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The late response of rat subependymal zone stem and progenitor cells to stroke is restricted to directly affected areas of their niche ☆

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ABSTRACT

Ischaemia leads to increased proliferation of progenitors in the subependymal zone (SEZ) neurogenic niche of the adult brain and to generation and migration of newborn neurons. Here we investigated the spatiotemporal characteristics of the mitotic activity of adult neural stem and progenitor cells in the SEZ during the sub-acute and chronic post-ischaemic phases. Ischaemia was induced by performing a 1 h unilateral middle cerebral artery occlusion (MCAO) and tissue was collected 4/5 weeks and 1 year after the insult. Neural stem cells (NSCs) responded differently from their downstream progenitors to MCAO, with NSCs being activated only transiently whilst progenitors remain activated even at 1 year post-injury. Importantly, mitotic activation was observed only in the affected areas of the niche and specifically in the dorsal half of the SEZ. Analysis of the topography of mitoses, in relation to the anatomy of the lesion and to the position of ependymal cells and blood vessels, suggested an interplay between lesion-derived recruiting signals and the local signals that normally control proliferation in the chronic post-ischaemic phase.

Introduction

The largest neurogenic area of the adult rodent and human brain is the subependymal zone (SEZ), located at the lateral wall of the lateral ventricles, in which relatively quiescent neural stem cells (NSCs) generate actively dividing progeny (Lois and Alvarez-Buylla, 1994). In rodents, SEZ-born neuronal progenitors have the capacity to migrate long distances, through a specialized route called rostral migratory stream (RMS), in order to reach their final destination within the olfactory bulb (OB) (Riquelme et al., 2008). Experimental studies have shown that neurons and glia are also born at the SEZ in response to focal ischaemic lesions that model stroke in humans (Li et al., 2010a; Zhang et al., 2001, 2004) with many of these newly-generated cells migrating towards the infarcted areas (Hou et al., 2008; Jin et al., 2010; Thored et al., 2006, 2007; Yamashita et al., 2006). Proliferation in the SEZ peaks at around 1 week post-ischaemia, though SEZ-driven striatal neurogenesis persists for at least 4 months and is thought to correlate with spontaneous recovery during this sub-acute phase (Thored et al., 2006). Although only limited evidence exists demonstrating the potential of SEZ-derived newborn cells to develop into viable and functional neurons (Hou et al., 2008; Li et al., 2010a; Thored et al., 2006), the experimental ablation of endogenous neurogenesis in a transgenic mouse in which progenitors of neuronal commitment were depleted, compromised early post-ischaemic neuroprotection (Jin et al., 2010; Sun et al., 2012; Wang et al., 2012). Conversely, exogenous stimulation of neurogenesis through increased Wnt-3A expression or administration of retinoic acid enhanced tissue protection (Plane et al., 2008; Shruster et al., 2012). These results indicate that neurogenesis from the SEZ stem cell niche may be important for enhanced tissue preservation after stroke by the generation of cells with neuroprotective properties, and that it therefore constitutes a valid target for therapeutic interventions. However, in order to fully appraise its potential to be used in post-ischaemia recovery strategies, further analysis of its response after such insults is required. This includes investigating: a) the identity of the cell populations that respond (stem cells and/or their progeny), as has been done in other adult stem cell systems (Mascre et al., 2012; Simons and Clevers, 2011), b) the level of response (time frame, cell numbers) and c) the anatomy of the response (e.g. the fraction of the niche that becomes activated). In this study we map and quantify the activation of the SEZ during sub-acute and late post-ischaemic stages (4–5 weeks and 1 year, respectively), which are under-investigated though medically

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relevant in terms of recovery (Markus et al., 2005). We calculate the fraction of the niche responding to focal ischaemia and explore separately the mitotic activation of stem and progenitor cells. Finally, we assess the effects of ischaemia on the structure of the specialized microenvironment of the niche, focusing on the positioning of dividing progenitors in relation to two major structural elements of the SEZ: blood vessels and the ependymal cell layer/cerebrospinal fluid interface; as well as on the response of macrophages of the innate and blood-born immune system.

Materials and methods

Animals, experimental stroke and AraC treatment

Adult male Sprague–Dawley rats were used and all experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. In order to model focal ischaemia stroke in humans, ischaemia was induced by middle cerebral artery occlusion (MCAO) for 1 h, as previously reported (Modo et al., 2002). Briefly, animals were anaesthetised with isofluorane and temporary ligatures were placed on the ipsilateral external and common carotid to stop the flow of blood to the internal carotid artery. The tip of the thread (Doccol) was advanced 18–20 mm from the cervical carotid bifurcation or until reaching resistance from the ostium of the middle cerebral artery in the circle of Willis. Occluded animals were re-anaesthetised in order for the thread to be removed. For the sub-acute post-surgery stage, 260–280 g adult rats (2–3 months old) were operated (sham and ischaemia groups) and were killed 4 or 5 weeks later. Because all analyses performed in tissue derived from the two sub-acute phase time-points produced similar results (data not shown), data from 4 and 5 weeks were pooled together. For the chronic post-injury phase, another two groups of animals were operated (sham and ischaemia) and killed 1 year later. All animals were included based on the presence of a T2-hyperintense lesion using MRI according to previously published protocols (Modo et al., 2009).

