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Bioluminescence imaging of stroke-induced endogenous neural stem cell response

Caroline Vandeputte [a,b,c,1], Veerle Reumers [a,1], Sarah-Ann Aelvoet [a,1], Irina Thiry [d], Sylvie De Swaef [a], Chris Van Den Haute [a,e], Jesus Pascual-Brazo [a,b], Tracy D. Farr [f], Greetje Vande Velde [b,g], Mathias Hoehn [f], Uwe Himmelreich [b,g], Koen Van Laere [b,c], Zeger Debyser [b,d], Rik Gijsbers [d,e,*], Veerle Baekelandt [a,b,**]

[a] KU Leuven, Laboratory for Neurobiology and Gene Therapy, Department of Neurosciences, KU Leuven, 3000 Leuven, Flanders, Belgium
[b] Molecular Small Animal Imaging Center, MOSAIC, KU Leuven, 3000 Leuven, Flanders, Belgium
[c] Division of Nuclear Medicine, University Hospital and KU Leuven, 3000 Leuven, Flanders, Belgium
[d] KU Leuven, Laboratory for Molecular Virology and Gene Therapy, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, 3000 Leuven, Flanders, Belgium
[e] Leuven Viral Vector Core, KU Leuven, 3000 Leuven, Flanders, Belgium
[f] In-vivo-NMR Laboratory, Max Planck Institute for Neurological Research, 50931 Cologne, Germany
[g] Biomedical MRI, Department of Imaging and Pathology, KU Leuven, 3000 Leuven, Flanders, Belgium

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ABSTRACT

Brain injury following stroke affects neurogenesis in the adult mammalian brain. However, a complete understanding of the origin and fate of the endogenous neural stem cells (eNSCs) *in vivo* is missing. Tools and technology that allow non-invasive imaging and tracking of eNSCs in living animals will help to overcome this hurdle. In this study, we aimed to monitor eNSCs in a photothrombotic (PT) stroke model using *in vivo* bioluminescence imaging (BLI). In a first strategy, inducible transgenic mice expressing firefly luciferase (Fluc) in the eNSCs were generated. In animals that received stroke, an increased BLI signal originating from the infarct region was observed. However, due to histological limitations, the identity and exact origin of cells contributing to the increased BLI signal could not be revealed. To overcome this limitation, we developed an alternative strategy employing stereotactic injection of conditional lentiviral vectors (Cre-Flex LVs) encoding Fluc and eGFP in the subventricular zone (SVZ) of Nestin-Cre transgenic mice, thereby specifically labeling the eNSCs. Upon induction of stroke, increased eNSC proliferation resulted in a significant increase in BLI signal between 2 days and 2 weeks after stroke, decreasing after 3 months. Additionally, the BLI signal relocalized from the SVZ towards the infarct region during the 2 weeks following stroke. Histological analysis at 90 days post stroke showed that in the peri-infarct area, 36% of labeled eNSC progeny differentiated into astrocytes, while 21% differentiated into mature neurons. In conclusion, we developed and validated a novel imaging technique that unequivocally demonstrates that nestin^{+} eNSCs originating from the SVZ respond to stroke injury by increased proliferation, migration towards the infarct region and differentiation into both astrocytes and neurons. In addition, this new approach allows non-invasive and specific monitoring of eNSCs over time, opening perspectives for preclinical evaluation of candidate stroke therapeutics.

Abbreviations: BLI, bioluminescence imaging; BrdU, 5-bromo-2′-deoxyuridine; CC, corpus callosum; Cre-Flex, Cre-mediated flip-excision; DCX, doublecortin; eNSCs, endogenous neural stem cells; Fluc, firefly luciferase; GFAP, glial fibrillary acidic protein; LV, lentiviral vector; MCAO, middle cerebral artery occlusion; MRI, magnetic resonance imaging; NeuN, neuronal nuclei; OB, olfactory bulb; PET, positron emission tomography; PT, photothrombotic; RMS, rostral migratory stream; SGZ, subgranular zone; SVZ, subventricular zone.

