Electroporation Loading of Calcium-Sensitive Dyes Into the CNS

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INTRODUCTION

During the last decade, calcium imaging has become a powerful tool for analyzing the spatiotemporal patterns of activity generated by neural networks (Bonnot et al. 2002a; Denk et al. 1994; Miyawaki 2003). Despite progress in this field, the methods used for loading neurons with calcium-sensitive dyes have two significant limitations: the restricted amount of tissue that can be labeled and/or the time taken for loading.

Many applications would benefit from the ability to label large regions of the nervous system. For example, in the spinal cord, retrograde loading has been used to successfully label two to three segments for visualizing the spatiotemporal organization of motoneuron recruitment during locomotor-like activity (Bonnot et al. 2002b). However, the network producing this activity spans many more segments, including those from the sacralcaudal and thoracic region, and consists of both motoneuron and interneuron populations. Voltage-sensitive dyes have been used to monitor the population activity over several centimeters of cortex (for review see Wu et al. 1998), but the signals are small and subject to photobleaching, and the method does not provide cellular resolution in the vertebrate brain.

Membrane-permeant dyes can be loaded quickly (1–2 h) by direct application to exposed neurons (O’Donovan et al. 1994; Yuste and Katz 1991) or by injection into neural tissue (Stosiek et al. 2003). Tissue injection has been used to label the spinal cord of the larval zebrafish (Brustein et al. 2003), but the method relies on the ability of the dye to diffuse throughout the limited volume of the larval cord. In the mammalian cortex, unless several injections are made, labeling is restricted to a sphere ~300 μm in diameter following a single bolus injection (Stosiek et al. 2003). Recently, membrane-impermeant dyes have been applied to tissues with a “gene gun” (Kettunen et al. 2002), allowing a rapid labeling of a few cells, but the targeted tissue remains superficial, and both the density and extent of the labeling are limited.

Another approach has been to apply membrane-impermeant (O’Donovan et al. 1993) or membrane-permeant dyes (Koshiba and Smith 1999) into an available tract of axons to allow retrograde transport to cells bodies. The most spatially extensive labeling (2–3 spinal cord segments) has been accomplished by retrogradely loading spinal motoneurons (Bonnot et al. 2002b) or spinal interneurons projecting into the white matter (A. Bonnot, unpublished data). However, the time required for transport to cell bodies in vitro (6–14 h) compromises the viability of the neural tissue. Furthermore, the target neurons must have a tract of axons available, and labeling is restricted to this specific population.

We now describe a new method—electroporation—that overcomes these limitations. In this technique, an electric field is imposed across a population of cells to transiently introduce membrane pores through which large molecules can enter. Electroporation has frequently been used to introduce genes, antibodies, dyes, and other molecules into cultured cells or cell suspensions (Neumann et al. 1982; Weaver 1993). In addition, it has been used in vivo to visualize cell migration and gene function in the developing nervous system (for reviews, see Ogura 2002; Trezise et al. 2003). While most studies have used electroporation for gene delivery, a few have used the technique to introduce fluorescent dyes into cultured neurons (Yang et al. 2004). However, with the exception of two studies in which calcium-sensitive dyes were electroporated into individual cultured neurons or neuronal precursors (Lundqvist et al. 1998; Teruel et al. 1999), no reports have described the use of electroporation to load these dyes into large populations of neurons either in vitro or in vivo. In particular, it was unclear if the technique would work for in vitro physiological studies in which neural function must be studied shortly after electroporation. In addition, the effectiveness of electroporation for loading neurons into a relatively intact tissue such as the spinal cord where the dyes do not have a direct access to exposed cells was unknown.

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Innovative Methodology

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In this study, we show the feasibility of using electroporation to introduce membrane impermeant calcium indicators into neuronal populations of the isolated mouse spinal cord in vitro. We show that the technique enables rapid loading of calcium dyes into a large number of neurons over several millimeters, throughout the thickness of the tissue, and at levels sufficient to signal neuronal activity. Furthermore, we show for the first time that calcium dyes can be electroporated into neurons after intravascular injection. A preliminary report of this work has appeared as an abstract (Bonnot et al. 2003).

