Experimental Neurology 242 (2013) 18–26

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

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

Review

Two-photon imaging of spinal cord cellular networks

Helge C. Johannssen [1], Fritjof Helmchen *

Department of Neurophysiology, Brain Research Institute, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

ARTICLE INFO

Article history:
Received 1 April 2011
Revised 27 March 2012
Accepted 21 July 2012
Available online 28 July 2012

Keywords:
Spinal cord
Two-photon
Calcium imaging
Sensorimotor
Dorsal horn

ABSTRACT

Two-photon microscopy enables high-resolution *in vivo* imaging of cellular morphology and activity, in particular of population activity in complex neuronal circuits. While two-photon imaging has been extensively used in a variety of brain regions in different species, *in vivo* application to the vertebrate spinal cord has lagged behind and only recently became feasible by adapting and refining the experimental preparations. A major experimental challenge for spinal cord imaging is adequate control of tissue movement, which meanwhile can be achieved by various means. One set of studies monitored structural dynamics of neuronal and glial cellular components in living animals using transgenic mice with specific expression of fluorescent proteins. Other studies employed *in vivo* calcium imaging for functional measurements of sensory-evoked responses in individual neurons of the dorsal horn circuitry, which at present is the only part of rodent spinal cord grey matter accessible for *in vivo* imaging. In a parallel approach, several research groups have applied two-photon imaging to sensorimotor circuits in the isolated spinal cord (*in vitro*) to provide complementary information and valuable new perspectives on the function of specific interneuron types in locomotor-related networks. In this review we summarize recent results from these types of high-resolution two-photon imaging studies in the spinal cord and provide experimental perspectives for improving and extending this approach in future applications.

Contents

Introduction

The vertebrate spinal cord harbors multiple types of neurons organized in many different functional circuits, which in turn are involved in fundamental behaviors including sensation, reflexes and locomotion (Garcia-Campmany et al., 2010; Schouenborg, 2008). These circuits can be broadly defined according to developmental origins and with respect to their specific functional role in particular behaviors. Single-cell electrophysiological recordings have helped to characterize the physiological properties of various spinal cord cell types, *e.g.* in the dorsal horn, both *in vitro* (Grudt and Perl, 2002; Lu and Perl, 2003, 2005; Ruscheweyh et al., 2004; Todd et al., 2002; Yoshimura and Jessell, 1989) and *in vivo* (Bester et al., 2000; Dalal et al., 1999; Furue et al., 1999; Graham et al., 2004a, 2004b; Light and Willcockson, 1999). However, this approach is usually limited to individual or very few cells and – in the case of *in vivo* studies – suffers from the difficulties of targeting

Abbreviations: ACSF, artificial cerebrospinal fluid; EAE, experimental autoimmune encephalitis; GFP, green fluorescent protein; OGB-1, Oregon Green Bapta-1; SR101, sulforhodamine 101; ROI, region of interest.

* Corresponding author. Fax: +41 44 6353303.
E-mail address: helmchen@hifo.uzh.ch (F. Helmchen).
[1] Present address: Department of Neuropharmacology, Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland.

0014-4886/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.expneurol.2012.07.014

and identifying particular neuronal cell types. Moreover, blind *in vivo* recordings may be biased towards active network elements and thus provide a narrow if not skewed view on circuit dynamics. To obtain a more comprehensive view on spinal cord circuits, imaging techniques are of great value as they can directly visualize various structural and functional aspects. In particular, two-photon microscopy provides sub-cellular resolution in intact neural tissue and can reveal activity patterns in local neuronal populations (for reviews see Garaschuk et al., 2006; Helmchen and Denk, 2005; Holtmaat and Svoboda, 2009; Kerr and Denk, 2008), making it a promising technique to reveal the cellular composition and dynamic interplay within spinal cord circuits in a descriptive, non-biased manner.

Two-photon imaging has been widely applied in various animal models and brain regions, either to study structural dynamics of dendrites, axons, and glial processes, or to reveal functional activity patterns in local neuronal populations. For example, in the rodent neocortex the turn-over of dendritic spines and axonal boutons and their structural rearrangements have been characterized by repeated imaging over long time periods, up to several months (Bhatt et al., 2009; Holtmaat and Svoboda, 2009). In addition, two-photon calcium imaging has enabled simultaneous monitoring of functional signals in tens to hundreds of cells in various cortical areas (reviewed in Garaschuk et al., 2006; Grewe and Helmchen, 2009). Both types of imaging studies, investigating either structural dynamics or cellular activities, have also been applied to various animal models of brain diseases in order to characterize pathological alterations (*e.g.*, Brown et al., 2010; Busche et al., 2008; Fuhrmann et al., 2010; Nimmerjahn et al., 2005). In recent years, two-photon microscopy in addition has seen an expansion in available scanning modes (Cheng et al., 2011; Göbel and Helmchen, 2007), improvements in speed (Grewe et al., 2010; Katona et al., 2012), and growing application of genetically-encoded indicators (Lütcke et al., 2010; Tian et al., 2009), all advances that should further extend the possibilities for studying neural circuits.

For spinal cord studies, two-photon imaging has been lately employed both *in vitro* and *in vivo*. Spinal cord circuits comprise various neuronal subpopulations that are often interconnected in a complex, translaminar fashion. *In vitro* preparations used for two-photon imaging include acute slices (Wilson et al., 2007a, 2007b) and in particular whole spinal cords isolated from neonatal mouse (Diaz-Rios et al., 2007; Kwan et al., 2009; Wilson et al., 2010; Zhong et al., 2010). In the latter case, neuronal network activity was monitored following electrical stimulation of afferents (O'Donovan et al., 2005) or during drug-induced fictive locomotion paradigms (Kwan et al., 2009; Wilson et al., 2007a, 2007b, 2010; Zhong et al., 2010). Moreover, two-photon calcium imaging was applied to *in vitro* preparations from transgenic mice with specific fluorescent protein expression to investigate the role of genetically defined spinal neuronal populations (Kwan et al., 2009; Wilson et al., 2007a, 2007b, 2010; Zhong et al., 2010).

High-resolution imaging in the spinal cord *in vivo* is more challenging compared to the brain because it faces special problems such as difficult surgical access and pronounced tissue movement artifacts. In addition, in adult animals typically used for these experiments, suboptimal optical conditions prevail due to high levels of myelination. Most two-photon imaging studies performed in the spinal cord of living rodents so far have focused on superficial dorsal white matter tracts, making use of sparsely labeled axons and the expression of fluorescent reporter proteins in different glial cell populations (Davalos et al., 2008; Dibaj et al., 2010a, 2010b; Dray et al., 2009; Farrar et al., 2012; Fenrich et al., 2012; Ylera et al., 2009). Especially in the field of imaging glial networks and their interactions with defined neuronal circuits, transgenic animals have proven highly beneficial to elucidate mechanism of structural plasticity. Because the superficial area of the spinal cord is often affected during severe human pathologies including traumatic injury and multiple sclerosis, imaging the concomitant structural plasticity of axonal fibers or glial cells in the respective animal models of such pathologies may enable a more detailed understanding of disease-related mechanisms.

Finally, further challenges exist for establishing functional two-photon imaging of mammalian spinal cord neuronal networks *in vivo*. Neuronal populations residing in the central grey matter are even more difficult to access optically than white matter cells. Specifically, the presence of superficially located myelinated fiber bundles hinders deep imaging. In addition, a high level of mechanical stability is paramount to resolve functional signals such as rapid calcium transients on a fast time scale. Only recently, using special approaches to achieve sufficient stability levels, fast calcium transients in mouse spinal cord neuronal populations were resolved (Johannssen and Helmchen, 2010; Laffray et al., 2011). In the following we first provide an overview of two-photon imaging in spinal cord *in vitro* preparations before we describe the recent progress in high-resolution imaging of spinal cord dorsal horn in living animals.

Two-photon imaging of sensorimotor spinal circuits *in vitro*

To investigate principles of motor and pre-motor neuronal circuit function with two-photon imaging, several groups recently used calcium imaging in spinal cord preparations from early postnatal mice *in vitro* (Kwan et al., 2009; Wilson et al., 2007a, 2007b, 2010; Zhong et al., 2010). The isolated spinal cord of neonatal mice can be superfused with oxygenated artificial cerebrospinal fluid (ACSF) and kept in healthy condition for more than 10 h (Fig. 1A). At this early age, optical access can be gained to deeper spinal cord laminae due to the lack of full myelination and the overall smaller dimensions of the neonatal spinal cord. In addition, the preparation can be arbitrarily placed to optimize access to either the dorsal or the ventral side. Calcium indicator delivery was accomplished either by retrograde labeling (O'Donovan et al., 2005), bulk loading with small molecule AM-ester dyes (Kwan et al., 2009; Wilson et al., 2007a, 2007b, 2010; Zhong et al., 2010) (Fig. 1B), or electroporation techniques (Bonnot et al., 2005). These experimental advantages come, however, with some limitations. First, the isolated spinal cord preparation is restricted to the first few postnatal days in mice, thus reflecting a developing system, which has to be considered for appropriate data interpretation. Second, the connectivity to other CNS areas, in particular the brainstem, which is known to modulate sensorimotor integration, is generally truncated, although it can be preserved using variants of the standard preparation protocol (Gordon and Whelan, 2008). Modulatory *in vivo* effects can be, however, mimicked to a certain degree, *e.g.*, by using neurotransmitters or electrical activation of the dorsal roots still attached to the preparation.

