Journal of Neuroscience Methods 299 (2018) 8–15

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

In vivo two-photon imaging of motoneurons and adjacent glia in the ventral spinal cord

Luciana Politti Cartarozzi[a,b], Phillip Rieder[a], Xianshu Bai[a], Anja Scheller[a], Alexandre Leite Rodrigues de Oliveira[b,*], Frank Kirchhoff[a,*]

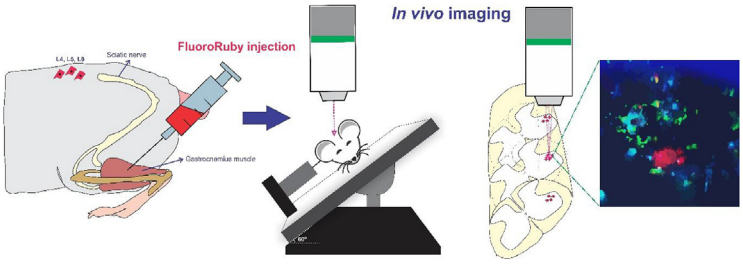
[a] *Molecular Physiology, Center for Integrative Physiology and Molecular Medicine (CIPMM), University of Saarland, Building 48, 66421 Homburg, Germany*
[b] *Laboratory of Nerve Regeneration, State University of Campinas – UNICAMP, Cidade Universitária "Zeferino Vaz", Rua Monteiro Lobato, 255, 13083970 – Campinas-SP, Brazil*

HIGHLIGHTS

- *In vivo* imaging of spinal motoneurons and adjacent glia by two-photon laser-scanning microscopy (2P-LSM).
- Cell-type identification by using transgenic mice with cell-specific expression of fluorescent proteins.
- Study of immediate glial responses to motoneuron axotomy.

GRAPHICAL ABSTRACT

Summary of a novel *in vivo* 2P-LSM imaging approach to study motoneurons of the ventral spinal cord. For accurate cell localization, tracer was injected in the hindlimb muscles of transgenic mice with selective expression of fluorescent proteins in microglia, astrocytes or neurons. Subsequently, motoneurons and their adjacent glial cells at the lumbar intumescence could be detected in the living mouse using a combination of careful surgery and 2P-LSM imaging.



ARTICLE INFO

Article history:
Received 24 November 2016
Received in revised form 11 January 2018
Accepted 28 January 2018
Available online 3 February 2018

Keywords:
Ventral spinal cord
Motoneurons
In vivo two-photon imaging
Glial cells

ABSTRACT

Background: Interactions between motoneurons and glial cells are pivotal to regulate and maintain functional states and synaptic connectivity in the spinal cord. *In vivo* two-photon imaging of the nervous system provided novel and unexpected knowledge about structural and physiological changes in the grey matter of the forebrain and in the dorsal white matter of the spinal cord.
New method: Here, we describe a novel experimental strategy to investigate the spinal grey matter, *i.e.* the ventral horn motoneurons and their adjacent glial cells by employing *in vivo* two-photon laser-scanning microscopy (2P-LSM) in anesthetized transgenic mice.
Results: After retrograde tracer labelling in transgenic mice with cell-specific expression of fluorescent proteins and surgical exposure of the lumbar intumescence groups of motoneurons could be visualized deeply localized in the ventral horn. In this region, morphological responses of microglial cells to ATP could be recorded for an hour. In addition, using in mice with expression of GCaMP3 in astrocytes, physiological Ca^{2+} signals could be recorded after local noradrenalin application.
Comparison with existing methods: Previous *in vivo* imaging protocols were restricted to the superficial dorsal white matter or upper layers of the dorsal horn. Here, we modified a multi-step procedure originally established for a root-crush injury. We adapted it to simultaneously visualize motoneurons and adjacent glial cells in living animals.

* Corresponding authors.
E-mail addresses: alroliv@unicamp.br (A.L.R.d. Oliveira), frank.kirchhoff@uks.eu (F. Kirchhoff).

https://doi.org/10.1016/j.jneumeth.2018.01.005

Conclusion: A modified surgery approach is presented to visualize fluorescently labelled motoneurons and glial cells at a depth of more than 200 μm in the grey matter ventral horn of the mouse spinal cord.

1. Introduction

Lesions to the spinal cord result in severe loss of motor and sensory function that involves neuronal degeneration as well as acute reactions of the adjacent glial cells, microglia and astrocytes. To date, the knowledge about *in vivo* immediate responses to injury occurring at the minute time scale such as shape changes of microglial processes or transients of intracellular Ca^{2+} changes in astrocytes remained largely elusive. So far, most of the morphological information on acute, trauma-associated spinal cord events is based on two-photon laser-scanning microscopy (2P-LSM) of superficial layers in the dorsal white matter. Electrophysiological approaches have been used to quantify the impairment and putative regeneration of axon function without direct information of the underlying structural alterations. However, parameters such as the acute activation of microglial cells or the visualization of intracellular signaling cascades could provide important mechanistic information on scar formation and subsequent degenerative or healing processes.

