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Enriched housing down-regulates the Toll-like receptor 2 response in the mouse brain after experimental stroke

Miriana Jlenia Quattromani [a,*], Pierre Cordeau [b], Karsten Ruscher [a], Jasna Kriz [b], Tadeusz Wieloch [a]

[a] Laboratory for Experimental Brain Research, Department of Clinical Sciences, Division of Neurosurgery, Lund University, BMC A13, 22184 Lund, Sweden
[b] Department of Psychiatry and Neuroscience, Laval University, Le Centre de Recherche de l'Institut Universitaire en Santé Mentale de Québec, 2601, de la Canardière, Québec G1J 2G3, Canada

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ABSTRACT

Post-ischemic inflammation plays an important role in the evolution of brain injury, recovery and repair after stroke. Housing rodents in an enriched environment provides multisensory stimulation to the brain and enhances functional recovery after experimental stroke, also depressing the release of cytokines and chemokines in the peri-infarct. In order to identify targets for late stroke treatment, we studied the dynamics of inflammation and the contribution of resident Toll-like receptor 2 (TLR2) expressing microglia cells.

We took advantage of the biophotonic/bioluminescent imaging technique using the reporter mouse-expressing luciferase and GFP reporter genes under transcriptional control of the murine TLR2 promoter (TLR2-luc/GFP mice) for non-invasive *in vivo* analysis of TLR2 activation/response in photothrombotic stroke after differential housing.

Real-time imaging at 1 day after stroke, revealed up-regulation of TLR2 in response to photothrombotic stroke that subsequently declined over time of recovery (14 days). The inflammatory response was persistently down-regulated within days of enriched housing, enhancing recovery of lost sensori-motor function in TLR2-luc mice without affecting infarct size. The number of YM1-expressing microglia in the peri-infarct and areas remote from the infarct was also markedly attenuated.

Using a live imaging approach, we demonstrate that multisensory stimulation rapidly, persistently and generally attenuates brain inflammation after experimental stroke, reducing the TLR2 response and leading to improved neurological outcome. TLR2-expressing microglia cells may provide targets for new stroke therapeutics.

Introduction

The evolution of brain injury after stroke and the subsequent recovery of lost brain function due to neuronal damage and neuronal dysfunction, encompass dynamic, complex, heterogeneous and interconnected processes (Endres et al., 2008; Wieloch and Nikolich, 2006), including brain inflammation (Iadecola and Anrather, 2011). These inflammatory events are triggered by the release of chemotactic compounds from the degenerating tissue in the infarct and from cells in the non-injured brain that participate in tissue repair and brain remodeling. This attracts blood-borne inflammatory cells to the injured tissue and activates resident glial cells, including microglia. The activation of microglia cells after stroke is prominent (Iadecola and Anrather, 2011; Kempermann and Neumann, 2003; Kriz and Lalancette-Hébert, 2009), and evident by a pronounced up-regulation of pathogen recognition receptors, such as Toll-like receptors (TLRs) (Akira and Takeda, 2004; Lalancette-Hébert et al., 2009; Ziegler et al., 2010). TLRs are expressed by cells of the innate immune system to identify pathogen-associated molecular patterns (PAMPs), which are associated with microbial pathogens or cellular stress (Medzhitov et al., 1997), as well as damage-associated molecular patterns (DAMPs), recognized in processes associated with cell components released during cell damage, such as ischemic injury (Arumugam et al., 2009; Hanisch et al., 2008; Kariko et al., 2004). Recent evidence suggests that TLRs, especially Toll-like receptor 2 (TLR2), may have a key role in the evolution of brain damage elicited by cerebral ischemia (Babcock et al., 2006; Lalancette-Hébert et al., 2009; Lehnardt et al., 2007; Tang et al., 2007; Ziegler et al., 2007, 2010). At present, the spatio-temporal dynamics and the potential long-term role of the TLR2 response to brain damage are not well understood.

