Psicothema 2003. Vol. 15, nº 2, pp. 216-220
www.psicothema.com

ISSN 0214 - 9915 CODEN PSOTEG

Maturation of astrocytes in the rat hippocampus: Potential behavioral implications

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Several studies have shown that astroglia plays an important role on the physiology of the central nervous system. However, still little research has focused on the analysis of the astroglial population during the postnatal development, and the possible influence of biological factors like sex. Using stereological methods, the total number of glial fibrillary acidic protein immunoreactive (GFAP-ir) astrocytes and their morphology, were estimated in CA1 and CA3 hippocampal areas from 21 and 90-day-old male and female rats. The number of GFAP-ir astrocytes were significantly increased according to age in both hippocampal areas from females, but the same result was obtained only in the CA3 area of males. In addition, no gender differences were found in both hippocampal areas at 21 days of age, but the opposite result was found at 90 days of age. Numerical changes and gender differences in the developing hippocampal astrocyte population are discussed as related to their possible functional meaning.

Maduración de los astrocitos del hipocampo de la rata: posibles implicaciones conductuales. Diversos trabajos han mostrado que la astroglía juega un papel relevante en la fisiología del sistema nervioso central. Sin embargo, aún existen pocos estudios que se hayan centrado en el análisis de la población astroglial durante el desarrollo postnatal y la posible influencia de factores biológicos como el sexo. Empleando métodos estereológicos, se estimó el número total de astrocitos inmunorreactivos a la proteína ácida de la glía fibrilar (GFAP-ir) y su morfología en las áreas CA1 y CA3 del hipocampo de ratas macho y hembra a los 21 y 90 días de edad. El número de astrocitos GFAP-ir se incrementa significativamente con la edad en ambas áreas hipocámpicas en las hembras, pero sólo en el área CA3 en los machos. Además, no se observaron diferencias de género en ambas áreas hipocámpicas a los 21 días de edad, pero sí a los 90 días. Los cambios numéricos y diferencias de género en la población astroglial hipocámpica durante el desarrollo, se discuten en relación con su posible significado funcional.

Traditionally, a crucial role has been attributed to neuronal systems as related to brain function by most researchers. In this regard, little attention is currently paid to the nonneuronal cellular components of the brain, which outnumber neurons by a factor of ten. Particularly, among glial cells, astrocytes have been attributed considerable functional significance in the developing and mature central nervous system (CNS). For example, astrocytes contribute to the maintenance of brain extracellular ionic homeostasis (Catalani et al., 2002), migration and maturation of neurons (Bezzi and Volterra, 2001), synthesis and reuptake of certain neurotransmitters (Inazu, Takeda, Ikoshi, Sugisawa, Uchida and Matsumiya, 2001), and are able to store energy in form of glycogen (Magistretti, Olivier and Martin, 1993). Interestingly, from a clinical point of view, astroglial cells are also involved in the maintenance of the tissue integrity following brain lesions (García-Segura, Naftolin, Hutchison, Azcoitia and Chowen, 1999) and modulate the synaptic function underlying behavior (Bacci, Verderio, Pravettoni and Matteoli, 1999; Pfrieger and Barres, 1997). Recent evidence suggest regional alterations in glial number in patients with depression and after receiving neuroleptic drugs (Selemon, Lidow and Goldman-Rakic, 1999; Ongur, Drevets and Price, 1998).

Regarding behavior, one of the brain regions that plays an essential role in spatial learning and memory is the hippocampus. Throughout its development, this structure is a main target for gonadal steroids, hormones involved in the sexual differentiation (O'Keefe, Li, Burgess and Handa, 1994). Hence, sex differences during development in behavioral tasks associated with hippocampal function have been reported extensively in rodents (Cimadevilla et al., 1999; Roof and Havens, 1992) and humans (Wang, Hermer and Spelke, 1999). It must be taken into consideration that astrocytes are clearly affected by gonadal steroids, because like neurons, they express receptors for these hormones (Jung-Testas, Renoir, Gasc and Baulieu, 1991). In this sense, it has been reported that astrocytes may alter their morphology in response to variations in the levels of estradiol (Chowen, Azcoitia, Cardona-Gómez and García-Segura, 2000). A cellular constituent responsible for maintaining the morphology of astrocytes is the glial fibrillary acidic protein (GFAP), a component of the intermediate filaments