2P-LSM has turned out as an important methodology for cellular investigations *in vivo* taking advantage of genetically modified mice with cell-specific expression of various fluorescent reporter proteins. In this way, novel and often unexpected findings about the dynamic behavior of cells have been described under physiological or pathological conditions (Davalos and Akassoglou, 2012). 2P-SLM is an optical imaging approach that relies on fluorescence emission detection (Denk et al., 1990). Initially it was restricted to cultured cells or acutely isolated brain slices. However, it was very quickly adapted to anesthetized rodents which represented an "intravital" preparation. The first observations of the brain revolutionized the field of neuroscience. Suddenly, researchers could visualize the dynamics of cellular processes in living animals (Nayak et al., 2012).

Several approaches have been introduced for imaging the spinal cord (Cupido et al., 2014; Davalos and Akassoglou, 2012; Dibaj et al., 2010; Dray et al., 2009; Fenrich et al., 2012; Johannssen and Helmchen, 2010, 2013), *e.g.* allowing observation of dorsal funiculus axons and glial cells. The use of Texas Red-dextran by injection into the tail vein was employed for the evaluation of the vasculature and its close relationship with astrocytes and microglia. Microlesioning neuronal fibers allowed real-time investigation of acute microglial changes, as well as chronic changes up to 6 months (Laskowski and Bradke, 2013). Imaging of the spinal cord, so far, has been largely restricted to the superficial white matter of the dorsal columns. The surgical complexity to reach ventral positions of the spinal cord prevented an adequate analysis. However, for a better understanding of the mechanisms that re-establish muscle innervation after a peripheral nerve injury, direct visualization of fast Ca^{2+} changes or structural alterations could be a significant advancement.

Therefore, the present work was designed to establish a novel approach to image spinal motoneurons (located at lamina IX of Rexed) and surrounding glial cells *in vivo* employing 2P-LSM.

2. Materials and methods

2.1. Transgenic mice and animal licenses

To visualize neurons and glial cells we used transgenic mice in which neurons were labelled by transgenic expression of the yellow fluorescent protein EYFP under the control of Thy1 promoter (TgN(Thy1-EYFP)) (Winter et al., 2007), astrocytes by the cyan fluorescent protein ECFP under control of the GFAP promoter (TgN(GFAP-ECFP)) (Hirrlinger et al., 2005) and microglia by the green fluorescent protein EGFP from the CX_3CR_1 gene locus (TgH(CX_3CR_1-EGFP)) (Jung et al., 2000). Double as well as triple transgenic mice were investigated. TgN(GFAP-CreERT2) and TgH(R26-stop-floxGCaMP3) mice were used for Ca^{2+} imaging of astrocytes (Hirrlinger et al., 2006; Paukert et al., 2014).

All animal procedures were carried out at the University of Saarland in strict accordance with the recommendations to European and German guidelines for the welfare of experimental animals, approved by the Saarland state's "Landesamt für Gesundheit und Verbraucherschutz" in Saarbrücken/Germany (animal license numbers: 71/2010 and 36/2016).

2.2. Tracer injection for retrograde labelling of spinal motoneurons

To unequivocally identify spinal motoneurons of the sciatic nerve pool, we unilaterally injected FluoroRuby (FR; dextran-conjugated tetramethylrhodamine, 10,000 MW, Molecular Probes) in the gastrocnemius muscle (GM) of transgenic mice. Such labelled dextrans are hydrophilic polysaccharides of low toxicity that are frequently employed as retrograde neuronal tracer. FR was diluted 10% in phosphate buffered saline (PBS) (0.1 mg/μl), kept at −20 °C and protected from light until use. For tracer injection, each animal was anesthetized with isoflurane (4%). Once immobile and with respiration rate slowed down, they were placed onto a heating pad. Anesthesia was maintained by isoflurane/nitrous oxide inhalation (2.5%). After shaving, a skin incision was performed in the posterior part of the left hind limb to expose the GM that is easily identified by the calcaneal tendon. The injection site was washed with PBS and the leg was gently pinched to stabilize the muscle group. A micro-needle coupled with a 10 μl Hamilton syringe containing the FR solution was inserted until the bevel of the needle was embedded in the muscle and the FR was applied. Four injections of 1 μl were applied in the distal, proximal, lateral and medial parts of the GM, separated by about 5 mm. After each injection, the needle was removed and pressure was gently applied for 10 s in the injection site to prevent leakage. In sequence, the skin was sutured, the animal received a subcutaneous injection of buprenorphine (1 mg/kg body weight) and was placed into a recovery cage where it was continuously monitored until waking up and walking normally. In general, 6 days after tracer injection the numerous motoneurons were retrogradely labelled, in line with the observation of others (Hayashi et al., 2007).

2.3. Surgical procedure

The surgical approach is based on previous work (Steffens et al., 2012a, b). Mice were anesthetized with an intraperitoneal injection ketamine (140 mg/kg body weight, Zoetis, Germany) and xylazine (10 mg/kg body weight, Bayer, Germany). Bepanthen (Bayer, Germany) ointment was applied onto the eyes of the mice to prevent drying. Mice were put in prone position onto a heating pad. The back was shaved and cleaned. A skin incision was made along the sagittal plane over the column. To recognize the region of interest in the lumbar spinal cord, morphological landmarks were