Indomethacin treatment reduces microglia activation and increases numbers of neuroblasts in the subventricular zone and ischaemic striatum after focal ischaemia

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Neuroblasts from the subventricular zone (SVZ) migrate to striatum following stroke, but most of them die in the ischaemic milieu and this can be related to exacerbated microglial activation. Here, we explored the effects of the non-steroidal anti-inflammatory indomethacin on microglial activation, neuronal preservation and neuroblast migration following experimental striatal stroke in adult rats. Animals were submitted to endothelin-1 (ET-1)-induced focal striatal ischaemia and were treated with indomethacin or sterile saline (i.p.) for 7 days, being perfused after 8 or 14 days. Immunohistochemistry was performed to assess neuronal loss (anti-NeuN), microglial activation (anti-Iba1, ED1) and migrating neuroblasts (anti-DCX) by counting NeuN, ED1 and DCX-positive cells in the ischaemic striatum or SVZ. Indomethacin treatment reduced microglia activation and the number of $ED1^+$ cells in both 8 and 14 days post injury as compared with controls. There was an increase in the number of DCX^+ cells in both SVZ and striatum at the same survival times. Moreover, there was a decrease in the number of $NeuN^+$ cells in indomethacin-treated animals as compared with the control group at 8 days but not after 14 days post injury. Our results suggest that indomethacin treatment modulates microglia activation, contributing to increased neuroblast proliferation in the SVZ and migration to the ischaemic striatum following stroke.

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1. Introduction

Continuous neuroblast formation takes place in two major regions of the adult central nervous system (CNS):the subventricular zone (SVZ), in the wall of the lateral ventricle (Doetsch *et al.* 1997, 1999), and the subgranular zone of the hippocampal dentate gyrus (Gage *et al.* 1998; Aimone *et al.* 2011). In physiological conditions, neuroblasts from SVZ migrate to the olfactory bulb (OB) through the rostral migratory stream in order to be integrated into functional OB interneurons (Curtis *et al.* 2009; Nissant and Pallotto 2011). Neural progenitors also migrate from the hippocampal subgranular zone (SGZ) to the granular cell layer to become adult hippocampal neurons (Gage *et al.* 1998;

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Aimone *et al.* 2011). Some functions of adult neurogenesis have been proposed (Aimone *et al.* 2011; Ming and Song 2011; Sahay *et al.* 2011), including maintenance and repair of adult circuitry (Imayoshi *et al.* 2008) as well as memory formation in the hippocampus (Imayoshi *et al.* 2008; Aimone *et al.* 2011; Sahay *et al.* 2011) or interpretation of new odorants in the OB (Lazarini and Lledo 2011; Breton-Provencher and Saghatelyan 2012).

Adult neurogenesis is affected by pathological conditions. It has been shown that neuroblasts migrate to ischaemic striatum, partially replacing lost neurons following middle cerebral artery occlusion (MCAO) (Arvidsson *et al.* 2002; Parent *et al.* 2002). These newborn cells are attracted to the ischaemic site by molecules released by astrocytes, microglia and blood vessels, including monocyte chemoattractant protein-1 (MCP-1) (Yan *et al.* 2006a), stroma-derived factor-1α (SDF-1α) (Thored *et al.* 2006), osteopontin (Yan *et al.* 2009) and meteorin (Wang *et al.* 2012).

Several studies suggest that microglia play a dual role on adult neurogenesis (Ekdahl 2012; Gomes-Leal, 2012). *In vitro* studies suggest that microglia may regulate the migration and survival of neural progenitor cells by releasing soluble factors (Aarum *et al.* 2003; Walton *et al.* 2006). These results are supported by *in vivo* studies showing that microglia may be proneurogenic in both hippocampus (Battista *et al.* 2006; Sierra *et al.* 2010) and SVZ (Thored *et al.* 2009). We have shown that microglia may display a proneurogenic phenotype contributing to long-lasting SVZ neurogenesis following MCAO (Thored *et al.* 2009). Nevertheless, other studies suggest that microglia may impair adult neurogenesis in different pathological conditions, including stroke (Hoehn *et al.* 2005; Liu *et al.* 2007), epilepsy (Ekdahl *et al.* 2003; Monje *et al.* 2003) and trauma (Lazarini *et al.* 2012).

Previous reports suggest that inhibition of microglia with the anti-inflammatories minocycline (Liu *et al.* 2007; Das *et al.* 2011) or indomethacin (Hoehn *et al.* 2005) increases adult neurogenesis following stroke, while recent investigations did not confirm these effects following selective ablation of microglia with saporin-conjugated Mac-1 antibody (Heldmann *et al.* 2011) or minocycline (Kim *et al.* 2009).

Indomethacin, a non-steroidal anti-inflammatory drug, may modulate microglial activation following stroke; however, only one report has investigated its action on stroke-induced neurogenesis (Hoehn *et al.* 2005). There are no studies investigating the effect of indomethacin on adult neurogenesis following endothelin-1 (ET-1)-induced striatal stroke. In the present stud, we explored the efficacy of indomethacin as microglial inhibitor as well as the effect of indomethacin treatment on the numbers of neuroblasts present in the SVZ or migrating to striatum following ET-1-induced stroke.

2. Material and methods

2.1 *Experimental animals*

Male adult Wistar rats (250–300 g) were obtained from the Federal University of Pará Central Animal Facility. All animals were housed under standard conditions with food and water freely available. All experimental procedures were carried out in accordance with the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985) and European Commission Directive 86/609/EEC for Animal Experiments under license of the Ethics Committee on Experimental Animals of the Federal University of Pará. All possible efforts were made in order to avoid animal suffering and distress.

2.2 *Surgical procedures and experimental model of focal ischaemia*

The focal ischaemia was induced by microinjections of the vasoconstrictor peptide ET-1 (Sigma-Aldrich, Saint Louis, MO, USA) according to a protocol routinely used in our laboratory (Dos Santos *et al.* 2007; Souza-Rodrigues *et al.* 2008). In brief, animals were anesthetized with a mixture of ketamine hydrochloride (72 mg/kg, i.p.) and xylazine hydrochloride (9 mg/kg, i.p.) and held in a stereotaxic frame after their corneal reflex was abolished. A homoeothermic blanket unit was used to maintain the animal's body temperature, as measured by a rectal thermometer. After craniotomy, 20 pmol of ET-1 in 1 μL of sterile saline were injected into ventral striatum close to the piriform cortex (n=4–5 per survival time/animal group) over a period of 2 min using a finely drawn glass capillary needle, using the following stereotaxic coordinates (in relation to bregma): +2.5 mm, lateral; +1.2 mm, posterior; and 4.0, mm deep from the pial surface in the dorsoventral axis (Paxinos *et al.* 1980). The capillary needle was left in position for 3 min before being slowly withdrawn. Control animals were injected with the same volume of sterile saline (n=5 per survival time). To identify the injection site, a small quantity of colanyl blue was added to both ET-1 and vehicle solutions. After surgery, animals were allowed to recover with free access to food and water for 8 and 14 days.

