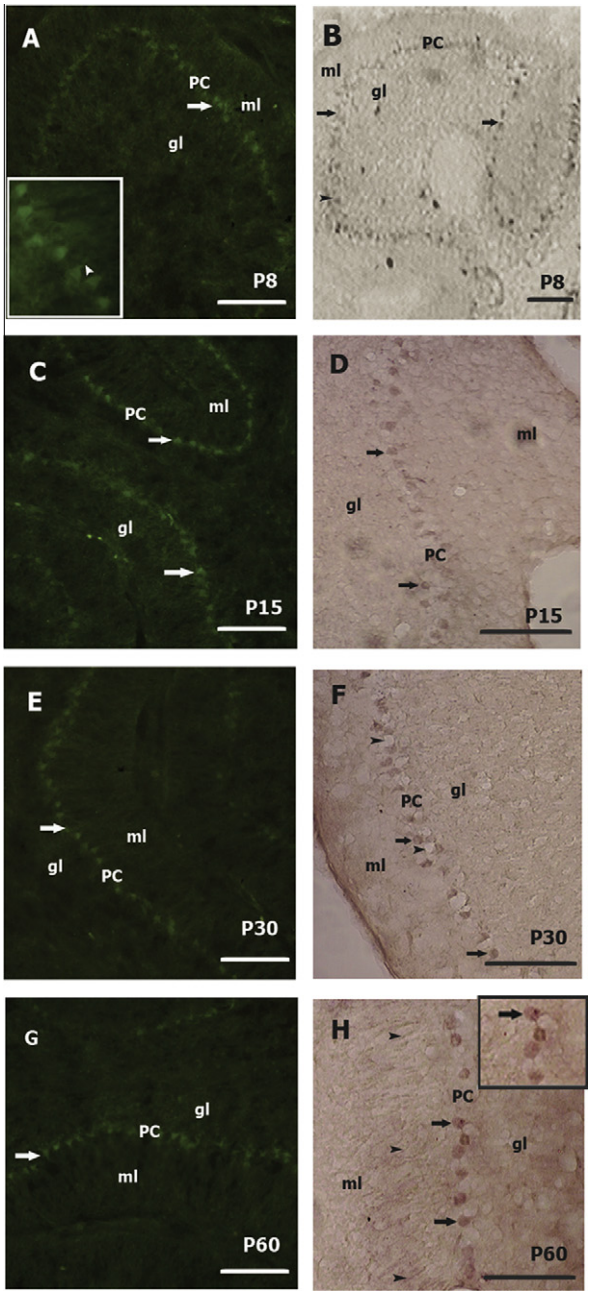

Fig. 4. Immunofluorescence and immunohistochemistry for AT_2 receptors in sections of the cerebellar cortex at different developmental stages. A, C, E and G: AT_2 receptor immunofluorescence (left) staining of PCs in parasagittal sections of P8, P15, P30 and P60 rat cerebellum, respectively. Arrows point to the AT_2-immunoreactive PC layer. Inset in A: higher magnification view of representative PCs with the soma immunostained with AT_2; arrowhead points to the PC process. B, D, F and H. Peroxidase–DAB immunostaining of AT_2 in parasagittal sections of P8, P15, P30 and P60 rat cerebellum, respectively. Arrows point to the AT_2-immunoreactive PC. (B) Arrowhead points to a Golgi cell. (F) Arrowhead points to unlabeled PCs. (H) Arrowheads point to Bergmann glial processes at the molecular layer. Inset in (H) shows an enlarged image of the PC layer. *Abbreviations:* ml, molecular layer; gl, granular layer; PC, Purkinje cells. Scale bar: 100 μm.

P30 and P60: Ang II AT_2 receptors in cerebellum of older animals have not been extensively studied and only recently, the presence of these receptors in adult cerebellum was suggested. Sagittal sections of P30 and P60 animals were stained with anti-AT_2 antibodies either by immunofluorescence (Fig. 4E and G) or immunohistochemistry (Fig. 4F and H), revealing a monolayer with the typical disposition of Purkinje cells (see Fig. 4 H inset).

At the stages P30 and P60, it appears that not all PCs are labeled (Fig. 4F, arrowhead). About 68–69% of PCs were immunopositive in P30 and P60 animals, quantified on immunohistochemical stained coronal sections (Bregma level −9.80 to −10.80 mm).