Fecha recepción: 24-10-02 • Fecha aceptación: 20-12-02
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that build the cytoskeleton. Actually, GFAP is the most widely used marker for identification of astrocytes (Bignami and Dahl, 1977). Changes in the expression of GFAP have been also observed in the dentate gyrus from the rat hippocampus with estrous cycle (Hajos, Halasy, Gerics, Szalay, Michaloudi and Papadopoulos, 2000). Furthermore, in the CA1 area of the hippocampus, not only the morphology but also the number of GFAP-immunoreactive (GFAP-ir) astrocytes change during the first two weeks after birth (Nixdorf-Bergweiler, Albrecht and Heinemann, 1994).

Only one recent study has analyzed changes in hippocampal GFAP-ir astroglial population during the postnatal development of male and female rats (Catalani et al., 2002), but an inaccurate counting method seemed to be used. We have recently applied steoreological methods to assess gender differences in the number of GFAP-ir astrocytes in adult rat hippocampus (Conejo, González-Pardo, Pedraza, Navarro, Vallejo and Arias, 2003 –in press–). The aim of the present study was to evaluate the possible changes in the number of hippocampal GFAP-ir astrocytes (CA1 and CA3 areas) in both weanling (21 days) and young (90 days) rats from both sexes. The optical fractionator (West, 1993) was used as an unbiased stererological counting method.

Materials and methods

Subjects

Twenty-four female and male Wistar rats from the University of Oviedo central vivarium were used in the present study. Six animals from both sexes were selected at the following ages: 21 days (weaning period) and 90 days (young adults). To avoid litter effect, a maximum of two or three animals were selected from the same litter to make up the four experimental groups. Estrous cycle was assessed in female adult rats analyzing vaginal smears, in order to select them during the same estrous phase (early proestrous). Animals were housed in groups of five of the same sex per cage and maintained on a 12:12 h light/dark cycle (light on at 08:00 h) a temperature of 22±2 ºC, and had ad libitum access to food and water. All experimental procedures followed strictly the EEC Council Directive 86/609 regarding care and use of laboratory animals.

Immunocytochemistry

Rats from the different groups were deeply anaesthetized with sodium pentobarbital (70 mg/kg for males and 45 mg/kg for females i.p.) and perfused through the left cardiac ventricle with 0.9% saline in 0.1 M phosphate buffer (PBS; pH 7.4) followed by 10% phosphate-buffered formalin. Brains were removed after perfusion and post fixed for at least one week in the same fixative. Brain blocks were cut coronally to select the hippocampal formation. Tissue blocks were then dehydrated with a graded series of ethanol solutions (70, 80, 96 and 100%) a xylene bath, and embedded in paraffin.

Coronal sections of the entire hippocampal formation contained in each paraffin block were serially cut at 20 µm with a rotary microtome (Leica, Germany), selecting one out of five sections on gelatinized slides. For each subject, the total numbers of sections containing the different hippocampal areas (CA1 and CA3) were taken into consideration, to estimate in a later analysis their volumes. A series of alternate sections were Nissl stained with a 0.5% cresyl violet solution to easily outline the boundaries of the hippocampal areas studied. The remaining sections were used to detect hippocampal astrocytes using an antibody to an intermediate filament protein of the cell cytoskeleton from astrocytes (glial fibrillary acidic protein or GFAP).

The immunocytochemical staining method used will be briefly described below. After deparaffinizing the sections, they were permeabilized in tris buffer saline (TBS) containing 0.1% Triton X-100, followed by a 30 min pre-incubation in 1% human serum (Sigma, USA) dissolved in TBS. After this blocking step, a polyclonal primary antibody (rabbit anti-GFAP) (Dako, Denmark) was applied at 1:800 dilution, and the sections were incubated for 24 h at 4 ºC. Sections were washed three times in TBS with Triton X-100, incubated in biotinylated secondary goat anti-rabbit IgG antibody (1:30 dilution; Pierce, USA) in 10% bovine serum for 30 min, and dipped three times in TBS with Triton X-100. The sections were then incubated with an avidin-biotin horseradish-peroxidase complex (Vectastain ABC-Ultrasensitive, Elite Kit; Pierce, USA) for 1 hr at room temperature, washed twice in TBS with Triton X-100, rinsed in TBS, and then visualized with diaminobenzidine (Sigma, USA). Finally, the sections were dehydrated in ethanol, cleared in xylene, and coverslipped with *Entellan* (®Merck, USA).