2.3 *Experimental groups*

To investigate the effects of indomethacin treatment on both microglia activation, neuroprotection and adult neurogenesis, animals were divided in four experimental groups: animals injected with ET-1, treated with sterile saline (i.p.) for 7 days and perfused at 8 days following ET-1 injection (group 1, n=4); animals injected with ET-1, treated with indomethacin (Sigma Company, Saint Louis, MO, 2.5

Table 1. Antibodies and normal serum used

Primary antibodies	Secondary antibodies	Normal serum (10%)	Labelling purpose
Anti-Iba1 (1:1000, Wako)	Goat anti-rabbit (1:100 Vector Laboratories)	Goat	Microglia/macrophages
Anti-ED-1 (1:200, Serotec)	Horse anti-mouse (1:100 Vector Laboratories)	Horse	Activated microglia/macrophages
Anti-NeuN (1:100, Chemicon)	Horse anti-mouse (1:100 Vector Laboratories)	Horse	Mature neurons
Anti-DCX (1:400, Santa Cruz Biotechnology)	Horse anti-goat (1:100 Vector Laboratories)	Horse	Neuroblasts

mg/kg, i.p) for 7 days and perfused at 8 days following ET-1 injection (group 2, n=4); animals injected with ET-1, treated with sterile saline (i.p.) for 7 days and perfused at 14 days following ET-1 injection (group 3, n=4); animals injected with ET-1, treated with indomethacin (2.5 mg/kg, i.p) for 7 days and perfused at 14 days following ET-1 injection (group 4, n=4).

Indomethacin has been shown to be an effective inhibitor of microglial activation (Monje *et al.* 2003; Hoehn *et al.* 2005). To inhibit microglial activation in the first week following ET-1-induced stroke, animals from groups 2 and 4 received daily injections (twice a day) of indomethacin (2.5 mg/kg, i.p) during 7 days. The first dose was administered at 24 h after the ET-1 microinjection. The concentration of indomethacin adopted here was the same used in a previous report addressing the role of microglia activation on adult neurogenesis following MCAO (Hoehn *et al.* 2005).

2.4 *Perfusion and tissue preparation*

After survival times of 7 or 14 days, animals were deeply anesthetized with a mixture of ketamine hydrochloride (72 mg/kg, i.p.) and xylazine hydrochloride (9 mg/kg, i.p.). After the verification of complete absence of both the corneal and the paw withdraw reflexes, the animals were transcardially perfused with heparinized 0.9% warm phosphate-buffered saline (PBS) followed by 4% cold paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 (Rocha *et al.* 2007). Brains were post-fixed for 24 h in the same fixative and cryoprotected in different gradients of sucrose-glycerol solutions over 7 days. The tissue was then frozen in an embedding medium (Tissue Tek, Sakura Finetek, Japan), and cut at 30 μm in the coronal plane using a cryostat (Carl Zeiss Micron, Jena, Germany). Sections were then mounted onto gelatinized slides and stored in a freezer at −20°C.

2.5 *Gross histopathology and imunolabeling protocol*

The lesion area was visualized in sections stained with cresyl violet (Sigma-Aldrich, Saint Louis, MO). The site of the ET-1 injection was recognized by the presence of colanyl blue, tissue pallor and necrosis induced by focal ischaemia (Souza-Rodrigues *et al.* 2008).

To evaluate the patterns of neuronal loss, microglia/macrophage activation and neuroblast migration in the different experimental groups, we performed a series of immunohistochemical procedures. Table 1 shows details regarding the antibodies used for this purpose. These antibodies have been used in previous investigations for labeling of activated microglia/macrophages (Dijkstra *et al.* 1985; Ito *et al.* 1998), mature neuronal cell bodies (Mullen *et al.* 1992) and neuroblasts (Gleeson *et al.* 1999).

