Overexpression of kynurenic acid and 3-hydroxyanthranilic acid after rat traumatic brain injury

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Abstract

Using an immunohistochemical technique, we have studied the distribution of kynuneric acid (KYNA) and 3-hydroxyanthranilic acid (3-HAA) in a rat brain injury model (trauma). The study was carried out inducing a cerebral ablation of the frontal motor cortex. Two mouse monoclonal specific antibodies previously developed by our group directed against KYNA and 3-HAA were used. In control animals (sham-operated), the expression of both KYNA and 3-HAA was not observed. In animals in which the ablation was performed, the highest number of immunoreactive cells containing KYNA or 3-HAA was observed in the region surrounding the lesion and the number of these cells decreased moving away from the lesion. KYNA and 3-HAA were also observed in the white matter (ipsilateral side) located close to the injured region and in some cells placed in the white matter of the contralateral side. The distribution of KYNA and 3-HAA perfectly matched with the peripheral injured regions. The results found were identical independently of the perfusion date of animals (17, 30 or 54 days after brain injury). For the first time, the presence of KYNA and 3-HAA has been described in a rat trauma model. Moreover, by using a double immunocytochemistry protocol, it has been demonstrated that both metabolites were located in astrocytes. The findings observed suggest that, in cerebral trauma, KYNA and 3-HAA are involved in tissue damage and that these compounds could act, respectively, as a neuroprotector and a neurotoxic. This means that, in trauma, a counterbalance occurs and that a regulation of the indoleamine 2,3 dioxygenase (IDO) pathway could be required after a brain injury in order to decrease the deleterious effects of ending metabolites (the neurotoxic picolinic acid). Moreover, the localization of KYNA and 3-HAA in the contralateral side of the lesion suggests that the IDO pathway is also involved in the sprouting and pathfinding that follows a traumatic brain injury.

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Introduction

Traumatic injuries are major health and socioeconomic problems affecting nowadays our societies.[1,2] Traumatic brain injury (trauma) is the leading cause of loss of human potential around the world, and especially in low and middle income countries.[3] Severe lesions of motor cortex produce devastating effects, affecting voluntary movements.[4] Almost one out of every ten deaths in the world were caused from injuries in 2010, higher than malaria, tuberculosis and immune deficiency syndrome combined together.[5,6]

Trauma occurring mechanisms are very similar to those occurring in neurodegenerative and ischemic processes. Ischemic stroke is characterized by glutamate excitotoxicity, excessive ROS production and inflammation,[7-10] these processes also occur in traumatic brain injury.[11] In stroke, indoleamine 2,3-dioxygenase (IDO) and nitric oxide (NO) pathways are activated and several intermediate metabolites of the IDO pathway such as kynurenic acid (KYNA) and 3-hydroxyanthranilic acid (3-HAA) are overexpressed during ischemic stroke.[8-10] KYNA, a tryptophan catabolite, is mainly synthesized by astrocytes,[12,13] acts as a scavenger in these cells[14] and, in ischemia, exerts a neuroprotective role against the neuronal loss.[15] By contrast, 3-HAA (another tryptophan catabolite) exerts a cytotoxic effect.[16-18] In fact, 3-HAA inhibits mitochondria respiration, promotes oxidative damage on proteins and induces apoptosis.[10,18-22]

To our knowledge, the neuroanatomical distribution of the immunoreactive structures containing KYNA or 3-HAA in the mammalian central nervous system has been studied in an ischemic stroke model only and, in this model, the involvement of the IDO pathway has been demonstrated.[8-10] Since mechanisms occurring in trauma are very similar to those appearing in stroke,[23-24] our main aim is to study in a trauma model (ablation of the motor cortex) the presence / overexpression of markers (KYNA, 3-HAA) of the IDO pathway. This pathway has been implicated in some pathological mechanisms (*e.g.*, inflammation).[25-28] Thus, the presence, absence or overexpression of these molecules belonging to the IDO pathway will serve to increase our knowledge on the underlying mechanisms occurring during traumatic processes (that is, the involvement of the IDO pathway in brain trauma) and to suggest possible therapeutic strategies for the treatment of brain trauma.