In P60 animals, a weak labeling can be observed in glial processes which enter into the molecular layer (Fig. 4H, arrowhead). The pattern seems to correspond to Bergmann glial processes, cells which are fully developed around P60 (Bellamy, 2006). These observations might account for the low Ang II AT_2 binding at this stage and apparent co-localization of AT_1 and AT_2 binding observed by autoradiography (see Fig. 1A).

3.3. Co-localization of AT_2 receptors and PC markers in P15 cerebella

Early after Purkinje cells are born, they express specific markers, such as calbindin (Morales and Hatten, 2006). Preliminary studies with calbindin antibodies and zebrin II immunolabeling suggested that AT_2 receptors labeled the PC layer (supplementary Fig. S1). To confirm our observations from immunostaining and to address the question whether AT_2 receptors co-localized with different cellular markers, we performed double immunolabeling and confocal analysis. Initially, double immunostaining was performed with the glial marker GFAP and AT_2 antibodies in coronal sections of P15 cerebella. Fig. 5A–C shows a clear and differential labeling of PCs with AT_2 antibodies, while GFAP was observed in the molecular layer. In this way, it becomes clear that the Bergmann cells bodies located next to the PC somata were labeled differentially.

Although the anatomical disposition and cellular structure indicate that AT_2 antibody labeled the monolayer corresponding to Purkinje cells, to confirm this assumption, we performed double immunolabeling with anti-AT_2 antibody and the specific PC marker zebrin II (Fig. 5D–G). Clearly, the monolayer recognized by the anti-AT_2 antibodies corresponded to the one identified by the selective PC marker zebrin II. However, Fig. 5D–F suggests a different subcellular localization for Ang II AT_2 receptors and the Purkinje cell marker zebrin II. Fig. 5G shows a wide image of the cerebellar cortex with zebrin positive and zebrin negative strips co-localized with AT_2 receptors. Zebrin II labels not only the somata but also the dendritic arbors of PCs (Fig. 5G). A similar pattern was observed at P8 and P30 (supplementary Fig. S2).

4. Discussion

In spite of the great deal of research devoted to study Ang II receptors in the brain and its physiological roles, very little effort has been dedicated to study the localization and potential role of Ang II receptors in the cerebellum during development. The development of mice with targeted disruption of the AT_2 receptor which showed attenuation of the exploratory behavior and movement prompted us to study Ang II receptors localization in the developing cerebellum.

In a previous study in developing rat hindbrain by autoradiography, we identified Ang II binding present in the cerebellum and several brainstem nuclei. A clear complementary pattern of AT_1 and AT_2 receptors within the cerebellar cortex was observed in P15 animals (Arce et al., 2001). In the present study we extended the autoradiographic study from P8 to P60 and demonstrated co-localization of AT_2 receptors with PC markers.

Binding by autoradiography evidenced an increase of AT_1 binding at the cerebellar cortex with development and a decrease of AT_2 binding, being maximal at P15. A detailed analysis of the cerebellar cortex revealed a complementary pattern in both Ang II receptor subtypes, present in P8 and P15 animals. At these stages, AT_2 binding was present in a narrow layer surrounding the AT_1 labeled area. In P30 and P60 animals, binding by autoradiography exhibited