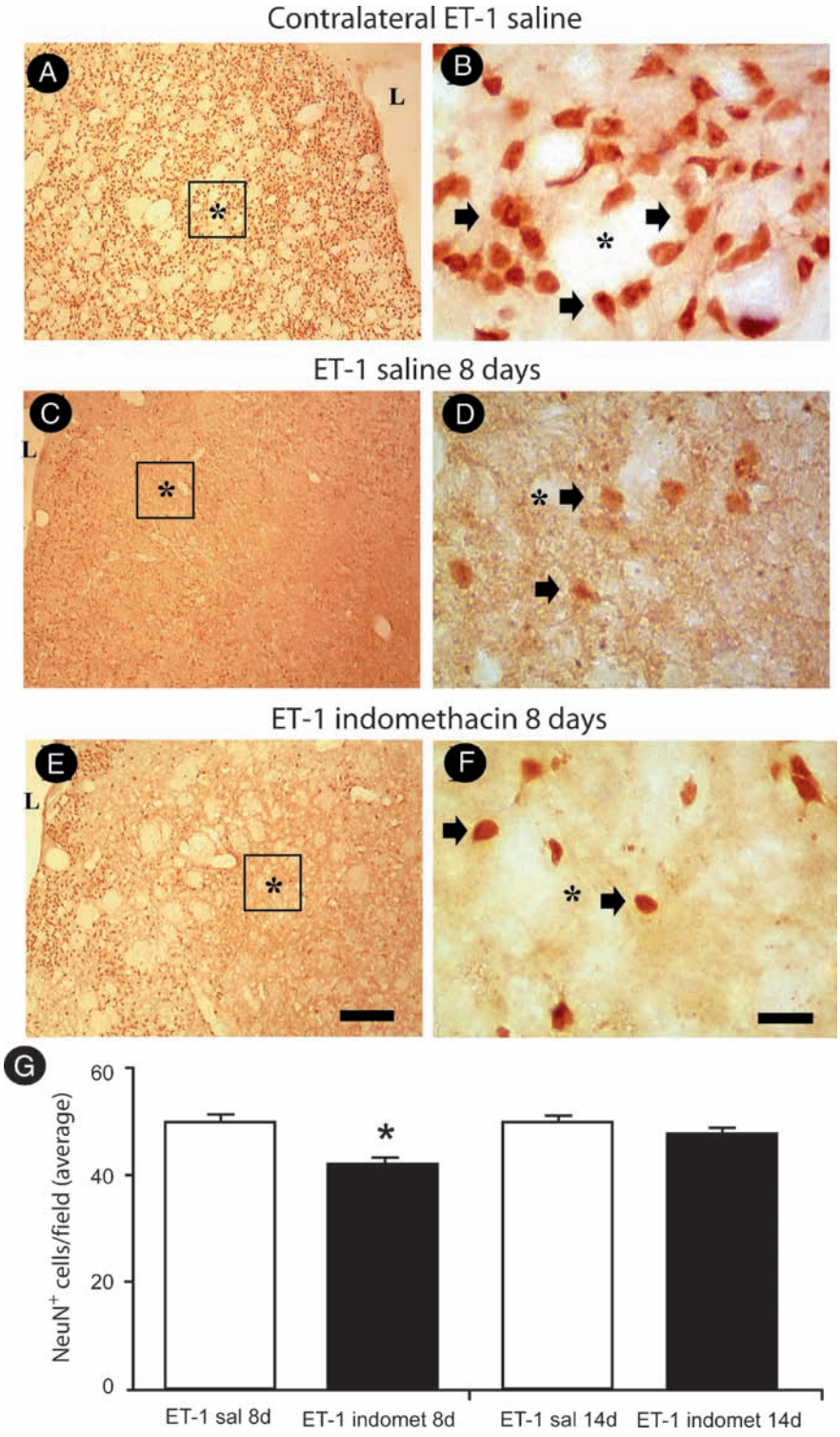

Figure 1. Neuronal loss following microinjections of ET-1 into the rat striatum as revealed by anti-NeuN immunohistochemistry. Contralateral side of a sterile saline-treated animal (**A–B**); Ischaemic animals treated with sterile saline (**C–D**) or indomethacin for 7 days and perfused at8 days following ET-1-induced stroke (**E–F**). A reduction of $NeuN^+$ cells was observed in the indomethacin-treated animals at 8 days (*p<0.05, **G**), but there was no difference between control and indomethacin-treated animals at 14 days post-ischaemia (**G**). Arrows point to $NeuN^+$ cells and boxes/asterisks to the location where higher-magnification pictures were obtained. Legend: L, lateral ventricle. Scale bars: 150 μm (**A**, **C**, **E**); 50 μm (**B**, **D**, **F**).

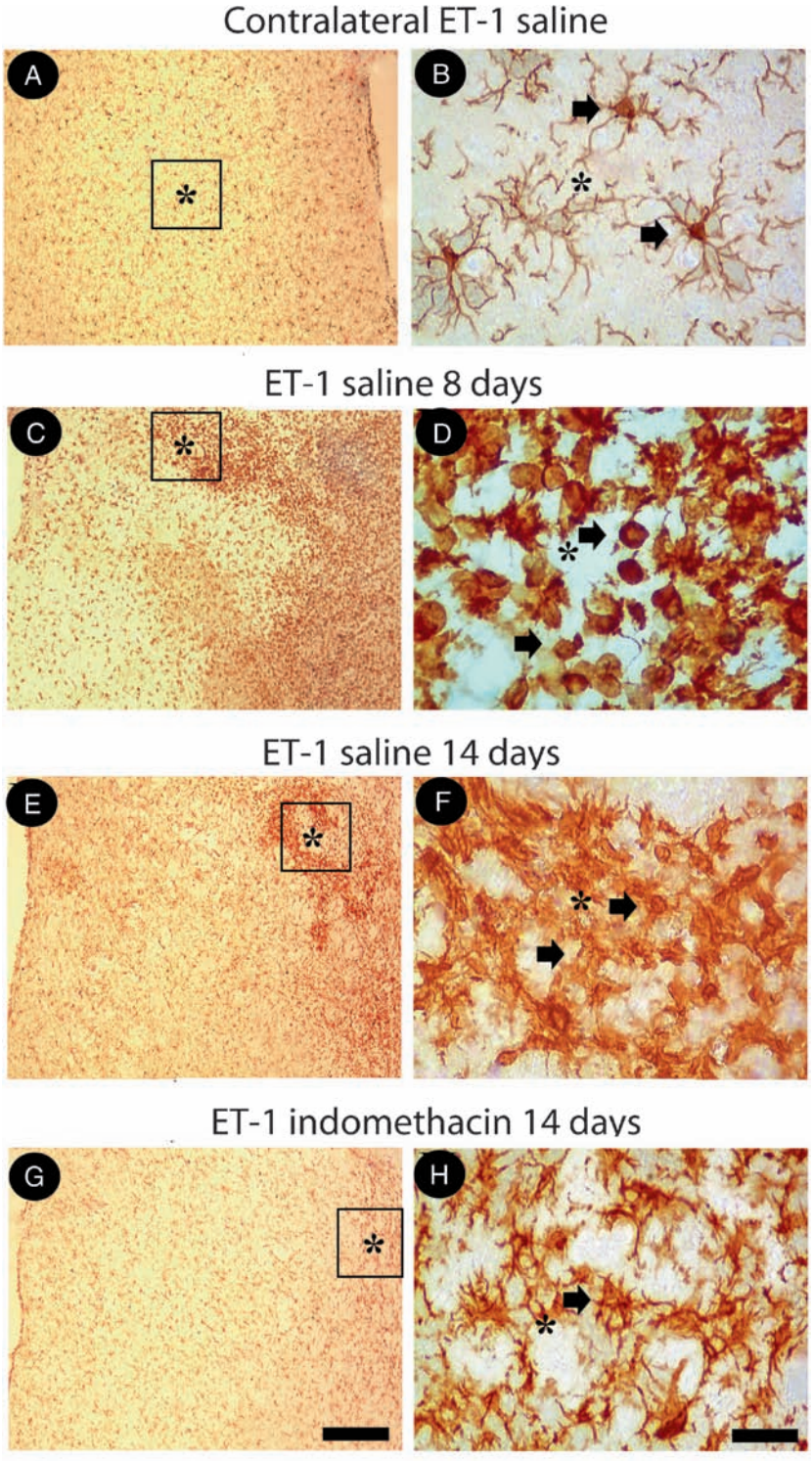

Figure 2. Effects of indomethacin treatment on microglia activation following striatal stroke, as revealed by anti-Iba1 immunohistochemistry. Contralateral side of a sterile saline-treated animal (**A–B**); Ischaemic animals treated with sterile saline or indomethacin for 7 days and perfused at 14 days following ET-1-induced stroke (**G–H**). Ramified microglia are observed predominantly in the contralateral side (**A–B**). Highly activated microglial (round cells) are present in great number at 8 days post-ischaemia. Indomethacin treatment reduced microglia activation (**G–H**). Arrows point to $Iba1^{+}$ microglia and boxes/asterisks to the location where higher-magnification pictures were obtained. Scale bars: 150 μm (**A**, **C**, **E**); 50 μm (**B**, **D**, **F**).