Materials and Methods

Animals and experimental groups

Twenty-four adult male Wistar rats (Charles River, 200-220 g at arrival) were used. Animals were housed under standardized conditions of light and temperature for 7 days before the experiments started. Animals were fed *ad libitum*, except when the paw-reaching-for-food task was carried out. For this procedure, animals were maintained at 86-88% of their initial *ad libitum* weight.[4,29] The experimental procedure of this work was performed under the guidelines of the legal and ethics recommendations of French, Spanish and European laws (2010/63/EU). Moreover, this study was approved by the research commission of the University of Salamanca (Spain).

Animals were divided into two groups: operated (surgical procedure with ablation

of the cortex, n=12) and "sham-operated" animals (surgical procedure without ablation of the cortex, n=12). Animals of both groups were perfused at 17, 30 and 54 days after the surgical intervention.

Ablation of frontal motor cortex

The ablation of deeply anesthetized animals was conducted by aspiration of the motor cortex. Thus, ablation of the contralateral cortex to the preferred paw was conducted by using a stereotaxic apparatus taking as reference the Bregma suture in order to conduct the same lesion to all animals (Figure 1). Sham-operated animals were anesthetized, according to their weights, and operated like the animals of the operated group (crania, meninges), but no ablation of the cortex was conducted. As previously described, lesion effectiveness was verified 7 days after the operation.[29]

Immunocytochemical study

Once the model was carried out and the animals perfused (17, 30 or 54 days), an immunocytochemical study was performed. As previously reported,[8-10,29] animals were deeply anaesthetized with Equithesin and perfused with paraformaldehyde (PAF) (4%); brains were dissected out after decapitation, post-fixed in 4% of PAF for 16 h and cryoprotected in sucrose. Using a freezing microtome, 40-45 µm-thick brain coronal sections were obtained and processed for immunocytochemistry.[8-10] As previously described[8-10,30,31] and in order to inactivate the endogenous peroxidases, sections were pre-incubated in a mixture solution of methanol/H_2O_2 (2v/1v) for 30 min. Then, sections were washed three times with PBS for 30 min and pre-incubated in a PBS solution containing 0.3% Triton X-100 and supplemented with 1% of normal horse serum (mixture solution) for 30 min. After that, sections were incubated for 1 h and 45 min at room temperature and overnight at 4ºC in the mixture solution containing the primary antibodies: monoclonal anti-3-HAA or anti-KYNA antibodies (diluted 1/1,000; Gemacbio, Saint Jean d'Illac, France), polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1/100; Dako, Glostrup, Denmark) or polyclonal goat anti-ionized calcium-binding adapter molecule 1 (IBA-1) antibody (1/1,500, Abcam, Cambridge, UK). Afterward, three washes were conducted in PBS for 30 min at room temperature. Then, sections were incubated with the corresponding biotinylated anti-mouse/goat/rabbit immunogammaglobulin (BA-1,000; BA-5,000; BA-9,200, Vector Labs, Burlingame, CA, USA) (1/200 in mixture solution) for 1 h at room temperature. Later, sections were washed three times with PBS for 30 min and incubated with the avidin-biotin-peroxidase complex (ABC, Vectastain PK-6,100) (1/100) for 1 h at room temperature. Then, sections were rinsed three times with PBS for 30 min, and a later wash of at least 10 min in Tris-HCl buffer. Finally, during 10 min in darkness and room temperature, the peroxidase was developed with H_2O_2, and 3, 3' diaminobenzidine (chromogen).

The immunological characteristics of the primary antibodies (anti-KYNA, anti-3-HAA) used here have been published previously.[8-10] In order to confirm the specificity of the immunoreactivity observed here, the following histological controls were also conducted: i) omission of the primary and/or secondary antibodies, and ii) pre-absorption of the anti-KYNA or anti-3-HAA antibodies with an excess (100 µg/mL) of their corresponding antigens (KYNA or 3-HAA). In both cases no residual immunoreactivity was found. The specificity of the anti-IBA-1 antibody has been previously demonstrated[9] and, here, the immunoreactivity also disappeared after its preabsorption with the corresponding antigen. Moreover, in order to demonstrate that the immunoreactivity found for KYNA and 3-HAA was located in astrocytes, a double-labelling was conducted in sections previously developed with DAB (brown precipitate) for KYNA or 3-HAA. We followed a previous published protocol.[8-10] Thus, 4-chloro-1-naphol was used to show the presence of GFAP (blue precipitate) in those cells containing KYNA or 3-HAA and the immunocytochemistry procedure showed that double-labelling cells (GFAP-KYNA or GFAP-3-HAA) were astrocytes.

