Two-Photon Calcium Imaging of Network Activity in XFP-Expressing Neurons in the Mouse

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INTRODUCTION

Fluorescent protein (XFP) expression in neurons, driven by promoters for various cellular proteins including transcription factors (Goulding et al. 2002; Tanabe and Jessell 1996) and calcium binding proteins (Meyer et al. 2002), is increasingly used to identify and study subsets of postnatal neurons in the CNS. Anatomical and physiological properties of XFP-expressing neurons can be studied using established techniques, such as immunohistochemistry and microelectrode recording; this was previously done in hippocampus (Oliva et al. 2000), cortex (Meyer et al. 2002), and spinal cord (Alvarez et al. 2005; Dougherty et al. 2005; Wilson et al. 2005). However, these methods are not generally adequate to address the spatiotemporal pattern of activity of XFP neurons within neuronal networks and thus their role in producing behaviors.

Rhythmic locomotor activity generated by spinal interneuron networks was previously studied using multiple microelectrode recordings in nonmammalian species such as lamprey (Buchanan and Grillner 1988) and Xenopus (Roberts et al. 1998). In these simpler networks, interneurons can be defined by their physiological properties and their roles in network function can be studied. However, obtaining similar recordings from the mammalian spinal cord presents more complex challenges because the interneurons are more numerous, less accessible, and not readily classified either morphologically or electrophysiologically.

The use of calcium imaging to study spinal locomotor network function was first investigated using charge-coupled detector cameras in the embryonic chick spinal cord (O’Donovan et al. 1994) and later using confocal microscopy (Fetz and O’Malley 1995) and 2PE LSM in the in vivo zebrafish spinal cord (Brustein et al. 2003) and in the isolated mouse spinal cord (O’Donovan et al. 2005). These studies focused on motoneurons, which were identified by selective application of retrogradely transported calcium indicators. In the zebrafish olfactory bulb, mitral cells were identified with the genetic marker HuC:cameleon, and subsequent 2PE LSM imaging of a calcium indicator loaded into these cells allowed the correlation of activity with cell type (Yaksi and Friedrich 2006). The application of 2PE LSM combined with calcium indicators to investigate the role of defined populations of interneurons in mammalian central networks has not yet been demonstrated.

A major obstacle in performing calcium imaging with 2PE LSM in labeled interneurons lies in the overlapping spectral characteristics of the optimal calcium indicators and XFPs. The properties of calcium indicators that make them most suitable for in situ physiological experiments include: ready introduc-
tion into the intracellular environment; favorable $K_d$ values for recording the intracellular calcium changes; and large signal change on calcium binding (Knopfel et al. 2006). However, indicators that fulfill these criteria, including Calcium Green-1 and Fluo-3, have similar excitation and emission spectra to the most commonly used XFPs: green and yellow fluorescent proteins (GFPs and YFPs, respectively) (Bolsover et al. 2001).

Here, we present the methodology to study 2PE LSM imaging of calcium activity in GFP- or YFP-expressing neurons in both the mouse spinal cord slice and the isolated intact spinal cord preparations. This allows for the identification and study of defined interneuron populations during network activity and fictive locomotion.