A widely used experimental paradigm to study locomotor circuits in the isolated spinal cord is a condition of rhythmic activity termed "fictive locomotion", which can be induced by bath application of a neuromodulatory cocktail (Cazalets et al., 1992; Kudo and Yamada, 1987). Combining calcium imaging techniques with suitable transgenic reporter lines, several groups lately investigated the activity patterns of discrete neuronal populations in the neonatal mouse spinal cord during fictive locomotion (Kwan et al., 2009; Wilson et al., 2007a, 2007b, 2010; Zhong et al., 2010). Since the summed output of the spinal cord locomotor circuit can be simultaneously recorded from the ventral roots (see Fig. 1A), the functional role of different neuron types in network activity could be assessed. Two-photon calcium imaging was for example used in acute slices and the whole spinal cord preparation of HB9:eGFP mice to monitor the extent of synchronous bursting activity and electrotonic coupling in the subpopulation of HB9 interneurons (Wilson et al., 2007a, 2007b). In another imaging study of spinal locomotor networks, two-photon imaging of HB9 interneuron activity patterns during fictive locomotion revealed spatio-temporal relationships of HB9-activities with respect to the locomotor network (Kwan et al., 2009). Other neuronal populations residing in more central laminae have been accessed by hemisecting the spinal cord (Wilson et al., 2010). In this study, two-photon imaging of dorsomedial GABAergic neurons in hemisected spinal cords from Gad65-GFP mice showed that these cells are

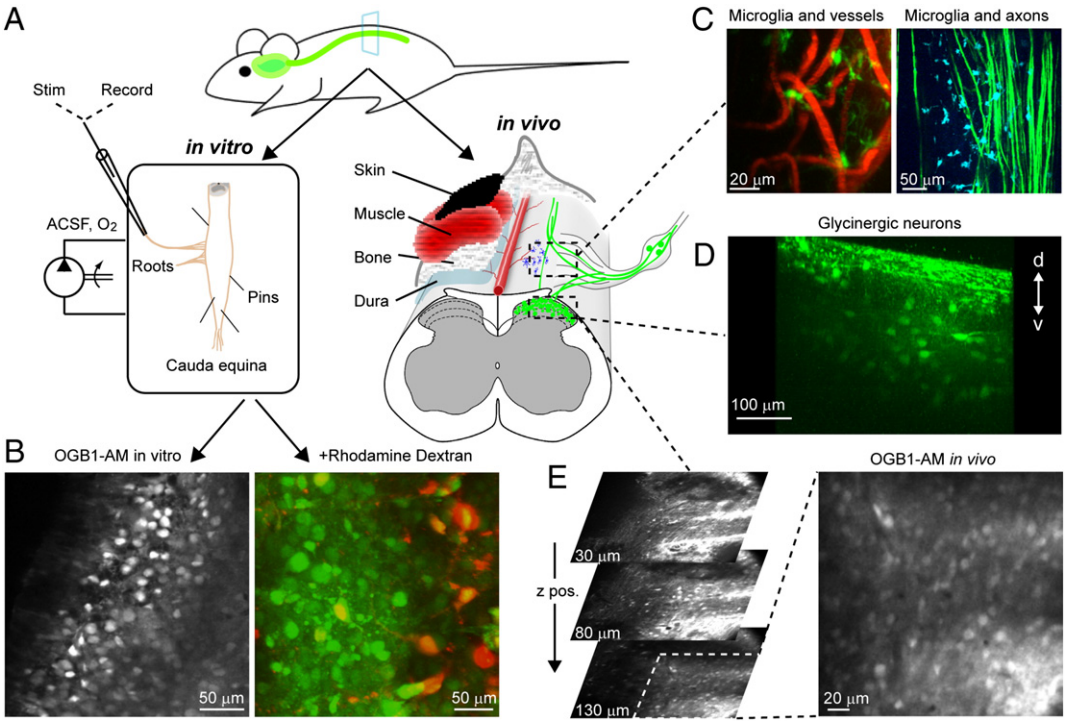

Fig. 1. High-resolution two-photon imaging of rodent spinal cord. (A) Preparations for spinal cord imaging. Left: Following isolation of the whole spinal cord from neonatal mouse or rat pups, the preparation is placed in a perfusion chamber and continuously perfused with oxygenated ACSF. Small pins stabilized the isolated cord. Glass pipettes can be used to record or stimulate spinal circuits *via* the ventral and/or dorsal roots. Right: For imaging in living animals surgical and optical access to the spinal cord requires resection of multiple layers of connective tissue. Typically, the osseous vertebral lamina is removed by laminectomy. For bulk loading of calcium indicators, the dura mater is incised or deflected. (B) Left: Example two-photon image of a neuronal population in the intermediate zone of a hemisected neonatal spinal cord bulk loaded with OGB-1AM. Right: Staining pattern in ventral horn locomotor circuits after OGB-1 loading. Motor neurons were in addition retrogradely labeled by intramuscular injection of Rhodamine Dextran one day before the imaging session. Note that primary neurite extensions can be resolved in many cases. (C) *In vivo* two-photon image examples. Left: Two-photon image of the spinal microvasculature (red) in close contact with microglial cells (green; 30-µm z-projection). Prior to image acquisition, the blood plasma of Cx3CR1-GFP mice was stained by tail vein injection of a red fluorescent dextran. Right: Sensory axons (green) and adjacent microglia cells (pseudo-colored in blue) imaged in the spinal cord dorsal white matter of Thy1-YFP/Cx3CR1-GFP double transgenic mice. (D) Side projection of glycinergic neurons imaged in the superficial dorsal horn of GlyT2-EGFP mice *in vivo*. (E) Series of two-photon images of OGB-1-labeled neuronal networks in the dorsal spinal cord of wild-type mice (left). Afferent fiber tracts entering the lateral grey matter at superficial levels are partly stained. Despite the occluding effects of these fiber tracts, individual neurons in the substantia gelatinosa can be well resolved (right).

rhythmically active during fictive locomotion and are likely to provide inhibitory drive towards motoneuron output. Similarly, two-photon calcium imaging was used in Chx10::CFP mouse spinal cords to visualize activation of V2a interneurons during ipsi- and contralateral episodes of ventral root output during fictive locomotion (Zhong et al., 2010). Kwan et al. recently introduced coherence analysis as a tool to analyze specifically large sets of cellular fluorescence traces with respect to rhythmic locomotor output (Kwan et al., 2010). Calculation of the coherence between cellular signal traces and the system's summed output provides information about amplitude and phase of individual neuronal activities relative to network activity. This approach seems to be a useful addition to other approaches used in population data analysis (like spike train reconstruction or principal component analysis, reviewed in Lütcke and Helmchen, 2011), especially when imaging data is acquired in parallel with a measure of more global network state (*e.g.* locomotor output, EEG recording). Taken together, two-photon imaging studies in the neonatal spinal cord have provided important insights into the functional role of genetically defined spinal cord subpopulations. The *in vitro* approaches thus are well suited to determine the intrinsic contributions of different cell classes towards shaping sensorimotor output on a circuit level.

Two-photon imaging of spinal cord *in vivo*

Experimental challenges

High-resolution optical imaging of neuronal and glial networks in living rodents requires minimization of translational tissue movements, in particular drifts of the focal plane. Tissue movements originate from the pulsations associated with heart beat and breathing. In most studies on neocortex or subcortical regions such as the hippocampus, tissue stabilization is achieved by either reducing invasiveness (preparation of a small craniotomy or a thinned skull preparation, *e.g.* Davalos et al., 2005; Drew et al., 2010; Nimmerjahn et al., 2005) or by mechanically dampening tissue pulsations with cover glass slides in combination with agar (Stosiek et al., 2003) or more specialized indention materials (Dombeck et al., 2007). With such approaches, the remaining tissue pulsations, which in the cortex are not influenced to large extents by breathing movements, can be well controlled allowing even fine-scale imaging of subcellular structures like dendritic spines (Bhatt et al., 2009; Holtmaat and Svoboda, 2009) and microglial filopodia (Davalos et al., 2005; Nimmerjahn et al., 2005). The degree of stability can be high enough to even permit functional imaging in awake, behaving animals (Dombeck et al., 2007).