Stereological quantification

The equipment used in our laboratory for obtaining stereological measures is composed of a binocular microscope (Olympus BH-2, Japan) equipped with a device that measures movements in the z-axis direction (Heidenhain MT-12 microcator, Germany) and connected via a high resolution CCD video camera to a black and white video monitor (Sony, Japan). A square-shaped counting frame was drawn on the screen of the monitor. The optical fractionator stereological method (West, 1993; West, Slomianka and Gundersen, 1991) was used to estimate the total number of astrocytes in selected hippocampal areas separately (CA1 and CA3). This statistically unbiased method is a combination of the optical disector, another stereological method in which objects (cells in our case) are counted using a three-dimensional probe and a fractionator sampling scheme, performed by placing this probe systematically throughout a known fraction of the tissue. Briefly, a series of a few equidistant sections per subject selected in a systematic-uniform random manner from all of the sections comprising the hippocampus were placed under a 100 X oil immersion objective. In each section, the microscopic fields including the regions of interest were visualized on the video monitor, and each hippocampal area was systematically sampled according to an x- and y-axes microscope stage movement sequence with a certain step. Additionally, only hippocampal astrocytes within the counting frame and focused below 2.5 µm (guard height) and above or equal to 15 mm in the z-axis direction were counted. An unbiased estimation of the total number of astrocytes (*N*) from a particular hippocampal area can be estimated using the following formula:

$$N = (\Sigma Q^{-}) \cdot (1 / ssf) \cdot (1 / asf) \cdot (1 / tsf)$$

where ΣQ^{-} is the total number of cells included in the counting frames from all of the selected sections counted, *ssf* is the section sampling fraction or the proportion of selected sections as regards to the total number of sections obtained from an hippocampal area

(1 out of 15 on average). The thickness sampling fraction or *tsf* is given by the section thickness (20 µm) divided by the height of the optical disector used (15 µm). Calculation of the area sampling fraction (*asf*) is given by the quotient between the area of each counting frame and the tissue area covered by each displacement of the microscope stage in x and y directions with a preset step (1 out of 3 microscopical fields selected in both directions).

An estimation of the total volume of both hippocampal areas was performed by applying the Cavalieri principle (Gundersen et al., 1988). Using a microscope equipped with a drawing tube, the anatomical boundaries of the hippocampal regions selected in each section used for the optical fractionator procedure were drawn on a paper. By superimposing randomly a transparent point grid on the images drawn on the paper, the number of points falling within the boundaries of each region profile is calculated. An unbiased estimate of the volume of each region is given by $t \cdot \Sigma P \cdot a(p)$, where t is the thickness of the brain region selected, derived from the total number of sections obtained from it and the thickness of each section (20 µm), ΣP is the total number of points counted for each region and $a(p)$ is the area associated with each point of the grid corrected for the microscopic magnification used.

The anatomical boundaries for each area followed the atlas by Paxinos and Watson (1986) using the Nissl stained sections as a reference for the GFAP stained alternate sections.

Statistical analysis

Differences in the number of GFAP-positive astrocytes in the experimental groups for each hippocampal area were analyzed by two-way ANOVAs with sex and age as main factors. Post hoc Tukey's tests were done to assess significant differences between group means when significant effects were obtained after performing an ANOVA. Differences were considered statistically significant when $p<0.05$. The coefficients of variation (CV) were calculated according to Gundersen and Jensen (1987) for each numerical stereological estimation.

Results

Morphology of GFAP-ir astrocytes

The GFAP-ir astrocytes of 21-day-old animals were located surrounding the pyramidal neuron cell layer of the hippocampus, but also this layer was partially covered by astrocytes in adult rats (Fig. 1). An analysis of the morphological types of GFAP-ir astrocytes found in these hippocampal layers showed clear differences related to the ages studied. Hence, following the morphological classification by Mong et al. (1996), 21-day-old males presented mostly type II astrocytes. These cells are characterized by an overall stellate morphology, but still retain a bipolar appearance and show strongly stained thick processes. However, in 90-day-old rats GFAP-ir astrocytes were characterized by a fully stellate morphology, with a small cell soma, with long and thin extended processes, which possessed a high degree of branching (types III and IV).