**Methods**

**Animal preparation**

All procedures and experiments were approved by both the Dalhousie and Cornell University Committees for Laboratory Animals and conform to the standards set by the Canadian and United States Council of Animal Care, respectively. Experiments involved the use of Hb9:eGFP (Wichterle et al. 2002; Wilson et al. 2005), GAD65:GFP [from the Genstat project (www.genstat.org; Heintz 2001), and Lhx3-Cre (Sharma et al. 1998) × Thy1-lox-STOP-lox-YFP (Feng et al. 2000) transgenic mice and ICR wild-type mice (Taconic Farms, Hudson, NY). Experiments were performed using spinal cords of postnatal day 3 (P3) to P6 mice. Animals were either deeply anesthe-
tized with ketamine (100 mg/kg administered intraperitoneally; Dal-
housie University) before decapitation or killed by decapitation with-
out prior anesthesia (Cornell University); laminectomies were per-
formed and the thoracolumbar spinal cords were removed under cold (<4°C) artificial cerebral spinal fluid (aCSF). The dissection of cords was undertaken in sucrose-substituted aCSF (Jiang et al. 1999). For the spinal cord slice experiments, the low-thoracic to midlumbar region was embedded in Agarose (Type IX-A, Sigma) in a custom-designed chamber and transversely slices were cut (200–300 μm) with a vibrating microtome (Vibratome 3000; Vibratome, St. Louis, MO). Either a slice or the whole or hemisected in vitro spinal cord prepara-
tion was then transferred to a chamber on the microscope superfused with warm aCSF at 30°C (Figs. 2–5) or about 25°C (Fig. 1). For the in vitro spinal cord preparation, suction electrodes were attached to bilateral (whole cord) or unilateral (hemisected spinal cord) lumbar ventral roots (Jiang et al. 1999) to monitor fictive locomotion. The aCSF, superfused during the experiment, contained (in mM): 127 NaCl, 1.9 KCl, 1.2 KH₂PO₄, 2.4 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃, and 10 g-glucose.

**Calcium dye loading**

An initial 1 mM stock solution of Fluo-3 AM (Invitrogen, Burling-
ton, Canada), dissolved in dimethyl sulfoxide and 20% pluronic plu-
ronium acid, was diluted with aCSF to obtain the final loading concentra-
tion. This final solution was filtered (0.2 μm) and immediately backloaded into a pipette for pressure injection. In the slice preparation, the loaded pipette was placed lateral to the GFP neurons of interest because neurons directly adjacent to the pipette tip were subject to pressure damage. To position the injection pipette in the whole cord prepara-
tion through the dorsal spinal cord, lumbar dorsal roots were removed. Using a technique similar to that described by Stosiek et al. (2003),
positive pressure (100–200 mmHg for an average of 2 min) was applied through the injection pipette. We injected the dye at a concen-
tration of 100 μM (or 1 mM from a 10 mM stock in some cases); initial studies using 10 μM dye gave reduced staining and calcium signals and were not used (data not shown) (Fig. 1: 1 mM, Figs. 2–5: 100 μM).

In the isolated spinal cord, the location of the GFP-positive neurons [i.e., ventrally located Hb9 interneurons, vs. dorsally located gluta-
mate decarboxylase (GAD) interneurons] determined the route of dye injection. The GAD65:eGFP neurons were most readily loaded and imaged in a hemisected spinal cord preparation in which the spinal cord is split through the midline, thus still allowing recording of locomotor output from the ipsilateral ventral roots. The cord was pinned cut side up so that the GAD cells were readily visible and the dye was injected through the dorsal white matter.

The Hb9 interneurons, located medially near the dorsal aspect of the ventral commissure (Hinkelley et al. 2005; Wilson et al. 2005), were initially labeled with dye injections through the ventral horn. However, this route proved unreliable because it affected ventral root output. Therefore Hb9 interneurons were labeled by injections through the dorsal horns. Although the Hb9 interneurons were not visible during these injections, measurements to their approximate location were used as a guide for pipette positioning.

To image calcium transients in slices containing YFP neurons, slices were incubated in Fura-2 AM (Invitrogen) following the meth-
ods of MacLean and Yuste (2005). Fura-2 AM (50 μg) was dissolved in 48 μl of DMSO and 2 μl of 20% pluronic acid to make a 1 mM stock solution. This stock solution was then “painted” over the slice in a chamber with a known volume of oxygenated aCSF to give a final concentration in the bath of 10 μM Fura-2.