Finally, the stereotaxic atlas of Paxinos and Watson[32] was used for nomenclature and mapping. Photomicrographs were obtained by using a Kyowa Unilux-12 microscope coupled to an Olympus DP50 digital camera. Only the brightness and contrast of the images were adjusted in order to improve the visualization of results with Adobe Photoshop Elements software.

Results

Timeline signal

In all animals of the operated group (surgical procedure with ablation of the motor cortex), the results found were identical, independently of the date in which rats were perfused (17, 30 or 54 days after the ablation). In all animals of the sham-operated group (surgical procedure without ablation of the cortex), the results observed were also identical, independently of the perfusion date. However, these results were very different when comparing sham-operated and operated animals (Table 1). In both groups, four markers have been studied: KYNA and 3-HAA (to demonstrate the involvement of the IDO pathway), IBA-1(to

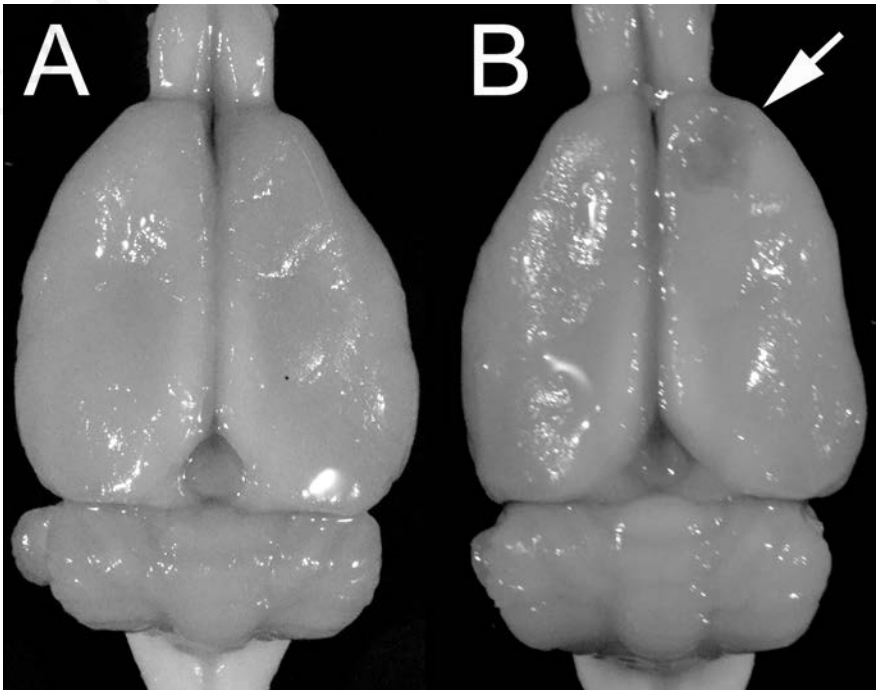

Figure 1. Low-power magnifications of brains after fixation. A) Sham operated animal; B) operated animal; the arrow indicates the lesion conducted on the motor cortex.

check microglia activation) and GFAP (to confirm the presence of KYNA and 3-HAA in astrocytes).

Sham-operated animals

No immunolabelling was observed for KYNA and 3-HAA (Table 1), whereas a normal distribution of microglia (immunoreactivity for IBA-1) and astrocytes (immunoreactivity for GFAP) in both ipsilateral and contralateral sides was visualized (Table 1). Thus, after the surgical procedure without ablation of the motor cortex, the expression of KYNA and 3-HAA was not observed.

