Expression of coat proteins changes during postnatal development in selected areas of the rat brain

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ABSTRACT

It is well known that clathrin-mediated endocytosis is crucial for the normal functioning and integrity of neurons in the central nervous system. In this study we attempted to correlate the expression of coat proteins with development in different areas of rat brain. By Western blot, we studied the expression of AP-2, GGA1 and GGA2 in striatum, cerebellum, brain stem, cerebral cortex and hippocampus of newborn rats and during post-natal development; 5, 15, 30, 60, 90 or 150 days after birth. We observed that the expression of the α2 subunit of AP-2 increased substantially between the 15th and 30th day after birth in all areas studied, excepting the cerebellum and cortex. On the other hand, the expression of the α1 subunit does not change significantly during the development in any of the areas under study. We also noted that the expression of the μ2 subunit did not follow the pattern of α2 during development. In general terms, the expression of GGA1 and GGA2 followed a similar pattern to that of AP-2, although these proteins increased significantly in the cerebral cortex from the 15th day after birth. Moreover, presenilin-1, a protein associated with aging and neurodegeneration, shows an expression pattern similar to coat proteins in the striatum and cortex. These results suggest that proteins that conform the intracellular transport machinery in the brain cells seems to accompany development, according to the maturation of the different brain areas.

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1. Introduction

Vesicle-mediated transport is one of the main ways to carry material from one area to another within the cell, or in the exchange with the extracellular environment or with other cells (Conner and Schmidt, 2003). In the central nervous system, the clathrin-mediated transport appears crucial for the proper functioning and maintenance of the integrity of neurons (Hirst and Robinson, 1998). Thus, the study of proteins that form the coat of the vesicles could give us some clues about the conditions of the cells on the different areas of the brain. These clathrin-coated vesicles (CCVs) are involved in endocytosis and transport from the trans-Golgi network (TGN) to endosomes (reviewed by Kirchhausen, 1999). Although clathrin is a major component of the coat of the vesicles, from the functional standpoint adaptins (APs) play a key role in the formation of the coat, as they confer selectivity. Four families of APs (AP 1–4) have been described so far, of which AP-2 is specific to the CCV-mediated endocytosis and plays an important role in membrane homeostasis of nerve terminals (Granseth et al., 2007; Granseth and Lagnado, 2008). AP-2 is a heterotetramer composed of two major subunits (α, β2), a medium subunit (μ2) and a small subunit (δ2). Interestingly, in nervous tissue two isotypes of α subunit have been described, α1 and α2, but the co-existence of these isotypes is not yet well understood. Other proteins functionally related to CCVs have been identified, such as the family of the Golgi-localised, gamma-ear containing, ARF-binding proteins (GGA1–3) (Robinson and Bonifacino, 2001; Hirst et al., 2007; Zhang et al., 2007). These proteins, along with a myriad of accessory proteins (Owen et al., 1999; Collins et al., 2002) form a complex machinery, leading to the formation of the CCVs. In addition, some proteins and lipids in membranes are the partners for the recruitment of coat proteins.

It has been shown that the dynamics of formation of CCVs changes during the growth of neurons in primary cultures, to the point that the cycle of assembly–disassembly of the coat is faster in young neurons (Blanpied et al., 2002). Furthermore, it seems that changes in the dynamics of uptake of molecules by endocytosis plays a considerable role in neuronal aging (Benzi and Moretti, 1995; Samson and Nelson, 2000), as well as in age-related diseases such as Parkinson's and Alzheimer's disease (Herbert et al., 1994;
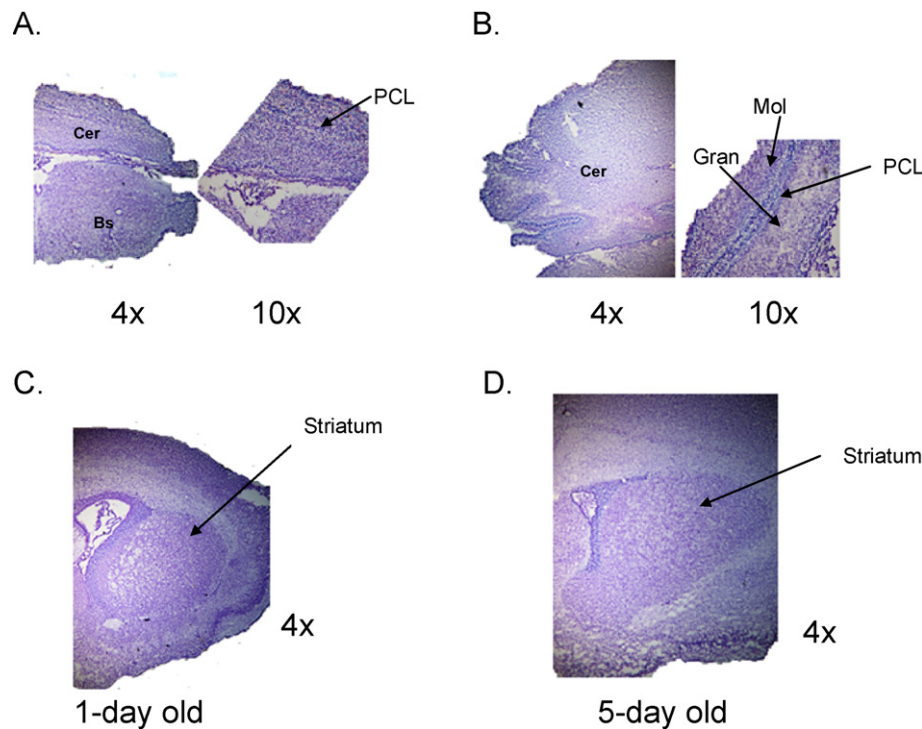
Fig. 1. Identification of brain areas from 1 and 5-day-old rat pups. Sections of 20 μm were cut with a cryostat and stained with cresyl violet. (A) The newborn animal shows an incipient cerebellum where Purkinje cells can be identified. (B) The 5-day-old cerebellum displays lobes and molecular and granular layers. (C) and (D) Striatum is a well defined brain area in both 1 and 5-day-old animals; Cer: cerebellum; PCL: Purkinje cell layer; Mol: molecular layer of the cerebellum; Gran: granular layer; Bs: brainstem.