**Data recording**

In all experiments, we waited ≥30 min for dye uptake before beginning to image. The intact spinal cord was placed ventral surface up in the chamber and suction electrodes were secured to flexor-related ventral roots (L1 or L2) and/or extensor-related roots (LS) to record motor activity. Synchronous motor bursting in all roots was induced by the addition of tetraethylammonium (TEA, 1–30 mM), 4-aminopyridine (4-AP, 1–4 mM), 2,3-dihydroxy-6-nitro-7-sulfa-
moyl-benzof[1,3]oxazine-2,3-dione (NBQX, 5 μM), 2-amino-5-
phosphonopentoanoic acid (AP-5, 10 μM), bicusculine (10 μM), and strychnine (1 μM). Alternatively, fictive locomotion (with alternating bursts between flexors and extensors) was induced by the addition of N-methyl-d-aspartate (NMDA, 5 μM), 5-hydroxytryptamine [seroto-
nin (5-HT), 10 μM], and/or dopamine (DA, 50 μM) as previously described (Jiang et al. 1999; Zhong et al. 2006). Interneurons were visualized with an upright Zeiss Axiom II microscope equipped with a LSM510 NLO Meta scan head (Carl Zeiss, Toronto, Canada) and a tunable near-infrared Ti:Sapphire laser (Chameleon XR, Coher-
et, Santa Clara, CA; Figs. 2–5) or using an upright Olympus BX50WI microscope equipped with a Radiance 2000 scan head (Bio-Rad) and a tunable near-infrared Ti:Sapphire laser (Spectra-
Physics; Fig. 1). We used both trans- and epi-collection using a H7422

**Calcium transients**

Calcium transients were recorded as changes in mean pixel inten-
sity in a defined region of interest (neuronal soma) over time and expressed as the change in fluorescence divided by the baseline

**Innovative Methodology**
fluorescence ($\Delta F/F$). These data were initially analyzed in Microsoft Office Excel 2003 and subsequently imported, along with the corresponding Axioscope file, into custom LabVIEW software developed at Cornell University or custom software developed at the Spinal Cord Research Centre, University of Manitoba (SCRC Data Capture and Analysis Software: http://www.scrum.umanitoba.ca/doc/) that ran on Mac OS X. These analysis packages were modified to allow the alignment of the calcium imaging waveform with the TTL pulses, and thus with ventral root recordings. For publication purposes only, the TIFF images (except those in Fig. 1) were filtered with an “A Trous” thresholding algorithm filter to remove the noise, using ImageJ software (WS Rasband, National Institutes of Health, http://rsb.info.nih.gov/ij/1997–2006). Figures were compiled using SigmaPlot 9, Adobe Illustrator, and Photoshop.

RESULTS

Calcium imaging in spinal interneurons

Based on recent methods to load large populations of neurons with calcium-sensitive dyes in intact preparations (Stosiek et al. 2003), we began by testing the suitability of these methods for recording neuronal population activity during fictive locomotion in intact spinal cords (lacking XFP-labeled neurons). We tested various dyes, loading times, and ejection pressures. Despite repeated attempts, neither Calcium Orange-AM nor Rhod2-AM was found to efficiently label groups of spinal cord neurons (Supplementary Fig. S1, A and B).1 Calcium Green-1-AM and Fluo-3-AM were found to have the highest labeling efficiency (Supplementary Fig. S1, C and D). This was assessed by noting the number of cells labeled, the uniformity of labeling, and the cell fluorescence versus background within a single preparation. Neurons filled with Fluo-3 typically had the highest signal-to-noise ratios (around 10, but as high as 30) for recording neural activity during fictive locomotion in vitro spinal cord (where the locomotor pattern-generating networks are thought to reside) (Cazalets et al. 1995; Kjaerulff and Kiehn 1996; y Ribotta et al. 2000) while simultaneously recording transmitter-induced fictive locomotor activity in the ventral roots ($n = 5$). An example of one of these recordings is seen in Fig. 1Bi, which was filtered and rectified in Fig. 1Bii. In this and other experiments, we found that cell activity could be subdivided into four general classes: 1) class 1: cells showing no significant activity above the noise level during the