The imunolabeling protocol used here was detailed elsewhere (Gomes-Leal *et al.* 2004). Briefly, slide-mounted sections were removed from the freezer, kept in a heating oven at 37°C for 30 min and rinsed in 0.1 M PBS for 5 min. To improve labelling intensity, sections were then pretreated in 0.2 M boric acid (pH 9.0) previously heated to 65°C for 25 min. This temperature was maintained constant over the pretreatment period. Sections were further allowed to cool down for 20 min in borate solution and were incubated under constant agitation in 1% hydrogen peroxide in methanol for 20 min. Sections were rinsed 3 times (5 min each) in 0.05% PBS/Tween (Sigma-Aldrich Company, Saint Louis, MO, USA) and incubated with normal serum (table 1) in PBS for 1 h. Without further rinsing, sections were then incubated with the primary antibody diluted in PBS for 24 h, rinsed in PBS/Tween solution for 5 min (3 times), and incubated with appropriate secondary antibody (table 1) for 2 h. All incubations were made at room temperature (20°C). As a negative control, PBS, rather than the primary antibody, was used in some randomly selected sections. Sections were rinsed again for 5 min (3 times) and incubated in avidin-biotin-peroxidase complex (Vectastain Standard ABC kit, Vector Laboratories, USA) for 2 h. Sections were then rinsed 4 times (3 min each) and DAB-reacted according to a protocol published elsewhere (Gomes-Leal *et al.* 2004). After DAB reaction, sections were rinsed 3 times (3 min each) in 0.1 M PB, dehydrated using alcohols and xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany). Some sections were also counterstained with cresyl violet.

2.6 *Qualitative and quantitative analysis*

All sections stained with the different histological methods were surveyed by light microscopy. Illustrative images from all experimental groups were obtained using a digital camera (Moticam 2500) attached to the microscope (Nikon Eclipse 50i, Nikon, Tokyo, Japan). We used coronal sections containing the damaged striatum to count the number of activated microglia/macrophages ($ED1^+$ cells), mature neuronal bodies ($NeuN^+$ cells) and neuroblasts (DCX^+ cells) per field using a square 0.25-mm-wide grid (objective 40) in the eyepiece of a microscope, corresponding to an area of 0.0625 mm^2. For counts of $ED1^+$, $NeuN^+$ and DCX^+ cells in the striatum, we counted 16 fields per section and 3 sections/animal (n=5 animals/survival time) according to a protocol published in a previous investigation of our group (Cardoso *et al.* 2013). The fields were located in the striatum, comprising about 80% of the striatal areas. For counts of SVZ neuroblasts, we used a protocol adapted from our previous investigations (Thored *et al.* 2009). Neuroblasts were counted in all dorsoventral extension of the SVZ per section (three sections per animal and 4 animals per experimental group).

2.7 *Statistical analysis*

Averages and standard deviations were calculated for all counts. Comparisons among different groups were assessed by analysis of variance (ANOVA) with Tukey *post-hoc* test. Statistical significance was accepted for $p<0.05$. All statistical analyses were performed using Prism 5.0 software (GraphPad Software Inc., USA).

3. Results

3.1 *Striatal microinjections of endothelin-1 induce focal ischaemia with locomotor impairment and neuronal loss*

ET-1 microinjections induced a lesion pattern similar to that described in previous reports (Gresle *et al.* 2006; Souza-Rodrigues *et al.* 2008). The infarct area comprised the lateral portion of the rat striatum (figure 1C-D). All ischaemic animals displayed abnormal postures characterized by ipsilateral trunk protrusion, circle running and retraction of the contralateral limbs (data not shown).

ET-1 microinjections induced conspicuous neuronal loss characterized by disappearing of $NeuN^+$ cell bodies mainly in the lateral striatum at 8 and 14 days post stroke (figure 1C–D), compared with the contralateral counterpart (figure 1A–B). The indomethacin treatment did not reduce the primary infarct area and neuronal loss in comparison to saline-treated animals (figure 1C–F). Surprisingly, the number of $NeuN^+$ cells was decreased in the indomethacin-treated animals at 8 days after ET-1 injection, although no significant statistical difference at 14 day post stroke had been observed (figure 1G).

3.2 *Indomethacin treatment reduced microglia activation after ET-1-induced focal ischaemia*

Striatal ET-1 microinjections induced intense microglia/macrophage activation, as revealed by both Iba1 (figure 2) and ED1 (figure 3) immunohistochemistries. An increased number of ramified microglial profiles were observed in the contralateral side to the ET-microinjections at 8 days post stroke (figure 2A–B). Several round phagocytic and amoeboid $Iba1^+$ cells were observed in the ischaemic striatum at the same survival time (figure 2C–D), being located mainly at the center of the striatal ischaemic core (figure 2C–D). The microglia/macrophage activation remained intense at 14 days post stroke (figure 2E–F). In this time point, a prominent amount of amoeboid microglia was observed in the peri-infarct area around the ischaemic core (figure 2E–F).

