

# Evaluation of glial cell proliferation with non-invasive molecular imaging methods after stroke

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**Glial proliferation:** For the last decades, glial cells have been wrongly believed to have a mere passive supporting role for neurons. Nevertheless, this notion has clearly changed and it is now admitted that these cells are essential for the correct development and regulation of the nervous system. Glia cell population are commonly subdivided in astrocytes, oligodendrocytes and microglia. During the development, neural stem cells (NSCs) (called neuroepithelial progenitor cells or NPCs) transform into radial glia, the primary progenitor cells for neurons, astrocytes and oligodendrocytes (Zuchero and Barres, 2015). Microglial cells, however, derive from a mesenchymal precursor infiltration, meaning that during brain development, precursors generated in the bone marrow invade the nervous parenchyma and differentiate into microglial cells (Zuchero and Barres, 2015). This proliferative capacity is preserved in the adult mammalian brain, and neurogenic NSCs are stored in two restricted regions of the central nervous system (CNS), the forebrain subventricular zone (SVZ) and the hippocampal dentate gyrus (subgranular zone). These cells continue to produce neurons and glial cells during the adulthood, being activated after certain signals and leaving the quiescent state (Urbán et al., 2019). This process, in which glial progenitor cells differentiate into mature glia during development and in the adult brain to maintain and regulate brain function, is called gliogenesis (Ardaya et al., 2020). Besides these two niches, oligodendrocyte progenitor cells (OPCs) are present all around the CNS, both in the white and gray matter. These cells are the major dividing cells in the CNS generating new myelinating oligodendrocytes, or to a lesser extent astrocytes and they are constantly scanning the environment and controlling brain homeostasis. In addition, there is evidence of generation of new astrocytes from proliferating mature astrocytes in the brain parenchyma (Frisén, 2016). In summary, the capacity of generation of new glial cells is preserved not only in the SVZ and subgranular zone niches, but in the parenchymal tissue of the adult brain. In fact, the proliferative capacity of glial cells is increased in the injured CNS following neurological diseases. Adult OPCs play an important role in demyelinating diseases, where they turn to an activated state and start proliferating and migrating to the demyelination areas. Once there, they differentiate into mature oligodendrocytes and renew the destroyed myelin (Kuhn et al., 2019). After brain ischemia, microglia and astrocytes play an important role, representing the primary defense line facing neuroinflammation. Different studies

using rodents have tried to disclose how microglia and astrocytes behave in this context and what triggers its activation. There is evidence of formation of a glial scar by reactive astrocytes originated from NSCs in the SVZ niche, but also generated through proliferation of local resident astrocytes (Nakafuku and Del Águila, 2020). Krishnasamy et al. (2017) showed the expression of nestin, a stem cell marker, in both reactive astrocytes and activated microglia after brain injury. These studies confirmed that glial cells differentiate and proliferate in order to restore the injured tissue, concluding that adult mammalian brain has the capacity of tissue repair following neuroinflammation.

Therefore, these findings raise some questions related to the usefulness of regenerative glial cells as a theranostic target for both diagnosis and therapy of brain diseases and its applicability to the clinical practice. For this reason, there is still a need for the development of molecular imaging methods to evaluate glial cell proliferation *in vivo*.

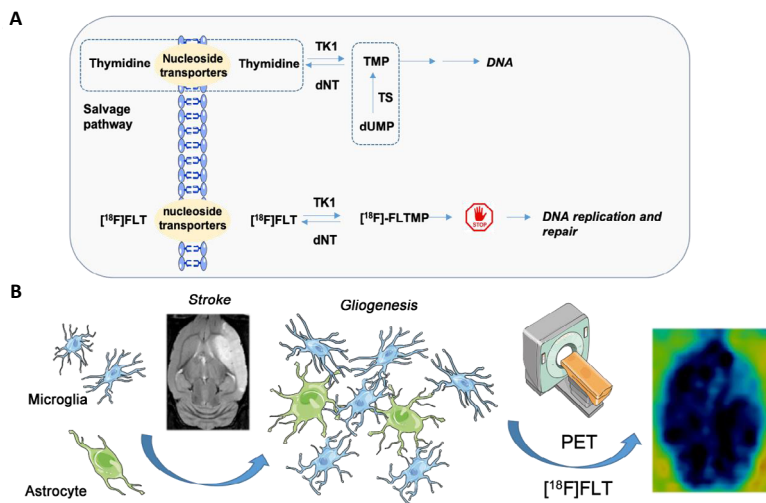
## Optical imaging detection of gliogenesis:

Recently, the effect of neuroinflammation on neurogenesis and gliogenesis has been studied using *in vivo* biophotonic/bioluminescence molecular imaging. This technique implies the use of a transgenic animal model, in this case, co-expressing the reporter genes luciferase and green fluorescent protein under the transcriptional control of the murine nestin gene promoter. This methodology allowed measuring the co-expression of both the photon emission of luciferase by bioluminescence, and the signal of green fluorescent protein by biofluorescence in those cells expressing nestin as a surrogate marker for proliferation of neurons and glia of transgenic mice. Nestin signal detected by bioluminescence was up-regulated after the ischemic lesion in those brain areas affected by cerebral ischemia. All these *in vivo* findings were validated by immunofluorescence showing nestin co-expression with both glial fibrillary acidic protein and Iba-1, markers of mature astrocytes and microglia. Therefore, this work suggested that neuroinflammatory conditions such as brain ischemia strongly up-regulated nestin signals as a surrogate biomarker of glial proliferation (Krishnasamy et al., 2017). Nevertheless, this technique showed high light scattering in tissues limiting the spatial resolution signals from deeper tissue imaging restricting its applicability to the preclinical evaluation of gliogenesis. For this reason, there is a need for novel methods based on nuclear or

magnetic resonance imaging modalities to study gliogenesis with higher resolution.

## Positron emission tomography (PET) imaging evaluation of glial proliferation:

During the past years, the use of [<sup>18</sup>F]-fluoro-3'-deoxy-3'-L-fluorothymidine ([<sup>18</sup>F]FLT) to trace proliferation with PET has been increased in oncology, where malignant tumor cells might be detected due to their relentlessly division rate. [<sup>18</sup>F]FLT is an analog of thymidine which is phosphorylated by thymidine kinase-1, an enzyme expressed during the DNA synthesis and up-regulated during the S phase of the cell cycle. Once phosphorylated, [<sup>18</sup>F]FLT cannot be incorporated into the DNA and is metabolically trapped inside the cells (Figure 1A). For this reason, the uptake and accumulation of [<sup>18</sup>F]FLT are used as indicative of cellular proliferation. Therefore, the main application of this radiotracer in neurology has been the diagnosis of glioma. Despite this, few studies have reported the usefulness of *in vivo* imaging of endogenous neural stem cells (NSCs) in ischemic rats with [<sup>18</sup>F]FLT-PET to monitor therapies aimed at expanding the neural stem cell niche (Rueger et al., 2010). In addition, Braun and colleagues (2016) tracked the mobility of NSCs in SVZ after cerebral ischemia in rats showing an increase of neurogenesis in the ipsilateral SVZ in relation to the contralateral hemisphere. Likewise, this study showed that [<sup>18</sup>F]FLT uptake was not specific for only endogenous NSCs, but for all proliferating cells including glial cells, so a comparison with the neuroinflammatory radioligand [<sup>11</sup>C]PK11195 for Translocator protein 18 kDa (TSPO) was carry out (Braun et al., 2016). In fact, *in vivo* PET imaging of neuroinflammation has been mainly focused on the evaluation of microglia/macrophage and astrocytic activation using radiotracers for the detection of TSPO overexpression. In rat models of cerebral ischemia, TSPO-PET imaging showed a progressive increase during 3–7 days after reperfusion followed by a decrease from days 15 to 28 in the region of infarction (Pulagam et al., 2017). In this sense, a paper published by our group evaluated the use of [<sup>18</sup>F]FLT to monitor de cellular proliferation belonging to the immune response after cerebral ischemia in rats (Ardaya et al., 2020; Figure 1B). This work showed the [<sup>18</sup>F]FLT uptake increase during the first week followed by a decline afterwards from the second to fourth week after cerebral ischemia displaying a similar profile to that showed by TSPO radioligands. Therefore, PET imaging data support that both activation and proliferative glial cells follow a similar temporal pattern after experimental stroke. [<sup>18</sup>F]FLT imaging findings were confirmed by the increase of cells co-expressing CD11b, a marker for macrophages and microglia, and Ki67, a marker for proliferation at day 7 after ischemia. Therefore, these results provide valuable information regarding the *in vivo* detection of proliferative microglia after stroke that might contribute to the discovery of novel diagnostic tools for stroke care. On the contrary, the increase of proliferative astrocytes cells at day 28 was not paralleled