FIG. 1. Calcium activity of many individual cells in the isolated intact spinal cord preparation [lacking fluorescent protein (XFP)–labeled neurons]. A: projection image (generated from a time-series movie) of the medial L1 region about 100 μm below the ventral surface showing many labeled cells after Fluo-3 dye injection. Spinal cord midline is located at the bottom of the image. Fluorescence activity of 20 cells (numbered in A; colored traces in B) was recorded in response to N-methyl-D-aspartate (NMDA, 6 μM) and 5-hydroxytryptamine [serotonin (5-HT), 9 μM] bath application and time was synchronized to the contralateral L2 ventral root activity (raw signal in B1; rectified and integrated in Bii). B: based on their calcium activity patterns, the Fluo-3–labeled cells were separated into 4 groups: those showing no significant activity above the noise level during the recording period (Biii), those showing slow changing oscillations of activity lasting ≥20 s (Biv), those showing a few bursts of activity (≤10–20 s) during the recording period (Bv), and those showing an oscillatory activity pattern with a set phase with respect to the locomotor pattern (Bvi) (boxed region expanded in inset). Ci: time projection image in the medial L2 region nearly 100 μm below the ventral surface shows many labeled cells after Calcium Green-1 dye injection. Midline is located toward the bottom of the image. Raw contralateral L2 ventral root activity is seen in Cii and the rectified and integrated in activity in Ciii. Labeled cells in Cii show an oscillatory fluorescence activity pattern that peaked out of phase with the contralateral ventral root bursts (Civ). Scale bars: 20 s, 10% $\Delta F/F$ for all fluorescence traces.

1 The online version of this article contains supplemental data.
recording period (Fig. 1Biii); 2) class 2: cells showing slowly changing activity (>20 s in duration) that were not correlated with the locomotor pattern (Figs. 1Biv); 3) class 3: cells showing a few bursts of activity (<10–20 s in duration), which were not correlated with the ongoing locomotor pattern during minutes of recording time (Fig. 1Bv); or 4) class 4: cells showing an oscillatory activity pattern with the same cycle period as that of the ventral root activity (Fig. 1Bvi). Based on previous reports (Stosiek et al. 2003), the activity seen in classes 3 and 4 are likely correlated with bursts of action potentials; the slow changing activity seen in class 2 may also arise from spiking activity, but these cells show a greater similarity to the slowly varying calcium activity of astrocytes (Nimmerjahn et al. 2004). Interestingly, the groups of interneurons that were rhythmically active in phase with fictive locomotion (Fig. 1Bv) were located in close proximity to one another, in clusters in the ventromedial region. These neurons had identical oscillatory phasing and were out of phase from the contralateral ventral root locomotor output. These observations demonstrate the suitability of the bulk dye-loading technique to record the activity of large populations of unidentified neurons in the spinal cord. However, they emphasize the lack of critical information concerning cell identity, making such recordings ambiguous with regard to understanding the networks that drive locomotion. For this reason, it was necessary to design methodology that enabled calcium imaging of neurons genetically identified by their expression of XFPs.

**Calcium imaging in GFP-labeled neurons**

To obtain wide spectral separation between GFP and the calcium indicators (Bolsover et al. 2001), we initially tested the red variant calcium indicators (Fura-red, X-Rhod-1). However, as noted earlier, difficulties with dye loading, intracellular compartmentalization, and lack of discernable activity in response to the chemicals that induce fictive locomotion showed that these dyes could not be used for monitoring the cytosolic calcium changes associated with network activity. Our alternative approach was based on the reasoning that: 1) the fluorescent changes recorded with a “good” calcium indicator would sufficiently override any background fluorescence from the GFP and 2) any remaining background GFP signal could be reduced further with careful selection of excitation wavelengths and emission filters. Using this approach along with the success demonstrated earlier (Fig. 1), we selected Fluo-3, which has a large signal-to-noise ratio and a peak two-photon excitation cross section at a shorter wavelength than that of GFP. We also took advantage of the short-tail emission spectra of GFP to separate the emission from the two dyes.