Indomethacin treatment reduced microglia/macrophage activation at 14 days (figure 2G–H), but not at 8 days (control: 7.92 ± 0.58 cells; indomethacin: 6.95 ± 0.50 cells;

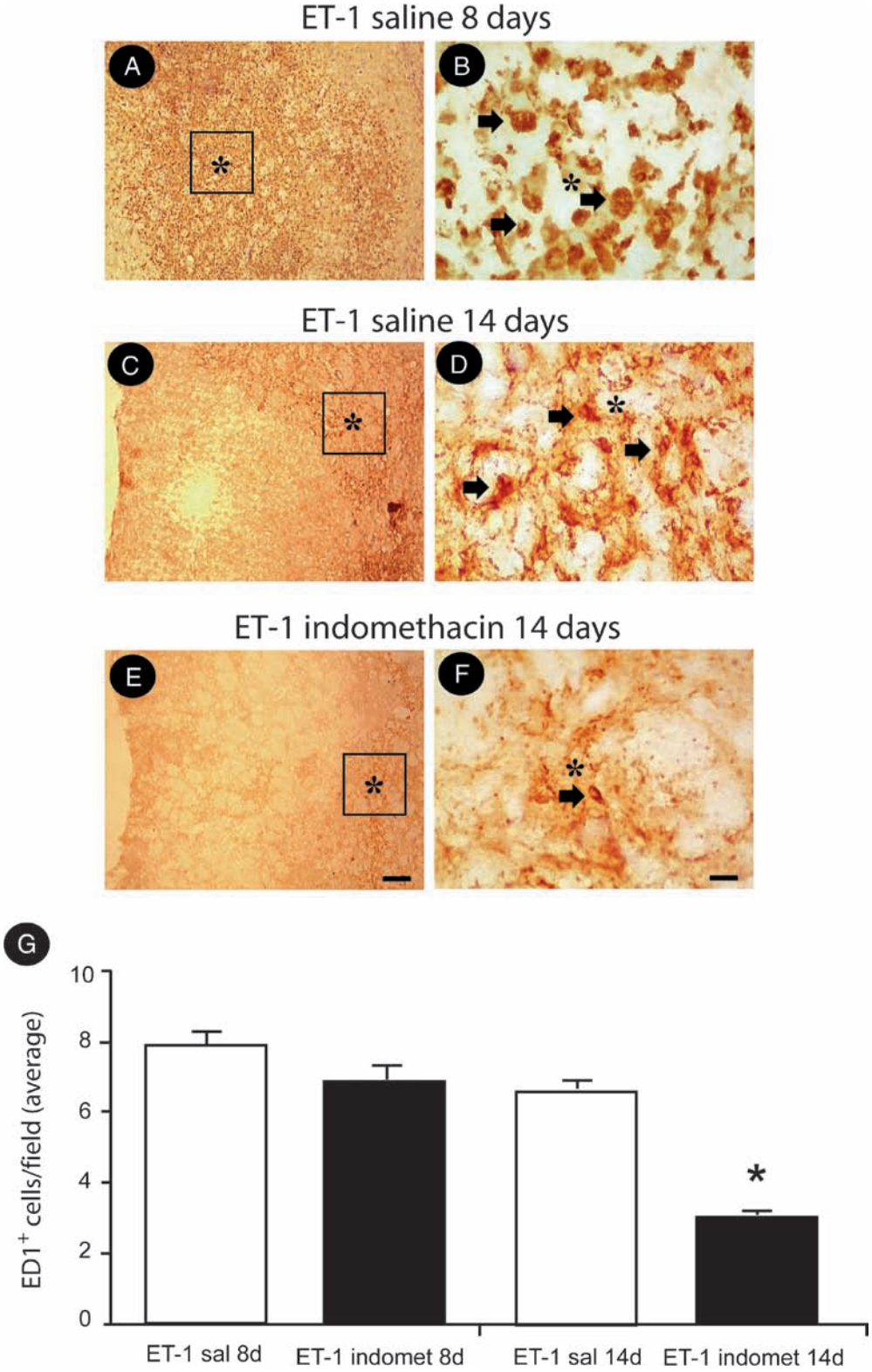

Figure 3. Effects of indomethacin treatment on microglia activation revealed by anti-ED1 immunohistochemistry following striatal stroke. Ischaemic animals treated with sterile saline (**A–D**) or indomethacin for 7 days and perfused at 14 days (**E–F**) following ET-1-induced stroke. Indomethacin treatment reduced microglia activation (**E–F**), as confirmed by quantitative analysis (*p<0.05, **G**). Arrows point to ED1$^+$ cells and boxes/asterisks to the location where higher-magnification pictures were obtained. Scale bars: 150 μm (**A**, **C**, **E**); 50 μm (**B**, **D**, **F**).

figure 2) after ET-1 induced stroke. This has been confirmed by quantitative analysis of the number of $ED1^+$ cells (figure 3). The number of $ED1^+$ cells/field was reduced ($p<0.05$, ANOVA-Tukey) in the animals treated with indomethacin as compared to control at 14 days post stroke (control: 3.00 ± 0.19 cells; indomethacin: 6.57 ± 0.36 cells; figure 3G).

3.3 *Indomethacin treatment enhances the number of neuroblasts in both SVZ and striatum after ET-1 microinjections*

In order to evaluate the influence of indomethacin treatment on the presence of immature neurons in both SVZ and striatum following ET-1 microinjections, neuroblasts were labeled with an anti-DCX antibody (Gleeson *et al.* 1999). There was an apparent increase in the number of SVZ DCX^+ cells in animals treated with indomethacin compared to saline-treated control (indomethacin: 71.7 ± 7.1 cells/field; control: 63.6±5. 62 cells/field), but without statistical significance at 8 days after ischaemia (figure 4; $p>0.05$). Nevertheless, indomethacin treatment induced a significant increase in the number of SVZ DCX^+ cells/field (125 ± 15.7), compared to control (51.70 ± 4.28) at 14 days post stroke (figure 4; $*p<0.05$). The average number of DCX^+ cells/field was higher at 14 days than in the 8 days' survival time (figure 4; $*p<0.05$). The data analysis revealed a 174% increase in the number of DCX^+ cells/field from 8 to 14 days post stroke (figure 4).

ET-1 microinjections induced neuroblast migration toward striatum, as previously reported following microinjections of ET-1 (figure 5). The indomethacin treatment increased the number of DCX^+ cells/field in the ischaemic striatum at both 8 (0.80 ± 0.053 cells/field or 16 cells/mm^2) and 14 days (1.45 ± 0.05 cells/field or 23.2 cells/mm^2) post stroke, compared with control animals (0.30 ± 0.03 cells/field or 4.8 cells/mm^2; 1.04 ± 0.04 cells/field or 16.6 cells/mm^2) (figure 5F). Nevertheless, the increase in the number of striatal DCX^+ cells/field was more significant at 14 days post stroke (figure 5E). The data analysis revealed an 82% increase in the number of striatal DCX^+ cells/field from 8 to 14 days following ET-1 microinjections.