* Correspondence to: R. Gijsbers, Laboratory for Molecular Virology and Gene Therapy, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Kapucijnenvoer 33, VCTB+5 bus 7001, B-3000 Leuven, Flanders, Belgium. Fax: +32 16 33 63 36.
** Correspondence to: V. Baekelandt, Laboratory for Neurobiology and Gene Therapy, Department of Neurosciences, KU Leuven, Kapucijnenvoer 33, VCTB+5 bus 7001, B-3000 Leuven, Flanders, Belgium. Fax: +32 16 33 63 36.
E-mail addresses: rik.gijsbers@med.kuleuven.be (R. Gijsbers), veerle.baekelandt@med.kuleuven.be (V. Baekelandt).
URL: http://www.kuleuven.be/molmed (V. Baekelandt).
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[1] These authors contributed equally.

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Introduction

The presence of endogenous neural stem cells (eNSCs) in the adult mammalian brain, including human brain, is now widely accepted (Altman, 1962, 1963; Curtis et al., 2005; Eriksson et al., 1998). Two brain regions, *i.e.* the SVZ of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus, are recognized as primary regions of adult neurogenesis (Ming and Song, 2005). Under physiological conditions, eNSCs in the SVZ divide and their progeny migrates tangentially *via* the rostral migratory stream (RMS) to the olfactory bulb (OB). Upon arrival in the OB, neuroblasts differentiate into local interneurons and integrate into the glomerular and granular layers (Alvarez-Buylla and Garcia-Verdugo, 2002). Pathological conditions, including brain injury and stroke, affect adult neurogenesis (Curtis et al., 2005; Gray and Sundstrom, 1998; Liu et al., 1998). Stroke, a common cause of morbidity and mortality worldwide, deprives the brain of oxygen and glucose (Flynn et al., 2008). Following stroke, neurogenesis augments the number of immature neurons in the SVZ (Jin et al., 2001; Zhang et al., 2008). Neuroblasts (positive for the marker doublecortin, DCX) migrate towards sites of ischemic damage and upon arrival, phenotypic markers of mature neurons can be detected (Arvidsson et al., 2002; Parent et al., 2002). On the other hand, retroviral labeling of the SVZ showed that cells migrated to the lesion and differentiated into glia (Goings et al., 2004), demonstrating that following injury, the SVZ can generate both neural cell types. Some studies showed that SVZ-derived progenitors can differentiate into medium spiny neurons in the striatum after stroke (Collin et al., 2005; Parent et al., 2002), whereas others claimed that the newborn cells are fate restricted to interneurons or glia (Deierborg et al., 2009; Liu et al., 2009). Whether SVZ neural progenitors can alter their fate, integrate in the injured circuits and survive for long time periods is still a matter of debate (Kernie and Parent, 2010). Up till now, specific labeling of eNSCs in the SVZ and the follow-up of the migration of their progeny to the ischemic area over time has not yet been shown.

Apart from the primary neurogenic niches, other brain regions, *e.g.* the cortex, contain cells that become multipotent and self-renew after injury (Komitova et al., 2006). Although mature astrocytes do not divide in healthy conditions, they can dedifferentiate and proliferate after stab wound injury and stroke (Buffo et al., 2008; Sirko et al., 2013). While these proliferating astrocytes remained within their lineage *in vivo*, they formed multipotent neurospheres *in vitro* (Buffo et al., 2008; Shimada et al., 2010). Therefore, these reactive astrocytes may represent an alternative source of multipotent cells that may be beneficial in stroke.

A major hurdle when studying endogenous neurogenesis is the lack of methods to monitor these processes *in vivo*, in individual animals over time. We and others attempted to label eNSCs by injection of iron oxide-based particles in the lateral ventricle or SVZ (Nieman et al., 2010; Shapiro et al., 2006; Sumner et al., 2009; Vreys et al., 2010), or by lentiviral vectors (LVs) encoding a reporter gene into the SVZ (Vande Velde et al., 2012) to monitor stem cell migration along the RMS with magnetic resonance imaging (MRI). Although MRI provides high resolution, it suffers from low *in vivo* sensitivity and gives no information on cell viability and non-specific signal detection cannot be excluded. Rueger et al. described *in vivo* imaging of eNSCs after focal cerebral ischemia *via* positron emission tomography (PET) imaging (Rueger et al., 2010), however, the cells responsible for the PET signal could not be identified. Alternatively, transgenic mice expressing Fluc driven by a DCX promoter allowed monitoring of adult neurogenesis using *in vivo* BLI (Couillard-Despres et al., 2008). However, the robust BLI signal emitted from the SVZ, leading to scattering and projection of these photons to the OB, impedes direct visualization of eNSC migration from the SVZ towards the OB. Moreover, when the DCX^+ neuroblasts differentiate into mature neurons, they lose the Fluc expression. In a first part of the present study, we generated inducible transgenic mice that express Fluc in the $nestin^+$ eNSCs, to monitor a stroke-induced eNSC response with BLI.