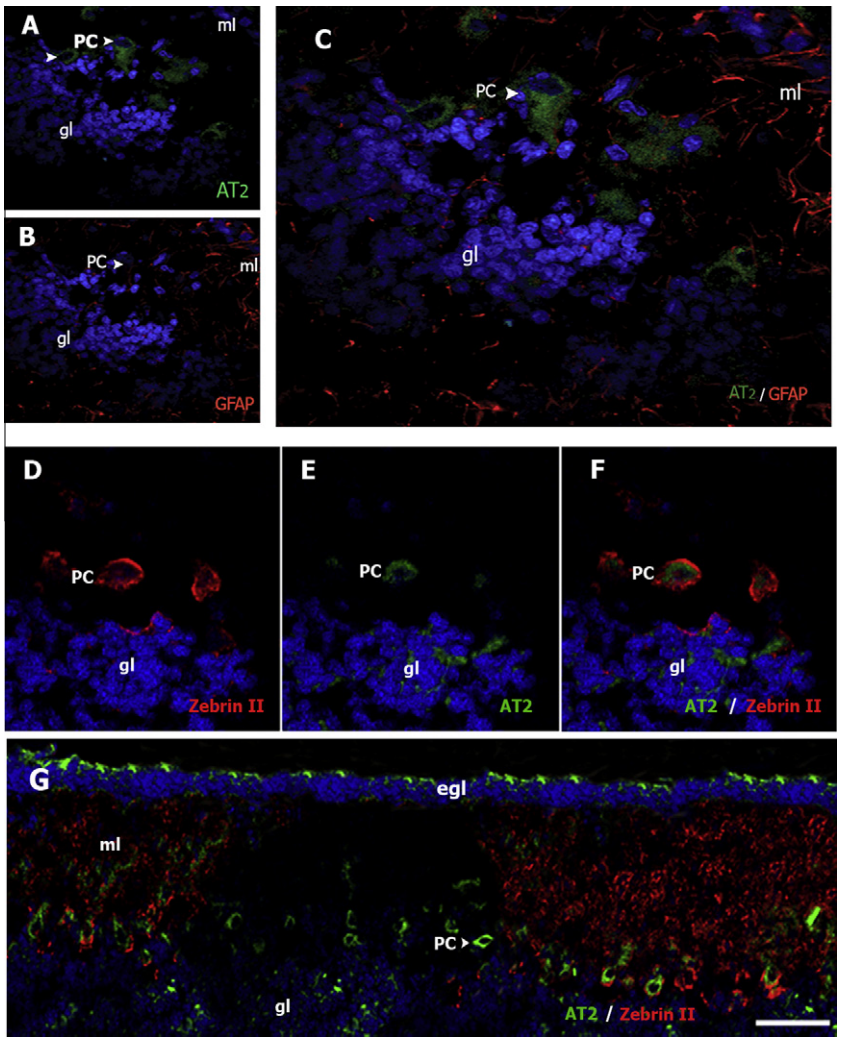

Fig. 5. Double immunolabeling of AT_2 receptors and molecular markers in cerebellar cortex of P15 rats. (A)–(C) Confocal images of coronal sections of P15 rat cerebellum labeled for AT_2 receptor (green, A) and GFAP (red, B). (C) Merged confocal image double-labeled for AT_2 receptor (green) and GFAP (red). (D)–(G) Confocal images of coronal sections of P15 rat cerebellum labeled for zebrin II (red, D) and AT_2 receptor (green, E) and F, G: Merged confocal image double-labeled for AT_2 receptor (green) and zebrin II (red). Arrowheads point to immunoreactive PCs. Nuclear DNA (blue) was stained with Hoechst. *Abbreviations:* ml, molecular layer; gl, granular layer; egl, external granular layer; PC, Purkinje cells. Scale bar: 20 μm.

an apparent co-localization of AT_1 binding at the cerebellar cortex with AT_2 binding. However, immunostaining with anti-AT_2 antibodies labeled selectively the PCs somata at these two stages.

There is a general concept of predominant AT_1 receptors after birth and AT_2 receptors before birth (Mao et al., 2009). However, in certain cells, tissues or organs, that view may not always be true. Particularly, in rodents, where some tissues evidence an important postnatal development, such as kidneys or cerebellum, this concept should be revised. In our experience, in those tissues where development continues after birth, AT_2 receptors are present in postnatal stages, maybe encompassing the developmental process (Ciuffo et al., 1993; Sánchez et al., 2008,2009). The present data, showing an increase of AT_1 receptors in postnatal stages until they reach an adult level, confirm this assumption.

Neurons of the cerebellar cortex do not grow simultaneously and a complex mechanism is required in order to establish the adult pattern of the cerebellar cortex. While PCs precursors are formed during the embryonic stage, most granule cells are generated postnatally. PCs migration in small rodents starts during the second half of gestation (E12 in mice and E14 in rats) and lasts until 2 or 3 days after birth. In rats, the dendritic arbors of PCs reach their maximal length by P30 (Sotelo and Dusart, 2009).