Operated animals

In the ipsilateral side, KYNA and 3-HAA were exclusively observed in the region around the ablated area and in the motor cortex white matter placed close to the injured region. A high immunoreactivity (overexpression) for KYNA (Figure 2 and Figure 3 A,B) and 3-HAA (Figure 3 C,D and Figure 4) was found around this region, but the number of immunoreactive cells decreased moving away from the injured region (in fact, immunoreactivity disappeared in the regions of the white matter located far from the lesion) (Figure 2A and Figure 4A). Thus, a higher number of immunoreactive cells (containing KYNA or 3-HAA) was observed close to the lesion. In the white matter of the contralateral side, some cells containing KYNA or 3-HAA were also observed (Table 1). In comparison with sham-operated animals, an overexpression of the four markers studied (there is a perfect match between them) was observed in the region around the ablation (ipsilateral side) (Table 1) (Figures 2, 4, 5). As KYNA and 3-HAA, the expression of IBA-1 and GFAP decreased moving away from the region surrounding the lesion. In the white matter of the motor cortex placed far from the injured region, the expression of IBA-1 or GFAP was similar (normal distribution) to that found in the contralateral side of the operated animals and in sham-operated animals (Table 1).

Astrocytes: Coexistence of GFAP with KYNA or 3-HAA

Cells containing GFAP, KYNA or 3-HAA showed the same morphological characteristics (Figures 2-4). In order to confirm the presence of KYNA and 3-HAA in astrocytes (GFAP), a double-immunolabelling technique was applied and GFAP-immunoreactivity was detected in astrocytes expressing KYNA or 3-HAA (Figure 3). The coexistence was observed in both ipsilateral and contralateral sides (operated animals).

Discussion

Here, it has been demonstrated for the first time that KYNA and 3-HAA were exclusively found in astrocytes of animals in which the ablation of the motor cortex was performed and that both molecules were overexpressed around the injured region. Moreover, the results observed (distribution/degree of the immunoreactivity) were identical in animals perfused 17, 30 or 54 days after the ablation.

Anti-KYNA and anti-3-HAA labelling

As previously described,[8,10] monoclonal anti-KYNA and 3-HAA antibodies have been fully characterized by ELISA. Both antibodies were considered of high affinity (10^{-10} M for KYNA; 10^{-9} M for 3-HAA) and highly specifics, since they do not cross-react with close chemical structures.[8,10] After the preabsorption of these antibodies with their corresponding antigens (anti-KYNA with KYNA, anti-3-HAA with 3-HAA) the immunoreactivity disappeared completely demonstrating that the immunolabelling observed was specific of the targeted molecules. Moreover, here, a double immunolabelling technique has been applied to demonstrate in astrocytes the coexistence of GFAP and KYNA or 3-HAA.[8-10,33] 4-chloro-1-napthol was used to

Table 1. Immunolabelling for kynurenic acid, 3-hydroxyanthranilic, ionized calcium-binding adapter molecule 1 and glial fibrillary acidic protein.

Marker	Sham-operated		Operated	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Kynurenic acid	-	-	+++	+*
3-hydroxyanthranilic	-	-	+++	+*
Ionized calcium-binding adapter molecule 1	+	+	+++	+
Glial fibrillary acidic protein	+	+	+++	+

-, absence of immunoreactivity; +, presence; +*, only observed in some astrocytes located in the white matter of the motor cortex; +++, very high: region around the ablation.