METHODS

Surgical procedures

Experiments were performed on Swiss Webster neonatal mice (P0–P4; Taconic Laboratory). The mice were anesthetized with methoxyflurane, transected at T5–T6, and eviscerated. The remaining tissue was placed in a dissecting chamber and continuously perfused with a normal (in mM: 128 NaCl, 4 KCl, 1.5 CaCl2, 1 MgSO4, 0.5 NaH2PO4, 21 NaHCO3, and 30 d-glucose) or low Ca2+/high Mg2+ artificial cerebrospinal fluid (ACSF; in mM: 0.1 CaCl2 and 5 MgSO4), bubbled with 5% O2–5% CO2, at a flow rate of 35 ml/min. Initially the temperature of the ACSF was ~17°C, and it was gradually allowed to rise to room temperature (23–25°C) during the dissection. A ventral laminectomy exposed the cord, which was removed from the vertebral column together with the attached roots and the cauda equina.

Dye application and electroporation

The isolated spinal cord was pinned down with the ventral side up, and a broken sharp glass electrode was used to pressure-apply 1–2 μl of calcium-sensitive dye solution under the dura matter in the caudal lumbar area (~L5/L6) at the level of the ventral commissure (Fig. 1B). This location was chosen because the space between the dura mater and the ventral commissure allowed the injection electrode to be inserted through the dura mater without penetrating into the spinal cord, thereby allowing the dye to enter the subdural space. Proper introduction of the dye into the ventral subdural space was assessed postexperimentally on fixed sections by the absence of tissue lesions and the ventral commissure allowed the injection electrode to be placed away from the injection site. Under these conditions, this was important not to remove the pia mater extensively, even though it improved visualization, because doing so compromised coccygeal-evoked locomotor-like activity. After electroporation, the cord was left for 1 h to allow both wash-out of the excess of dye from the extracellular space and better visualization of the labeled cells. We took several precautions to minimize the electroporation-induced depression of neural activity: 1) voltage, duration, and number of electroporation pulses were set as low as possible while still allowing enough penetration of the dye into the cells, 2) the temperature was generally kept below 20°C—a temperature at which motor networks remain mostly silent—during electroporation, and 3) we used a low Ca2+/high Mg2+ solution in most (19/27) of our injected, electroporated, and recorded preparations to block electroporation-induced transmitter release.

Electrophysiology

Tight-fitting plastic suction electrodes were placed on individual ventral and dorsal roots to record either motoneuron electrical activity or to stimulate afferent or efferent axon bundles. They were placed within the electroporated area (L6) as well as more rostrally (L7–L3) outside the electroporated area. The recorded signals were filtered (DC to 3 kHz), digitized (NeuroData), and recorded on videotape. Episodes of data were acquired off-line using Clampex v.9 and analyzed with Clampfit (Axon Instruments) and Excel software. Spinal motoneurons were activated antidromically by square wave stimulation of the ventral roots (stimulus duration: 500 μs, trains 5–50 Hz, stimulus intensity: 50–80 μA, train duration: 2 s). To assess the recovery of function after electroporation, we recorded monosynaptic and polysynaptic reflex ventral root potentials evoked by a single electrical stimulus applied to a lumbar dorsal root (stimulus duration: 200 or 500 μs, stimulus intensity: 18–99 μA). Several electroporation protocols were sometimes performed on the same preparation. The monosynaptic response was measured from the peak-to-peak amplitude of the initial reflex spike, and the polysynaptic response was measured as the peak amplitude of the electronic potential within the first 50 ms after the stimulus. We also compared the potentials evoked and recorded within the electroporated area (L6) with those evoked and recorded outside the electroporated area (L7). We took several precautions to minimize the electroporation-induced depression of neural activity: 1) voltage, duration, and number of electroporation pulses were set as low as possible while still allowing enough penetration of the dye into the cells, 2) the temperature was generally kept below 20°C—a temperature at which motor networks remain mostly silent—during electroporation, and 3) we used a low Ca2+/high Mg2+ solution in most (19/27) of our injected, electroporated, and recorded preparations to block electroporation-induced transmitter release.