4. Discussion

In the present study, we induced focal ischaemia through microinjections of ET-1 into rat striatum to investigate the hypothesis that indomethacin treatment may improve neuroblast migration to ischaemic striatum. Our results were threefold. First, focal ischaemia was associated with intense neuronal loss and microglia/macrophage activation in the lateral portion of the striatum in both post-stroke evaluated survival times (8 and 14 days), as previously reported (Gresle *et al.* 2006; Souza-Rodrigues *et al.* 2008). Second, indomethacin treatment reduced microglia/macrophage activation at both time points, although this had not effectively resulted in conspicuous neuroprotection. Third, indomethacin treatment enhanced the number of neuroblasts in both SVZ and striatum, mainly at 14 days following ET-1 microinjections.

Here, we showed that indomethacin treatment reduced striatal microglia activation in about 62% of the cases following ET-1 microinjections. A few studies have investigated the effect of this anti-inflammatory on microglial activation following CNS diseases (Monje *et al.* 2003; Hoehn *et al.* 2005). Hoehn and colleagues have shown that indomethacin treatment reduces the percentage of CD11b/$ED1^+$ cells in both cortex and striatum following MCAO, but with no decrease in the total number of ED1+ cells (Hoehn *et al.* 2005), but these authors had not evaluated the effects of indomethacin treatment on the morphological patterns of microglial activation. Here we filled this gap by labelling both resting and activated microglia/macrophage with Iba1, a more suitable microglial marker (Ito *et al.* 1998). The indomethacin treatment decreased the absolute number of round microglia/macrophages ($ED1^+$ cells) following ET-1 microinjections and the activated microglia/macrophage remained in a more ramified stage of activation. This might be fairly associated with an ischaemic environment more permissive to neurogenesis and repair.

The mechanisms by which indomethacin inhibits microglia/macrophage activation are not fully established. This drug may acts blocking the activation of cyclooxygenase enzymes, which can decrease the formation of oxygen-derived species (Takahashi *et al.* 2004) or modulating some transcription factors in microglia, thus decreasing the release of pro-inflammatory cytokines by these cells (Jiang *et al.* 1998).

The indomethacin treatment did not reduce the neuronal loss in any survival time after ET-1-induced striatal stroke in the present study. These results are rather similar to those described following MCAO (Hoehn *et al.* 2005) or experimental irradiation (Monje *et al.* 2003). Nevertheless, it has been reported that indomethacin treatment (10 mg/kg) induces neuronal preservation in the hippocampus following global ischaemia (Sasaki *et al.* 1988). The reasons for these discrepant results are not wholly clear, although differences on the experimental model (focal *vs* global ischaemia) and CNS regions (cortex, striatum *vs* hippocampus) may be involved.

Minocycline, an antibiotic/anti-inflammatory tetracycline (Guimaraes *et al.* 2010), reduces infarct area following rat MCAO in both cortex and striatum (Yrjanheikki *et al.* 1999). In a previous study, we have reported that minocycline

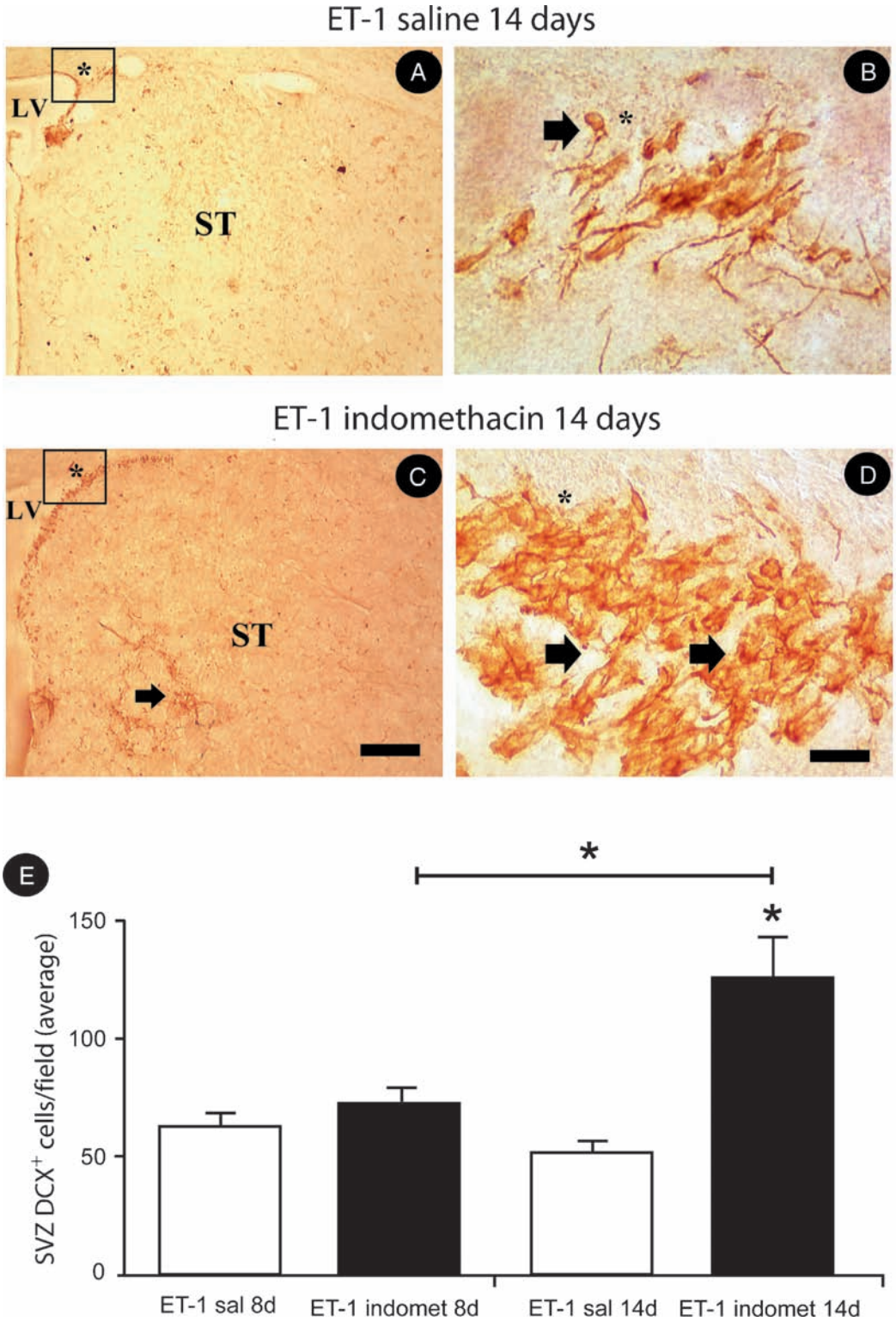

Figure 4. Effects of indomethacin treatment on the number of DCX^+ cells in the SVZ following striatal stroke. Ischaemic animals treated with sterile saline (**A**–**B**) or indomethacin (**C**–**D**) at 14 days post-stroke. Indomethacin treatment increased the number of neuroblasts in the SVZ at 14 days post-stroke as compared with both control and 8 days post stroke (**E**, $*p<0.05$). Arrows point to neuroblasts (DCX^+ cells) and asterisks/boxes to the location where higher-magnification pictures were obtained, in the SVZ flanking the border of the lateral ventricle (LV). Scale bars: 150 μm (**A**, **C**); 50 μm (**B**, **D**).