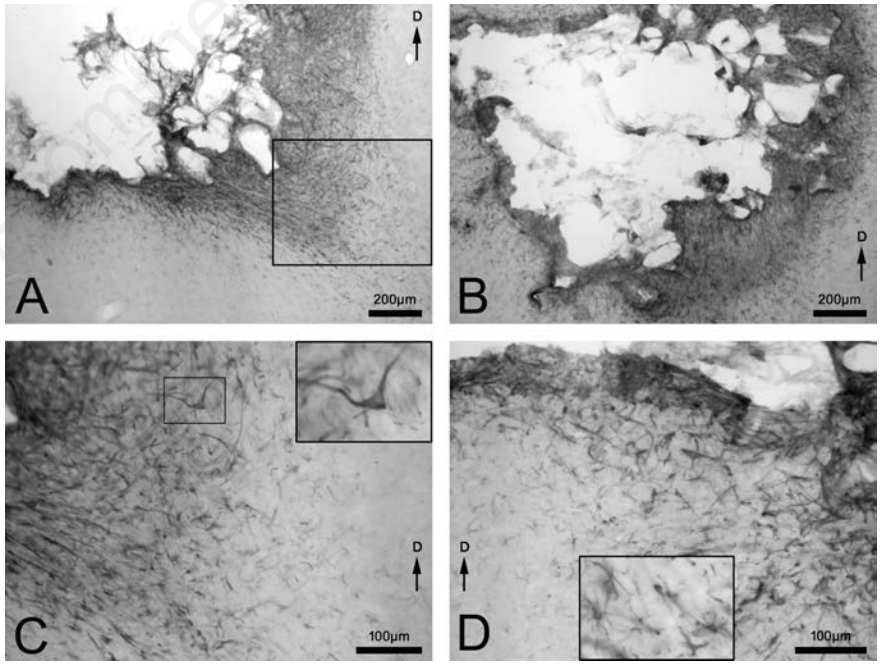

Figure 2. Immunoreactivity for KYNA. Ipsilateral side of the ablated motor cortex (A-D). A) Note the expression of KYNA in the region close to the lesion; the number of immunoreactive cells decreased moving away from the injured region. The region delimited by a rectangle is shown, at a higher magnification, in panel C. B) Picture of another region showing the immunoreactivity all around the lesion; C) The rectangle in the upper right corner is a higher magnification of the small left rectangle. D) High power magnification image, note the morphology of the cells containing KYNA. Small arrow D, dorsal orientation.

show GFAP immunoreactivity in those sections in which the presence of KYNA or 3-HAA had been observed by using diaminobenzidine. It is known that the diaminobenzidine reaction masks the antigen and catalytic sites of the first sequence of the immunoreagents, preventing an interaction of the second sequence with the reagents.[8-10,33] Thus, in a neurotrauma model and thanks to the use of specific antibodies, it has been visualized for the first time the presence of KYNA and 3-HAA in rat astrocytes. This means that the IDO pathway is involved in the mechanisms occurring in brain trauma.

Trauma *versus* transient middle cerebral artery occlusion

Expression of KYNA and 3-HAA (peripheral area of the injured region and in the white matter of the ipsilateral and contralateral sides) occurred from at least 17 days after the ablation procedure. From early phases of stroke, it has been also described the expression of both molecules.[8,10] Thus, in the transient middle cerebral artery occlusion model (tMCAO, stroke model), the expression of KYNA and 3-HAA was exclusively found around the infarcted region.[8,10] However, in the trauma model, KYNA and 3-HAA were found around the injured region (there was a perfect anatomical match between both molecules) but also in astrocytes placed in the white matter of the ipsilateral and contralateral hemispheres. The immunolabelling found in the contralateral side was visualized only in a few cells. The presence of astrocytes (containing KYNA or 3-HAA) in the white matter could be due to a possible involvement of both molecules in mechanisms of neurogenesis and new axonal connections occurring after cortical ablation.[29] The presence of KYNA and 3-HAA in the contralateral hemisphere supports the involvement of these molecules in the compensation mechanisms developed after a motor cortex injury. In a stroke model, it has been reported that sprouting neurons have many genes activated which are linked to axonal pathfinding or sprouting.[34] This pathfinding processes are in close association with astrocytes and surrounding milieu.[35-37] Thus, the role of the IDO pathway molecules (KYNA, 3-HAA) in the previous mentioned processes should be studied in-depth since it seems that this pathway plays an important role in the pathological processes involved in brain injury and repair.

In the neurotrauma model, the overexpression of KYNA, 3-HAA, IBA-1 and GFAP was observed around of the ablated cortex; this is in agreement with the results found in the tMCAO model in which a per-