AraC infusions were performed in an additional group of non-operated rats at an age equivalent to that of the sub-acute post-stroke time-point group and according to previously published protocols (Kazanis et al., 2010). AraC was infused on the surface of the brain via a cannula (BIK-II, Alzet) that was fixed on the skull (1 mm lateral and 0.5 mm rostral to bregma) and was connected to a subcutaneously implanted mini-osmotic pump (1007D, Alzet). 4% AraC (Sigma, UK) in saline, or saline alone, was infused for 7 days and animals were sacrificed 2 days after the end of the infusion. The success of the treatment was evaluated by immunostaining for the neuroblast marker Dcx, as has been previously reported in mice (Kazanis et al., 2010). In all rats included in the study AraC resulted in the complete depletion of Dcx+ cells in the ipsilateral SEZ niche (as shown in Fig. S3).

Tissue processing, imaging, data collection and statistical analysis

Animals were sacrificed by transcardial infusion of 4% paraformaldehyde (under terminal anaesthesia) and tissue was post-fixed overnight in 4% paraformaldehyde (at 4 °C). The brains were subsequently sectioned using a vibratome in 70 μm thick slices and were stored at −20 °C in an anti-freezing solution (containing 30% glycerol, 30% Ethylene glycol in 0.5% phosphate buffered saline; reagents purchased from Sigma-Aldrich UK). Immunohistochemistry was performed using a free-floating slice protocol. Initially, the slices were treated for antigen retrieval by being incubated in a 10 mM citrate buffer (pH = 6) at 90 °C for 15 min. Slices were then incubated in blocking solution (3% BSA, 0.1% Triton X-100 in PBS) at room temperature for a minimum of 5 h. Subsequently they were incubated overnight with the primary antibodies diluted in blocking solution at room temperature, on a rocking plate. The antibodies used were the following: rabbit anti-PH3 (1/500, Millipore) and mouse anti Ki67 (1/500, Novocastra Labs, UK) to label mitotic cells, mouse anti-GFAP (1/500, Sigma) for astrocytes, rabbit anti-Dcx (1/500, Abcam, UK) for neuroblasts, rabbit anti-pan-laminin (1/100, Sigma, UK) for blood vessels and mouse anti-ED1 (CD68, 1/500, Millipore) for phagocytotic macrophages and microglia. The final step was the incubation – for a minimum of 4 h – at room temperature with the respective Alexa-conjugated secondary antibodies (Invitrogen, UK) diluted in PBS. In the case of triple stainings for GFAP, PH3 and laminin, the first two primary antibodies were incubated together and then with the respective secondary antibodies; the anti-laminin staining was performed subsequently. Biotinylated isolectin IB-4 (Invitrogen, UK) was used to stain all microglia and macrophages. Images were acquired using a Zeiss fluorescence microscope or a Leica SP5 confocal microscope and were processed using Photoshop (Adobe) software.

A range of sections was analysed from each animal, taken from the same rostro-caudal area [from bregma (=0 mm), to 1.5 mm rostrally]. In each rat, a minimum of 7 slices taken from respective anatomical ares, as judged by the shape of the lateral ventricles, of the corpus callosum and of the anterior commissure, were immunostained for GFAP/PH3 and laminin. In these slices all mitotic (PH3+) cells were identified and counted within a distance of 100 μm of the ventricular wall; this being the normal maximal width of the neurogenic niche revealed by the distribution of Ki67+ cells (Kazanis and ffrench-Constant, 2011). For each one mitotic cell, at least 3 confocal-microscopy generated optical sections were taken (using the ×63 objective and a further ×4 digital zoom) in order to assess whether it was GFAP positive or negative (see the example in Fig. S2). The boundaries of the lesion were estimated and delineated on the same slices based on the increased expression of GFAP (astrogliosis), blood vessel swelling and laminin deposition and the dorsoventral lengths of the affected areas were calculated using Image Processing and Analysis in Java software (ImageJ). For the analysis of the anatomical characteristics of the SEZ response to stroke, the neurogenic niche was divided in two halves along the dorso-ventral axis (see Fig. 5). For the analysis of PH3+ cells within the penumbra, at least three optical fields were selected in each section positioned entirely within the penumbra (as it was defined by the above-mentioned histological criteria) and at a distance higher than 500 μm from the wall of the lateral ventricle. Statistical analysis of the densities of mitotic cells for the effects of treatment (sham, ischaemia and AraC) and of the area was performed separately for NSCs and progenitors and for the two time-points, using one-way or two-way ANOVA, as required.

For the analysis of microglia/macrophages, 3 sections per animal were stained for Ki67 and IB-4, one section per animal for PH3 and IB-4 and another 3 sections per animal for ED1 and Dcx. For the analysis of the positioning of mitotic cells, measurements were performed as described previously (Kazanis and ffrench-Constant, 2011; Kazanis et al., 2010) in the same triple-stained (for PH3, GFAP and laminin to mark blood vessels) slices. For the calculation of the distance from the nearest blood vessel, an area spanning at least 30 μm in each direction around a mitotic cell was scanned with the confocal microscope, all blood vessels were identified and the shortest distance between the mitotic centre of the nucleus and the surface of the blood vessel was measured using ImageJ. Mitotic cells located near the surface of the sections (hence without a 30 μm area around them, which were less than 10% of all PH3+ cells identified) were excluded from this analysis in order to avoid missing the nearest blood vessel that might be outside the section. For the calculation of the distance from the ventricular wall, the shortest distance between the centre of the mitotic nucleus and the wall of the ventricle (the ventricular surface of the nearest ependymal cell, or the line linking the nuclei of two neighbouring ependymal cells) was calculated using ImageJ.