Previous studies have suggested that AT_2 binding in the cerebellar cortex of young rats localized to the molecular layer and it was attributed to the climbing fibers from the inferior olivary complex (Jöhren et al., 1998). Other authors identified AT_2 receptor-immunoreactivity in the adult cerebellum associated with the Purkinje cell layer and the glia (Reagan et al., 1994; Fogarty and Matute, 2001). Gendron et al. (2003) reported the presence AT_2 receptor-immunoreactivity in granule cells in culture. Recently, a non-AT_1, non-AT_2 binding was described in the adult rat cerebellum, attributed to the granular layer (Karamyan and Speth, 2008). Thus, there is still controversy regarding the neuronal layer in the cerebellar cortex which expresses AT_2 receptors.

In the present study, we demonstrated that AT_2 antibodies recognized postmitotic Purkinje cells, labeling the somata of these cells at all the stages studied, from P8 to P60, suggesting that PCs express these receptors from early stages of development until adulthood. In P8 and P15 animals, we observed a clear correspondence between immunolabeling and the well-defined layer observed by binding autoradiography. At these stages, PCs are still differentiating, and dendritic processes are in a primary stage. To verify the co-localization of PCs with AT_2 receptors, we performed a double immunostaining at the P15 stage. Confocal analysis

allowed us to discard the co-localization of AT_2 receptors with the glial marker GFAP and verify the co-localization with the PC marker zebrin II. Zebrin II exhibited a different subcellular localization than AT_2 receptors within PCs.

The function of the Bergmann glial cell changes during development, allowing cell migration, support migration of PC dendrites and become a clear anatomical partner to PC in the adult cerebellum (Bellamy, 2006). In P60 rats, where the Bergmann glia exhibits elongated cellular processes within the molecular layer, a weak AT_2 immunolabeling of glial processes was observed. This observation is in agreement with previous ones (Fogarty and Matute, 2001) and with the weak AT_2 binding observed in the P60 cerebellar cortex.

The differential localization of Ang II AT_1 and AT_2 receptors in rat P15 cerebellum was described in previous studies using binding autoradiography (Arce et al., 2001; Jöhren et al., 1998; Sánchez et al., 2009). Jöhren et al. (1998), on the basis of chemical lesions of the IO, attributed AT_2 binding to the climbing fibers originated in the IO. The use of specific markers for postmitotic PCs, allowed us to clearly establish the localization of Ang II AT_2 receptors to the somata of PCs. Our present results thus clarify the specific localization of Ang II receptors: AT_1 receptors at the molecular layer and AT_2 receptors at the PC layer. No Ang II binding was observed in the granular layer.

Early on cerebellar development, the granular cells originate from a proliferative external layer (EGL) which later becomes the internal granular layer (IGL). In P0 animals we have shown high Ang II binding expressed at the EGL, binding that disappeared in animals born from mothers treated with the AT_2 antagonist PD123319 (Sánchez et al., 2009). In PD123319-treated animals, an increased number of cells was observed at the EGL, with a loss of binding (Sánchez et al., 2009), suggesting an over proliferation of granule cells. Since treatment was performed during late pregnancy (E13–E21), a critical stage during PCs migration, we can speculate that blockade of AT_2 receptors prevents cell migration or the differentiation of cells expressing AT_2 receptors, such as the PCs. These observations are in agreement with previous studies about the potential role of Ang II AT_2 receptors in cell migration (Côté et al., 1999).

In the present study it becomes clear that Ang II AT_2 receptors are localized on PC layer at different developmental stages. Double immunolabeling with specific cell markers confirm the localization of AT_2 receptors at the PC somata. Targeted disruption of AT_2 receptors in knock-out mice evidenced attenuation of the exploratory behavior and movement. Since PCs are the sole output signal from the cerebellar cortex and considering the role of cerebellum in movement control, the specific receptor localization suggests a potential role for AT_2 receptors in the cerebellar function.

Acknowledgements

The authors thank Dr. R. Hawkes for providing the zebrin II antibody and helpful comments on the manuscript. G.M. Ciuffo and A.M. Seltzer are members of the researcher's career of CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas, Argentina). ME. Arce and F. López Aguilera are fellows from the ANPCYT (Agencia Nacional de Promoción Científica y Tecnológica), grant numbers 15-32350 and 2006-451 respectively. This work was supported by grants from CONICET, PICT15-32350 (ANPCYT) and Universidad Nacional de San Luis, Argentina.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.npep.2010.11.002.