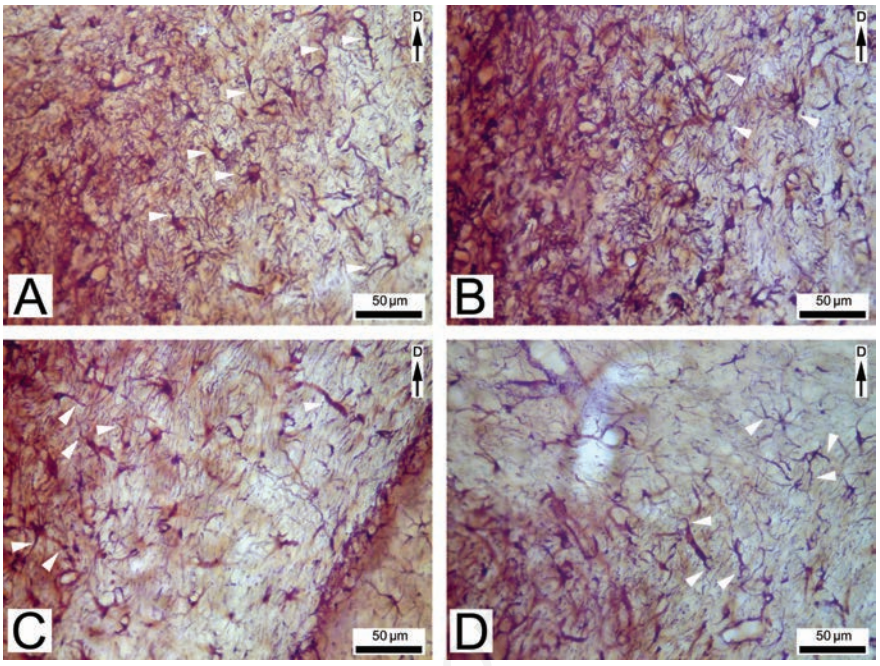

Figure 3. Photographs taken from the region around the lesion. Double-labelling of astrocytes containing GFAP and KYNA (A, B) or GFAP and 3-HAA (C, D). The immunolabelling for GFAP appears in blue (chloronapthol) and that of KYNA or 3-HAA in brown (diaminobenzidine). Note the blue staining in the astrocyte projections (arrowheads) and the brown staining in the cell bodies. Small arrow D, dorsal orientation.

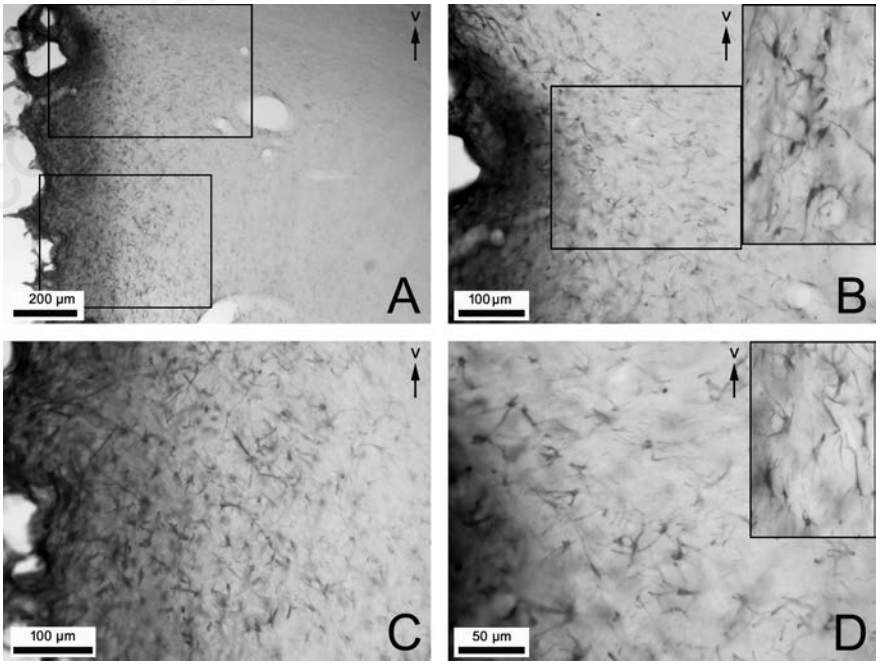

Figure 4. Immunoreactivity for 3-HAA. Ipsilateral side of the motor cortex. A) Note the expression of 3-HAA in the region close to the lesion; the number of immunoreactive cells decreased moving away from the injured region; the region delimited by the upper rectangle is shown at higher magnification in panel B, and that of the lower rectangle in panel C. B) High-power magnification of the region delimited by the left rectangle is shown in panel D; right rectangle: a detail of another region showing immunoreactive cells. D) Rectangle, detail of cells containing 3-HAA. Small arrow V, ventral orientation.

fect match of the four markers was also found in the ischemic area. Moreover, as previously described in the tMCAO model,[8-10] here in astrocytes, it has been also demonstrated the coexistence GFAP and KYNA or 3-HAA.