Jellinger, 1999; Gaidarov and Keen, 1999; Foley and Riederer, 2000; Double et al., 2000; Halliwell, 2001).

In recent years, studies on endocytosis in nerve terminals became a priority, as it could have implications for neuromodulation and neuronal growth (Nagappan and Lu, 2005; Yamashita et al., 2010; Hines et al., 2010; Assaife-Lopes et al., 2010). The importance of the endocytic machinery in the maintenance of neuronal integrity is also supported by evidence showing changes in the expression of AP-2 and loss of its affinity for membranes in certain brain areas when subjected to the effect of excitotoxic drugs (Borgonovo et al., 2009).

This study attempted to determine whether the intracellular transport machinery accompanies neuronal development in different areas during postnatal development. To address this, by applying the immunoblot and immunohistochemistry methodologies, we studied the expression of proteins involved in the intracellular transport, in the cerebral cortex, *Corpus striatum*, hippocampus, cerebellum and brain stem from newborn rats and at different ages after birth.

2. Materials and methods

2.1. Subjects

Wistar rats used in this study were newborn (less than 1 day), 5, 15, 30, 60, 90 and 150 days after birth. The different brain areas from newborn (24 pups) and 5–15-day-old animals were pooled into three independent groups, (n = 3). Older animals (age 30–150 days) were processed individually (three animals for each age). The newborn were sacrificed 3 h after birth, and older pups were kept with their mothers and siblings until sacrifice. The rats aged 15–150 days were kept under controlled temperature, humidity and light–dark cycles with food and water available *ad libitum*. All experiments were carried out in accordance with the rules set in the National Institute of Health guide for the care and use of laboratory animals and the local animal-ethic instructions set by the CICUAL (Comité Institucional para el cuidado y uso de Animales de Laboratorio) de Argentina.

2.2. Materials and reagents

The monoclonal IgG against α-adaptin (AP-2) (Sigma Chemicals, St. Louis, MO, USA, A 4325) was prepared in mouse against AP-2 adaptor polypeptides from bovine

brain, and it reacts with the 105 and 110 kDa α-subunits of adaptor complex AP-2 in bovine and rats. The mouse monoclonal antibody against the N-terminus (R11-29; amino acids 11–29) of μ2 subunit was kindly provided by Dr. Bonifacino (Bethesda, USA). The goat polyclonal IgG against GGA-1 was purchased from Santa Cruz (sc-23261) raised against an epitope mapping at the N-terminus of human GGA1, and it recognizes a unique band arround 67 kDa. The goat polyclonal IgG against GGA2 (Santa Cruz, sc-19326) was raised against an epitope
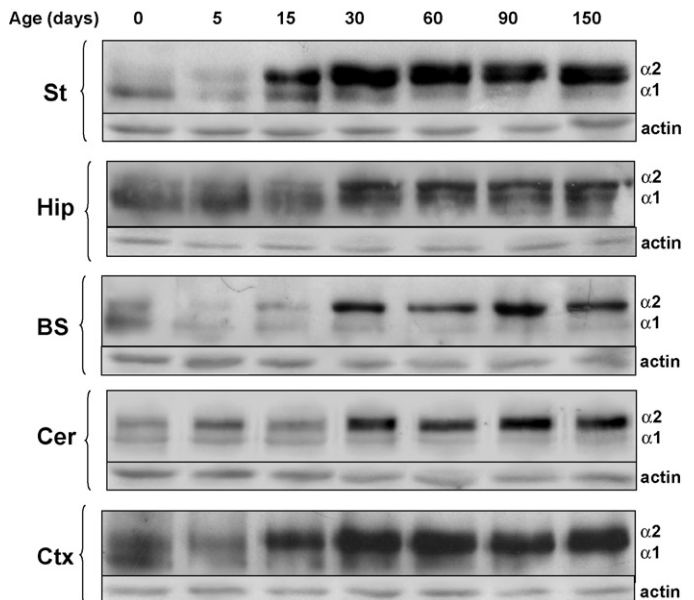
Fig. 2. Immunoblot analysis of AP-2 (α subunits) in different areas of rat brain. Proteins (45 μg) from homogenates of each brain area, at the indicated ages, were subjected to electrophoresis, transferred to nitrocellulose membranes and tested for α1 and α2 subunits of AP-2 with the specific monoclonal antibody that recognize both isoforms. The image shows a representative immunoblot from three independent experiments at each age, as detailed in Section 2. The α1 and α2 isoforms of AP-2 are indicated in the figure. St: striatum; Hip: hippocampus; BS: brainstem; Cer: cerebellum; Ctx: cerebral cortex.
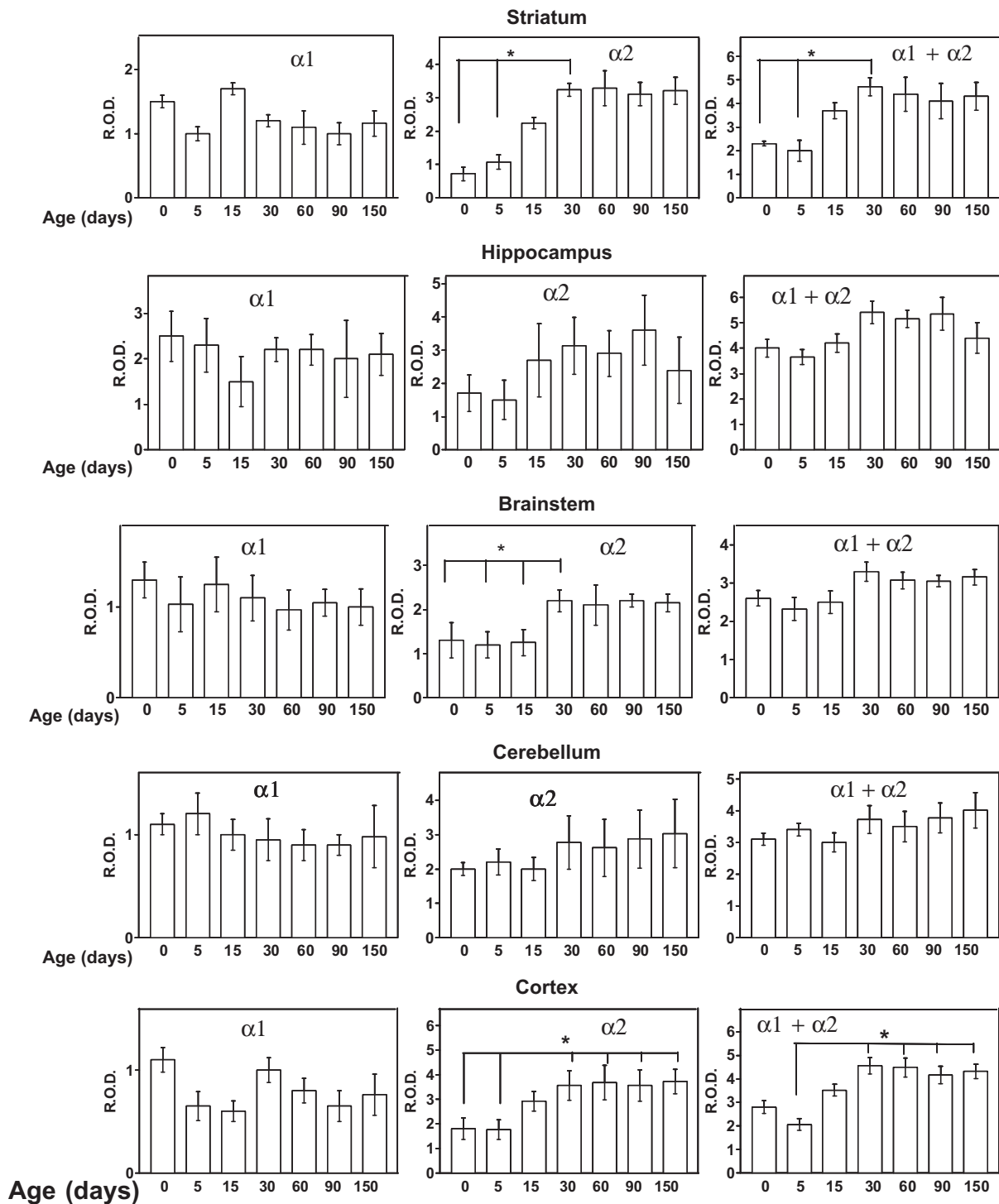
Fig. 3. Quantification of the bands from Fig. 2. The signals were detected by a chemiluminescent method and the bands were quantified by densitometry and normalized to actin expression as reference. Bars represent the means of relative optical densities (ROD) ± SD ($n = 3$ for each age). (*) Significantly different between the maximal and minimal values ($P < 0.05$).