Given the spinal cord's inherent mechanical flexibility and the level of invasiveness that is often required for optical access during the experiment, achieving sufficient stability of the preparation is particularly important for spinal cord imaging. Typically, spinal cord movement is first of all minimized by fixating the vertebral column with metal clamps. For mice, the optimal age for clamp fixation appears to be >6–8 weeks, which ensures sufficient rigidity of the vertebral column. Relatively stable conditions can be achieved by optimizing the animals' posture, as has been demonstrated in various single-cell patch-clamp recording studies probing dorsal horn cells in rats (Bester et al., 2000; Dalal et al., 1999; Furue et al., 1999; Light and Willcockson, 1999) and mice (Graham et al., 2004a, 2004b), as well as

in recent imaging studies (Davalos et al., 2008; Dibaj et al., 2010a, 2010b; Drdla et al., 2009; Ikeda et al., 2006; Johannssen and Helmchen, 2010). Spontaneous breathing has a major effect on spinal cord movements in intact rodents and its impact appears to be larger in rats when compared to age-matched mice. To mechanically isolate the spinal cord from breathing related movements, the fixated animal can be slightly elevated from the heating pad (Davalos and Akassoglou, 2012; Davalos et al., 2008) although great care has to be taken to avoid hypothermia in this case, especially in mice. Periodic clearance of accumulated mucus is advisable to maintain stable breathing over time (Johannssen and Helmchen, 2010) and the anesthesia regime used can affect recording stability as well (Davalos et al., 2008).

A further method for stabilization is artificial ventilation, which does not seem to be absolutely required but helps to stabilize spinal cord *in vivo* preparations in several ways (Dibaj et al., 2010a, 2010b; Ikeda et al., 2006; Kerschensteiner et al., 2005). Within their physiological range, ventilation frequency and inhalation volume can be adjusted to minimize their influence on spinal cord movement. There are several ways to implement artificial ventilation in mice and rats such as endotracheal intubation techniques and intubation after tracheotomy, which are usually combined with paralyzing agents making anesthesia surveillance by reflex assessment challenging (alternative methods to monitor anesthesia levels in this case could be recording of the electrocardiogram, ECG, and/or electroencephalogram, EEG, previously calibrated to the actual anesthetic regime used; see for example (Dibaj et al., 2010a)). Although less invasive when compared to the tracheotomy approach, endotracheal intubation is more difficult to master in mice due to smaller upper airway dimensions. When relatively slow events are monitored, data acquisition can be triggered to the artificial breathing cycle when using artificial ventilation. Similarly triggering on the cardiac cycle can help to circumvent heart-beat induced movement artifacts.

Optical imaging of the spinal cord *in vivo* obviously involves surgical procedures that enable direct tissue access (Fig. 1A). The extent and complexity of the surgery, however, largely depend on the specific experimental questions. If target structures are pre-labeled, *e.g.*, by expression of fluorescent marker proteins in subsets of neurons (Zeilhofer et al., 2005) or glial cells (Jung et al., 2000), the dura does not need to be removed but only cleared from connective tissue to allow high-resolution imaging (Dibaj et al., 2010a). For optical access to the dorsal spinal cord surface, the intervertebral space can be exploited without microsurgical removal of one or more vertebrae (Kim et al., 2010). While this minimally invasive approach is limited to a rather restricted area, it is especially well suited for repeated imaging over days, in part because the necessary post-operative care regime does not have to deal with a major surgery as in the case of a laminectomy. On the other hand, if imaging is performed repeatedly after conducting a laminectomy, adequate analgesic management is required and the surgery site needs to be protected between imaging sessions by means of an artificial dura substitute (Kerschensteiner et al., 2005) or a protective agar pad (Dray et al., 2009). Only recently, time-lapse imaging of axons, glia and immune cells in the living mouse spinal cord was reported by using chronically implanted imaging chambers (Farrar et al., 2012; Fenrich et al., 2012). In these studies, implanted animals could be re-anesthetized for each imaging session without the need of repeated surgery. So far, most spinal cord *in vivo* imaging studies in intact rodents focused on medially located, superficial white matter tracts to visualize their structural plasticity or interaction with glial cells (Davalos et al., 2008; Dibaj et al., 2010a, 2010b; Dray et al., 2009; Farrar et al., 2012; Fenrich et al., 2012; Kerschensteiner et al., 2005; Ylera et al., 2009). In contrast, for two-photon calcium imaging of dorsal horn neurons, the grey matter comprising neuronal circuits of interest needs to be accessed. By slightly rotating the animal, direct, lateral access to the superficial laminae of the lumbar dorsal horn is facilitated (Johannssen and Helmchen, 2010). To maximize imaging depth, it is beneficial to avoid myelinated structures like prominent fiber tracts as far as possible because these efficiently absorb and scatter the excitation light, severely decreasing resolution and contrast. In combination with bulk loading of the calcium indicator OGB-1AM, activity of neuronal populations located in Laminae I and II in the spinal cord of adult mice could be monitored *in vivo* (Johannssen and Helmchen, 2010). Since other factors including signal-to-noise ratio and the degree of neuropil staining of a given dye also influence imaging depth (Göbel and Helmchen, 2007), bright labeling of relatively few structures might be especially suited to gain access to even deeper spinal cord laminae *in vivo* (see Fig. 1D for example).

Imaging structural dynamics in vivo

Given the spinal cord's cellular diversity, techniques for specific labeling of neuronal subpopulations are indispensable to investigate specific spinal cord circuits. Classical anatomical approaches like tracing techniques to identify neuronal populations based on target innervation have been used both *in vitro* (O'Donovan et al., 2005) and *in vivo* (Ikeda et al., 2006). Fluorescent tracers like DiI or dextrans can then also be combined with bulk loading of AM-type calcium indicators during the imaging experiment, for example to retrogradely identify Lamina I projection neurons *in vivo* (Ikeda et al., 2006) (see also Fig. 3B) or motoneurons *in vitro* (Fig. 1B).

A particularly valuable and surgically non-invasive way to identify specific subtypes of spinal cord neurons is transgenic expression of fluorescent proteins (see examples in Figs. 1C and D). Depending on several factors, including expression levels and sparseness of labeling, genetically encoded markers provide good contrast and importantly, allow repeated imaging (Dray et al., 2009; Farrar et al., 2012; Fenrich et al., 2012; Kerschensteiner et al., 2005). However, many current promoters used to drive fluorescent protein expression result in labeling of large neuronal populations, which share a certain transmitter system or receptor type. Transgenic mice expressing fluorescent proteins only in subsets of spinal cord neurons could be an increasingly valuable tool to investigate spinal circuits on a finer scale (Belle et al., 2007; Heinke et al., 2004; Torsney et al., 2006).

Several recent studies imaging spinal cord neurons *in vivo* used Thy1-GFP transgenic mice for acute and repeated imaging of single axons (Dray et al., 2009; Farrar et al., 2012; Fenrich et al., 2012; Kerschensteiner et al., 2005; Ylera et al., 2009). Sparse expression of fluorescent reporter proteins in spinal cord projection neurons enabled faithful monitoring of individual regenerating axons with high resolution after experimental spinal cord injury (Dray et al., 2009; Fenrich et al., 2012; Kerschensteiner et al., 2005; Ylera et al., 2009). From a technical perspective, these structural changes are relatively slow, typically occurring on the minutes to hours time scale, and therefore allow offline correction of lateral movements and focal plane shifts, given that they do not exceed the dimensions of image stacks acquired over time. After spinal cord injury, central sensory axon regeneration capacity depends on the timing of peripheral nerve injuries, which was recently observed with *in vivo* two-photon imaging of fibers growing through an acute lesion site (Ylera et al., 2009). In a related study using the lamprey spinal cord model of axonal regeneration, two photon imaging was used to assess the overall growth-promoting effect of cAMP after spinal cord injury *in vivo*, highlighting its differential role of inhibiting the early phase of retraction and increasing the later outgrowth speed (Jin et al., 2009).

Following a local mechanical dorsal column lesion, Dray et al. (2009) monitored the parallel dynamics of regenerating, GFP-positive sensory axon populations and the local microvasculature repeatedly for up to 4 months. Blood vessels in this case were stained by i.v. administration of Rhodamine dextran (see also Fig. 1C). Long-term imaging revealed an early phase of enhanced vessel growth during a longer lasting period of axonal regrowth past the lesion site. In this case, the laminectomy site could be sufficiently protected in between trials by agar cushioning,

making repeated surgery necessary but also allowing periodic cleaning of the imaging site to optimize optical access. Two groups recently employed custom-made, implanted imaging chambers for chronic spinal cord imaging in transgenic mice (Farrar et al., 2012; Fenrich et al., 2012). Farrar et al. (2012) repeatedly imaged microglia dynamics associated with axonal injury and regeneration. With this approach, the authors visualized and quantified the time course of microglia activation after focal laser injury on a daily basis and also observed differential axonal plasticity over several weeks. Importantly, it now became possible to repeatedly image the spinal cord without additional surgery (Farrar et al., 2012; Fenrich et al., 2012). However, great care is necessary to avoid deterioration of the imaging window over time due to inflammation or tissue regrowth. Taken together, these chronic imaging approaches open new avenues towards examining the long-term regeneration of axons during spinal cord pathologies as well as their interaction with other tissue compartments with high resolution *in vivo*.