GFP-positive neurons could be initially identified before Fluo-3 labeling by two-photon excitation of GFP at 900 nm (Mainen et al. 1999; Xu et al. 1996) and emissions collected either through a 515-nm long pass (LP) filter or a 465- to 495-nm band-pass (BP) filter. After Fluo-3 loading, GFP interneurons could be readily identified by excitation of Fluo-3 at 900 nm and collection of emissions through a 465- to 495-nm BP filter (Fig. 2A). Using this combination, Fluo-3 fluorescence cannot be discerned. To visualize the Fluo-3 loading of neurons, the emission fluorescence was filtered through a 500- to 550-nm BP filter, allowing both GFP and Fluo-3 to be visualized (Fig. 2B). To selectively monitor calcium changes in the GFP-positive neurons, the excitation wavelength was then changed to 800 nm and the laser power reduced. This reduced the excitation of GFP (Xu et al. 1996). The fluorescence emission from the Fluo-3 was collected through a 500- to 550-nm BP filter (Fig. 2C). At this excitation wavelength, the calcium-free form of Fluo-3 is minimally excited, whereas the calcium-bound form is intensely fluorescent (Eilers and Konnerth 2000; Minta et al. 1989). Therefore any increase in calcium activity will be readily observed as an increase in fluorescence.

**Hb9 interneurons**

The Hb9 interneurons are a subset of medially clustered interneurons that are rhythmically active during fictive locomotion (Hinckley et al. 2005) and that have intrinsic, conditional oscillatory properties in the presence of NMDA, 5-HT, DA, and tetrodotoxin (Wilson et al. 2005). Thus they are of interest as potential components of the spinal locomotor pattern generator. In the whole cord preparation (n = 4), Hb9-eGFP interneurons (n = 8) were located and imaged 130–180 μm deep to the ventral surface, simultaneously with nearby cells (Fig. 3A; Wilson et al. 2005). Addition of blockers of synaptic transmission and of potassium currents is known to evoke a synchronous oscillatory motor pattern in all the ventral roots (Bracci et al. 1998; Taccola and Nistri 2004). Under these conditions of synchronous ventral root output, both GFP-positive Hb9 interneurons and GFP-negative interneurons in this region were found to show strong rhythmic oscillations of calcium signals. In addition, all the neurons recorded in this region under these conditions were synchronously active (Fig. 3B). These calcium signals were readily detected even when co-localized with GFP, allowing the activity of Hb9 interneurons to be recorded and correlated with ventral root output. The onset of each calcium transient was coincident with the start of the ventral root burst (Fig. 3C, arrow). Termination of the calcium transient, however, outlasted the ventral root burst (Fig. 3C, arrowhead) likely as a result of slow cytosolic calcium buffering capacity combined with slow dye–calcium dissociation times rather than long-duration action potential bursting (Eilers and Konnerth 2000).
GABAergic interneurons

In GAD65-GFP mice, a subpopulation of GABAergic neurons that express the synthetic enzyme GAD65 are labeled with GFP; these GFP-positive interneurons are located in the dorsal horn and clustered bilaterally near Clarke’s column (E Blagovechtchenski, J. Wilson, and R. Brownstone, unpublished observations). In the midline hemisected spinal cord preparation (n/H110052), GFP-positive interneurons (n/H110057) were identified after bulk loading with Fluo-3 (Fig. 4A), and the rhythmic activity of these GAD65 neurons was imaged during fictive locomotion (Fig. 4B). Although the signal-to-noise ratio in these particular recordings was lower than that shown in Fig. 3, it was clear that the GAD65 interneurons were active with a relatively fixed phase compared with the ipsilateral flexor ventral root activity: in the example of Fig. 4B, each peak of activity of the GAD65 neuron occurred out of phase with the ventral root discharges. Other nearby GFP-negative neurons were also rhythmically active with different phase relationships to the ventral root discharges. This can readily be seen in the overlay shown in Fig. 4C.