mapped within an internal region of the human GGA2 that reacts with a unique band of 85 kDa in rats. The antibody against presenilin-1 (PS-1) (Sigma Chemicals, St. Louis, MO, USA, P7854), was produced in rabbit (IgG fraction), raised against a synthetic peptide (17 aminoacids) corresponding to the C-terminal of human PS-1 and reacts against a band of 48 kDa from rat brain (full length t protein). The anti-mouse IgG (Fc specific) biotin conjugate (B9904), anti-rabbit IgG (whole molecule) biotin conjugate (B8895), anti-goat IgG (whole molecule) biotin conjugate (A9294), and avidin-peroxidase conjugate (A7419) were all purchased from Sigma Chemicals. Chemiluminescent reagents were from Pearce (Rockford, IL, USA).

2.3. Sample processing

Rats were sacrificed by decapitation and the following brain areas were identified and dissected: cerebral cortex, striatum (bilateral), hippocampus (bilateral), brainstem and cerebellum. Tissues were weighed and homogenized with a glass homogenizer and Teflon pestle, in 20 mM HEPES buffer pH 7.4 (1:3 w/v), containing 0.25 M sucrose, 2 mM EDTA and a mixture of protease inhibitors (1 mM PMSF, 10 mM NaF, 1 μM pepstatin, 100 μM leupeptin, 0.1 TIU/ml aprotinin) purchased from Sigma Chemicals. The homogenates were then centrifuged at 800 × g for 10 min, and the post-nuclear supernatants were recovered and stored at −20 °C up to use.
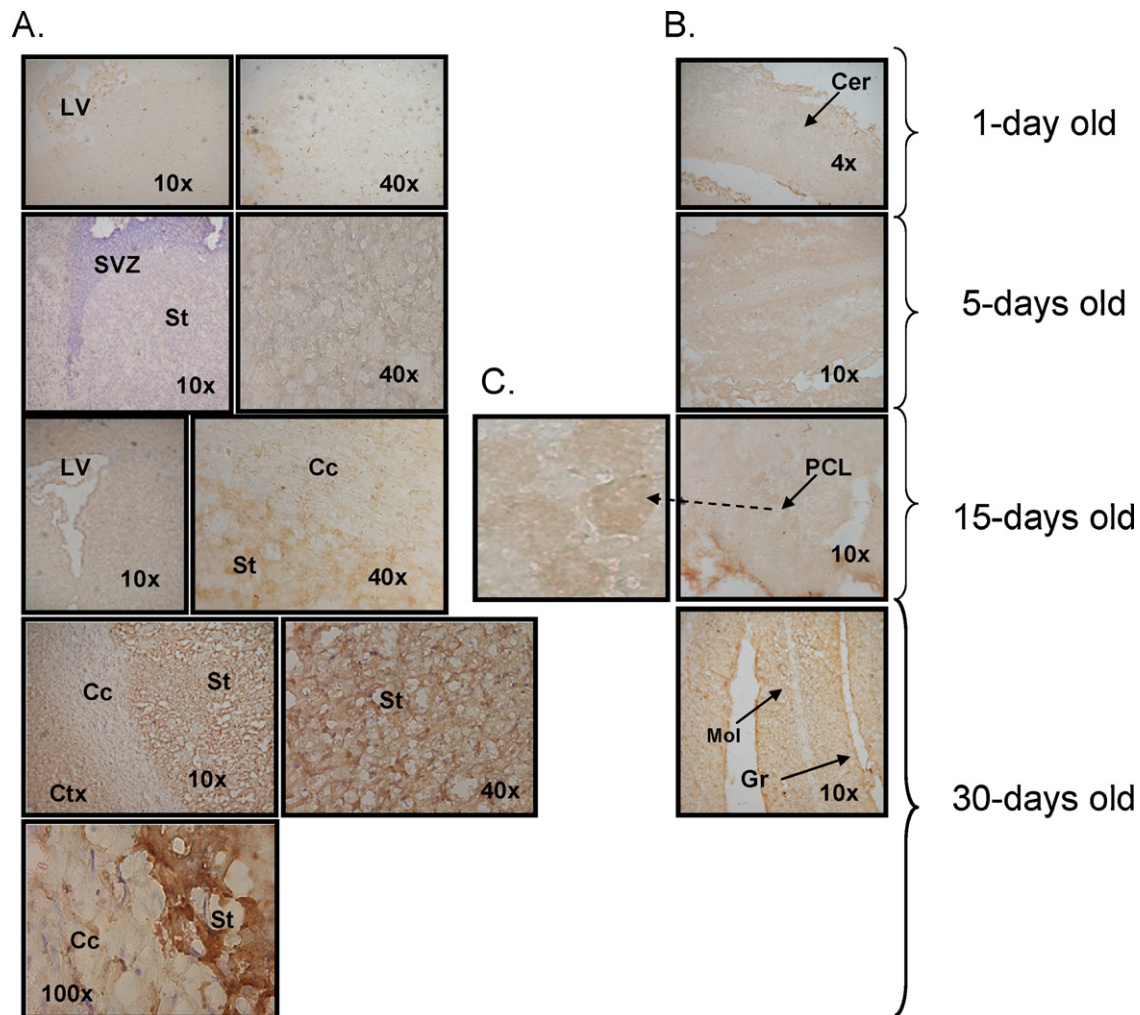
Fig. 4. Immunohistochemistry (IHC) of AP-2 in the brain of rats during development. (A) Images of the *Corpus striatum* at ages 1, 5, 15 and 30 days. Positive immunoreactivity increases with age. By day 30th the IHC revealed intense stained cells and fibers present in the striatosomes. (B) Immunoreactivity to AP2 in the cerebelum increases with age. By the day 15th a clear labeling is observed in the cytoplasm of the Purkinje cells, which are shown in detail at a larger magnification in C. LV: lateral ventricle; St: striatum; Ctx: cerebral cortex; Cc: *Corpus callosum*; Cer: cerebellum; PCL; Purkinje cell layer; Gr: granular cell layer of the cerebellum; Mol: molecular cell layer. 10×, 40× and 100×: magnification of the microscope objective.

2.4. Electrophoresis and immunoblotting