Stereological quantification of astroglial cell numbers

The total number of GFAP-ir astrocytes found in all layers (*stratum oriens, radiatum* and *lacunosum-moleculare*) of the selected hippocampal areas were not significantly different regarding sex and the hippocampal areas studied in 21-day-old animals. However, a significant interaction was found between gender and hippocampal area in adult rats [$F(1,20)= 14.96$; $p<0.001$]. Particularly, the number of GFAP-ir astrocytes was significantly higher in the CA3 area of males as compared with age-matched females [Tukey's test; $p<0.05$]. Moreover, females showed significantly more GFAP-ir astrocytes in the CA1 area as compared to males [Tukey's test; $p<0.05$]. In addition, the magnitude of the differen-

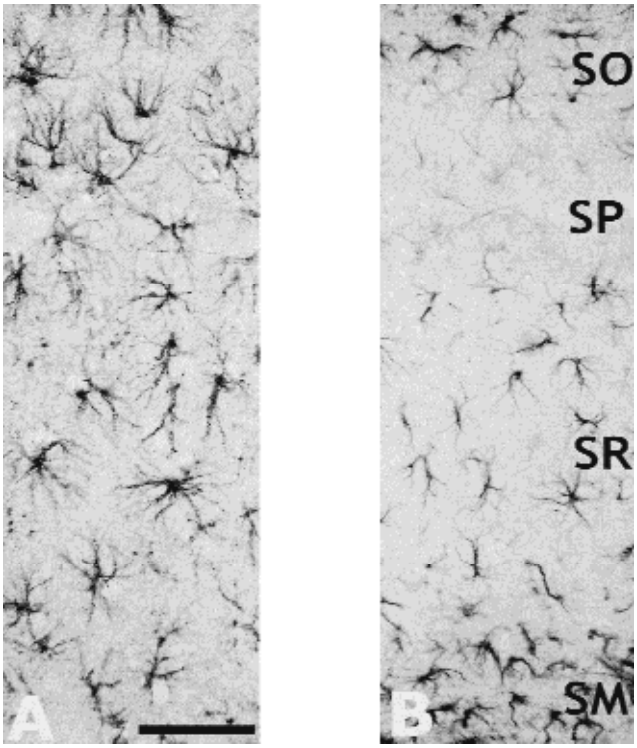

Figure 1. *Photomicrographs showing GFAP-immunoreactivity in the CA1 area of the rat hippocampus at 90 days (**A**) and 21 days (**B**) of age. Changes in the density and morphology of stained astrocytes can be clearly observed between both ages studied. **SR**: stratum radiatum, **SP**: stratum pyramidale, **SO**: stratum oriens, **SM**: stratum lacunosum-moleculare. Scale bar: 50 µm*

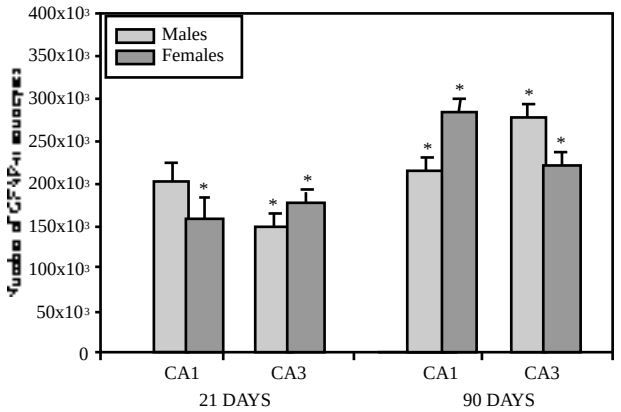

Figure 2. *Total number (mean + S.E.M.) of GFAP-ir astrocytes quantified in the hippocampus (CA1 and CA3 areas) of male and female rats from both sexes. * $p<0.05$ males versus females, and $^{+}$ $p<0.05$, comparison between 21 and 90 days of age (Tukey's post-hoc tests)*

ces between the hippocampal areas studied was similar in males and females (mean numerical differences of about 22%).

