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Evidence for sexual difference in astrocytes of adult rat hippocampus

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Abstract

We quantified the number of glial fibrillary acidic protein immunoreactive (GFAP-IR) astrocytes in the CA1 and CA3 areas of the adult rat hippocampus. The dorsal and ventral regions of the hippocampus were taken into account to estimate the GFAP-IR cells using unbiased stereological techniques. Males had a higher number of GFAP-IR astrocytes in the CA3 area, whereas females had more in the CA1 area. No sex difference was found between dorsal and ventral regions, although most GFAP-IR astrocytes were located in the dorsal hippocampus.

Keywords: Glial fibrillary acidic protein; Hippocampus; Sex differences; Optical fractionator; Astrocytes; Rat

The hippocampus represents a heterogeneous structure, functionally differentiated along its dorsoventral axis as recent behavioural studies suggest [5,8]. In addition, sexual differences at both morphological and physiological levels of the hippocampal formation have been reported [1,12]. Data accumulated over the last years have focused mainly on the neuronal population and the synaptology of this brain region [12,13].

However, not only neurons but also glial cells are targets for sex hormones such as estrogen and testosterone [10]. In this regard, the growth of astrocytic processes, the extent to which neuronal membranes are covered by astroglial processes and the distribution of glial fibrillary acidic protein (GFAP), which is the major intermediate filament protein in differentiated astrocytes, are sexually dimorphic and can be altered by the level of these hormones [3,7]. For example, changes in the distribution of GFAP and in the branching of astrocytic processes have been detected in the hilus of the dentate gyrus of the rat [4]. However, in animals pre-treated with estradiol, when analyzing GFAP glial cell morphology in the CA3 area of the hippocampus, as well as the number of astroglial cells in CA3 and CA1, GFAP expression was found to be reduced [11].

The aim of the present study was to evaluate gender differences in the total number of GFAP-immunoreactive (GFAP-IR) astrocytes in CA1 and CA3 hippocampal areas, estimated by unbiased stereological counting methods. In particular, the dorsoventral anatomical subdivision of these hippocampal areas was taken into account.

Adult male and female Wistar rats (3 months of age, $n = 6$ per group) obtained from the University of Oviedo central vivarium were used. The animals were maintained on a 12:12 h light/dark cycle, and a temperature of 22 ± 2 °C, with food and water available ad libitum. All experimental procedures followed strictly the EEC Council Directive 86/609 regarding the care and use of laboratory animals.

Vaginal smears were taken from virgin females to determine the different stages of the estrous cycle. The subjects were selected at the morning of the proestrous phase. This arbitrary criterion was primarily chosen to avoid unpredictable variations in the results due to different levels of sex steroids in circulation.

The rats were anaesthetized with sodium pentobarbital (70 mg/kg for males and 45 mg/kg for females, i.p.) and intracardially perfused with 0.9% saline in 0.1 M phosphate buffer (PBS; pH 7.4) followed by 10% phosphate-buffered

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formalin. After perfusion, brains were cut coronally to obtain the hippocampus and embedded into paraffin blocks. Using a rotary microtome (Leica, Germany), 20 μm-thick sections were cut through the hippocampal formation. For each subject, the total numbers of sections containing the hippocampus were taken into consideration, to calculate in a later analysis the hippocampal volume. A series of alternate sections were Nissl stained with a 0.5% cresyl violet solution to easily discriminate the hippocampal regions studied. The remaining sections were processed for GFAP immunocytochemistry.

Briefly, 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 °C. 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 h at room temperature, washed twice in TBS with Triton X-100, rinsed in TBS, and then visualized with DAB (Sigma, USA). Finally, the sections were dehydrated in ethanol, cleared in xylene, and cover-slipped with Entellan (Merck, USA).