Spinal cord glial cell populations, in addition to their established roles in neurotrophic support, electrical insulation and immunological surveillance (Hanisch and Kettenmann, 2007; Nave, 2010), have emerged as important players in influencing neuronal activity patterns by release of various modulatory factors (Bardoni et al., 2010; Coull et al., 2005). In certain pathological settings, *e.g.* after spinal cord injury, astrocytes are known to undergo reactive gliosis impeding the regeneration of injured axons (Silver and Miller, 2004). For imaging astrocyte signaling under various experimental conditions, it would be desirable to identify this cell population with a rather simple staining technique. In the neocortex, brief application of sulforhodamine 101 (SR101) to the brain surface is such a method, selectively staining astrocytes *in vivo* (Nimmerjahn et al., 2004). In the spinal cord, however, SR101 application resulted in more complex staining patterns (Johannssen and Helmchen, 2010). Although topical application of SR101 to the dorsal surface *in vivo* resulted in preferential astroglial dye uptake, control experiments using the fixable analogue Texas Red hydrazide (TRH) or tissue injection of SR101 caused labeling of neuronal cells as well (Johannssen and Helmchen, 2010). *In vitro*, bath application of SR101 has been reported to label spinal cord neurons in isolated, neonatal preparations in an activity-dependent manner (Kjaerulff et al., 1994; Lavallee and Pflieger, 2009). This rather wide range of SR101 uptake in the spinal cord could be due to several factors including the type of preparation used, the mode, duration and concentration of dye application and the animals' age. As an alternative, selective genetic labeling techniques can be exploited to study morphological interactions of spinal cord astrocytes with neurons, for example in models of spinal cord edema (Dibaj et al., 2007), which frequently occurs after spinal cord injury.

Recent imaging studies of microglia dynamics in the neocortex shed new light on the role of these cells in constant and active surveillance of the surrounding brain tissue and also demonstrated purinergic signaling as a key chemoattractant pathway for microglia migration (Davalos et al., 2005; Nimmerjahn et al., 2005). These studies used a Cx3CR1-GFP mouse line, in which microglia express GFP (Jung et al., 2000). Mechanisms of how microglia interact with neurons during regeneration after injury and during plasticity of pain networks are a field of major interest in current spinal cord research (Coull et al., 2005; Dibaj et al., 2010a; Farrar et al., 2012). In the spinal cord, microglial cells have been shown to tune neuronal firing in spinal pain-processing circuits by release of BDNF (Coull et al., 2005). Specifically, ATP-dependent signaling mechanisms have been identified (Tsuda et al., 2010), which are especially important in spinal cord injury paradigms and in models of neuropathic pain (Coull et al., 2005). Recently, *in vivo* time lapse imaging in double-transgenic Cx3CR1/Thy1-mice was used to visualize the structural dynamics of microglia in spinal white matter before and after focal laser injury (Dibaj et al., 2010a) as well as in acute post-mortem tissue (Dibaj et al., 2010b) (see also Fig. 1C). After injury, microglia showed increased rates of directional motility towards the lesion site, which – in addition to ATP – depended on NO signaling (Dibaj et al., 2010a), which had been previously shown to influence microglia migration behavior in the leech nerve cord (Duan et al., 2003). These signaling pathways are likely to be critically involved in the enhanced activation and recruitment of macrophages during pathological conditions like spinal cord injury (Schnell et al., 1999), multiple sclerosis (Monif et al., 2010) and chronic pain (Tsuda et al., 2010). Using new methodological improvements, especially in repeated spinal cord imaging techniques (Dray et al., 2009; Farrar et al., 2012; Fenrich et al., 2012; Kerschensteiner et al., 2005), mechanistic details of microglia plasticity can now be further explored *in vivo* in the context of various experimental settings.

In addition to the roles of microglia in spinal cord trauma and chronic pain states, *in vivo* imaging approaches can be used to visualize interactions of monocytic immune cells with the spinal cord white matter and vasculature in the context of experimental autoinflammatory encephalitis (EAE), which is an animal model of human multiple sclerosis. *In vivo* imaging of pre-labeled T cells directly revealed mechanistic details about their interaction with the spinal cord blood brain barrier in EAE mice (Vajkoczy et al., 2001). In the same disease model, two-photon imaging of Cx1CR3-positive microglia or CxCR6-positive immune cells expressing GFP was used to observe the directional migration of CxCR6-cells within the dorsal white matter *in vivo* (Kim et al., 2010). In summary, high resolution *in vivo* imaging of neuronal and glial structural plasticity in the intact spinal cord recently provided detailed insights into mechanisms of disease and regeneration.

Two-photon calcium imaging of spinal cord neurons

Functional imaging of cellular activity in the intact spinal cord obviously would be of great value to complement the studies on structural changes. At least for the superficial dorsal horn, progress has been made recently to establish two-photon calcium imaging of grey matter neurons and glial cells, complementing earlier *in vivo* electrical recordings of spinal cord neurons. The main method that has been used so far for calcium indicator labeling in spinal cord is bulk loading by injection of a calcium indicator in its AM-ester form (Brustein et al., 2003; Drdla et al., 2009; Ikeda et al., 2006; Johannssen and Helmchen, 2010), similar to previous studies in various brain areas (Kerr et al., 2005; Schultz et al., 2009; Stosiek et al., 2003; Sullivan et al., 2005). Injection of the indicator typically results in a quick, functional labeling of neuronal and glial populations covering an area of several hundred microns (Fig. 1E). Although brightness of staining typically broadly segregates neuronal and glial cells (Stosiek et al., 2003), labeling of cellular structures is virtually non-selective and also accompanied by a relatively high background due to neuropil staining raising the possibility of signal contamination (Göbel and Helmchen, 2007; Kerr and Denk, 2008). In principle, various other techniques for calcium indicator labeling could also be applied to the spinal cord, such as intracellular loading of individual cells or local electroporation of salt- or dextran-forms of small molecule indicators, which can stain individual or groups of dozens of cells with high signal-to-background ratio (Nagayama et al., 2007; Nevian and Helmchen, 2007). In the isolated neonatal spinal cord bulk electroporation has been shown to efficiently label cellular networks, albeit with unclear cell type-specificity (Bonnot et al., 2009). The feasibility and the degree of specificity of these methods yet remain to be determined for the intact, adult spinal cord.

In the first study using *in vivo* bolus loading of calcium indicator and high-resolution two-photon calcium imaging of spinal cord neuronal populations, zebrafish larvae were used to monitor responses to sensory stimulation and drug application (Brustein et al., 2003) (Fig. 2A). Zebrafish larvae have great genetic potential and are highly transparent and thus advantageous for optical techniques, as previously

