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A simple method to fluorescently label pericytes in the CNS and skeletal muscle

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Abstract

Pericytes play important roles in vascular control and may form an important part of the blood brain barrier. Here we introduce a simple method for fluorescently labelling pericytes to enable further studies in live or fixed tissue of rats and mice. Following intraperitoneal injection, the fluorescent tracer Fluorogold was rapidly taken up into vascular endothelial cells, and within 3 hours in the central nervous system appeared within small perivascular cells with a morphology consistent with pericytes. These Fluorogold labelled cells were pericytes since they displayed immunoreactivity for platelet derived growth factor receptor beta and were closely associated with isolectin B4 binding to endothelial cells. Pericytes in skeletal muscle were also labelled with this method, but not those within the heart, lungs or kidney. This simple method could therefore be applied for labelling pericytes in a wide variety of studies, including live cell imaging or immunohistochemistry.

Introduction

Pericytes are perivascular cells found in close association with vascular endothelial cells of small diameter vessels and capillaries. Within the CNS, pericytes play an important role in angiogenesis, vascular stability, control of capillary blood flow and macrophage activity (Rucker et al., 2000). Through either the use of transgenic mice lacking pericytes, or in mice where the maturation of pericytes is inhibited, they have been shown to be involved in the development of diabetic retinopathy (Enge et al., 2002), neonatal intracranial haemorrhage (Li et al., 2011), some forms of neurodegeneration (Bell et al., 2010) and in the development of the glial scar following spinal cord injury (Goritz et al., 2011). There are currently many markers for pericytes including: NG2 (Ozerdem et al., 2001), α-smooth muscle actin (Verbeek et al., 1994), CD13 (Kunz et al., 1994), and platelet derived growth factor beta (PDGFRβ, (Lindahl et al., 1997; Winkler et al., 2010)). Unfortunately, none of these markers appear to be universal pericyte markers, rather they pick out pericytes in a particular stage of maturation or they may be effective in a particular technique or tissue (Armulik et al., 2011).

One approach used to circumvent issues labelling pericytes is to use genetically altered animals which express marker proteins, but this relies on breeding and maintenance of specific lines. An alternative approach that could be applied to all mice, and indeed rats, would therefore be beneficial in promoting the study of pericytes in physiological conditions and disease models. Progress towards this aim, at least in the CNS, was made when cells with the morphology of pericytes were labelled following intracerebroventricular injection of fluorescently conjugated dextrans (Sarkar and Schmued, 2012). However, the use of intracerebroventricular injection means a surgical step is required with related expense, time and welfare requirements. Achieving similar pericyte labelling without surgery would therefore represent an advance.

A potential candidate for labelling pericytes is the commonly used neuronal tracer Fluorogold (FG) (Schmued and Fallon, 1986). FG is unable to cross the blood brain barrier but when delivered peripherally via an intravascular or subcutaneous route results in the labelling of discrete groups of neurones within the CNS, which send axons out into the periphery (Ambalavanar and Morris, 1989). However, during our

previous studies (e.g.(Edwards et al., 2013; Milligan et al., 2006)) we noticed that FG injections result in labelling of cells other than neurones.

Here we identify that a single intraperitoneal injection of FG fluorescently labels pericytes throughout the CNS and in skeletal muscle, rendering them visible for further studies. This technique may therefore offer a simple method for labelling pericytes without the need for surgery or the maintenance of transgenic animals.

Materials and Methods

All procedures were carried out in accordance with the UK Animals Scientific Procedures Act 1986.

Animals

C57/BL6 mice (6 – 8 weeks, n = 12) were injected i.p. with FG (0.05 ml 1% in water, 25 mg/ kg, Fluorochrome, CO, USA). At 1, 3, 24 and 48 hours post- injection (n = 3 per group), animals were terminally anaesthetised with sodium pentobarbitone (60 mg / kg i.p.) and transcardially perfused with 200 ml of 4% paraformaldehyde. The CNS, heart, lungs, kidneys and skeletal muscle were removed and post fixed for 24 hours in the same fixative. Tissue samples were then sectioned at 50 µm using a vibratome (Leica VT1000M, UK). For all experiments n refers to the number of animals used.

