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Neurovascular unit remodelling in the subacute stage of stroke recovery

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ABSTRACT

Brain plasticity following focal cerebral ischaemia has been observed in both stroke survivors and in preclinical models of stroke. Endogenous neurovascular adaptation is at present incompletely understood yet its potentiation may improve long-term functional outcome. We employed longitudinal MRI, intracranial array electrophysiology, Montoya Staircase testing, and immunofluorescence to examine function of brain vessels, neurons, and glia in addition to forelimb skilled reaching during the subacute stage of ischemic injury progression. Focal ischemic stroke (~100 mm^3 or ~20% of the total brain volume) was induced in adult Sprague-Dawley rats via direct injection of endothelin-1 (ET-1) into the right sensori-motor cortex, producing sustained impairment in left forelimb reaching ability. Resting perfusion and vascular reactivity to hypercapnia in the peri-lesional cortex were elevated by approximately 60% and 80% respectively seven days following stroke. At the same time, the normal topological pattern of local field potential (LFP) responses to peripheral somatosensory stimulation was abolished and the average power of spontaneous LFP activity attenuated by approximately 50% relative to the contra-lesional cortex, suggesting initial response attenuation within the peri-infarct zone. By 21 days after stroke, perilesional blood flow resolved, but peri-lesional vascular reactivity remained elevated. Concomitantly, the LFP response amplitudes increased with distance from the site of ET-1 injection, suggesting functional remodelling from the core of the lesion to its periphery. This notion was further buttressed by the lateralization of spontaneous neuronal activity: by day 21, the average ipsi-lesional power of spontaneous LFP activity was almost twice that of the contra-lesional cortex. Over the observation period, the peri-lesional cortex exhibited increased vascular density, along with neuronal loss, astrocytic activation, and recruitment and activation of microglia and macrophages, with neuronal loss and inflammation extending beyond the peri-lesional cortex. These findings highlight the complex relationship between neurophysiological state and behaviour and provide evidence of highly dynamic functional changes in the peri-infarct zone weeks following the ischemic insult, suggesting an extended temporal window for therapeutic interventions.

1. Introduction

Although ischemic stroke is the leading cause of adult disability worldwide, most stroke survivors report some improvement with time and some show significant recovery even in the absence of treatment (Seil 1997; Steinberg and Augustine, 1997; Hallett, 2001; Krishnamurthi et al., 2013). Recent work has investigated the determinants of prognosis post stroke and there is widespread recognition of the importance of understanding the mechanisms of endogenous recovery as a means of guiding the development of new treatments (Lee and van Donkelaar, 1995; Seil, 1997; Steinberg and Augustine, 1997; Hallett 2001). A prominent topic of current enquiry is injury-induced vascular remodelling. An increase in vascular density on post-mortem neuropathological examination in stroke patients correlates

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with functional improvement and longer survival after ischemic injury (Krupiński et al., 1992, 1994; Szpak et al. 1999). A number of preclinical studies have reported that histopathological evidence of angiogenesis correlates with transient in vivo increases in cerebral blood flow (CBF) and/or cerebral blood volume (CBV) in the peri-lesional tissue during the subacute phase (one to three weeks following stroke) (Lin et al., 2002; Dijkhuizen et al., 2003; Lin et al., 2008; Hayward et al., 2011; Martin et al., 2012; Chang et al., 2013). In preclinical models, a transition from hypo- (in the initial 24–48 h) to hyper-perfusion is associated with angiogenesis one to two weeks following injury (Dijkhuizen et al., 2003; Hayward et al., 2011; Zhang et al., 2013) and improved performance on neurological test batteries, beam walking and cylinder tests (Lin et al., 2002; Dijkhuizen et al., 2003; Lin et al., 2008; Hayward et al., 2011; Martin et al., 2012; Zhang et al., 2013). The details of spatio-temporal changes in neurovascular morphology and function post ischaemia remain uncertain yet are critical for the design of more effective therapeutic approaches in the subacute stage of stroke.

To address this gap, we injected endothelin-1 (ET-1), a potent vasoconstrictor, into the forelimb region of the right sensorimotor cortex to produce a robust, focal region of ischaemia. The ET-1 model was chosen as it produces a spatially targeted, focal lesion, similar in volume to what is observed in stroke survivors (Carmichael, 2005); and exhibits flow impairment and resolution kinetics similar to those observed in human stroke (Olsen and Lassen, 1984; Mohr et al., 1986; Heiss et al., 2000; Biernaskie et al., 2001; Carmichael, 2005). Brain morphology and vascular function were assessed using *in situ* T_2-weighted magnetic resonance imaging (MRI) and continuous arterial spin labelling (CASL) functional MRI; neuronal activity was evaluated using intracortical array electrophysiology, and neuronal, glial, and vascular morphology were assessed using pathological analysis. These biological assays were related to skilled reaching ability on the Montoya Staircase test. Assays were conducted over 21 days following ischemic insult to examine the evolution of functional and structural changes in the neurovascular unit.

2. Methods

All animals within this study were adult male Sprague-Dawley rats. All data processing was performed blinded to surgery group allocation (stroke or sham) and injury status (seven or 21 days after stroke or sham-surgery). Inclusion criteria are summarized in Supplementary Table 1 for each assay.