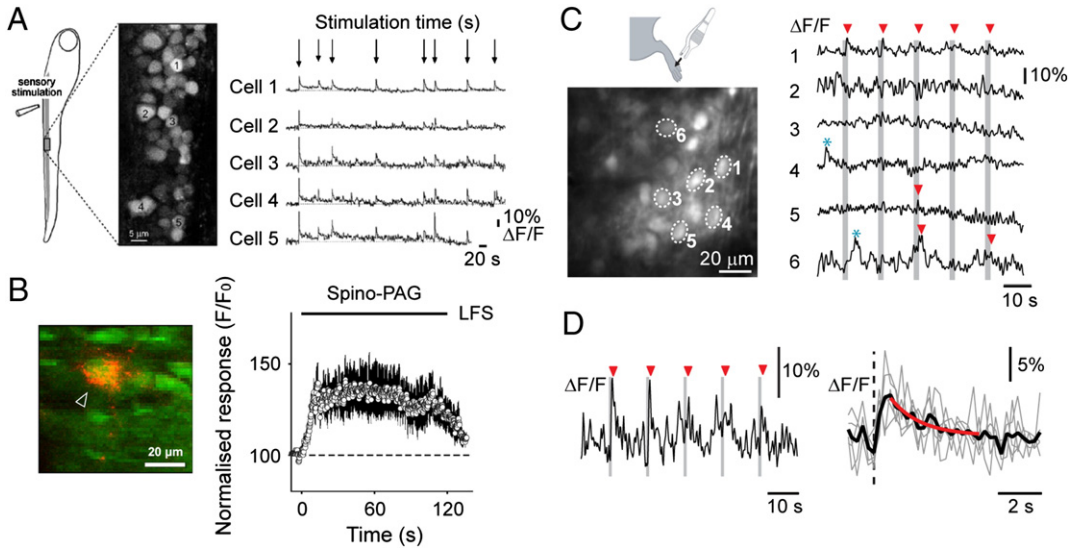

Fig. 2. *In vivo* two-photon calcium imaging in the spinal cord of three different model organisms. In all cases neuronal circuits were bulk-loaded with AM-type calcium indicators and fluorescence changes in individual cells were imaged during sensory stimulation of different modalities. (A) Mechanical stimulation evokes calcium transients in spinal cord neurons of zebrafish larvae. Left, schematic of the stimulation paradigm and two-photon image of spinal cord populations loaded with Fura PE3 AM. Right, sensory-evoked calcium transients in the five marked cells. (B) Left, Lamina I projection neuron identified by injection of the tracer DiI into the periaqueductal grey (PAG) prior to imaging. Right, average changes in OGB-1 fluorescence within PAG-projecting Lamina I neurons (n = 7) in rats. The calcium elevation is evoked by low-frequency electrical stimulation (LFS) of the sciatic nerve at C-fiber intensity *in vivo* and persists throughout the stimulation period. (C) Pinch-evoked calcium transients in subsets of dorsal horn cells *in vivo*. Two-photon image showing OGB-1 loaded neuronal populations in the mouse superficial dorsal horn. White circles indicate ROIs for which the corresponding fluorescence traces are shown on the right. Repeated mechanical stimulation induced time-locked calcium transients (red triangles) in a subset of cells and intermittent spontaneous events (blue asterisks) were detected as well. (D) Left, expanded view of the pinch-evoked calcium transients observed in cell 1 (uppermost trace in panel C) illustrating precise time-locking of the responses in this cell and variations of signal magnitude during repeated stimulation. Right, overlay of the sensory-triggered calcium transients. The dashed line marks pinch stimulation and the red curve is the exponential fit used to approximate signal decay.
Panel A adapted from Brustein et al. (2003). Panel B adapted from Ikeda et al. (2006). Panels C and D adapted from Johannssen and Helmchen (2010).

exploited for confocal *in vivo* imaging of the embryonic ventral spinal cord with genetically encoded calcium indicators (Higashijima et al., 2003; Warp et al., 2012). It remains yet to be determined how exactly findings from developing zebrafish spinal circuits relate to experiments in adult rodents, which are frequently used as model systems to investigate mechanisms of sensory processing and pain. In recent pioneering experiments in spinal cord of young rats, *in vivo* calcium imaging was performed after bulk loading of OGB-1, involving extensive adaptations to stabilize the preparation including artificial ventilation, cardiac triggering of data acquisition and cardio-pulmonary bypass techniques (Drdla et al., 2009; Ikeda et al., 2006). Using these procedures, Ikeda et al. (2006) could image sciatic nerve stimulation-induced calcium gradients in several classes of Lamina I neurons *in vivo* (Fig. 2B). Similarly, in another publication from the same group, calcium gradients were observed after opioid application *in vivo* in rats (Drdla et al., 2009). The strengths of this approach are clearly the established and well-defined functional anatomy of the somatosensory system and its behavioral correlates in the rat as a model organism. However, in contrast to the zebrafish study of sensory-evoked activity (Brustein et al., 2003), elementary calcium transients, triggered by a single or a few action potentials, could not be resolved in these two studies, possibly because spinal cord movement is more difficult to control in rats when compared to smaller model organisms.

To resolve individual calcium transients in dorsal horn neurons in living rats and mice, spinal cord movements need to be further reduced down to the microscopic level. While electrical recording configurations seem to tolerate certain amounts of movement relative to the pipette (after seal formation), high-resolution calcium measurements are highly sensitive to drifts of only a few microns occurring in the z-direction (Kerr and Denk, 2008). While lateral shifts within a single focal plane can be corrected offline, such correction is usually difficult or impossible for focus shifts (Greenberg et al., 2008). It is therefore paramount to improve the preparation stability as much as possible in order to optimize the raw data quality. Recently, we demonstrated *in vivo* calcium imaging of dorsal horn neurons in mice (Johannssen and Helmchen, 2010) (Figs. 2C, D). In this study we effectively dampened tissue pulsations that remained after posture optimization with simple mechanical means by covering the laminectomy with a sheet of agarose and a coverglass (Fig. 3A). With this method, sensory-evoked elementary calcium transients could be resolved in single cells within the mouse superficial dorsal horn (Figs. 2C, D). Importantly, the dampening material can be temporarily removed if required, e.g. for the local application of drugs, either topical or by intraspinal injection.

An alternative method could be to compensate focal shifts by online corrective movements of the microscope objective, as demonstrated recently (Laffray et al., 2011). Furthermore, remaining minor motions can be dealt with by using ratiometric imaging. In principle, this can be either achieved with ratiometric indicator dyes or by co-injection of OGB-1 and a red fluorescent marker such as Calcein red/orange AM, which itself does not exhibit activity-dependent fluorescence intensity changes (Fig. 3B). The cellular signal is in this case evaluated as the ratio of the fluorescence intensities in the green (OGB-1) and the red channel (Calcein), respectively. This ratio is much less sensitive to tissue shifts so that the calcium transients can be extracted even under noisy conditions (Fig. 3B). Ratiometric imaging thus might be especially well suited for functional imaging studies in the intact spinal cord.

Conclusions and outlook

In summary, two-photon imaging in the spinal cord is now applied using both *in vitro* and *in vivo* preparations. For *in vivo* imaging, key methodological advances have been made that now enable high-resolution studies of both spinal cord structure and function. In particular, functional imaging of neuronal calcium transients in the intact spinal cord has just emerged. This approach can be expected to find extended applications in the future as it opens new opportunities to study circuit activity with cellular resolution. The

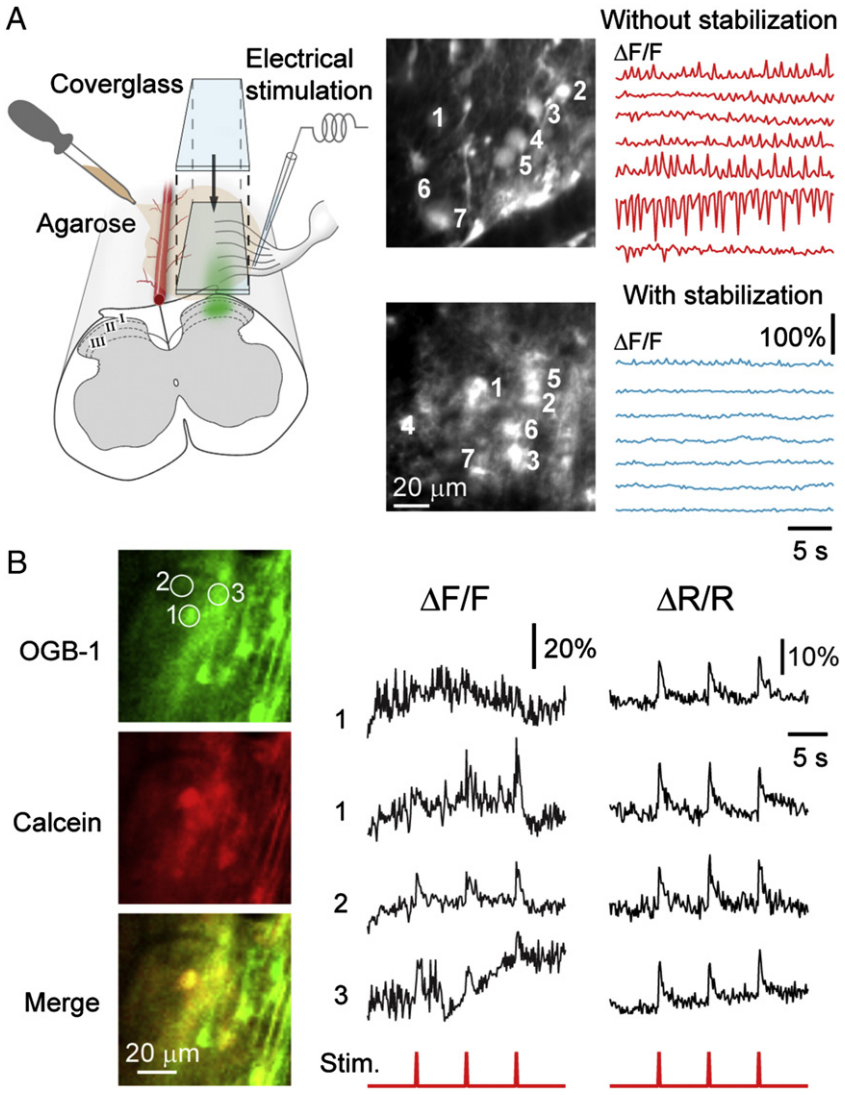

Fig. 3. Stabilization of spinal cord fluorescence signals using mechanical or ratiometric approaches. (A) Dampening tissue pulsations by a layer of agarose and a cover glass significantly reduces the extent of noisy fluorescence fluctuations during calcium imaging in the mouse dorsal horn. On the right several example traces from neurons filled with OBG-1 are shown before (top) and after (bottom) stabilization. (B) Alternatively ratiometric imaging can be used, which is less sensitive to movement artifacts. In this example, OGB-1AM was co-injected in the dorsal spinal cord together with the non-functional dye CellTrace calcein red-orange AM. The fluorescence changes induced by electrical afferent stimulation *in vivo* are shown for example cells in two different ways (two trials are shown for cell 1). Relative percentage changes of OGB-1 fluorescence (ΔF/F from the green channel alone) contain many movement-induced artifacts superpositioned on the calcium signals. When the same calcium signals are evaluated as relative percentage changes of the ratio between the two dye channels (ΔR/R), signals appear much cleaner.
Panel A modified from Johannssen and Helmchen (2010).

main limitation of *in vivo* calcium imaging experiments currently is the restriction to the superficial dorsal horn due to heavy myelination precluding optical access to deeper laminae. Even if optical conditions in terms of light scattering and absorption could be improved, *e.g.* by using substantially longer excitation wavelengths (Kobat et al., 2009), two-photon microscopy cannot be expected to yield a maximal depth penetration beyond 1 mm (Helmchen and Denk, 2005). Thus, imaging of deeper layers and especially ventral laminae *in vivo* would necessarily involve more invasive approaches, for example the use of fiber optics and microlenses for fluorescence excitation and collection. The feasibility of directly accessing the spinal cord of living rats with a commercially available microlens system was recently demonstrated (Wang et al., 2007). Similarly, fiber- or microlens-based miniature microscopes (Engelbrecht et al., 2008; Flusberg et al., 2005; Göbel et al., 2004) might be inserted deeper into spinal tissue. However, since spinal sensorimotor circuits are heavily interconnected across laminae and rely on feedback inputs for proper functioning, such approaches would require careful interpretation.