An alternative strategy to efficiently and stably introduce Fluc in the eNSCs is by stereotactic injection of LVs into the SVZ, which allowed us and others to monitor the migration of eNSCs and their progeny towards the OB with BLI (Guglielmetti et al., 2013; Reumers et al., 2008). However, since LVs transduce both dividing and post-mitotic cells, not only eNSCs but also neighboring astrocytes and mature neurons are labeled after injection of constitutive LVs in the SVZ (Geraerts et al., 2006). As a result, in line with the data described in transgenic mice, a high BLI signal emerges from the site of injection that interferes with the measurement of the migrating cells (Reumers et al., 2008). To overcome the latter, we developed new conditional Cre-Flex LVs in a second part of this study. These Cre-Flex LVs incorporate Cre-lox technology, allowing that Fluc and eGFP are restrictively expressed in eNSCs after injection in the SVZ of transgenic Nestin-Cre mice. While numerous research groups have previously described stroke-induced eNSC behavior (Arvidsson et al., 2002; Parent et al., 2002), we here report for the first time successful *in vivo* imaging and characterization of long-term eNSC responses after stroke.

Materials & methods

Animals

Animal studies were performed in accordance with the current ethical regulations of the KU Leuven. Nestin-$CreER^{T2}$ mice (a kind gift from Dr. Amelia J. Eisch (University of Texas Southwestern Medical Center, Dallas, TX) (Lagace et al., 2007)) and B6.Cg-Tg(Nes-cre)1Kln/J (Jax labs stock nr 003771, (Tronche et al., 1999)) were crossbred with C57BL/6-Tyr^{c-2J}/J (Jax labs, stock nr 000058), creating white furred albino mice in a C57BL/6 genetic background. White furred inducible Nestin-$CreER^{T2}$ mice were crossbred with ROSA26-LoxP-stop-LoxP(L-S-L)-luciferase transgenic mice (Safran et al., 2003) (Jax labs, stock nr 005125), indicated as Nestin-$CreER^{T2}$/Fluc mice. To induce Fluc expression, mice received tamoxifen intraperitoneally (ip) or orally at 180 mg/kg dissolved in 10% EtOH/90% sunflower oil for 5 consecutive days. BrdU was administered as previously published (Geraerts et al, 2006). For the stroke follow-up, Fluc expression was induced in 11 Nestin-$CreER^{T2}$/Fluc mice by oral tamoxifen treatment. Four days later, the animals were divided into 2 groups: 8 mice received a PT stroke and 2 mice received a sham treatment; one mouse died during tamoxifen induction. Three Cre-negative littermates that received a stroke were added as controls.

White furred B6.Cg-Tg(Nes-cre)1Kln/J mice, here referred to as Nestin-Cre mice, were stereotactically injected with Cre-Flex LV in the SVZ at the age of 8 weeks. One week after stereotactic injection, Nestin-Cre mice received a PT stroke ($n = 33$) or sham treatment ($n = 10$). A Cre-negative littermate that received a stroke was added as control.

Mice were genotyped by PCR using genomic DNA and primers previously described (Lagace et al., 2007).

Lentiviral vector construction and production

We designed a new conditional LV system based on the Cre/loxP mechanism, here referred to as Cre-Flex (Cre-mediated flip-excision). The Cre-Flex LVs carry a reporter cassette encoding eGFP and Fluc flanked by a pair of mutually exclusive lox sites. The reporter cassette is activated after Cre recombination (flip-excision, Fig. 3A). For the construction of the Cre-Flex plasmids, we used the pCHMWS-eGFP plasmid as a backbone (Geraerts et al., 2006). As illustrated in Fig. 3A, pairs of heterotypic *lox*P_*loxm*2 recombinase target sites were cloned respectively, upstream and downstream of eGFP using synthetic oligonucleotide adaptors. To enable efficient recombination, 46-bp spacers were inserted in between both lox sites. In this plasmid, eGFP was replaced by the coding sequence for eGFP-T2A-Fluc (Ibrahimi et al., 2009). All cloning steps were verified by DNA sequencing. Cre-Flex LVs were

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