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Short communication

Astrocyte morphology after ischemic and hemorrhagic experimental stroke has no influence on the different recovery patterns

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HIGHLIGHTS

- Long-term astrocyte morphology has no influence on the different recovery patterns of stroke.
- Ischemic and hemorrhagic stroke subtypes have similar long-term astrocyte morphology in perilesional sensorimotor cortex and dorsolateral striatum.
- Long-term GFAP immunoreactivity profile is similar between ischemic and hemorrhagic stroke.

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ABSTRACT

Stroke, broadly subdivided into ischemic and hemorrhagic subtypes, is a serious health-care problem worldwide. Previous studies have suggested ischemic and hemorrhagic stroke could present different functional recovery patterns. However, little attention has been given to this neurobiological finding. Coincidently, astrocyte morphology could be related to improved sensorimotor recovery after skilled reaching training and modulated by physical exercise and environmental enrichment. Therefore, it is possible that astrocyte morphology might be linked to differential recovery patterns between ischemic and hemorrhagic stroke. Thus, we decided to compare long-term GFAP-positive astrocyte morphology after ischemic (IS, $n = 5$), hemorrhagic (HS, $n = 5$) and sham (S, $n = 5$) stroke groups (induced by endothelin-1, collagenase type IV-S and salina, respectively). Our results showed ischemic and hemorrhagic stroke subtypes induced similar long-term GFAP-positive astrocyte plasticity ($P > 0.05$) for all evaluated measures (regional and cellular optical density; astrocytic primary processes ramification and length; density of GFAP positive astrocytes) in perilesional sensorimotor cortex and striatum. These interesting negative results discourage similar studies focused on long-term plasticity of GFAP-positive astrocyte morphology and recovery comparison of stroke subtypes.

Stroke, broadly subdivided into ischemic and hemorrhagic subtypes, is a serious health-care problem worldwide [1]. Some clinical studies have shown that by the time of hospital discharge hemorrhagic stroke presents a better functional improvement compared to ischemic stroke [2]. Moreover, ischemic stroke patients showed a longer functional recovery time window than those with the hemorrhagic etiology [3]. Yet, these recovery differences are not completely understood due to stroke heterogeneity [4]. Animal models aid researchers to control some factors and provide an unbiased analysis of stroke subtypes. We have recently published a study, using two controlled lesion "site and size" rat models, showing the spontaneous recovery pattern is better in hemorrhagic than in ischemic stroke [5]. Unfortunately, the neurobiological explanations for this finding remain poorly understood.

Coincidentally, our research group has also shown astrocyte morphology could be related to improved sensorimotor recovery after a rehabilitation protocol [6] and modulated by physical

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exercise and environmental enrichment [7,8]. For example, skilled reaching training after collagenase-induced intracerebral hemorrhage increases the length of primary astrocytic GFAP-positive processes in perilesional tissue. This finding was correlated with functional forelimb recovery [6]. Moreover, enriched environment [8] and physical exercise [7] induce changes in the polarization of GFAP-positive astrocytes in healthy animals. Therefore, it is possible that astrocyte morphology might be linked to differential recovery patterns between ischemic and hemorrhagic stroke.

Thus, we decided to compare long-term GFAP-positive astrocyte morphology after ischemic (IS) and hemorrhagic (HS) stroke using coronal sections left over from the study "Behavior outcome after ischemic and hemorrhagic stroke, with similar brain damage, in rats", previously published in Behavioural Brain Research [5]. This was done in an effort to reduce the animals needed in research, according to the replacement, reduction and refinement principle (3R's) [9].

The sections were taken randomly from 15 brains of Wistar rats divided into the following groups: Sham ($n = 5$), ischemic (IS) ($n = 5$) or hemorrhagic (HS) ($n = 5$). Brain ischemia or hemorrhage was induced by endotelin-1 (ET-1) and collagenase type IV-S microinjections, respectively (see Mestriner et al., 2013 for detailed description) [5]. The following morphological parameters were evaluated in GFAP positive astrocytes: (1) semi-quantitative analysis of GFAP imunohistochemistry intensity, measuring regional and cellular optical density; (2) astrocytic morphology and polarization (primary processes ramification and length), using Sholl concentric circles analysis; (3) density of GFAP positive astrocytes (number of GFAP positive astrocytes/mm^2), using planar morphometry. All measurements were made in the perilesional sensorimotor cortex and striatum at 30 days post-surgery (Fig. 1).

For immunohistochemistry, coronal brain sections (50 μm) were obtained using a cryostat (Leica, Germany). They approximately ranged (relative to Bregma) from +1.80 mm (rostrally) to −0.70 mm (caudally). Briefly, brain sections were post-fixed in 4% PF for 15 min. After three washes in cold phosphate buffered saline (PBS, pH 7.4), GFAP immunohistochemistry was performed as previously described [7]. Sections were blocked for endogenous peroxidases (3% hydrogen peroxide in PBS) for 30 min, washed in PBS containing 0.4% Triton X-100 (PBS-Tx) and blocked with 2% bovine serum albumin (BSA) in PBS-Tx for 30 min. Sections were then incubated with anti-GFAP polyclonal antibody raised in rabbit (Z033401-2 – Dako), diluted 1:500 in PBS-Tx for 48 h at 4 °C. After two washes in PBS-Tx, the sections were incubated in peroxidase-conjugated goat anti-rabbit IgG antibody (A0545 – Sigma–Aldrich), diluted 1:200 in PBS-Tx at room temperature for 2 h.