A most promising future direction is the combination of two-photon microscopy with genetically encoded calcium indicators (Mank and Griesbeck, 2008; Miyawaki, 2003). Over the last decade, the performance of GECIs has been improved substantially, *e.g.* with respect to dynamic range, sensitivity, and kinetics, so that they now are a key tool to study network function *in vivo* (Dombeck et al., 2010; Lütcke et al., 2010; Mank et al., 2008; Tian et al., 2009). GECIs can be expressed in the cells of interest using various methods, including generation of transgenic animals (Hasan et al., 2004) and in particular viral delivery (Lütcke et al., 2010; Tian et al., 2009). The two major classes of GECIs are single fluorescent proteins and tandem fluorescent proteins, which are linked *via* a calcium-binding domain. The latter type might be particularly interesting, since its functional signals rely on changes in fluorescence resonance energy transfer (FRET) efficiency, which is typically read out ratiometrically in two spectral windows. In a recent study, action potential-induced calcium signals were resolved in mouse neocortex using the FRET-based indicator Yellow Cameleon 3.60 (YC3.60; Lütcke et al., 2010). The

CFP-YFP FRET-pair in YC3.60 is linked by a calmodulin domain and a M13 peptide, such that FRET efficiency changes as a function of calcium binding and protein conformation. Since the intact spinal cord is particularly prone to instabilities, ratiometric indicators such as YC3.60 could likely be advantageous in minimizing confounding motion artifacts *in vivo*.

By combining recent improvements of chronic preparations (Dray et al., 2009; Farrar et al., 2012; Fenrich et al., 2012; Kerschensteiner et al., 2005) with the latest developments in the field of GECIs, even long-term, repeated functional imaging might become feasible in the intact spinal cord. In addition, inducible and targeted GECI expression could allow population-specific expression of these indicators. Future cell-type specific functional imaging experiments thus promise further insights into the dynamic interplay within complex spinal circuits. To conclude, two-photon imaging has reached the spinal cord and exciting developments are taking place towards addressing more detailed and sophisticated questions regarding fundamental aspects of spinal cord physiology and pathology.

Acknowledgments

We thank D. Ladle for help with isolated spinal cord preparations and retrograde tracings, H. U. Zeilhofer for providing GlyT2-eGFP mice, E. Hochreutener for assistance with illustrations and M. Thallmair for critical comments on the manuscript. This work was supported by the NCCR “Neural Plasticity and Repair” and the EU FP7 program (PLASTICISE, project number 223524).

References

Bardoni, R., Ghirri, A., Zonta, M., Betelli, C., Vitale, G., Ruggieri, V., Sandrini, M., Carmignoto, G., 2010. Glutamate-mediated astrocyte-to-neuron signalling in the rat dorsal horn. J. Physiol. 588, 831–846.

Belle, M.D., Pattison, E.F., Cheunsuang, O., Stewart, A., Kramer, I., Sigrist, M., Arber, S., Morris, R., 2007. Characterization of a thy1.2 GFP transgenic mouse reveals a tissue-specific organization of the spinal dorsal horn. Genesis 45, 679–688.

Bester, H., Chapman, V., Besson, J.M., Bernard, J.F., 2000. Physiological properties of the lamina I spinoparabrachial neurons in the rat. J. Neurophysiol. 83, 2239–2259.

Bhatt, D.H., Zhang, S., Gan, W.B., 2009. Dendritic spine dynamics. Annu. Rev. Physiol. 71, 261–282.

Bonnot, A., Mentis, G.Z., Skoch, J., O'Donovan, M.J., 2005. Electroporation loading of calcium-sensitive dyes into the CNS. J. Neurophysiol. 93, 1793–1808.

Bonnot, A., Chub, N., Pujala, A., O'Donovan, M.J., 2009. Excitatory actions of ventral root stimulation during network activity generated by the disinhibited neonatal mouse spinal cord. J. Neurophysiol. 101, 2995–3011.

Brown, C.E., Boyd, J.D., Murphy, T.H., 2010. Longitudinal *in vivo* imaging reveals balanced and branch-specific remodeling of mature cortical pyramidal dendritic arbors after stroke. J. Cereb. Blood Flow Metab. 30, 783–791.

Brustein, E., Marandi, N., Kovalchuk, Y., Drapeau, P., Konnerth, A., 2003. “*In vivo*” monitoring of neuronal network activity in zebrafish by two-photon Ca(2+) imaging. Pflugers Arch. 446, 766–773.

Busche, M.A., Eichhoff, G., Adelsberger, H., Abramowski, D., Wiederhold, K.H., Haass, C., Staufenbiel, M., Konnerth, A., Garaschuk, O., 2008. Clusters of hyperactive neurons near amyloid plaques in a mouse model of Alzheimer's disease. Science 321, 1686–1689.

Cazalets, J.R., Sqalli-Houssaini, Y., Clarac, F., 1992. Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. J. Physiol. 455, 187–204.

Cheng, A., Goncalves, J.T., Golshani, P., Arisaka, K., Portera-Cailliau, C., 2011. Simultaneous two-photon calcium imaging at different depths with spatiotemporal multiplexing. Nat. Methods 8, 139–142.

Coull, J.A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., Gravel, C., Salter, M.W., De Koninck, Y., 2005. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438, 1017–1021.

Dalal, A., Tata, M., Allegre, G., Gekiere, F., Bons, N., Albe-Fessard, D., 1999. Spontaneous activity of rat dorsal horn cells in spinal segments of sciatic projection following transection of sciatic nerve or of corresponding dorsal roots. Neuroscience 94, 217–228.

Davalos, D., Akassoglou, K., 2012. *In vivo* imaging of the mouse spinal cord using two-photon microscopy. J. Vis. Exp. (59), e2760 (2012 Jan 5).

Davalos, D., Grutzendler, J., Yang, G., Kim, J.V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L., Gan, W.B., 2005. ATP mediates rapid microglial response to local brain injury *in vivo*. Nat. Neurosci. 8, 752–758.

Davalos, D., Lee, J.K., Smith, W.B., Brinkman, B., Ellisman, M.H., Zheng, B., Akassoglou, K., 2008. Stable *in vivo* imaging of densely populated glia, axons and blood vessels in the mouse spinal cord using two-photon microscopy. J. Neurosci. Methods 169, 1–7.

Diaz-Rios, M., Dombeck, D.A., Webb, W.W., Harris-Warrick, R.M., 2007. Serotonin modulates dendritic calcium influx in commissural interneurons in the mouse spinal locomotor network. J. Neurophysiol. 98, 2157–2167.

Dibaj, P., Kaiser, M., Hirrlinger, J., Kirchhoff, F., Neusch, C., 2007. Kir4.1 channels regulate swelling of astroglial processes in experimental spinal cord edema. J. Neurochem. 103 (6), 2620–2628.

Dibaj, P., Nadrigny, F., Steffens, H., Scheller, A., Hirrlinger, J., Schomburg, E.D., Neusch, C., Kirchhoff, F., 2010a. NO mediates microglial response to acute spinal cord injury under ATP control *in vivo*. Glia 58, 1133–1144.

Dibaj, P., Steffens, H., Nadrigny, F., Neusch, C., Kirchhoff, F., Schomburg, E.D., 2010b. Long-lasting post-mortem activity of spinal microglia *in situ* in mice. J. Neurosci. Res. 88, 2431–2440.

Dombeck, D.A., Khabbaz, A.N., Collman, F., Adelman, T.L., Tank, D.W., 2007. Imaging large-scale neural activity with cellular resolution in awake, mobile mice. Neuron 56, 43–57.

Dombeck, D.A., Harvey, C.D., Tian, L., Looger, L.L., Tank, D.W., 2010. Functional imaging of hippocampal place cells at cellular resolution during virtual navigation. Nat. Neurosci. 13, 1433–1440.