Intracellular recordings

Isolated spinal cords from P2–P3 neonatal mice were pinned down with the ventral side up, and the pia mater was removed before obtaining whole cell recordings from motoneurons under current clamp. Intracellular electrodes were pulled from borosilicate glass capillaries (1B100F-4, WPI) to a resistance ranging between 4–8 MΩ. The intracellular solution contained (in mM; all chemicals obtained from Sigma) 10 NaCl, 130 K-glucanate, 10 HEPES, 11 EGTA, 1 MgCl2, 0.1 CaCl2, and 1 Na3ATP; pH was adjusted with
FIG. 1. Electroporation-induced loading of calcium dyes into spinal neurons. A: schematic showing the transverse plane of the spinal cord together with different sites for dye injection. B: schematic showing the injection site for subdural dye injections and location of electroporation electrodes. C: high power view of a cell located dorsally in L1 following electroporation of calcium green salt (T12–L3). D: labeling pattern on a transverse section at L6 following electroporation of the calcium green salt (L4–S2). E: transverse section at L5 following dye injection in the ventral subdural space but no electroporation. Note absence of cellular labeling and presence of fluorescent blood vessels, particularly the central branch of the anterior spinal artery (arrowhead). F: midline sagittal section showing the labeling following electroporation of the calcium green salt (L4–S2). Vertical dotted lines indicate the approximate border between spinal segments. In all figures, dorsal is up and ventral is down.
In the confocal mode, the tissue was excited with a two-photon laser (excitation filter: 470–490 nm, emission filter: 520–560 nm) and imaged with a spinning disk confocal system (CARV, Atto Bioscience) coupled to the inverted microscope, and images were taken with a digital CCD camera (Roper Scientific). When motoneurons were labeled with fluorescein dextran, the labeling on fixed sections was checked using a confocal microscope (LSM 510, Carl Zeiss). The strength of the two-photon laser varied between 6.5 and 15% depending on the depth of the neurons. Analysis of the electrical and optical signals was performed by off-line analysis using MATLAB.

Two-photon optical imaging

To visualize neuronal calcium signals from cells embedded deep in the intact spinal cord, we used a confocal microscope (LSM 510, Carl Zeiss) equipped with a two-photon laser (Coherent). The confocal system consisted of an upright microscope (Axioskop 2 FS MOT, Carl Zeiss), allowing the emitted light to be directed to either a digital CCD camera (CoolSNAP HQ, Roper Scientific), or a photo-multiplier tube (PMT) in the scan head of the microscope (internal descanned 100 neuros activity) was measured in a grid superimposed on the visualized frame store. The fractional change in fluorescence (ΔF/F with respect to baseline fluorescence in the absence of evoked or spontaneous activity) was measured in a grid superimposed on the visualized field (~100 × 100 μm). At 10× magnification, the grid divided the cord in six columns dorsoventrally, and the average activation of the ventral third, the intermediate part, and the dorsal third were compared during different stimulation protocols (ventral root train, dorsal root single, coccyeal train). One-way ANOVA statistical tests were used to establish the probability that variations of fluorescence induced by a given stimulus were significantly different in ventral, intermediary, and dorsal parts of the cord.

Sync signal indicated the beginning of the scanning for each frame and was acquired on clampex v.9 along with ventral root signals. Frame acquisition was performed at 12-bit resolution with scan times from 37.7 to 294.9 ms. To limit photobleaching, a low magnification 10× water-immersion objective was used (Achromat with 0.3 NA, Carl Zeiss). The strength of the two-photon laser varied between 6.5 and 15% depending on the depth of the neurons. Analysis of the electrical and optical signals was performed by off-line analysis using MATLAB.

Results

The experiments reported in this study were performed on 70 isolated spinal cords of the neonatal mouse (P0–P4). In the first section of this paper, we present data from 49 experiments in which the cords were fixed, sectioned (transverse or sagittally), and examined with confocal microscopy. In 41 of these experiments, electroporation was performed after dye injection in one of several locations: the subdural space at the level of the ventral commissure (lumbar levels L4–L6, n = 33), the anterior spinal artery on the ventral surface of the cord (L3, n = 4), or the central canal (L3, n = 4; Fig. 1A). The electroporation electrodes were typically positioned within the L4–S1 region on either side of the cord (Fig. 1B), except for one experiment in which electroporation was performed ventro-dorsally. We used

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The cord was placed in a perfusion chamber with a cover slip as its base. The chamber was mounted on an inverted epifluorescence microscope (Axioskop Nikon) and continuously perfused with ACSF. The tissue was illuminated with a 75 W Xenon light source (excitation filter: 470–490 nm, emission filter: 520–560 nm) and viewed through the lateral white matter (see Fig. 6A). Suction electrodes were placed as indicated above, and activity-dependent changes in fluorescence were detected using an intensified CCD camera (Stanford Photonics) and stored on videotape (SONY SVO 9500MD). Changes in fluorescence were monitored on-line using a PC-based CCD camera (CoolSNAPHq, Roper Scientific), or a photo-multiplier tube (PMT) in the scan head of the microscope (internal descanned 100 neuros activity) was measured in a grid superimposed on the visualized frame store. The fractional change in fluorescence (ΔF/F with respect to baseline fluorescence in the absence of evoked or spontaneous activity) was measured in a grid superimposed on the visualized field (~100 × 100 μm). At 10× magnification, the grid divided the cord in six columns dorsoventrally, and the average activation of the ventral third, the intermediate part, and the dorsal third were compared during different stimulation protocols (ventral root train, dorsal root single, coccyeal train). One-way ANOVA statistical tests were used to establish the probability that variations of fluorescence induced by a given stimulus were significantly different in ventral, intermediary, and dorsal parts of the cord.