All procedures were carried out according to Alberdi et al. (2005). Briefly, 45 μg of protein from each sample were analyzed on 8% acrylamide gels (SDS-PAGE), and then electrotransfered onto nitrocellulose membranes and processed for detection of the proteins under study: $\alpha 1$-$\alpha 2$ (1:5000) and $\mu 2$ (1:1000) subunits of AP-2, GGA1 (1:1000), GGA2 (1:1000), and PS-1 (1:500), with the corresponding monoclonal antibodies. After incubating with biotin-conjugated antibody and subsequently with avidin-peroxidase, the specific bands were detected by a chemiluminescence method, and the bands were quantified from the films (Kodak X-Omat) by densitometric scanning and analyzed with the Scion Image program. The values of density were normalized for equal loadings in the gels by comparing with actin expression as follows: protein O.D. (AP-2, GGAs or PS-1)/actin O.D. × 1.2 (Correction factor to approximate values to 1).

2.5. Immunohistochemistry

Animals were deeply anesthetized with chloral hydrate, perfused intracardially with a washing solution (0.8% NaCl, 0.8% sucrose, 0.4% glucose) and then with 4% paraformaldehyde in 0.01 M phosphate buffer pH 7.3 and 0.35 M Na_2SO_3. The brains were cryoprotected and stored at −70 °C. Slices (20 μm) were mounted on gelatin- alum-chrome coated slides and treated for antigen retrieval by placing the sections on 10 mM sodium citrate buffer, pH 6.0 at 95 °C, incubated in blocking solution (10 mM phosphate buffer, 0.9% NaCl, pH 7.4, with 0.1% Triton X-100, 1% goat serum and 1% bovine serum albumin). The primary antibody was a rabbit monoclonal anti α-adaptin (Sc-10761, from Santa Cruz Biotechnology, Inc.) 1:100, diluted in 10 mM phosphate buffer, 0.9% NaCl, Triton 0.1%. The slides were then incubated with biotin-conjugated anti-rabbit IgG (Sigma, B8895, 1:200) for 2 h at room temperature. Subsequent incubation was carried out with HRP-conjugated ExtrAvidin® (Sigma, Mouse ExtrAvidin® Peroxidase Staining Kit, 1:100) and diaminobenzidine (DAB)/H_2O_2 for color development. As a negative control, the primary antibodies were omitted and no specific labeling was identified in these sections. Slices were counterstained with cresyl violet, mounted, dehydrated and coverslipped with Bálsamo de Canadá (Biopack). Sections were photographed with a light microscope (NikonAlphaphot-2 and the Software ACT2U).

2.6. Other procedures

Protein concentration was measured according to Lowry et al. (1951).

2.7. Statistics

The data obtained were subjected to Tukey's Multiple Comparisons Test and the level of significance was set at $P \leq 0.05$.