treatment enhances neuronal preservation in the first 3 weeks following ET-1-induced cortical ischaemia (Franco *et al.* 2012). PJ34, a poly(ADP-ribose) polymerase inhibitor, inhibits microglia activation and enhances neuronal preservation in about 84% after global ischaemia. These results suggest that indomethacin and minocycline may act by different molecular pathways affording different degrees of neurogenic (see below) and neuroprotective effects. Nevertheless, it has been suggested that minocycline neuroprotection is not afforded in female rodents (Li and McCullough 2009) and Humans (Amiri-Nikpour *et al.* 2015), although such a suggestion has not been made for indomethacin.

Indomethacin treatment reduced neuronal bodies at 8 days following striatal stroke. This is a surprising result, considering the beneficial effects of indomethacin already described. The explanation for these results remains an open question, but inhibition of neuroprotective microglia may be involved. It has been established that microglia can also be highly protective after stroke (Neumann *et al*. 2006; Lalancette-Hebert *et al.* 2007; Neumann *et al.* 2008; Thored *et al.* 2009; Franco *et al.* 2012), by the release of growth factors and anti-inflammatory cytokines that contribute to neuronal preservation after experimental stroke (Lalancette-Hebert *et al.* 2007; Thored *et al.* 2009). In addition, microglia can protect neurons by engulfing neutrophils (Neumann *et al.* 2008; Guimaraes *et al.* 2009). Whether indomethacin treatment inhibits or not beneficial population of microglial cells is not known. Further studies should establish which specific microglial populations are affected by the indomethacin treatment.

Indomethacin treatment increased the number of neuroblasts in the SVZ and enhanced neuroblast migration to the ischaemic striatum, mainly at 14 days after ET-1-induced stroke. These findings are supported by previous reports showing that indomethacin treatment enhances adult neurogenesis following stroke (Hoehn *et al.* 2005) or experimental irradiation (Monje *et al.* 2003) in distinct CNS regions, including striatum, cortex and hippocampus. Hoehn and colleagues reported that indomethacin treatment enhanced the number of neuroblasts migrating to ischaemic striatum after MCAO (Hoehn *et al.* 2005). Nevertheless, these authors did no quantify the numbers of neuroblasts in the SVZ. We have done so in the present study, showing that indomethacin treatment enhances SVZ neurogenesis, which is likely related to increased striatal neuroblast migration. Other study suggests that hippocampal neurogenesis is also enhanced by indomethacin treatment (Kluska *et al.* 2005), which renders further support to our results.

It can be argued that inhibition of microglia beyond 14 days may affect adult neurogenesis and neuronal replacements through endogenous precursors. Following stroke in rodents, maximum damage occurs around 7 days (Morioka et al., 1993), decreasing from this time point. Maximum peak of inflammation occurs around 7 days, as well (Morioka *et al*. 1993). It has been shown that intense neuroblast migration to striatum occurs in the first 2 weeks after MCAO (Arvidsson *et al.* 2002), which parallels the peak of striatal inflammation (Thored *et al.* 2009). It follows, that the use of indomethacin to inhibit microglia in the first week is a suitable approach. Further studies are necessary to address the effect of indomethacin on both microglia activation and adult neurogenesis in later time points up to one month. There is an ongoing investigation in our laboratory addressing this issue.

A potential drawback of this investigation is the absence of BrdU labeling to study proliferation of neuroblasts. Nevertheless, our intention was to evaluate the effect of minocycline treatment on the numbers of neuroblasts and not necessarily on their proliferation. A nuclear marker, like BrdU, would allow us to study the effects of stroke on the proliferation of neuroblasts. Several groups have studied that, including ours, using an experimental model of middle cerebral artery occlusion (Thored *et al.* 2009). Here, we aimed to evaluate how indomethacin treatment influences number of neuroblasts in both SVZ and striatum following stroke, regardless their proliferative pattern. Considering that DCX is a specific marker for immature neurons (neuroblasts) in both SVZ and ischaemic striatum, it follows that the absence of nuclear marker does not preclude our analysis. In addition, in the human brain, in the absence double immunofluorescence for BrdU/DCX, DCX immunohistochemistry was used as a marker for neuroblasts (Sanai *et al.* 2011) and neuroblasts are easily recognized by their morphology (Gleeson *et al.* 1999).

Studies using minocycline to inhibit microglia activation also suggest a detrimental role for these glial cells for stroke-induced neurogenesis (Liu *et al.* 2007; Kim *et al*. 2009). Chronic treatment using small doses of minocycline decreased microglial activation in the hippocampus, concomitant with neuron preservation and enhanced adult neurogenesis (Liu *et al.* 2007). Similar to our present results, there was no effect of the anti-inflammatory treatment on the primary infarct area and neuroprogenitor proliferation was not affected.

Despite the results discussed above, the role of microglia on adult neurogenesis remains an open field. Recent studies suggest that microglia have both beneficial and detrimental roles on adult neurogenesis (Ekdahl *et al.* 2009; Ekdahl 2012). We have shown that there is a long-lasting activation of SVZ microglia after MCAO (Thored *et al.* 2009). At least in the neurogenic niche, proneurogenic microglia may release growth factors, like IGF-1, which can contribute to the long-lasting adult neurogenesis described after MCAO (Thored *et al.* 2006). In addition, a recent paper suggests that inhibition of microglia activation with minocycline reduces neurogenesis after MCAO (Kim *et al.* 2009) and selective