References

Altman, J., 1972. Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer. J. Comp. Neurol. 145, 399–463.
Arce, M.E., Sánchez, S., Seltzer, A., Ciuffo, G.M., 2001. Autoradiographic localization of angiotensin II receptors in developing rat cerebellum and brainstem. Regul. Pep. 99, 53–60.
Bellamy, T.C., 2006. Interactions between Purkinje neurons and Bergmann glia. Cerebellum 5, 116–126.
Brochu, G., Maler, L., Hawkes, R., 1990. Zebrin II: a polypeptide antigen expressed selectively by Purkinje cells reveals compartments in rat and fish cerebellum. J. Comp. Neurol. 291, 538–552.
Ciuffo, G.M., Viswanathan, M., Seltzer, A.M., Tsutsumi, K., Saavedra, J.M., 1993. Glomerular Angiotensin II receptor subtypes during development of rat kidney. Am. J. Physiol. 265, F264–F271.
Côté, F., Do, T.H., Laflamme, L., Gallo, J.M., Gallo-Payet, N., 1999. Activation of the AT_2 receptor of angiotensin II induces neurite outgrowth and cell migration in microexplant cultures of the cerebellum. J. Biol. Chem. 274, 31686–31692.
De Gasparo, M., Catt, K.J., Inagami, T., Wright, J.W., Unger, T.H., 2000. International Union of Pharmacology XXIII. The angiotensin receptors. Pharmacol. Rev. 52, 415–472.
Ezquer, M.E., Valdez, S.R., Seltzer, A.M., 2006. Inflammatory responses of the substantia nigra after acute hypoxia in neonatal rats. Exp. Neurol. 197, 391–398.
Fogarty, D.J., Matute, C., 2001. Angiotensin receptor-like immunoreactivity in adult brain white matter astrocytes and oligodendrocytes. Glia 35, 131–146.
Gallinat, S., Busche, S., Raizada, M.K., Summers, C., 2000. The angiotensin II type 2 receptor: an enigma with multiple variations. Am. J. Physiol. Endocrinol. Metab. 278, 357–374.
Gendron, L., Payet, M., Gallo-Payet, N., 2003. The Angiotensin type 2 receptors of Angiotensin II and neuronal differentiation: from observations to mechanisms. J. Mol. Endocrinol. 31, 359–372.
Guimond, M.O., Roberge, C., Gallo-Payet, N., 2010. Fyn is involved in angiotensin II type 2 receptor-induced neurite outgrowth, but not in $p42/p44^{mapk}$ in NG108-15 cells. Mol. Cell. Neurosci. doi:10.1016/j.mcn.2010.06.011.
Hawkes, R., 1997. An anatomical model of cerebellar modules. Prog. Brain Res. 114, 39–52.
Hein, L., Barsh, G.S., Pratt, R.E., Dzau, V.J., Kobilka, B.K., 1995. Behavioral and cardiovascular effect of disrupting the angiotensin II type-2 receptors in mice. Nature 377, 744–747.
Ichiki, T., Labosky, P.A., Shiota, C., Okuyana, S., Imagawa, I., Fogo, A., Niimura, F., Ichikawa, I., Hogan, B.L., Inagami, T., 1995. Effects on blood pressure and exploratory behavior of mice lacking angiotensin II type 2 receptor. Nature 377, 748–750.
Jöhren, O., Saavedra, J.M., 1996. Gene expression of angiotensin II receptor subtypes in the cerebellar cortex of young rats. Neuroreport 7, 1349–1352.
Jöhren, O., Hauser, W., Saavedra, J.M., 1998. Chemical lesion of the inferior olive reduces [^{125}I] Sarcosine1–Angiotensin II binding to AT_2 receptors in the cerebellar cortex of young rats. Brain Res. 793, 176–186.
Karamyan, V.T., Speth, R.C., 2008. Distribution of the non-AT_1, non-AT_2 angiotensin-binding site in the rat brain: preliminary characterization. Neuroendocrinology 88, 256–265.
Kilian, P., Campbell, S., Bilodeau, L., Gumond, M., Roberge, C., Gallo-Payet, N., Payet, M.D., 2008. Angiotensin type 2 receptor stimulation increases the rate of NG108–15 cell migration via actin depolymerization. Endocrinology 149, 2923–2933.
Laflamme, L., De Gasparo, M., Gallo, J.M., Payet, M.D., Gallo-Payet, N., 1996. Angiotensin II induction of neurite outgrowth by AT_2 receptors in NG108–15 cells-effect counteracted by the AT_1 receptors. J. Biol. Chem. 271, 22729–22735.
Mao, C., Shi, L., Xu, F., Zhang, L., Xu, Z., 2009. Development of fetal brain renin–angiotensin system and hypertension programmed in fetal origins. Prog. Neurobiol. 87, 252–263.
Maul, B., und Halbach, O., Becker, A., Sterner-Kock, A., Voigt, J.P., Siems, W.E., Grecksch, G., Walther, T., 2008. Impaired spatial memory and altered dendritic spine morphology in angiotensin II type 2 receptor-deficient mice. J. Mol. Med. 86, 563–571.
Morales, D., Hatten, M.E., 2006. Molecular markers of neuronal progenitors in the embryonic cerebellar anlage. J. Neurosci. 26, 12226–12236.
Nuyt, A.M., Lenkei, Z., Palkovits, M., Corvol, P., Llorens-Cortes, C., 1999. Ontogeny of angiotensin II type-2 receptor mRNA expression in fetal and neonatal rat brain. J. Comp. Neurol. 407, 193–206.
Okuyama, S., Sakagawa, T., Chaki, S., Imagawa, Y., Ichiki, T., Inagami, T., 1999. Anxiety-like behavior in mice lacking the angiotensin II type-2 receptor. Brain Res. 821, 150–159.
Paul, M., Poyan Mehr, A., Kreutz, R., 2006. Physiology of local renin–angiotensin. Physiol. Rev. 86, 747–803.
Paxinos, G., Watson, C., 1986. The Rat Brain in Sterotaxic Coordinates. Academic Press, New York, USA.
Reagan, L.P., Flanagan-Cato, L.M., Yee, D.K., Ma, L.Y., Sakai, R., Fluharty, S.J., 1994. Immunohistochemical mapping of angiotensin type 2 (AT2) receptors in rat. Brain Res. 662, 45–59.
Saavedra, J.M., 2005. Brain angiotensin II: new developments, unanswered questions and therapeutic opportunities. Cell Mol. Neurobiol. 25 (3–4), 485–512.