Calcium imaging in YFP-labeled neurons

Because the nuances of GFP excitation and emission spectra that enabled the separation of GFP and Fluo-3 (see above) do not exist in the spectra of YFP, we found that it was not possible to definitively identify YFP-positive neurons in the presence of Fluo-3. Specifically, there is no “shoulder” on the YFP emission spectrum <500 nm, making it impossible to collect any signal in this wavelength range (e.g., using a 465- to 495-nm BP filter). It was therefore necessary to collect fluorescence >500 nm (e.g., using either a 500- to 550-nm BP or an LP 515-nm filter). However, in neurons labeled with Fluo-3, the Fluo-3 emissions in the 500- to 550-nm range occluded the YFP emissions, thus rendering impossible the definitive identification of YFP neurons after loading. Therefore it was necessary to use a different calcium indicator in YFP-expressing transgenic mice.

Based on published two-photon excitation cross sections, we selected Fura-2 because its two-photon excitation spectra peak at significantly shorter wavelengths (about 750 nm) than YFP (about 970 nm) (Heikal et al. 2000; Xu et al. 1996). Therefore...

FIG. 4. 2-Photon excitation calcium imaging of GAD65:GFP interneurons in the hemisected spinal cord. A: 2 GAD interneurons (GFP positive) were identified with excitation wavelengths of 900 nm and imaging of Fluo-3 was performed with 800-nm excitation. B: ventral root output was bilaterally synchronous in the presence of TEA, 4-AP, NBQX, AP5, bicuculline, and strychnine. Many interneurons, including the Hb9:GFP interneurons (1–3), could be imaged simultaneously with ventral root recording and were observed to be bursting in phase with ventral root output. Other nearby GFP-negative interneurons (4–7) also showed rhythmic bursting activity that was correlated to the ventral root motor output. C: overlay of the calcium transients with the ventral root bursts (taken from the region underlined in B) indicates the correlation of the onset of the bursts (arrow) and the discrepancy in the off times (arrowhead). Scale bar in A: 20 μM; scale bars in B represent 40 s, 100% ΔF/F.
these two fluorophores could be distinguished based on excitation wavelengths. In spinal cord slices that contained YFP-positive neurons, Fura-2-AM was bath loaded as in previous studies (MacLean and Yuste 2005). YFP-positive neurons were identified with the longest available excitation wavelengths on our laser (940–960 nm) and emissions collected through either a 500- to 550-nm BP or a LP 515-nm filter. The signal, although weak, was well above background, allowing unambiguous identification of the YFP neurons (Fig. 5A).

Fura-2 was excited at 780–800 nm and emissions were collected with a 435- to 485-nm BP filter (Fig. 5A). The power required to excite YFP at 780–800 nm was found to be at least four times that required to excite Fura-2, so at low laser powers, YFP fluorescence did not interfere with the Fura-2 recordings. Although Fura-2 is a ratiometric dye, 780 nm is past the isosbestic point and therefore any increase in calcium activity is observed as a decrease in fluorescence.

In the slice, we induced a generalized increase in excitability by addition of the potassium channel blockers TEA and 4-AP (Bracci et al. 1998; Taccola and Nistri 2004). Coincident with this increase in excitability, downward calcium transients were observed, indicating an increase in calcium activity in the YFP-positive neurons (Fig. 5B). These were not synchronous between the two YFP neurons recorded simultaneously. We also spent considerable effort to load Fura-2-AM into the whole cord of YFP-positive mice. Unfortunately, few cells readily loaded and, in those that did, activity changes could not be readily discerned.