Adult male Wistar rats (250 g, n =3) were injected i.p with FG (0.1ml 1% in water, 5 mg / kg, Fluorochrome, CO , USA). 24 hours post injection animals were terminally anaesthetised with sodium pentobarbitone and transcardially perfused with 500 ml of 4% paraformaldehyde. 9 day old, male wistar rats were also injected i.p. with FG (0.1ml 1% in water, 50 mg / kg, n = 3). 24 hours post injection animals were terminally anaesthetised using 60 mg / kg sodium pentobarbitone i.p. and then transcardially perfused with ice cold aerated (95% O_2, 5% CO_2) sucrose artificial cerebrospinal fluid containing (in mM): 217 sucrose, 26 NaCHO3, 3 KCl, 2 MgSO4, 2.5 NaH2PO4, 2 CaCl2, and 10 glucose. The brain was then removed and sliced at 200 µm using a vibrating microtome (Leica, VT1200S). Slices were then kept in aerated artificial cerebrospinal fluid containing (in mM): 124 NaCl, 26 NaHCO3, 3 KCl, 2 MgSO4, 2.5 NaH2PO4, 2 CaCl2, and 10 glucose. Slices were then viewed using an upright Olympus BX50WI microscope equipped for epifluorescence and images captured using Q Capture pro6 (Scientifica, UK).

Immunohistochemistry

Tissue sections were blocked in donkey serum (10 % in PBS) containing 3 % triton X-100 (PBS-T) for 1 hour at room temperature. All primary antibody incubations were performed overnight at 4 ºC in 3 % PBS-T containing 5 % donkey serum and secondary antibody incubations were performed at room temperature in PBS for 1-3 hours (Invitrogen Alexafluor secondaries) or 4 hours (Jackson Immunoresearch biotinylated secondaries). Pericytes were identified by incubating tissue sections with goat antibodies against PDGFRβ (1:500; R&D Systems, UK (Winkler et al., 2010)) and visualised using donkey anti goat Alexafluor[555] (1:1000; Invitrogen, UK) or with rat anti CD140b (1:250; eBioscience, INC) which was detected by biotinylated secondary antibody (donkey anti rat; Jackson Immunoresearch, PA USA) and then with Streptavidin Alexa[488] (1:1000; Invitrogen, UK). To distinguish endothelial cells, sections were labelled with biotinylated isolectin B4 (IB4) from *Griffonia simplicifolia* (1:1000; Invitrogen) and detected using Strepavidin Alexa[488] (1:1000;

Invitrogen).Sections were washed three times in PBS and mounted onto slides using Vectashield mounting medium (Vector Labs Ltd, UK) and viewed using a Nikon E600 epifluorescent microscope and Acquis image capture system (Synoptics, UK) or an Upright Zeiss LSM510 confocal microscope.

Results

Optimal time for Fluorogold labelling

FG is used to retrogradely label neurons and is given as an injection 3 - 12 days before perfusion (Anderson and Edwards, 1994; Milligan et al., 2006; Schmued and Fallon, 1986). Here we explored whether a shorter time period would be sufficient to label cells associated with blood vessels. Mice were injected i.p. with FG 1, 3, 24 or 48 hours prior to perfusion (n = 3 for each time point). Samples from throughout the CNS showed that, after a period of 1 hour, there was dispersed labelling of tissue with FG, which appeared to be associated with blood vessels (an example of a putative FG labelled pericyte is shown in Fig. 1A). However, between 3 hours and 24 hours (Fig. 1B and 1C) distinct cells labelled with punctate, intracellular FG were visible on blood vessels. The number and intensity of such FG labelled cells however decreased 48 hours post injection (Fig. 1D). The FG labelling at 3 – 24 hours was concentrated in the cell bodies with the morphology of pericytes, which were oval to oblong in shape, although processes associated with these cells followed the contours of the adjacent blood vessels. The method can extend also to rats since i.p. FG labelled cells with the morphology of pericytes in CNS of adult (n=3) and juvenile (n=3) rats (Fig. 1E-H). In addition, FG can be retained and imaged in these perivascular cells in living CNS slices (n=3, Fig. 1G,H).