Dray, C., Rougon, G., Debarbieux, F., 2009. Quantitative analysis by *in vivo* imaging of the dynamics of vascular and axonal networks in injured mouse spinal cord. Proc. Natl. Acad. Sci. U. S. A. 106, 9459–9464.

Drdla, R., Gassner, M., Gingl, E., Sandkuhler, J., 2009. Induction of synaptic long-term potentiation after opioid withdrawal. Science 325, 207–210.

Drew, P.J., Shih, A.Y., Driscoll, J.D., Knutsen, P.M., Blinder, P., Davalos, D., Akassoglou, K., Tsai, P.S., Kleinfeld, D., 2010. Chronic optical access through a polished and reinforced thinned skull. Nat. Methods 7, 981–984.

Duan, Y., Haugabook, S.J., Sahley, C.L., Muller, K.J., 2003. Methylene blue blocks cGMP production and disrupts directed migration of microglia to nerve lesions in the leech CNS. J. Neurobiol. 57, 183–192.

Engelbrecht, C.J., Johnston, R.S., Seibel, E.J., Helmchen, F., 2008. Ultra-compact fiber-optic two-photon microscope for functional fluorescence imaging *in vivo*. Opt. Express 16, 5556–5564.

Farrar, M.J., Bernstein, I.M., Schlafer, D.H., Cleland, T.A., Fetcho, J.R., Schaffer, C.B., 2012. Chronic *in vivo* imaging in the mouse spinal cord using an implanted chamber. Nat. Methods 9, 297–302.

Fenrich, K.K., Weber, P., Hocine, M., Zalc, M., Rougon, G., Debarbieux, F., 2012. Long-term in vivo imaging of normal and pathological mouse spinal cord with subcellular resolution using implanted glass windows. J. Physiol. http://dx.doi.org/10.1113/jphysiol.2012.230532 (Electronic publication ahead of print May 28, 2012).

Flusberg, B.A., Cocker, E.D., Piyawattanametha, W., Jung, J.C., Cheung, E.L., Schnitzer, M.J., 2005. Fiber-optic fluorescence imaging. Nat. Methods 2, 941–950.

Fuhrmann, M., Bittner, T., Jung, C.K., Burgold, S., Page, R.M., Mitteregger, G., Haass, C., LaFerla, F.M., Kretzschmar, H., Herms, J., 2010. Microglial Cx3cr1 knockout prevents neuron loss in a mouse model of Alzheimer's disease. Nat. Neurosci. 13, 411–413.

Furue, H., Narikawa, K., Kumamoto, E., Yoshimura, M., 1999. Responsiveness of rat substantia gelatinosa neurones to mechanical but not thermal stimuli revealed by *in vivo* patch-clamp recording. J. Physiol. 521 (Pt 2), 529–535.

Garaschuk, O., Milos, R.I., Grienberger, C., Marandi, N., Adelsberger, H., Konnerth, A., 2006. Optical monitoring of brain function *in vivo*: from neurons to networks. Pflugers Arch. 453, 385–396.

Garcia-Campmany, L., Stam, F.J., Goulding, M., 2010. From circuits to behaviour: motor networks in vertebrates. Curr. Opin. Neurobiol. 20, 116–125.

Göbel, W., Helmchen, F., 2007. *In vivo* calcium imaging of neural network function. Physiology (Bethesda) 22, 358–365.

Göbel, W., Kerr, J.N., Nimmerjahn, A., Helmchen, F., 2004. Miniaturized two-photon microscope based on a flexible coherent fiber bundle and a gradient-index lens objective. Opt. Lett. 29, 2521–2523.

Gordon, I.T., Whelan, P.J., 2008. Brainstem modulation of locomotion in the neonatal mouse spinal cord. J. Physiol. 586, 2487–2497.

Graham, B.A., Brichta, A.M., Callister, R.J., 2004a. An *in vivo* mouse spinal cord preparation for patch-clamp analysis of nociceptive processing. J. Neurosci. Methods 136, 221–228.

Graham, B.A., Brichta, A.M., Callister, R.J., 2004b. *In vivo* responses of mouse superficial dorsal horn neurones to both current injection and peripheral cutaneous stimulation. J. Physiol. 561, 749–763.

Greenberg, D.S., Houweling, A.R., Kerr, J.N., 2008. Population imaging of ongoing neuronal activity in the visual cortex of awake rats. Nat. Neurosci. 11, 749–751.

Grewe, B.F., Helmchen, F., 2009. Optical probing of neuronal ensemble activity. Curr. Opin. Neurobiol. 19, 520–529.

Grewe, B.F., Langer, D., Kasper, H., Kampa, B.M., Helmchen, F., 2010. High-speed *in vivo* calcium imaging reveals neuronal network activity with near-millisecond precision. Nat. Methods 7, 399–405.

Grudt, T.J., Perl, E.R., 2002. Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. J. Physiol. 540, 189–207.

Hanisch, U.K., Kettenmann, H., 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat. Neurosci. 10, 1387–1394.

Hasan, M.T., Friedrich, R.W., Euler, T., Larkum, M.E., Giese, G., Both, M., Duebel, J., Waters, J., Bujard, H., Griesbeck, O., Tsien, R.Y., Nagai, T., Miyawaki, A., Denk, W., 2004. Functional fluorescent Ca^{2+} indicator proteins in transgenic mice under TET control. PLoS Biol. 2, e163.

Heinke, B., Ruscheweyh, R., Forsthuber, L., Wunderbaldinger, G., Sandkuhler, J., 2004. Physiological, neurochemical and morphological properties of a subgroup of GABAergic spinal lamina II neurones identified by expression of green fluorescent protein in mice. J. Physiol. 560, 249–266.