Sections were then washed two times in PBS, and the GFAP immunostaining was performed by incubating the sections in a medium containing 0.06% 3,3′-diaminobenzidine (DAB, Sigma–Aldrich) dissolved in PBS for 10 min, and in the same solution containing 1 μL of 3% H_2O_2 per mL of DAB medium for an additional 10 min. After the DAB + H_2O_2 revelation, the sections were rinsed in PBS, dehydrated in series of increasing ethanol concentrations (70, 90 and 100%), cleared with xylene and covered with Entellan (Merck) and coverslips. As a control to rule out unspecific binding, in a few sections the primary antibody was omitted and replaced by PBS-Tx. In order to minimize differences in the staining of astrocytes and in background levels, the brains in all experimental groups were fixed, cryoprotected and post-fixed in identical solutions for the same length of time, processed at the same time and incubated in the same immunostaining medium for the same period of time.

The number of GFAP-immunoreactive astrocytes per mm^2 in the surrounding damaged tissue was estimated using an Olympus BX 50 microscope coupled to a Motic Images Plus 2.0 camera and Image Pro Plus (Image Pro-Plus 6.1, Media Cybernetics, Silver Spring, EUA) software. Our interested regions were sensorimotor cortex and dorsolateral striatum. For this analysis, four digitized images (20×) from surrounding injured tissue (two for each cortex and striatum) were obtained from each section (Fig. 1). Three sections from each animal were analyzed (a total of 12 images analyzed per animal – six from cortex and six from striatum). Two randomized squares measuring 5828 μm^2 and named areas of interest (AOIs) were overlaid on each image (Fig. 1). The astrocytes located inside this square or intersected by the upper and/or right edges of the square were counted. Astrocytes intersected by the lower and/or left edges of the square were not counted [7,8].

We established an area of analysis beginning at approximately 50 μm laterally to lesion border for all morphological measurements to avoid errors related to complex overlapping of astrocytes bodies and process, blood vessels and artifacts in an immediately surrounding tissue to lesion (Fig. 1).

The intensity of GFAP immunoreactivity was measured using semi-quantitative densitometric analysis [7,8] with the same software employed to estimate the astrocytic density. The same images and AOIs (5828 μm^2) used to estimate astrocytic density were used in the analysis of regional optical density (OD). The images were converted to an 8-bit gray scale (256 gray levels) and the AOIs were overlaid on each image.

For the analysis of cellular OD, two astrocytes GFAP-positive located inside the first AOI (5828 μm^2) were randomly selected to cellular OD assessment. Thus, a new AOI measuring 10.37 μm^2 was placed over analyzed astrocytic soma in each image (processes were not measured). A number of 12 astrocytes per structure of interest were analyzed by animal. All lighting conditions and magnifications were kept constant during the process of capturing the images. Blood vessels and other artifacts were avoided and the background correction was performed according to the formula previously described [10].

The morphological analysis was done using the same images employed to measure cellular optical density. For the analysis of astrocytic ramification, an adaptation of Sholl's concentric circles technique was used [6–8]. Briefly, seven virtual circles with 3.91 μm intervals were drawn around each astrocyte. The degree of ramification of the astrocytes was measured by counting the number of times the astrocytic processes intersected with each virtual circle around the astrocytes. Primary process quantification was performed by counting the processes extending directly from the soma in both the lateral and central quadrants of astrocytes in the same sections. The longest primary process in each quadrant was measured by tracing the process with a manual measurement tool found in the Image Pro Plus software.

All morphological data were obtained and analyzed by researchers blind to group identity (images capture and measurements). Data normality distribution was tested using the Kolmogorov–Smirnov test and showed a parametric profile. Data was analyzed using one-way ANOVA followed by Bonferroni post hoc test, when appropriate. Pearson correlation coefficient and analysis of co-variance (ANCOVA) were performed to assess the influence of astrocyte features on ladder walk performance. Behavioral data was obtained from our previous study [5]. All variables were expressed as mean ± standard error of the mean (SEM). Results were considered significant when $P \leq 0.05$. SPSS 16.0 (Statistical Package for the Social Sciences, Inc., Chicago, USA) and G*Power 3.1 software were used for data analysis.

In our study, GFAP-positive astrocytes surrounding damaged sensorimotor cortex and dorsolateral striatum (approximately from 50 μm to 210 μm laterally to border of tissue lost) were analyzed. In the qualitative analysis, it was possible to observe individual astrocyte's soma and proximal processes for all groups. As expected, a highly complex network of GFAP-positive cells was observed in the tissue adjacent to the core of damage for both stroke