In the tMCAO model, a great variability in the extension of the infarcted region has been reported,[9,38] but in the neurotrauma model this great variability does not occur because the extension of the injured region is almost always the same. This does not occur because of the stereotaxic apparatus used when conducting the ablation; thus, the variability in the extension of the mechanical lesion is very limited in comparison to that observed in stroke.

2,3 indoleamine dioxygenase pathway

Both IDO pathway markers (KYNA and 3-HAA) have been found from 17 days after the surgical procedure to late phases (54 days after ablation). This pathway plays an important role in the immune response[39-41] and alterations of this pathway in several diseases (stroke, multiple sclerosis, Parkinson, Alzheimer, schizophrenia) have been reported.[18,21,42,43]

In the IDO pathway, KYNA and 3-HAA are products of the catabolism of tryptophan and it is known that they exert opposite actions: KYNA is a neuroprotector and 3-HAA (a free radical generator) a cytotoxic.[9,21,43-48] In the neurotrauma model used here, the expression of catabolites (KYNA, 3-HAA) of the IDO pathway is in agreement with the data reported in the tMCAO model.[8-10] In the latter model, it is known that the administration of a new drug candidate (GEMST) reverted the expression of both KYNA and 3-HAA.[9] Thus, based on the opposed exerted effects of both molecules, a mechanism directed to counterbalance the cytotoxic effect of the 3-HAA by an expression of KYNA could occur in both models (stroke, brain trauma). Moreover, KYNA acts as a glutamate receptor antagonist[9] and hence the overexpression of KYNA in the neurotrauma model could block the deleterious actions exerted by glutamate. Our data also suggest that, after a brain injury, the enzymes kynureninase and microsomal hydroxylase (involved in the synthesis of 3-HAA) and kynurenine aminotransferases (involved in the synthesis of KYNA) are up-regulated in astrocytes. In the future, the inhibitors/activators of these enzymes might be investigated. It is also important to note that 3-HAA is an intermediary metabolite of the quinolinic acid, which is much more cytotoxic than 3-HAA.[47,49] Quinolinic acid induces the damage/death of glia cells and neurons and it is known that inhibitors of the enzyme 3-HAA oxidase (involved in the synthesis of quinolinic acid from 3-HAA) decreased the tissue damage promoted by the quinolinic acid.[10] Therefore, after the ablation of the cortex an augmentation of the level of quinolinic acid could occur as it has been previously reported after spinal cord injury.[50] Because of the intrinsic toxicity of quinolinic acid, in the future this point must be studied in-depth.

Our findings are in agreement with previous works.[51-53] In the present study and in the tMCAO model,[8,9] we have suggested that KYNA acts as a neuroprotector. In rodents, it has been reported that KYNA and kynurenate (a salt or ester of KYNA and an excitatory amino acid receptor antagonist) also exerted beneficial effects.[51,53] In this sense, it is known that after a traumatic brain injury, the kynurenate decreased edema formation, improved motor deficits and attenuated the trauma-induced cognitive dysfunction[51] and that, before performing a traumatic brain injury, the *in situ* administration of KYNA attenuated rapid astroglial and microglial responses.[53] It is known that, after a traumatic brain injury, a rapid response of both astrocytes and microglia occurred.[53] Moreover, the results found here, in a rat traumatic brain injury model, are in agreement with those observed in humans, since after a traumatic brain injury the levels of both KYNA and quinolinic acid increased in the human cerebrospinal fluid.[52] In schizophrenic patients, an increase in the levels of KYNA and picolinic acid has been also reported.[54] In the latter case, the concentrations of both metabolites decreased when memantine (a non-competitive NMDA receptor antagonist) and galantamine (an acetylcholinesterase inhibitor and a modulator of the alpha-7 nicotinic acetylcholine receptor) were co-administered.[54] This co-administration also improved cognition.[54,55] It is important to note that, in the tMCAO model,[9] the administration of GEMST also decreased the expression of both KYNA and 3-HAA. This could also occur in the traumatic brain injury model. Finally, it has been reported that mismatch negativity is generated automatically in patients with traumatic brain injury[56] and that, in schizophrenic patients, the administration of N-acetylcysteine (a glutathione precursor and antioxidant) improved mismatch negativity and protected the integrity of the white matter.[57,58] In the future, the action of GEMST on the mismatch negativity/white matter should be investigated. In this sense, it has been demonstrated that GEMSP (a GEMST analogue and a safe and well-tolerated drug candidate against multiple sclerosis) exerted a myelin-protecting role.[59]