**DISCUSSION**

This research describes the methodology by which two-photon excitation LSM, calcium imaging, and genetic identification of neurons can be combined to simultaneously study the activity of more than one identified neuron in the slice. This technique also permits imaging the activity of genetically identified neurons located deep in intact spinal cords and correlation of their activity with locomotor network output. This method complements single-cell electrophysiological analysis of neurons during locomotor activity.

There are two major benefits to using 2PE LSM in addition to conventional electrophysiological techniques [either blind patch-clamp techniques or through the use of infrared DIC video microscopy (IR-DIC; Dodt and Zieglgansberger 1990)] to investigate network function in the mammal. First, it is possible to study neurons deep in the spinal cord with 2PE microscopy because the scattering of fluorescence photons does not reduce image quality so long as they reach a detector. This results in the improved visualization of neurons under study, which in turn results in the ability to target specific neurons for study. Second, the activity of many such identified neurons can be recorded simultaneously, as demonstrated here.

The methods described here can be readily generalized to functional recordings of genetically identified neuronal populations throughout the mammalian CNS. We used a spinal cord preparation that allowed for the correlation of the behavioral rhythmic output of the network (ventral root locomotor rhythms) with the activity of identified neurons. Although it has been known for almost a century that spinal cord interneuronal networks generate the rhythm and pattern of locomotor function (Brown 1911), the unambiguous identification and subsequent study of these interneurons continues to pose many challenges (see Jankowska 1992). Recent work to understand the transcription factor coding of neuronal identity, combined with transgenic technology, enables the expression of fluorescent proteins in subsets of developmentally defined neurons (Briscoe et al. 2000; Wichterle et al. 2002). In addition to the anatomical and electrophysiological properties of these neurons that can be studied with conventional techniques (Alvarez et al. 2005; Gosgnach et al. 2006; Wilson et al. 2005), we demonstrate here that the activity of populations of these neurons can be determined at single-cell resolution with 2PE LSM calcium imaging techniques. This technique lays the foundation for mapping locomotor-related networks in the spinal cord. Although network mapping was previously undertaken in the cerebellar cortex (Sullivan et al. 2005), entorhinal cortex (Hafting et al. 2005), visual cortex (Ohki et al. 2005), neocortex (MacLean et al. 2005), and pre-Bötzing complex (Fisher et al. 2006), the technique presented here adds the potential to identify specific classes of neurons in each of these structures and to monitor their activity. Therefore in the context of spinal cord networks that organize locomotion, we can begin investigations into the roles of identified populations of neurons in different aspects of the behavior, including rhythm and pattern generation.

The primary challenge in combining calcium imaging with identified XFP-expressing neurons concerns the spectral characteristics of the fluorophores. Ideally, to achieve complete spectral separation, a red indicator (X-rhod-1) could be combined with GFP or Fura-red with blue fluorescent proteins (Bolsover et al. 2001). However, because of the positive charge of the rhod-2 derivatives including X-rhod-1, they often preferentially accumulate in mitochondria and other subcellular compartments (Minta et al. 1989; Rutter et al. 1996) and cannot be used to monitor changes in cytosolic calcium. Rhod-2 was