2.1. Stroke induction

All animals underwent the same stroke induction or sham surgical procedure under isoflurane anaesthesia (5% induction and 2–2.5% maintenance). Animals were secured in a small animal stereotaxic apparatus (David KOPF Instruments, Tujunga California, USA). Under aseptic condition, a midline incision was made, and two burr holes, 0.8 mm in diameter, drilled at 0.0 mm A-P (anterior-posterior), −2.5 mm M-L (medial-lateral); and at 2.3 mm A-P, −2.5 mm M-L over the right sensorimotor cortex using a high-speed micro-drill (Foredom Electric Co., Bethel Connecticut, USA). A 10 μl Hamilton Syringe (Model 80366, needle size 26 s gauge with beveled tip) was used to inject 800 picomoles of ET-1 (Sigma-Aldrich, St. Louis Missouri, USA) suspended in 4 μl of phosphate buffered saline (PBS) or PBS alone (sham-surgery) at −2.3 mm D-V (dorsal-ventral). One 2 μl aliquot was injected at each location, for a total of 4 μl. After lowering the needle to −2.5 mm and retracting to −2.3 mm D-V, a one-minute delay was allowed before injection began. A further one-minute delay was kept between the delivery of each μl, and a two-minute delay preceded needle retraction. Injections were made at a rate of 1 μl/min, for a total delivery time (including 4 one-minute delays, and 2 two-minute delays) of 12 min. Burr holes were closed with bone wax and the scalp sutured over the skull. For analgesia, animals were given a subcutaneous dose of Marcaine (0.2 mg/kg) at the beginning and end of surgery (Windle et al., 2006).

2.2. Magnetic resonance imaging

All animals were imaged seven and 21 days following stroke induction or sham-surgery on a 7T animal system (Bruker, BioSpec, Etlingen, Germany). Animals were immobilized with ear bars and an incisor bar, and a stable plane of anaesthesia maintained with an intravenous infusion of 45 mg/kg/hr of propofol (Pharmascience Inc., Montreal Quebec, Canada). Propofol anaesthesia was chosen in light of its being well tolerated and allowing rapid recovery, thus being well suited to longitudinal experiments; in addition to having been successfully used for cerebral blood flow quantification via ASL in rats (Griffin et al., 2010).

2.2.1. T_2-weighted MRI

A birdcage body coil was used for signal excitation and a quadrature receive-only coil for signal detection. Forty-five coronal images were obtained with a rapid acquisition with relaxation enhancement (RARE) sequence (RARE factor of eight, repetition time/echo time TR/TE of 5500/47 ms, and a matrix size of 128×256), with a nominal in-plane spatial resolution of 0.1×0.1 mm^2 and a slice thickness of 0.5 mm in under 12 min. Images were imported into ImageMagick [ImageMagick Studio LLC, 2013. http://www.imagemagick.org/script/index.php] for semi-automated segmentation. Following earlier work (Neumann-Haefelin et al., 2000; Virley et al., 2000; Kidwell et al., 2003; van der Zijden et al., 2008; Jiang et al., 2006), stroke regions were segmented using a predetermined signal intensity threshold of greater than two standard deviations (SDs) above the mean contra-lesional cortical signal intensity. Following segmentation, stroke volumes were calculated for each time point for each animal.

2.2.2. CASL

A custom built labelling coil was positioned at the level of the carotid arteries and a quadrature receive-only coil was used for signal detection. Using a 1.5-s adiabatic labelling pulse, and a 0.4-s post-labelling delay, single-average, single-shot echo-planar images (EPI) were obtained from five 1.5 mm thick coronal slices positioned over the sensorimotor cortex, with a 0.25×0.25 mm^2 in-plane resolution, TR/TE of 2000/8.3 ms, and inter-slice gap of 0.5 mm. Sixty EPI frames were collected to estimate resting perfusion. For vessel reactivity measurements, animals were tracheostomized, mechanically ventilated, and challenged by six presentations of a hypercapnic mixture in ON:OFF periods of 1:4 min (ON: 10% CO_2, 31% O_2 and 59% N_2, OFF: 0% CO_2, 31% O_2 and 69% N_2). Inspired mixture composition and delivery were controlled by a programmable GasMixer (GSM-3, CWE Inc., Boston MA).

2.2.3. CASL data processing

All CASL data were motion corrected (AFNI, Analysis of Functional NeuroImages (Cox, 1996), *2dImReg*), masked (to isolate grey matter), and spatially blurred within the grey matter mask (AFNI *3dBlurToFWHM*, full-width-half-maximum 0.55 mm) prior to fitting the data using a Generalized Linear Model (AFNI *3dDeconvolve*). Subject-specific hemodynamic response functions were produced by averaging the signal in the left (contra-lateral) cortical grey matter (Kang et al., 2003). A threshold was applied to resulting maps of perfusion signal changes elicited by hypercapnia and resting perfusion to correct for multiple comparisons (false discovery rate $q < 0.01$). In each animal, we manually identified a training set of approximately 40–60 voxels in both perfusion response and resting perfusion maps residing in the contra- and ipsi-lateral cortices. The pial surface and boundary with corpus callosum were excluded, along with major vessels. Using these training data, classification was performed with a