Stained slides were visualized using a system composed of a binocular microscope (Olympus BH, Japan) equipped with a digital *z*-axis gauge (Heidenhain microcator, Germany) connected via a video camera to a video monitor. The optical fractionator stereological method [19] was used to quantify the GFAP-IR cells in the CA1 and CA3 areas of the hippocampus. Thus, the total number of GFAP-IR astrocytes was estimated for each animal using eight to ten equidistant sections selected in a systematic-uniform random manner from all of the sections comprising the hippocampus (150 sections on average). In each section, cells were counted using an oil immersion objective (100×, NA 1.25) according to a systematic sampling scheme in each hippocampal area from one brain hemisphere. The sampling step used for the stage movement was 300 μm for the *x*-axis and 300 μm for the *y*-axis in CA1 and CA3 areas. Only the cell bodies included in four equidistant counting frames (frame size 0.025 × 0.025 mm) of each microscopic field were selected. In addition, cells coming into focus within each counting frame through a depth of 15 μm from the total section thickness as measured with the microcator were counted (height of the optical disector). The guard height was 2.5 μm, i.e. a *z*-axis distance from the upper and bottom surfaces of the section where no counts were performed to avoid overestimation errors. The reference volume and area of each brain region were estimated according to Cavalieri's principle using a point-grid (area per point 20.25 mm^2) superimposed on drawings of each region profile obtained from the same sections selected for the optical fractionator procedure. In sections where the hippocampus extended all along its dorsoventral axis, the limit between both regions corresponded to the dorsoventral co-ordinate −5.0 mm from bregma point according to Moser et al. [15].

Differences between the dorsal and ventral hippocampus were analyzed by Student's *t*-tests. To compare the differences among the areas (CA1, CA3) and gender, a two-way repeated measures analysis of variance (ANOVA) was applied. Post-hoc comparisons between means were done with Tukey's HSD tests when significant ($P < 0.05$) main effects were found.

Most GFAP-IR astrocytes were found in the stratum oriens, radiatum and lacunosum-moleculare from CA1 and CA3 areas. As regards astrocyte morphology, the pattern of immunoreactivity was similar in both sexes. GFAP-IR glia was characterized by a fully stellate morphology, with a small cell soma, with long and thin extended processes, which possessed a high degree of branching.

A significant interaction between gender and hippocampal area on the number of GFAP-IR astrocytes was found ($F(1, 23) = 32.9$, $P < 0.001$). In particular, the number of GFAP-IR cells was significantly higher in CA1 as compared to CA3 in female rats (Tukey's test; $P < 0.05$). In contrast, the pattern is reversed in males, showing significantly more GFAP-IR cells in the CA3 area (Tukey's test; $P < 0.01$) (Fig. 1). Furthermore, the magnitude of the differences between the hippocampal areas studied was similar in males and females (mean numerical differences of about 22%). Similarly, the total number of GFAP-IR astrocytes was 20% smaller in the CA3 area from females as compared to males. Nevertheless, females had 24% more GFAP-IR astrocytes in the CA1 area.

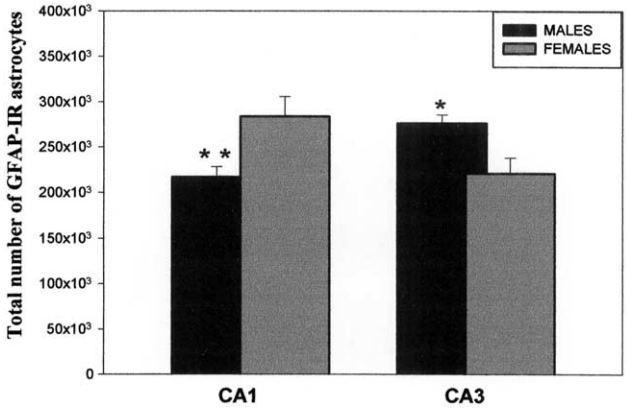

Fig. 1. Stereological estimation of the total number of GFAP-IR astrocytes (mean ± SEM) found in all layers (stratum oriens, radiatum and lacunosum-moleculare) of the selected hippocampal areas. The CV calculated for CA1 and CA3 areas were 0.13 and 0.10 (males), and 0.18 and 0.19 (females). The mean CE for CA1 and CA3 were 0.07 and 0.06 (males), and 0.07 and 0.06 (females). *$P < 0.05$, **$P < 0.01$, males versus females, Tukey's post-hoc test. CE, coefficient of error; CV, coefficient of variance.