Multisensory stimulation by changes in the environment as well as task-driven experience induce brain remodeling and plasticity, in the normal (Draganski and May, 2008), as well as in the injured brain (Johansson, 2011; Nithianantharajah and Hannan, 2006; van Praag et al., 2000). Environmental enrichment refers to housing conditions that facilitate enhanced sensory, cognitive, motor and social stimulation relative to standard conditions. Enriched housing of the rodents is a strong plasticity inducing agent that can restore lost functions following neurodegenerative disorders or brain damage, such as stroke (Nygren

* Corresponding author. Fax: +46 46 2220615.
E-mail addresses: miriana.quattromani@med.lu.se (M.J. Quattromani), petermavic@gmail.com (P. Cordeau), karsten.ruscher@med.lu.se (K. Ruscher), jasna.kriz@fmed.ulaval.ca (J. Kriz), tadeusz.wieloch@med.lu.se (T. Wieloch).
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and Wieloch, 2005; Ohlsson and Johansson, 1995). Recently this intervention has been translated to the clinical setting (Janssen et al., 2012). The recovery-promoting effect of enriched housing can be initiated 2 days after ischemia, without affecting the final infarct volume (Ohlsson and Johansson, 1995). The mechanisms by which enriched housing influences recovery are still poorly understood, but recently it has been proposed to modulate central brain inflammation by depressing the levels of cytokines and chemokines (Ruscher et al., 2009, 2013).

In order to investigate the spatio-temporal profile of the inflammatory response after photothrombotic stroke and the influence of multisensory stimulation provided by an enriched environment, we assessed the TLR2 response/microglia activation in the TLR2-luc/GFP-expressing mice. We took advantage of the minimally invasive *in vivo* biophotonic/bioluminescence imaging (Kriz and Lalancette-Hébert, 2009) which allows unique and simple longitudinal studies of brain inflammation (TLR2-luc expression) in the same animal for extended periods of time.

Materials and methods

Experimental animals

The transgenic TLR2sm-fluc-GFP mice were generated and characterized as previously described (Lalancette-Hébert et al., 2009). A total of 32 male TLR2 +/− mice, weighing 20–30 g and 2–4 months old, were used in the study. All animals were housed under a 12 h light/dark cycle with *ad libitum* access to food and water. Experimental procedures were approved by the Laval University Animal Care Ethics Committee and are in accordance with the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care.

Photothrombotic stroke

Mice were subjected to unilateral photothrombotic (PT) cortical infarct in the left sensori-motor cortex. During surgery, the body temperature of the animals was kept at 37 °C using a self-regulating heating pad. Infarct was induced using a modified method initially described by Watson et al. (1985), and adapted for mice by Schroeter et al. (2002). Briefly, animals were anesthetized using 1.5–2.5% isoflurane (Sigma) in 100% O_2. The skin above the skull was incised; a fiber optic bundle with a 2.5 mm aperture diameter was connected to a cold light source (CL 1500 ECO, Zeiss, Germany) and adjusted to the stereotaxic coordinates of the sensori-motor cortex (Paxinos and Franklin, 2001), 0.5 mm anterior to bregma and 1.5 mm lateral to midline. One intraperitoneal (i.p.) injection of 0.25 mL Rose Bengal (10 mg/mL in 0.9% saline; Sigma-Aldrich, Germany) was given 5 min before the light was turned on for 20 min. Care was taken not to exceed 38 °C at the skull surface, underneath the fiber optic bundle. Post surgery, animals were allowed to awake from anesthesia while on a heating pad and returned to the cages with free access to food pellets and water. Temperature and body weight were recorded (Supplementary Table 1). Control mice, after skin incision on the skull, were either subjected to Rose Bengal injection without subsequent illumination, or subjected to 20-minute illumination without dye injection. Out of the 32 animals subjected to photothrombosis, one animal spontaneously died at 24 h of recovery.