**Figure 1 | In vivo imaging detection of gliogenesis with  $[^{18}\text{F}]$ FLT after cerebral ischemia in rats.**

(A) Mechanisms of cellular 3'-deoxy-3'- $[^{18}\text{F}]$ fluorothymidine ( $[^{18}\text{F}]$ FLT) retention. Similar to thymidine in the salvage pathway,  $[^{18}\text{F}]$ FLT is taken up from the extracellular milieu by specialized nucleoside transporters. The key enzymes of this pathway include  $[^{18}\text{F}]$ FLT;  $[^{18}\text{F}]$ FLT-monophosphate ( $[^{18}\text{F}]$ FLTMP); thymidine kinase 1 (TK1); 5'(3')-deoxyribonucleotidase (dNT); thymidine monophosphate (TMP); thymidylate synthase (TS); deoxyuridine monophosphate (dUMP). (B) Graphical representation of imaging of glial proliferation with  $[^{18}\text{F}]$ FLT after stroke. PET: Positron emission tomography.

by increased  $[^{18}\text{F}]$ FLT uptake, which displayed pseudo-control values in both cerebral cortex and striatum one month after ischemia. In fact, these differences could be explained by the higher resolution showed by confocal microscopy in relation to PET imaging which allows for a more accurate detection of proliferative cells in the region of infarction (Tichauer et al., 2015). An alternative explanation for these differences could be due to the status of the blood-brain barrier (BBB). Nowosielski et al. (2014) observed that BBB disruption was necessary for  $[^{18}\text{F}]$ FLT uptake, thus, if the BBB was restored at day 28 after stroke, this radiotracer would not be able to be phosphorylated or included in the DNA. Despite this, it is necessary to better establish the pattern of BBB disruption after ischemia and its involvement on the  $[^{18}\text{F}]$ FLT passage to the brain.

Finally, bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is another analog of thymidine in which the methyl group of thymidine has been replaced by bromine. BrdU has been commonly used in immunohistochemistry to label neural stem cells due to its capacity to incorporate into newly synthesized DNA during the S-phase, in a similar way as FLT does. Likewise, BrdU labeled with  $^{76}\text{Br}$  showed an extensive metabolism rate to  $^{76}\text{Br}$ -bromide, reducing the incorporation to the DNA fraction and limiting its usefulness for imaging cellular proliferation *in vivo*. In addition to this issue, cytotoxic effects of BrdU on NSCs could cause a decrease in proliferation, what definitely made this molecule an unpromising PET radiotracer (Gudjonsson et al., 2001).

**Summary and perspectives:** In summary, the development of new methods for *in vivo* imaging of gliogenesis has been slowly growing over the last decade. Preclinical studies have evidenced the preserved capacity of proliferation in the adult brain,

paving the way for clinical applications of novel therapies. So far, the use of  $[^{18}\text{F}]$ FLT in PET imaging has demonstrated to be the more accessible method and potentially translatable to the clinics. Despite this,  $[^{18}\text{F}]$ FLT has shown certain limitations such as (i) the ability to discriminate proliferative cell types, (ii) the low cellular resolution of PET imaging, or (iii) the restrictions of the BBB when studying the brain. Moreover, the glial proliferation observed by this imaging method still needs to be validated by *ex vivo* immunohistochemistry studies limiting its application to the preclinical research field. Finally, magnetic resonance imaging approaches might offer higher resolution that nuclear imaging with a strong translational potential. Magnetic resonance imaging reporter genes and superparamagnetic iron oxide particles labeling proliferative cells are being tested to study neurogenesis and gliogenesis *in vivo*. Therefore, each molecular imaging modality has shown its advantages and limitations; however, during the last decades considerable improvements have been made in this field, reducing the gap between preclinical and clinical studies.

*This work was supported by grants from the Spanish Ministry of Education and Science (RYC-2017-22412, PID2019-107989RB-I00 and MDM-2017-0720), the Basque Government (BIO18/IC/006) and Fundació La Marató de TV3 (17/C/2017).*

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Date of submission: October 29, 2020

Date of decision: December 18, 2020  
Date of acceptance: February 6, 2021  
Date of web publication: March 25, 2021

<https://doi.org/10.4103/1673-5374.310681>  
**How to cite this article:** Joya A, Martín A (2021) Evaluation of glial cell proliferation with non-invasive molecular imaging methods after stroke. *Neural Regen Res* 16(11):2209-2210.

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C-Editors: Zhao M, Wang L; T-Editor: Jia Y