3. Results

3.1. Identification of brain areas in newborn and 5-day-old rat pups

As it is known, rat offspring are born before total completion of several nervous system organs, but around birth most of them are delineated. In order to corroborate the presence of brain area boundaries in very young animals we obtained 20 μm sections of
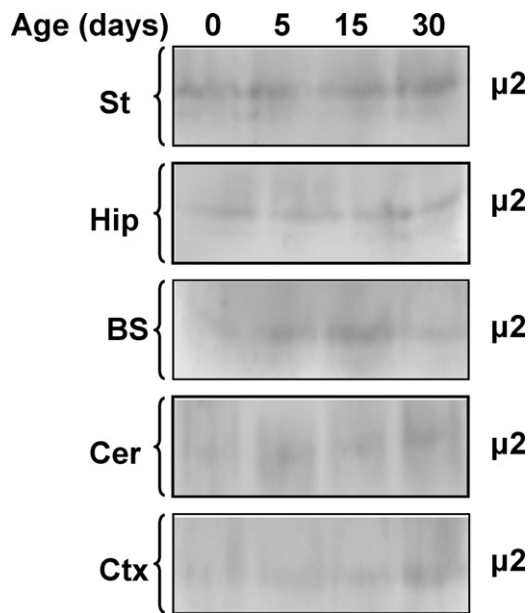
Fig. 5. Immunoblot analysis of μ2 subunit in different areas of rat brain. Proteins (45 μg) from homogenates of each brain area, at the indicated ages, were subjected to electrophoresis, transferred to nitrocellulose membranes and tested for μ2 subunit of AP-2 with the specific monoclonal antibody. St: striatum; Hip: hippocampus; BS: brainstem; Cer: cerebellum; Ctx: prefrontal cortex.

the brains containing the areas of interest, stained them with cresyl violet and identified their boundaries with the help of a low magnification (4×) microscopic objective (Fig. 1).

3.2. Expression of AP-2 during postnatal development

It was observed that expression of AP-2 changes specifically during perinatal development according to the brain area (Fig. 2). Interestingly, the expression of the α2 subunit is almost undetectable in the striatum of newborn and up to 5-day-old rats. However, on the 15th day, the expression of this protein increases substantially, reaching a maximum around the 30th day. This level is maintained during 150 days after birth. In contrast, α1 isoform does not change significantly throughout perinatal development. A similar trend was observed in hippocampus, prefrontal cortex and brainstem (Fig. 3), meanwhile, in the cerebellum, both α isoforms of AP-2 show little change during postnatal development. However, when both α isoforms were quantified together as representative of AP-2 the differences between early and late ages were significant only in the striatum and cortex (Fig. 3). The expression of actin was used as load control (Fig. 2). Immunohistochemistry of α subunits of AP-2 confirmed the results found by immunoblot (Fig. 4). On the other hand, the expression of the μ2 subunit did not vary between the newborn and the other postnatal ages, in any of the studied areas (Fig. 5). This was somehow expected, as this subunit interacts indistinctly with both, α1 and α2 isoforms of AP-2.

3.3. Expression of GGA1 and GGA2 during rat postnatal development

In general, it was noted that GGA1 expression is very low at the early stages of development, and tends to increase around the 30th day after birth (Fig. 6). However, only in the hippocampus and prefrontal cortex the differences between younger and older ages were significant (Fig. 6B). As with GGA1 and AP-2, the expression of GGA2 is low in newborn and 5-day-old pups, and tends to increase around the day 15th in the striatum and cortex, and by the day 30th

in the hippocampus (Fig. 7). Meanwhile, in brainstem and cerebellum the expression of this protein does not change significantly during development (Fig. 7).

3.4. Expression of presenilin during rat postnatal development

We set as objective to explore whether presenilin (PS-1), a protein related to aging and neurodegeneration, evolves similarly to the coat proteins. By studying the distribution of PS-1 in brain tissue during development in comparison with the evolution of coat proteins, we attempted to determine whether the endocytic processes and the vesicular transport are developmentally associated. PS-1 is not similarly distributed among the areas studied, since the expression of this protein is very high in the striatum compared to other areas, even from the 15th day of age, whereas in the cortex reaches the maximum around the 30th day after birth (Fig. 8A). In turn, the expression of PS-1 (full lenght ~48 kDa) varies differently in each brain area during development; in the striatum followed a similar trend to that observed for coat proteins, whereas in the hippocampus, cerebellum and cortex, the expression of this protein remains unchanged during postnatal development. A striking case is the brainstem, where PS-1 reaches a maximum on the 30th day after birth, and then it tends to decline (Fig. 8B).

4. Discussion

Coated vesicles are the main vehicles to transport macromolecules between cellular compartments. In the central nervous system (CNS), it is thought that clathrin-coated vesicles (CCVs) are actively involved in maintaining the integrity and neuronal plasticity. Among the large number of coat proteins, the adaptor complexes (APs) confer specificity to the CCVs, so that AP-2 is unique to the CCVs in the receptor-mediated endocytosis in most cell types. Recent findings have suggested that clathrin-mediated endocytosis might be altered in neurodegenerative processes, as well as in the mechanisms of excitotoxicity caused by drugs (Wu and Yao, 2009; Borgonovo et al., 2009). However, it is not known whether these alterations in the endocytic machinery are cause or consequence, or both, of neural disorders. AP-2 complexes consist of two major subunits, α and β2, a midsized subunit μ2, and a small subunit δ2. In the nervous system, two isoforms of α subunits have been described, α1 and α2 (or αA and αC adaptin respectively) (Ball et al., 1995; Hirst and Robinson, 1998), but co-existence of both forms have not yet been explained. We believe that the α1 subunit would be involved in clathrin-mediated endocytosis in the cell body, whereas α2 might be specific for vesicles of nerve terminals. This suggestion is based on the fact that α1 is common to all tissues, while α2 is mostly found in the CNS neurons (Ball et al., 1995).