Sánchez, S., Seltzer, A., Fuentes, L., Forneris, M., Ciuffo, G., 2008. Inhibition of Angiotensin II receptors during pregnancy induces malformations in developing rat kidney. Eur. J. Pharmacol. 588, 114–123.

Sánchez, S.I., Arce, M.E., Fuentes, L.B., Ciuffo, G.M., 2009. Prenatal blockade of Ang II receptors affects neonatal rat hindbrain structure and receptor localization. Exp. Neurol. 220, 246–254.

Sarna, J.R., Marzban, H., Watanabe, M., Hawkes, R., 2006. Complementary stripes of phospholipase Cß3 and Cß4 expression by Purkinje cell subsets in the mouse cerebellum. J. Comp. Neurol. 496, 303–313.

Sotelo, C., Dusart, I., 2009. Intrinsic versus extrinsic determinants during the development of Purkinje cell dendrites. Neuroscience 162 (3), 589–600.

Swinny, J.D., van der Want, J.J.L., Gramsbergen, A., 2005. Cerebellar development and plasticity: perspectives for motor coordination strategies, motor skills, and therapy. Neural Plast. 12, 153–160.

Terada, N., Banno, Y., Ohno, N., Fujii, Y., Murate, T., Sarna, J., Hawkes, R., Zea, Z., Baba, T., Ohno, S., 2004. Compartmentation of the mouse cerebellar cortex by sphingosine kinase. J. Comp. Neurol. 469, 119–127.

Vervoort, V.S., Beachem, M.A., Edwards, P.S., Ladd, S., Miller, K.E., de Mollerat, X., Clarkson, K., DuPont, B., Schwartz, C.E., Stevenson, R.E., Boyd, E., Srivastava, A.K., 2002. AGTR2 mutations in X-linked mental retardation. Science 296, 2401–2403.