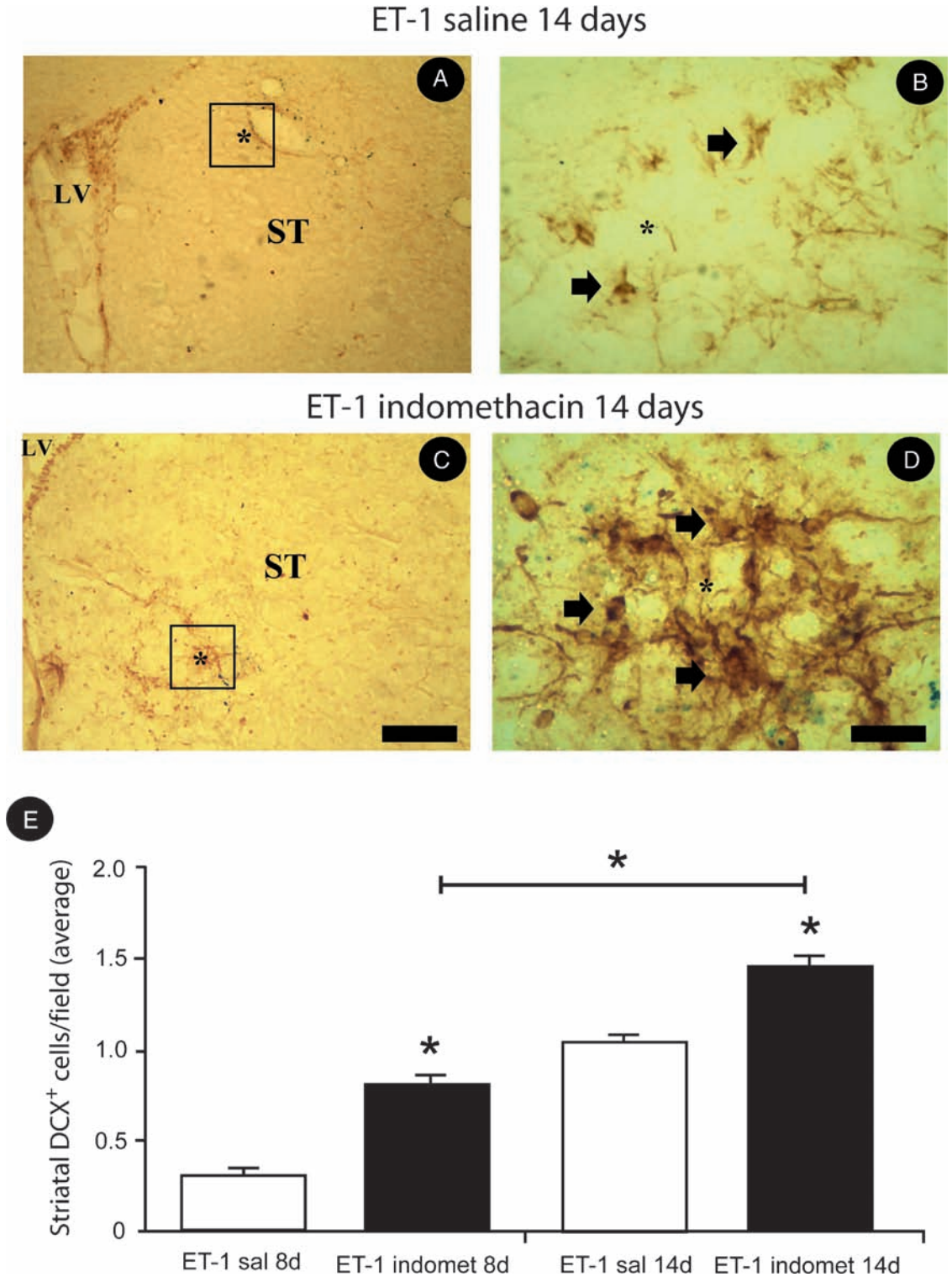

Figure 5. Effects of indomethacin treatment on the number of DCX^+ cells in the ischaemic striatum. Ischaemic animals treated with sterile saline (**A**–**B**) or indomethacin (**C**–**D**) for 7 days and perfused at 14 days post-striatal stroke. Indomethacin treatment increased the number of neuroblasts in the SVZ at both 8 and 14 days post stroke compared with control (**E**, $*p<0.05$). Arrows point to neuroblasts (DCX^+ cells) and asterisks to the location where higher-magnification pictures were obtained in the striatum. Legends: LV, lateral ventricle; ST, striatum. Scale bars: 150 μm (**A**, **C**); 50 μm (**B**, **D**).

ablation of microglia with Mac-1 saporin conjugated antibody did not interfere with neuroblast migration to the ischaemic striatum (Heldmann *et al.* 2011). These authors state that microglial cells are not important for neuroblast migration toward the ischaemic striatum. However, this work is not supported by other studies, suggesting that astrocytes and microglia may release soluble factors important for attracting immature neurons to the site of ischaemic damage (Yan *et al.* 2006b; Yan *et al.* 2009). Furthermore, the conclusion of this paper is questionable considering that the selective microglial ablation was restricted to the SVZ and did not affect striatal microglia. Proneurogenic microglial cells in the ischaemic striatum may contribute to neuroblast migration, regardless microglial activity in the subventricular zone.

It is likely that microglial cells may perform both beneficial and detrimental roles for adult neurogenesis depending on the CNS location (for example, SVZ *vs* striatum). Recently, we have hypothesized that in the same ischaemic striatum, microglia displaying anti or proneurogenic phenotypes may impair or even contribute to striatal neuroblast migration after stroke (Gomes-Leal 2012). The indomethacin treatment could modulate the excessive microglia activation that impairs adult neurogenesis, while preserving the microglial Proneurogenic phenotypes. The mechanisms by which indomethacin or minocycline enhances stroke-induced neurogenesis may be beyond direct actions on microglia, but might be related to direct actions on neural stem cells (Sakata *et al.* 2012).

5. Conclusion

The results suggest that indomethacin treatment is a suitable inhibitor of microglia activation in the first 2 weeks after striatal stroke. This effect is positively correlated with increased numbers of neuroblasts in both SVZ and ischaemic striatum mainly at 14 days post stroke, but not with changes in the infarct area or neuronal density. Anti-inflammatory treatments should be investigated as possible adjuvant therapies to enhance adult neurogenesis after stroke, by acting on microglial cell activity or through their pleiotropic effects, including direct actions on adult neural stem cells.

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Compliance with ethical standards

Ethical statement The experimental procedures were performed in accordance with European Community (EU Directive 2010/63/EU for animal experiments) and National Institutes of Health guidelines for the care and use of laboratory animals. The study was approved by the ethical committee of the Federal University of Pará (Brazil). The authors declare no conflict of interest that can influence this work. The work described has not been published previously. All authors have materially participated in the research or in the article preparation.

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