Fluorogold labelling co-localises with PDGFRβ in all investigated regions of the CNS

We used immunoreactivity (IR) for PDGRFβ as a marker for pericytes to confirm whether i.p. FG labelled these cells. In all experiments in which mice were injected with FG 3 hours and 24 hours prior to perfusion we found FG positive cells that were also PDGFRβ immunoreactive. These FG positive PDGFRβ immunoreactive cells were observed in all regions of the CNS that were investigated (olfactory bulbs, hippocampus [Fig. 2A-A3], prefrontal cortex, hypothalamus [Fig. 2B-B3], area postrema, medulla oblongata, and spinal cord, n = 6). To further confirm that this FG labelling was present in pericytes, FG labelled cells were also probed for dual labelling with an antibody raised against a different portion of PDGFRβ (CD140b, n = 6). Again co-localisation of FG labelled pericytes and CD140b immunoreactivity was observed throughout the CNS. To demonstrate the close association between the FG labelled cells and the vasculature, the endothelial lining of the vessels was labelled with IB4 (n=6). The FG labelled cells were always observed on the abluminal surface of blood vessels.

Fluorogold labelling in various tissues and organs differs to that seen in the CNS

As 3 hours post i.p. injection of FG is sufficient to label CNS pericytes we determined whether this technique also labelled pericytes in other tissues and organs of the mouse. Within skeletal muscle FG injection labelled small cells in close association with the vasculature, which were also immunoreactive for PDGFRβ (n= 6, Fig. 2C-C3). Within the heart FG strongly labelled the myocardium, and was totally absent from the PDGFRβ-IR cells that were in close association to IB4 positive endothelial

cells (n = 6, Fig. 2D-D3). In the kidney strong FG labelling was observed exclusively in glomeruli, whereas PDGFRβ immunoreactivity was observed in mesangial cells around and supporting the FG labelled glomerular capillaries. Endothelial cells within the glomeruli and in the surrounding basement membrane were characterised with IB4 (n = 6, Fig. 2E-E3).

Discussion

Here we report a novel method for labelling pericytes in rats and mice *in* vivo using i.p. injection of FG. Although conventionally used as a neuronal tracer, FG is not taken up by neuronal cells alone. Indeed, injection of FG into the dorsal columns of rats resulted in the labelling of small cells surrounding the injection site, presumed to be glial cells of the glial scar (Dado and Giesler, 1990). Further, 'perivascular cells' have been reported to be labelled with FG following injection into the corpus callosum (Pennell and Streit, 1998) or the facial nucleus (Streit and Graeber, 1993).

IP administration of FG results in labelling of CNS pericytes within 3 hours, with a punctate intracellular pattern suggesting it is localised to lysosomes as it is in neurones (Persson and Havton, 2009). FG labelling of pericytes can still be observed up to 48 hours post i.p. injection, although at a diminished level to that observed at 3 or 24 hours. At 3 and 24 hours post i.p. injection, FG labelled pericytes are clearly distinguishable from vascular endothelial cells within the CNS and skeletal muscle. Within the heart, lungs, and kidney, FG labelling at the time points investigated here no longer appears to be associated with perivascular structures. It is unclear why a difference in the labelling of pericytes within different tissues was observed. One possibility may be due to local differences in the pericyte populations e.g. brain pericytes have a strong phagocytic ability, which may account for their concentrating FG from the vasculature (Kristensson and Olsson, 1973). Another option is that not all endothelial cells may be able to accumulate or transcytose the FG since endothelial cells are well known to exhibit organ-specific phenotypes (Aird, 2007).

As FG does not permeate the blood brain barrier, it is interesting that pericytes are able to accumulate the tracer at all. At the earlier time points we investigated, there was clear labelling of the endothelial lining of blood vessels, which diminished over time – co-incident with an increase in the intensity of the labelling of pericytes. In transgenic mice with reduced numbers of brain pericytes, circulating tracers seem to endocytose through the endothelial layer into neurons (Armulik et al., 2010). It appears that the presence of pericytes slows the process of transcytosis across the endothelial lining of microvessels. 2 hours after IV injection of horseradish peroxidase (HRP), there appears to be a greater accumulation of HRP within the endothelial cells of mutant mice with reduced pericyte numbers than their wild type littermates. An alternative hypothesis may be that, when present, pericytes intercept the HRP and play a role in its degradation, which would be consistent with the apparent shift in FG labelling from endothelial cells into pericytes at the 3 hour time point used here.