Table 1
Differences in the total number of GFAP-IR astrocytes (mean ± SEM) between dorsal and ventral hippocampal regions of male and female rats

		Males	Females
CA1	Dorsal	167,772 ± 6499* (0.09/0.06)	190,303 ± 8739* (0.11/0.05)
	Ventral	84,956 ± 6903 (0.19/0.07)	102,756 ± 4101 (0.10/0.07)
CA3	Dorsal	194,110 ± 5924* (0.07/0.05)	172,826 ± 9885* (0.14/0.04)
	Ventral	91,223 ± 6225 (0.16/0.06)	81,572 ± 6379 (0.19/0.06)

*$P < 0.01$, dorsal versus ventral region, Student's *t*-test; CV and CE are shown respectively in parentheses.

By taking into consideration the dorsal and ventral regions of the hippocampal areas studied, the number of GFAP-IR cells in both groups was significantly higher in the dorsal region of CA1 and CA3 areas (Student's *t*-test; $P < 0.01$). Males and females had on average 36% more GFAP-IR cells in the dorsal hippocampal regions (Table 1).

Our present findings demonstrate direct evidence of sexual dimorphism in the number of GFAP-IR astrocytes in CA1 and CA3 hippocampal areas of adult rats. These data are consistent with the view that astrocytes respond to changing hormone levels in both sexes, since it is known that sex hormones modulate astroglial proliferation and GFAP levels [4,7]. Particularly, our results showed that adult females have more GFAP-IR astrocytes than males in the CA1 area. In agreement with our results, many authors reported a higher vulnerability of the CA1 area to the action of estrogens as compared to other hippocampal areas [14]. Conversely, the rat CA3 area seems to be more sensitive to the action of androgens [12,18]. In fact, our results show that males have more GFAP-IR astrocytes in this area. However, a recent study [2] reported an absence of sex differences in the same hippocampal area in 90-day-old rats. In order to explain this apparent discrepancy with our results, it must be pointed out that in the mentioned study only the stratum radiatum was analyzed, whereas our study included additional hippocampal layers like the stratum oriens and lacunosum-moleculare.

The sex difference found in glial cells may obviously reflect the differences reported previously in hippocampal neuron numbers given the well-known relationship between neurons and glial cells in the CNS. However, this explanation appears to be too simplistic, as the number of neurons in CA1 and CA3 areas in both sexes [1] follows the opposite pattern regarding glial cell population. Thus, it seems difficult to explain why regionally specific differences in the number of GFAP-IR cells are observed. Although the ultimate causes of these sex differences still remain obscure for us, much experimental evidence could account for our results. For example, high physiological levels of the female gonadal steroid 17-beta-estradiol seem to protect the hippocampal CA1 area against ischemic insults [9]. Since astrocytes are a source of numerous neurotrophic factors involved in nervous tissue repair, a higher number of astrocytes in the CA1 area of females would explain the less significant susceptibility of this hippocampal area in females as compared with males after ischemic insults.

As regards the CA3 area, some authors reported a trophic action of testosterone on the hippocampal formation associated with the better performance of males in spatial learning tasks in rodents [16]. Some authors have found an interesting role of GFAP expression in astrocytes in long-term potentiation [17]. An increase in the astrocyte population of the hippocampus may cause in turn an increase in the production of particular trophic factors that would promote neuronal plasticity involved in memory-related synaptic events like long-term potentiation, synaptic turnover or the release of neurotransmitters [20].

Concerning the dorsoventral differences found, our study showed for the first time that GFAP-IR astrocytes are more numerous at the dorsal level of the hippocampus in both sexes. Our results add new evidence at the glial level to other studies focused on neurons about the dorsoventral heterogeneity of the hippocampus at anatomical and functional levels [5,6]. Moreover, these data could be related with behavioural studies reporting a particular involvement of the dorsal hippocampus in spatial learning processes [5,8].

The sexual dimorphism found on the number of GFAP-IR astrocytes of rat CA1 and CA3 hippocampal areas would be related to the differential organizational action of gonadal steroids during the postnatal development on glial cells. Our results would help explain the neuroprotective effects of sex steroids after brain injury by acting not only on neurons, but also on glial cells. Additionally, we should not discard the hypothesis of a critical involvement of the astroglial population on behaviour, as our results correlate with previous studies based on neuron–glia interaction regarding hippocampal function.

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