Helmchen, F., Denk, W., 2005. Deep tissue two-photon microscopy. Nat. Methods 2, 932–940.
Higashijima, S., Masino, M.A., Mandel, G., Fetcho, J.R., 2003. Imaging neuronal activity during zebrafish behavior with a genetically encoded calcium indicator. J. Neurophysiol. 90, 3986–3997.
Holtmaat, A., Svoboda, K., 2009. Experience-dependent structural synaptic plasticity in the mammalian brain. Nat. Rev. Neurosci. 10, 647–658.
Ikeda, H., Stark, J., Fischer, H., Wagner, M., Drdla, R., Jager, T., Sandkuhler, J., 2006. Synaptic amplifier of inflammatory pain in the spinal dorsal horn. Science 312, 1659–1662.
Jin, L.Q., Zhang, G., Jamison Jr., C., Takano, H., Haydon, P.G., Selzer, M.E., 2009. Axon regeneration in the absence of growth cones: acceleration by cyclic AMP. J. Comp. Neurol. 515, 295–312.
Johannssen, H.C., Helmchen, F., 2010. *In vivo* Ca^{2+} imaging of dorsal horn neuronal populations in mouse spinal cord. J. Physiol. 588, 3397–3402.
Jung, S., Aliberti, J., Graemmel, P., Sunshine, M.J., Kreutzberg, G.W., Sher, A., Littman, D.R., 2000. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol. Cell. Biol. 20, 4106–4114.
Katona, G., Szalay, G., Maak, P., Kaszas, A., Veress, M., Hillier, D., Chiovini, B., Vizi, E.S., Roska, B., Rozsa, B., 2012. Fast two-photon *in vivo* imaging with three-dimensional random-access scanning in large tissue volumes. Nat. Methods 9, 201–208.
Kerr, J.N., Denk, W., 2008. Imaging *in vivo*: watching the brain in action. Nat. Rev. Neurosci. 9, 195–205.
Kerr, J.N., Greenberg, D., Helmchen, F., 2005. Imaging input and output of neocortical networks *in vivo*. Proc. Natl. Acad. Sci. U. S. A. 102, 14063–14068.
Kerschensteiner, M., Schwab, M.E., Lichtman, J.W., Misgeld, T., 2005. *In vivo* imaging of axonal degeneration and regeneration in the injured spinal cord. Nat. Med. 11, 572–577.
Kim, J.V., Jiang, N., Tadokoro, C.E., Liu, L., Ransohoff, R.M., Lafaille, J.J., Dustin, M.L., 2010. Two-photon laser scanning microscopy imaging of intact spinal cord and cerebral cortex reveals requirement for CXCR6 and neuroinflammation in immune cell infiltration of cortical injury sites. J. Immunol. Methods 352, 89–100.
Kjaerulff, O., Barajon, I., Kiehn, O., 1994. Sulphorhodamine-labelled cells in the neonatal rat spinal cord following chemically induced locomotor activity *in vitro*. J. Physiol. 478 (Pt 2), 265–273.
Kobat, D., Durst, M.E., Nishimura, N., Wong, A.W., Schaffer, C.B., Xu, C., 2009. Deep tissue multiphoton microscopy using longer wavelength excitation. Opt. Express 17, 13354–13364.
Kudo, N., Yamada, T., 1987. N-methyl-D, L-aspartate-induced locomotor activity in a spinal cord–hindlimb muscles preparation of the newborn rat studied *in vitro*. Neurosci. Lett. 75, 43–48.
Kwan, A.C., Dietz, S.B., Webb, W.W., Harris-Warrick, R.M., 2009. Activity of Hb9 interneurons during fictive locomotion in mouse spinal cord. J. Neurosci. 29, 11601–11613.
Kwan, A.C., Dietz, S.B., Zhong, G., Harris-Warrick, R.M., Webb, W.W., 2010. Spatiotemporal dynamics of rhythmic spinal interneurons measured with two-photon calcium imaging and coherence analysis. J. Neurophysiol. 104, 3323–3333.
Laffray, S., Pages, S., Dufour, H., De Koninck, P., De Koninck, Y., Cote, D., 2011. Adaptive movement compensation for *in vivo* imaging of fast cellular dynamics within a moving tissue. PLoS One 6, e19928.
Lavallee, A., Pflieger, J.F., 2009. Developmental expression of spontaneous activity in the spinal cord of postnatal opossums, *Monodelphis domestica*: an anatomical study. Brain Res. 1282, 1–9.
Light, A.R., Willcockson, H.H., 1999. Spinal laminae I–II neurons in rat recorded *in vivo* in whole cell, tight seal configuration: properties and opioid responses. J. Neurophysiol. 82, 3316–3326.
Lu, Y., Perl, E.R., 2003. A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input. J. Neurosci. 23, 8752–8758.
Lu, Y., Perl, E.R., 2005. Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II). J. Neurosci. 25, 3900–3907.
Lütcke, H., Helmchen, F., 2011. Two-photon imaging and analysis of neural network dynamics. Rep. Prog. Phys. 74.
Lütcke, H., Murayama, M., Hahn, T., Margolis, D.J., Astori, S., Meyer zum Alten Borgloh, S., Göbel, W., Yang, Y., Tang, W., Kügler, S., Sprengel, R., Nagai, T., Miyawaki, A., Larkum, M.E., Helmchen, F., Hasan, M.T., 2010. Optical recording of neuronal activity with a genetically-encoded calcium indicator in anesthetized and freely moving mice. Front. Neural Circuits 4, 9.
Mank, M., Griesbeck, O., 2008. Genetically encoded calcium indicators. Chem. Rev. 108, 1550–1564.
Mank, M., Santos, A.F., Direnberger, S., Mrsic-Flogel, T.D., Hofer, S.B., Stein, V., Hendel, T., Reiff, D.F., Levelt, C., Borst, A., Bonhoeffer, T., Hubener, M., Griesbeck, O., 2008. A genetically encoded calcium indicator for chronic *in vivo* two-photon imaging. Nat. Methods 5, 805–811.
Miyawaki, A., 2003. Fluorescence imaging of physiological activity in complex systems using GFP-based probes. Curr. Opin. Neurobiol. 13, 591–596.
Monif, M., Burnstock, G., Williams, D.A., 2010. Microglia: proliferation and activation driven by the P2X7 receptor. Int. J. Biochem. Cell Biol. 42, 1753–1756.
Nagayama, S., Zeng, S., Xiong, W., Fletcher, M.L., Masurkar, A.V., Davis, D.J., Pieribone, V.A., Chen, W.R., 2007. *In vivo* simultaneous tracing and Ca(2+) imaging of local neuronal circuits. Neuron 53, 789–803.
Nave, K.A., 2010. Myelination and support of axonal integrity by glia. Nature 468, 244–252.
Nevian, T., Helmchen, F., 2007. Calcium indicator loading of neurons using single-cell electroporation. Pflugers Arch. 454, 675–688.
Nimmerjahn, A., Kirchhoff, F., Kerr, J.N., Helmchen, F., 2004. Sulforhodamine 101 as a specific marker of astroglia in the neocortex *in vivo*. Nat. Methods 1, 31–37.
Nimmerjahn, A., Kirchhoff, F., Helmchen, F., 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. Science 308, 1314–1318.
O'Donovan, M.J., Bonnot, A., Wenner, P., Mentis, G.Z., 2005. Calcium imaging of network function in the developing spinal cord. Cell Calcium 37, 443–450.
Ruscheweyh, R., Ikeda, H., Heinke, B., Sandkuhler, J., 2004. Distinctive membrane and discharge properties of rat spinal lamina I projection neurones *in vitro*. J. Physiol. 555, 527–543.
Schnell, L., Fearn, S., Klassen, H., Schwab, M.E., Perry, V.H., 1999. Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. Eur. J. Neurosci. 11, 3648–3658.
Schouenborg, J., 2008. Action-based sensory encoding in spinal sensorimotor circuits. Brain Res. Rev. 57, 111–117.
Schultz, S.R., Kitamura, K., Post-Uiterweer, A., Krupic, J., Hausser, M., 2009. Spatial pattern coding of sensory information by climbing fiber-evoked calcium signals in networks of neighboring cerebellar Purkinje cells. J. Neurosci. 29, 8005–8015.
Silver, J., Miller, J.H., 2004. Regeneration beyond the glial scar. Nat. Rev. Neurosci. 5, 146–156.
Stosiek, C., Garaschuk, O., Holthoff, K., Konnerth, A., 2003. *In vivo* two-photon calcium imaging of neuronal networks. Proc. Natl. Acad. Sci. U. S. A. 100, 7319–7324.
Sullivan, M.R., Nimmerjahn, A., Sarkisov, D.V., Helmchen, F., Wang, S.S., 2005. *In vivo* calcium imaging of circuit activity in cerebellar cortex. J. Neurophysiol. 94, 1636–1644.
Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R., Bargmann, C.I., Jayaraman, V., Svoboda, K., Looger, L.L., 2009. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat. Methods 6, 875–881.
Todd, A.J., Puskar, Z., Spike, R.C., Hughes, C., Watt, C., Forrest, L., 2002. Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance p-containing afferents and respond to noxious stimulation. J. Neurosci. 22, 4103–4113.
Torsney, C., Anderson, R.L., Ryce-Paul, K.A., MacDermott, A.B., 2006. Characterization of sensory neuron subpopulations selectively expressing green fluorescent protein in phosphodiesterase 1C BAC transgenic mice. Mol. Pain 2, 17.
Tsuda, M., Tozaki-Saitoh, H., Inoue, K., 2010. Pain and purinergic signaling. Brain Res. Rev. 63, 222–232.
Vajkoczy, P., Laschinger, M., Engelhardt, B., 2001. Alpha4-integrin-VCAM-1 binding mediates G protein-independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. J. Clin. Invest. 108, 557–565.
Wang, H., Huff, T.B., Fu, Y., Jia, K.Y., Cheng, J.X., 2007. Increasing the imaging depth of coherent anti-Stokes Raman scattering microscopy with a miniature microscope objective. Opt. Lett. 32, 2212–2214.
Warp, E., Agarwal, G., Wyart, C., Friedmann, D., Oldfield, C.S., Conner, A., Del Bene, F., Arrenberg, A.B., Baier, H., Isacoff, E.Y., 2012. Emergence of patterned activity in the developing zebrafish spinal cord. Curr. Biol. 22, 93–102.
Wilson, J.M., Cowan, A.I., Brownstone, R.M., 2007a. Heterogeneous electrotonic coupling and synchronization of rhythmic bursting activity in mouse Hb9 interneurons. J. Neurophysiol. 98, 2370–2381.
Wilson, J.M., Dombeck, D.A., Diaz-Rios, M., Harris-Warrick, R.M., Brownstone, R.M., 2007b. Two-photon calcium imaging of network activity in XFP-expressing neurons in the mouse. J. Neurophysiol. 97, 3118–3125.
Wilson, J.M., Blagovechtchenski, E., Brownstone, R.M., 2010. Genetically defined inhibitory neurons in the mouse spinal cord dorsal horn: a possible source of rhythmic inhibition of motoneurons during fictive locomotion. J. Neurosci. 30, 1137–1148.
Ylera, B., Erturk, A., Hellal, F., Nadrigny, F., Hurtado, A., Tahirovic, S., Oudega, M., Kirchhoff, F., Bradke, F., 2009. Chronically CNS-injured adult sensory neurons gain regenerative competence upon a lesion of their peripheral axon. Curr. Biol. 19, 930–936.
Yoshimura, M., Jessell, T.M., 1989. Primary afferent-evoked synaptic responses and slow potential generation in rat substantia gelatinosa neurons *in vitro*. J. Neurophysiol. 62, 96–108.
Zeilhofer, H.U., Studler, B., Arabadzisz, D., Schweizer, C., Ahmadi, S., Layh, B., Bösl, M.R., Fritschy, J.M., 2005. Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. J. Comp. Neurol. 482 (2), 123–141.
Zhong, G., Droho, S., Crone, S.A., Dietz, S., Kwan, A.C., Webb, W.W., Sharma, K., Harris-Warrick, R.M., 2010. Electrophysiological characterization of V2a interneurons and their locomotor-related activity in the neonatal mouse spinal cord. J. Neurosci. 30, 170–182.