In summary, in a neurotrauma model (ablation of the frontal motor cortex): i) it

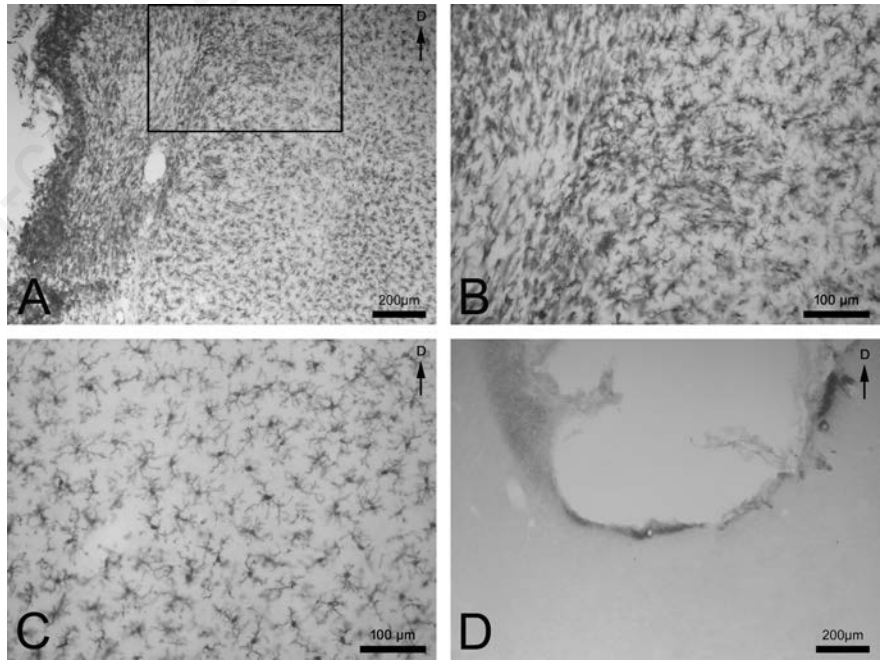

Figure 5. Immunoreactivity for IBA-1. A) High density of immunoreactive cells around the injured region. B) High-power magnification of the region delimited by the rectangle in A. C) Representative image of the labeling with IBA-1 observed in the contralateral side of the motor cortex or in sham-operated animals; compare the immunolabelling appearing in A and B. D) Control: absence of labelling when the first antibody was omitted in the immunocytochemical technique. Small arrow D, dorsal orientation.

has been demonstrated the presence of astrocytes containing KYNA or 3-HAA; ii) KYNA and 3-HAA were exclusively found (overexpression) in the region around the ablation and in the white matter of both ipsilateral and contralateral sides; iii) there is a perfect match between KYNA and 3-HAA; the region around the ablation is also characterized by the overexpression of GFAP and IBA-1; iv) KYNA and 3-HAA are expressed from mild phases (17 days after the surgical procedure) to 54 days after ablation; v) the neuroprotective metabolite KYNA could be overexpressed in order to counterbalance the cytotoxic effect of 3-HAA and its product (quinolinic acid), and vi) the presence of astrocytes containing KYNA and 3-HAA in the contralateral hemisphere means that they could be involved in the pathfinding and/or compensation mechanisms occurring after the ablation of the motor cortex. Finally, a suitable future experimentation could be the treatment of ablated animals with GEMST, a drug candidate for the treatment of stroke, since GEMST reverted the neuroanatomical changes (diminished the overexpression of KYNA, 3-HAA, IBA-1 and GFAP and exerted a general beneficial action) induced by an ischemic insult.

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