When changes in the number of GFAP-ir astrocytes with age were studied, a significant interaction between age and sex on the number of GFAP-ir astrocytes was found in CA1 area [$F(1,20)= 7$; $p= 0.016$] and CA3 area [$F(1,20)= 8.23$; $p<0.009$]. Particularly, an increase in the number of GFAP-ir astrocytes was observed for females in both the CA1 [Tukey's test; $p= 0.003$] and CA3 areas [Tukey's test; $p<0.05$]. However, only a significant increase in the number of GFAP-ir astrocytes of CA1 area [Tukey's test; $p<0.001$] was found in males. Lastly, no numerical changes were detected in CA3 area of male rats.

The CV calculated for CA1 and CA3 areas at 21 days were: 0.28 and 0.24 (males), 0.34 and 0.22 (females). At 90 days of age, the CV for CA1 and CA3 areas were: 0.13 and 0.09 (males), 0.18 and 0.19 (females).

Discussion

Our results on the quantification of the total number of GFAP-ir astrocytes have shown that the rat hippocampus undergoes sex-related regionally specific changes during the postnatal development between 21 and 90 days of age. Similar results were obtained by Catalani et al. (2002), who reported a significant increase in the number of GFAP-ir astrocytes between 16 and 90 days of age in both the CA1 and CA3 areas of male and female rats. However, our data indicate that only female rats show this increase, since only the CA3 area presents analogous changes in males. A likely explanation for this discrepancy would be the hippocampal layers analyzed, because their results were based on cell counts performed only in the stratum radiatum. In agreement with our results, other authors have found that adult number of astrocytes are reached at about 21 days of age in the CA1 area of male rats (Nixdorf-Bergweiler et al., 1994), as well in rat cerebral cortex (Ling and Leblond, 1973).

On the other hand, no numerical differences regarding sex and the selected hippocampal areas were observed at 21 days of age, the weaning period in rats. Moreover, their GFAP-ir astrocytes showed an undifferentiated morphology, a sign of immaturity of this brain region. At the behavioral level, it is precisely at this age when no sex differences in spatial learning have been reported (Cimadevilla et al., 1999; Champillon, Roullet and Lassalle, 1995). By taking into consideration the extensive neuronal-glial interactions, astrocytes may be essential for the development of synaptic plasticity in the hippocampus (Laming et al., 2000). Actually, GFAP-ir astrocytes are specifically involved in neural physiology, since they are related with the development of long-term potentiation properties by hippocampal synapses (McCall et al., 1996). Therefore, not only neurons alone, but also astrocytes would play a significant role in the development of spatial learning abilities.

In our present study sex differences were found at 90 days, when GFAP-ir astrocytes showed a fully differentiated morphology. This result would be related with the organizational influence of gonadal steroids at both the morphological and physiological levels. The widely known lesser susceptibility of the CA1 area to ischemic insults in females (He, He, Day and Simpkins, 2002) would be supported by the fact that higher numbers of astroglia would exert a greater neuroprotective effect. An unexpected finding was that male astrocytes outnumbered females in the CA3 area. However, it has been demonstrated that testosterone affects mainly CA3 area and dentate gyrus of male hippocampus (Roof, 1993). Particularly, the CA3 area has been recently involved in spatial memory formation (He, Yamada and Nabeshima, 2002) and may underlie the sex differences reported in spatial learning (Bimonte and Denenberg, 2000; Roof and Stein, 1999).

From a psychopathological point of view, major depression (Muller, Lucassen, Yassouridis, Hoogendijk, Holsboer and Swaab, 2001) and the deleterious effects of stress in anxiety disorders (Rajkowska, 2000) have been linked to changes in GFAP-ir astrocytes of prefrontal cortex and hippocampus respectively. Thus, the study of changes in astrocyte population would be interesting, since it could contribute to our understanding of the traditionally neglected role of glia in both the brain disorders and neural function.

Acknowledgements

We thank the laboratory technicians Piedad Burgos and Begoña Valdés for their contribution to this work. This work was supported by grants MCT BSO 2001-2757 (Ministry of Science and Technology, Spain) and PR-01-GE-2 (Principado de Asturias, Spain).

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