In this study, we found that the expression of both subunits varies differently during postnatal development in some areas of rat brain. In striatum, α1 can be detected even at early ages, whereas α2 is highly expressed on the 15th day after birth. A similar trend was observed in other areas of the brain, such as hippocampus, brainstem and cortex. However, in the hippocampus and cortex, the appearance of α2 was observed from the day 30 after birth onwards. From these observations, we might assume that the period between the 15th and 30th day after birth (depending on the area) could be crucial for the establishment and/or expansion of neural networks, and for new synapses during development of nervous tissue. Although the subunit μ2 did not follow the pattern of α2, this apparent discrepancy could be explained by the ability of μ2 to interact indistinctly with both isoforms of α subunit. Other proteins involved in intracellular transport mediated by vesicles, such
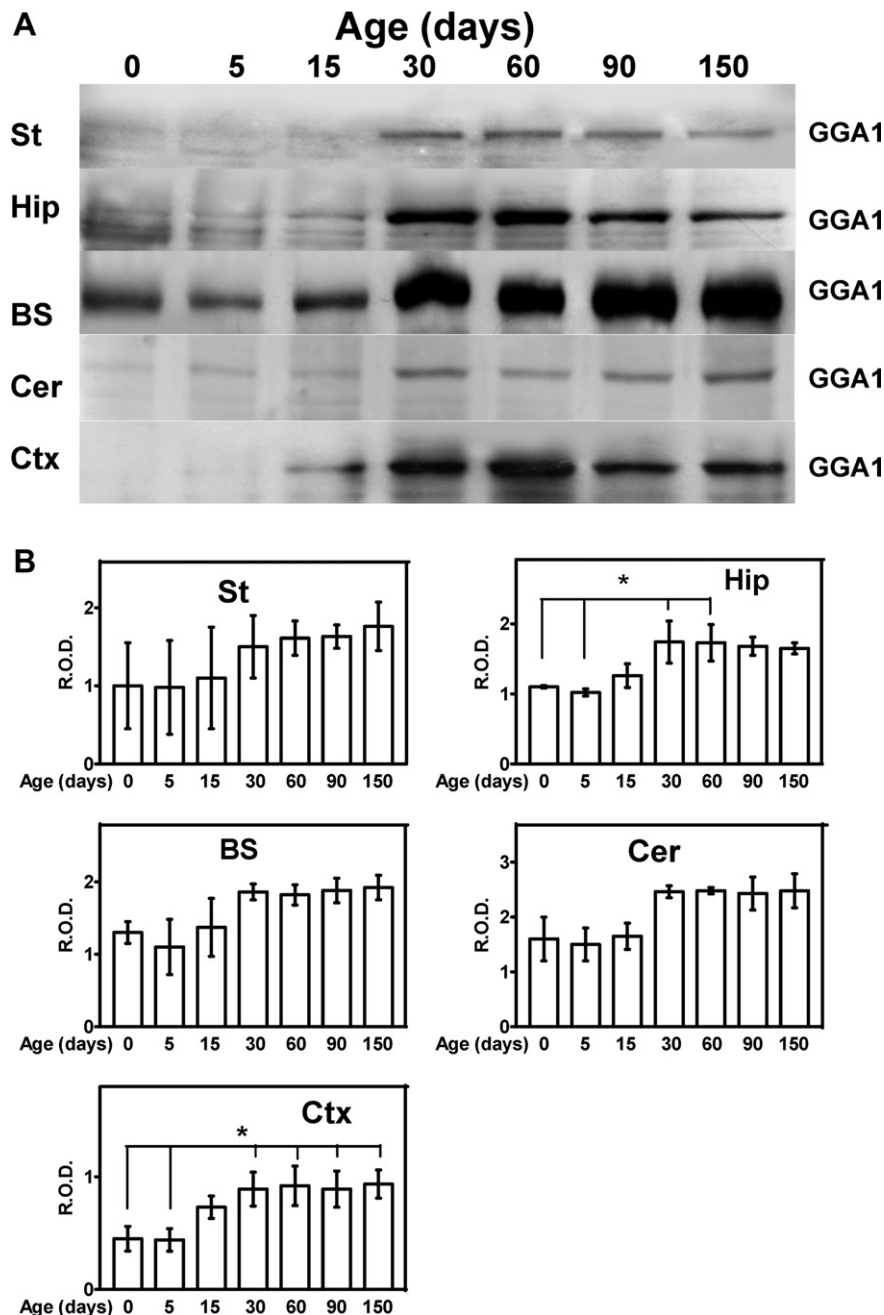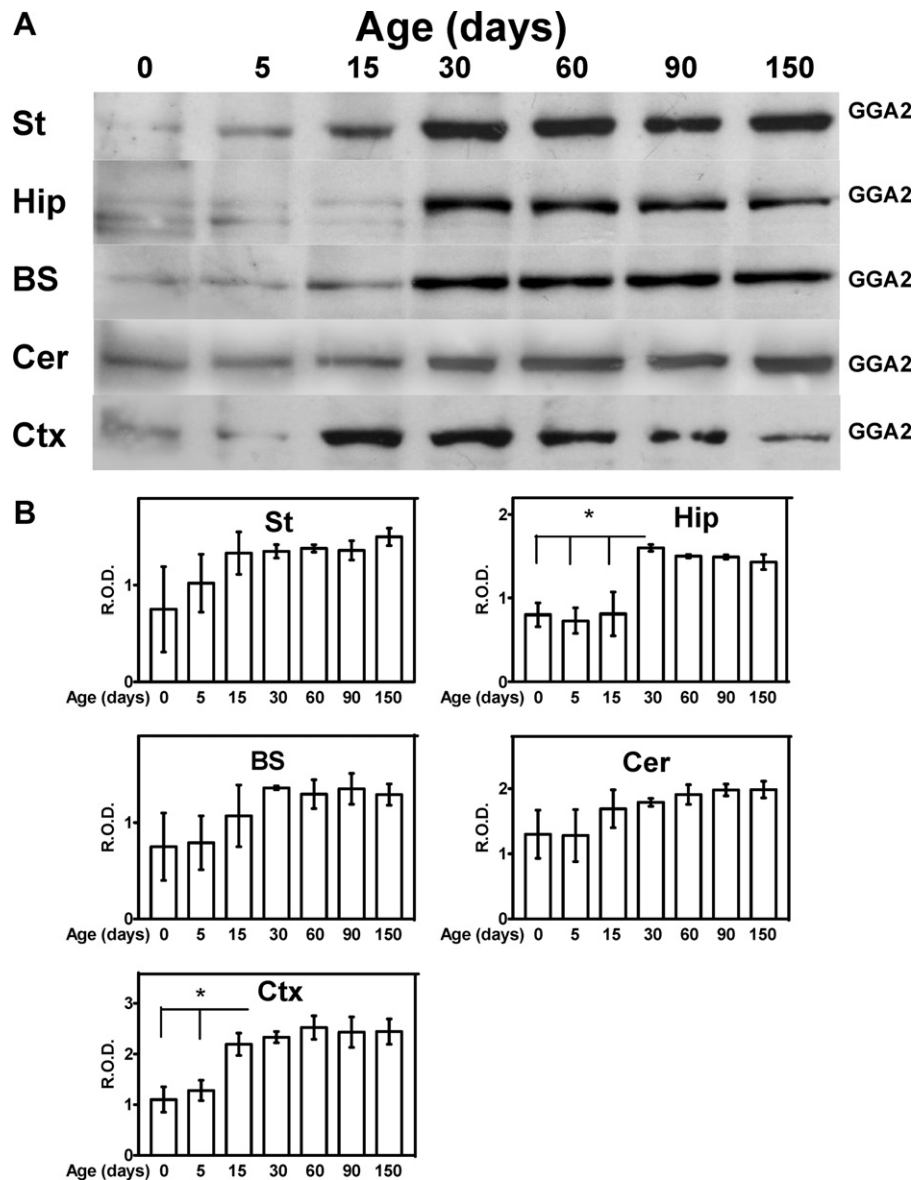
Fig. 6. Immunoblot analysis of GGA1 in different areas of rat brain. Proteins (45 μg) from homogenates of each brain area, at the indicated ages, were subjected to electrophoresis, transferred to nitrocellulose membranes and tested for GGA1 with the corresponding polyclonal antibody (see details in Section 2). (A) The image shows a representative immunoblot from three independent experiments at each age, as detailed in Section 2. (B) Quantification of the bands. The signals were detected by a chemiluminescent method and quantified by densitometry and normalized to actin expression. Bars represent the mean of relative optical densities (ROD) ± SD ($n = 3$ for each age). (*) Significantly different between the maximal and minimal values ($P < 0.05$). St: striatum; Hip: hippocampus; BS: brainstem; Cer: cerebellum; Ctx: cerebral cortex.