This method for labelling pericytes *in vivo* may prove to be a powerful tool to further reveal the important roles that these cells play in the central nervous system and musculature. This approach could easily be adapted to allow for further intravital imaging of pericytes, or even to allow for simple cell selection in *in vitro* electrophysiological studies – providing a distinct advantage over the currently used pericyte markers.

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Figure Legends

Figure 1. **Optimal time for labelling CNS pericytes with Fluorogold (FG)**

Time course for FG labelling at 1 (A), 3 (B), 24 (C), and 48 hours (D) after i.p. administration of FG. After 1 hour FG was mainly visible in the endothelial lining of blood vessels, becoming concentrated within pericytes over 3-24 hours (labelled pericytes marked with arrows), whilst the endothelial associated labelling had decreased. Neuronal labelling (N) becomes apparent at 48 hours. FG also labels CNS pericytes in the rat, cortex (E) and thalamus (F). FG labelled pericytes are also visible in live slices of rat brain (G) and DIC shows that they are confined to blood vessels and are not associated with neighbouring healthy neurones (H).
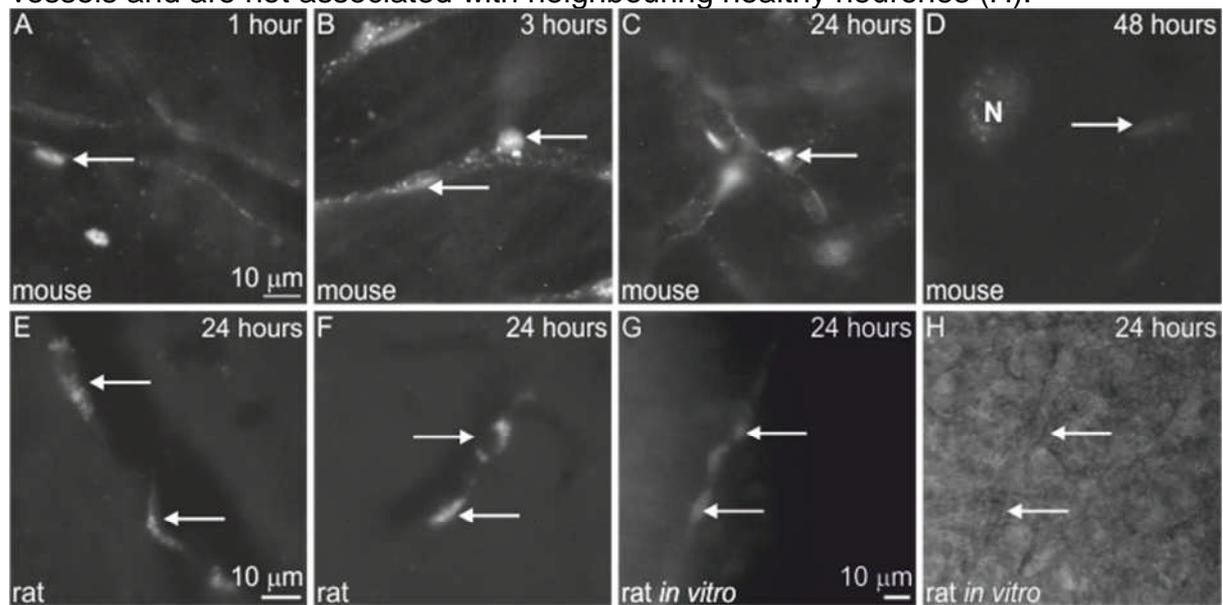
Figure 2. Fluorogold labels pericytes in the CNS, but not other organs

FG labelled PDGFRβ IR cells in the hippocampus (HPX, A-A3) and hypothalamus (HYP, B-B3) of the CNS and skeletal muscle (C-C3). FG did not label pericytes within cardiac (D-D3) and renal (E-E3) tissue.