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**FIG. 5.** 2-Photon imaging of yellow fluorescent protein (YFP) interneurons in the spinal cord slice. **A:** after Fura-2 loading, low power imaging with laser excitation wavelength of 960 nm detects the YFP interneurons. Fura-2-calcium signal can be visualized and recorded using an excitation wavelength of 800 nm through an emission filter of 435- to 485-nm BP. **B:** calcium transients recorded in the 2 YFP-positive neurons. Note that with Fura-2, the calcium transients are downward (Fig. 5B). These transients were not synchronous between the 2 YFP neurons recorded simultaneously.
successfully loaded into the olfactory bulb of zebrafish to monitor the activity of genetically defined mitral cells (Yaksi and Friedrich 2006). Unfortunately, our attempts at multicell bolus loading using Rhod-2, Calcium Orange, or Fura-red in the spinal cord resulted in poor and/or compartmentalized labeling. As an alternative to using red calcium indicators, a red fluorescent protein (dsRed) in lieu of GFP would permit imaging to be combined with the high signal-to-noise green calcium indicators used here (e.g., Fluo-3 or Calcium Green-1). Although there have been difficulties in the incorporation of dsRed into neuronal populations in mice (Gross et al. 2000; Hadjantonakis et al. 2002; Matz et al. 1999), we hope that future attempts with this or other long-wavelength XFPs are successful because these would greatly facilitate calcium imaging studies when combined with this technique. In particular, the growing number of new colored XFPs may facilitate spectral separation (Shaner et al. 2004). In the meantime, the most widely used XFPs and calcium indicators have closely related excitation and emission spectra in the green/yellow range (GFP and YFP, Calcium Green-1, Fluo-3), which present the challenge of combining and separating these indicators.

Although Fluo-3 has emission spectra with considerable overlap with GFP emissions (Bolsover et al. 2001), we were able to identify the GFP-labeled cells based on the combination of adjusting the excitation wavelength and the use of narrow-band emission filters. Of the many calcium dyes tested, Fluo-3 resulted in the most reliable dye loading, highest signal-to-noise ratio, and least photobleaching. As seen in Figs. 3 and 4, these recordings could be used to monitor rhythmic oscillations of HB9 and GAD65 interneurons during rhythmic motor output in the intact and hemisected spinal cord, respectively. This should greatly enhance further studies into the roles of these and other neurons in the generation of the locomotor rhythm.

Fura-2 and YFP could be distinguished by their differences in excitation and emission spectra, allowing us to separate and identify YFP interneurons and image their calcium signals in the slice. However, despite considerable effort, the method of loading Fura-2 into neurons in the slice (bath applied) could not readily be transferred to the whole cord, using the pressure-injection bulk-loading method. Few cells readily loaded and, in those that did, activity could not be readily discerned. The reasons for this are not clear, but may be related to the reduced signal-to-noise ratio of Fura-2 compared with that of Fluo-3 and Calcium Green.

An advantage of studying a relatively slow rhythmic behavior like locomotion is that neurons within the spinal network exhibit oscillations in membrane potentials with bursts of action potentials. Therefore to determine activity patterns within the step cycle, the resolution of single action potentials with calcium dyes is not required. Should this information be required in other preparations, it is possible to apply temporal deconvolution algorithms to the calcium signals (Yaksi and Friedrich 2006). Of note, in both the HB9 and GAD positive neuronal populations studied here, the fast rise times of the calcium signals were sufficient to determine the onset of activity in relation to ventral root output. Thus further studies and analyses of their activity will yield valuable information about their involvement in the locomotor network.

The development and use of genetically encoded calcium indicators such as the cameleons and the pericams (e.g., Miyawaki et al. 1997; Nagai et al. 2001) combines the genetic identification of neurons with the ability to monitor the activity of those neurons. This would eliminate the need for using two separate fluorophores as demonstrated here. However, their expression and use in vivo in mammals is not trivial (Hasan et al. 2004; Tsai et al. 2003). In addition, the current generation of genetic calcium indicators have lower signal-to-noise ratios than those of exogenous dyes. Further, they exhibit nonlinear kinetics with respect to both action potential firing and calcium concentration and are considerably “less bright” than both exogenous dyes and XFPs (Pologruto et al. 2004). Therefore at present these indicators are not optimal for studying mammalian network activity. Until genetically encoded calcium indicators are optimized for use in mammals, the methodology presented herein—demonstrating 2PE calcium imaging in genetically identified neurons—is the best available technique for investigating network activity of defined populations of neurons in the mammalian CNS.

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NOTE ADDED IN PROOF

Garaschuk et al. (2006) have recently demonstrated 2PE LSM imaging of calcium dye loaded GFP+ neurons in the visual cortex.

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