as GGA1 and GGA2 also vary in different brain areas during postnatal development, accompanying somehow the evolution of the α2 subunit.

It is known that the striatum consists mainly of medium spiny neurons (~95%) and only 10% of interneurons (reviewed by Jain et al., 2001). The GABAergic interneurons modulate the cortical input, and mediate inhibition of striatal medium-sized spiny projection neurons. Recently, it has been shown that interneurons are under the cortical control during the first days of life, and they mature considerably in the striatum of rats between 12–19 postnatal days (Chesselet et al., 2007). It is also likely that the development

and maturation of the medium spiny neurons may occur in these critical days of development, in parallel with the interneurons and expansions of neurons in the cortex.

A similar tendency was observed in the hippocampus, indicating that the endocytic machinery could reach the maximum development around 30 days after birth. As a precedent, both AP-180 and CALM, other proteins in CCVs that play a critical role in establishing neuronal polarity, are essential for the growth of dendrites and axons in hippocampal neurons (Bushlin et al., 2008). It has been shown that by the end of embryonic development and continuing into the postnatal period in the murine, there is a secondary
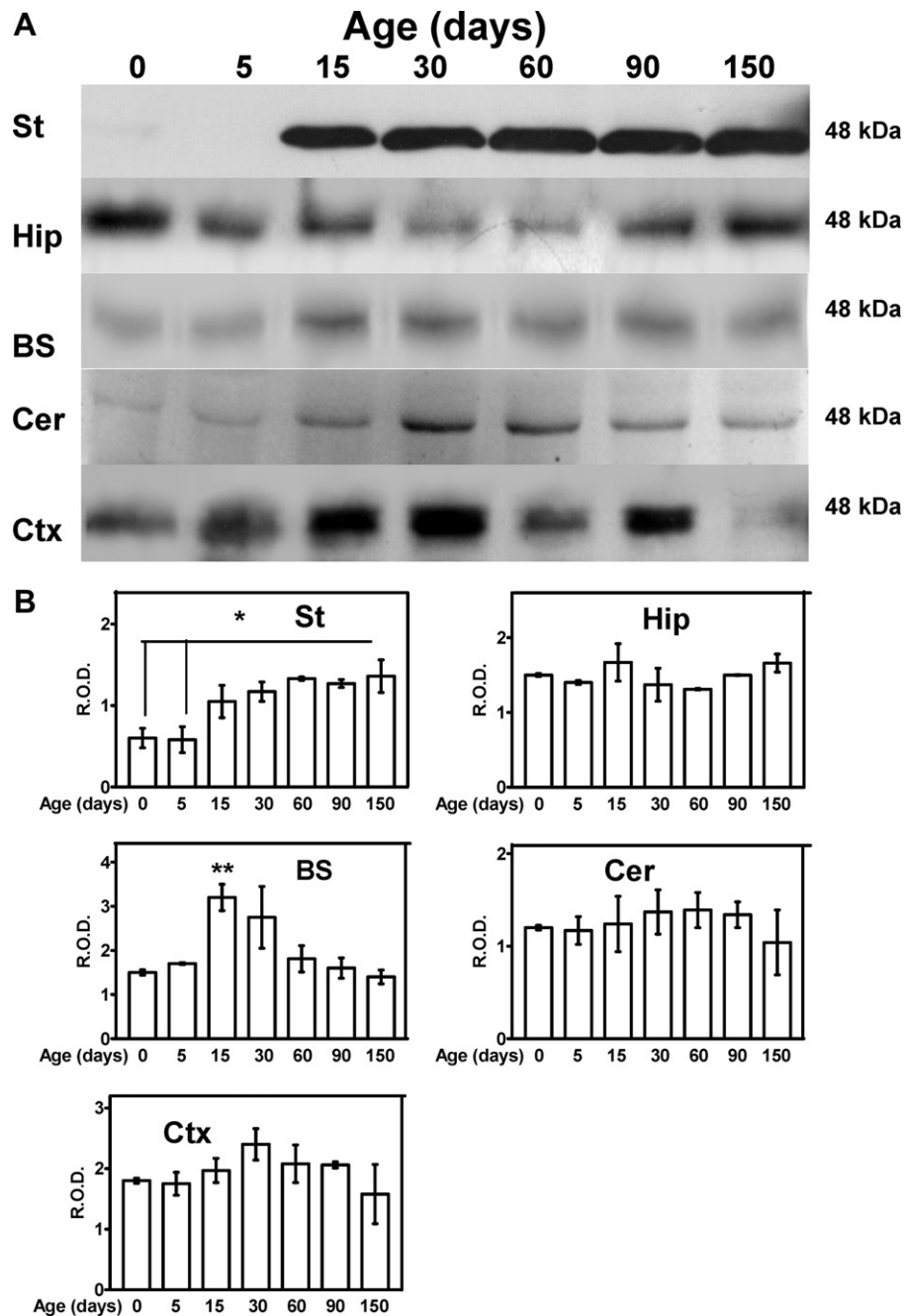
Fig. 7. Immunoblot analysis of GGA2 in different areas of rat brain. Proteins (45 µg) from homogenates of each brain area, at the indicated ages, were subjected to electrophoresis, transferred to nitrocellulose membranes and tested for GGA2 with the corresponding polyclonal antibody (see details in Section 2). (A) The image shows a representative immunoblot from three independent experiments at each age, as detailed in Section 2. (B) Quantification of the bands. The signals were detected by a chemiluminescent method and quantified by densitometry, normalized for actin detection. Bars represent the means of relative optical densities (ROD) $\pm$ SD ($n = 3$ for each age). (*) Significantly different between the maximal and minimal values ($P < 0.05$). St: striatum; Hip: hippocampus; BS: brainstem; Cer: cerebellum; Ctx: prefrontal cortex.

neurogenesis in the subventricular area, in the dentate gyrus and in the cerebellar external lamina, being mainly restricted to the olfactory bulb, hippocampus and cerebellar cortex (Abrous et al., 2005).

The fact that most granule cells are generated during the first week after birth and that a massive migration of neuronal precursors towards the olfactory bulb and cortical and subcortical areas occurs around the third week after birth could be consistent with the evolution of the endocytic machinery observed in this study. In cerebellum, the expression of the α2 subunit was different from other areas, as this protein is expressed already at birth and remains almost unchanged throughout life. Increases in the expression of the α2 subunit during postnatal development were corroborated by immunohistochemistry, noting a more intense staining at later ages (30th day). Not only the endocytic machinery evolves during development in some brain areas, but also the intracellular transport mediated by vesicles could increase around 15–30 days of age.