In vivo bioluminescence imaging

The images were gathered using IVIS® 200 Imaging System (Xenogen, Alameda, CA, USA) as previously described (Lalancette-Hébert et al., 2009). Prior to the imaging session, the mice received an i.p. injection of the luciferase substrate D-luciferine (150 mg/kg, Xenogen), dissolved in 0.9% saline. The mice were then anesthetized with 2% isoflurane in 100% O_2 with a flow rate of 2 L/min and placed in a heated, light-tight imaging chamber. Images were collected with a high-sensitivity CCD camera. Exposure time for imaging was 2 min. The bioluminescence emission was normalized and displayed in physical units of surface radiance (photons s^{-1} cm^{-2} $steradian^{-1}$). Light output was quantified by determining the total number of photons emitted per second with the use of Living Image 2.5 acquisition and imaging software (Xenogen). Region-of-interest (ROI) measurements on the images were used to convert surface radiance (photons s^{-1} cm^{-2} $steradian^{-1}$) to source the total flux of photons in photons/s. The data are represented as pseudocolor images indicating light intensity (red and yellow, most intense), which were superimposed over gray-scale reference photographs. IVIS images were acquired for each animal, prior to stroke (baseline) and at days 1, 3, 6, 10 and 14 of recovery (Fig. 1).

Behavioral assessment and randomization

Studies were subjected to randomization and carried out in a blinded fashion to the investigators who performed the surgeries and behavioral assessment.

Paw placement test: The mouse was held along its rostro-caudal extension on the edge of a bench and both the unilateral front and hind paws were gently pushed along it. The placement of each paw was recorded when the mouse was moved towards the edge (modified from De Ryck et al., 1989; Hunter et al., 2000). Sensori-motor dysfunction was assessed by using a score of 1, 0.5, and 0: (1) — the paw is immediately placed on the table surface; (0.5) — the limb is extended, but with some movements and attempts to place the paw on the surface of the table; (0) — the paw is totally immobilized, hanging down, with no movement. To obtain a neurological assessment during recovery, each mouse was tested at 2, 7 and 14 days after PT stroke.

In order to obtain groups with a similar functional deficit prior to placement of the mice into either standard or enriched cages, selective sorting was performed on day 2 after experimental stroke, a time point of recovery when the infarct development had subsided. Hence, only animals that showed a severe deficit in the paw placement (score = 0) entered the study ($n = 14$). After selective sorting, mice were randomly distributed into standard (STD, 17 cm × 16 cm × 34.5 cm) or enriched cages (EE, 30 cm × 27 cm × 43 cm). Multilevel-enriched cages were equipped with various colored objects such as plastic tunnels, small houses, slides and rodent running wheels; the disposition of the objects was changed every second day (Nygren and Wieloch, 2005). Mice were housed in either standard $n = 6$ (2 animals/cage) or enriched cages $n = 8$ (5 animals/cage; animals excluded from the study were used to fill up the cages) for 12 days (Fig. 1). Animals in standard cages were handled once a day.

Tissue collection

At 14 days after surgery, mice laying in the IVIS® imaging chamber and anesthetized with 2% isoflurane in 100% O_2, were lethally injected with chloral hydrate (i.p., 150 mg/kg, concentration 10 mg/mL). They were then transcardially perfused with 0.9% saline followed by ice-cold phosphate-buffered 4% paraformaldehyde (PFA, Sigma) at pH 7.4. Tissue samples were post-fixed overnight in 4% PFA and equilibrated in phosphate-buffered 30% sucrose for 48 h at 4 °C. The brains were then sectioned on a microtome at 30-μm thickness in the coronal plane. Sections were collected in 8 subsequent series and stored at −20 °C in an antifreeze solution made in phosphate buffer containing 30% glycerol and 30% ethylene glycol.

Infarct volume measurements

For each animal, seven coronal brain sections with a distance of 1.0 mm were immunostained with the NeuN antibody (Neuronal Nuclei, Millipore, Hampshire, UK; dilution 1:1500). Infarcted areas, the non-lesioned area of the infarcted hemisphere and the non-lesioned contralateral hemisphere were outlined on each brain section using the ImageJ software (National Institute of Health, USA). Infarct volume