This assumption arises from the fact that GGAs expression (GGA1 and GGA2) tended to increase between 15 and 30 days after birth in the areas studied. The increase of the coat protein expression in the cerebellum seems to initiate as early as postnatal day 17, when granule cell proliferation and migration decline in this area and from postnatal day 30 onward, after the end of the histogenetic processes (Altman, 1982) in the rat.

Presenilin 1 (PS-1) is an integral component of the complex of high molecular weight gamma-secretase, which participates in the cleavage of type I membrane proteins such as amyloid precursor protein. Recent studies have shown that proteolytic fragments of PS-1 are enriched in clathrin-coated vesicles from somatodendritic membranes (Efthimiopoulos et al., 1998), suggesting a regulatory role of presenilin in the constitutive secretion and endocytosis. We confirmed that the expression of PS-1 changes during postnatal development in different areas of the brain, although these changes were dissimilar from one area to another. Even though the

Fig. 8. Immunoblot analysis of PS-1 in different areas of rat brain. Proteins (45 μg) from homogenates of each brain area, at the indicated ages, were subjected to electrophoresis, transferred to nitrocellulose membranes and tested for PS-1 with the corresponding polyclonal antibody (see details in Section 2). (A) The image shows a representative immunoblot from three independent experiments at each age, as detailed in Section 2. (B) Quantification of the bands. The signals were detected by a chemiluminescent method and quantified by densitometry, normalized for actin detection. Bars represent the means of relative optical densities (ROD) ± SD (n = 3 for each age). St: striatum; Hip: hippocampus; BS: brainstem; Cer: cerebellum; Ctx: prefrontal cortex. (*) and (**), significantly different between the maximal and minimal values ($P < 0.05$ and $P < 0.01$, respectively).

expression of PS-1 in the cerebellum and hippocampus during postnatal development was similar to that observed by other authors (Ribaut-Barassin et al., 2003), we found no correlation with the evolution of AP-2 nor GGAs at all ages studied However, in the striatum the expression of PS-1 coincided with the appearance of coat proteins and with development of vesicular transport. Clathrin-coated vesicles as vehicle for PS-1 in this area, will be subject of study in further research. In the brainstem, the expression of PS-1 is contrasted completely with the expression of coat proteins, since PS-1 increased progressively from birth until day 15th of age and then

decline to values similar to the early ages. We may assume that this phenomenon takes place because PS-1 plays a crucial role in the development of this area during the first two weeks of life. We conclude that, the intracellular transport machinery and endocytic could evolve in line with neurogenesis, suggesting that both routes are essential for neuronal development.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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