Widefield epifluorescent optical imaging

To visualize neuronal calcium signals from cells embedded deep in the intact spinal cord, we used a confocal microscope (LSM 510, Carl Zeiss) equipped with a two-photon laser (Coherent). The confocal system consisted of an upright microscope (Axioskop 2 FS MOT, Carl Zeiss), allowing the emitted light to be directed to either a digital CCD camera (CoolSNAP HQ, Roper Scientific), or a photo-multiplier tube (PMT) in the scan head of the microscope (internal descanned 100 neuros activity) was measured in a grid superimposed on the visualized field (~100 × 100 μm). At 10× magnification, the grid divided the cord in six columns dorsoventrally, and the average activation of the ventral third, the intermediate part, and the dorsal third were compared during different stimulation protocols (ventral root train, dorsal root single, coccyeal train). One-way ANOVA statistical tests were used to establish the probability that variations of fluorescence induced by a given stimulus were significantly different in ventral, intermediary, and dorsal parts of the cord.

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Retrograde labeling

In some experiments, on termination of the recordings, the ventral roots belonging to the electroporated area were retrogradely loaded with Texas red 10,000 MW (Molecular Probes) using standard techniques (O’Donovan et al. 1993). For this purpose, several adjacent lumbar ventral roots were drawn into a suction electrode containing ~6 μl solution of the dye (10 mM) dissolved in distilled H2O with 0.2% Triton X-100. The cord was left at 17°C for 7–13 h to allow labeling of motoneuron cell bodies and dendrites.

Confocal microscopy on fixed sections

At the end of the experiment, most spinal cords electroporated with a calcium-sensitive dye were fixed in 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC; Sigma-Aldrich; 40 mg/ml) as described previously (Bonnot et al. 2002a). Cords injected with fluorescein dextran were fixed in 4% paraformaldehyde (PAF). The preparations we recorded from (n = 25) were usually fixed many hours after electroporation (5–20 h), causing the labeling to be less intense because of both photobleaching and leakage of the dye out of the cells (Takahashi et al. 1999). Therefore some preparations (25) were dedicated to neuroanatomical observation (no electrical or optical recordings) and fixed within 10 min to 2 h following application of the dye, allowing optimal preservation of the labeling. The cords were cut sagittally or transversely (70 μm) using a Vibratome. Sections were viewed on an Olympus FX300 confocal microscope (Argon laser 488 nm) using Fluoview software to establish the labeling pattern in control or electroporated cords. In some initial experiments, the labeling on fixed sections was checked using a spinning disk confocal system (CARV, Atto Bioscience) coupled to the inverted microscope, and images were taken with a digital CCD camera (Roper Scientific). When motoneurons were labeled with Texas red, z-stacks were acquired at two wavelengths (argon laser, 488 nm; krypton laser, 568 nm) using the sequential mode to avoid bleed-through of the calcium green labeling into the red channel. In these double-labeling experiments, counts were made from stacks of images acquired at 40× (20–50 slices at 1 μm intervals). Images presented in this study are two-dimensional projection images from the z-stacks.

Results

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three different dyes: the calcium-sensitive dyes calcium-green dextran \((n = 15)\), the calcium green salt \((n = 25)\), or fluorescein dextran \((n = 8)\). Following electroporation, their labeling patterns in fixed sections were similar, although use of the salt appeared to produce the most homogenous labeling throughout the ventro-dorsal extent of the cord, whereas labeling obtained with the dextran tended to be clustered into particular areas of the cord. In seven control experiments, the dyes were applied as described above, but the cords were not electroporated. Under these conditions, no labeled cells were detected in fixed sections, and fluorescence could only be seen at the surface of the cord, around the central canal, and within some of the cord vasculature (Figs. 1E, 3, A and B, and 4A). In the absence of dye application \((n = 1)\), tissue autofluorescence was extremely low and did not significantly contaminate the fluorescent labeling due to the dye.

**Labeling pattern following subdural dye injection and electroporation**

Few cells were labeled if the electroporation voltage was \(< 15 \text{ V} \ (n = 2)\). In contrast, reliable cellular labeling was observed when the electroporation voltage was between 18 and 27 V, with the most extensive labeling achieved at the highest voltages \((n = 31)\). We also determined that the use of paddle electrodes \((3 \times 5 \text{ mm})\) that covered the ventro-dorsal extent of the spinal cord gave better results than thinner wire electrodes \((0.5 \text{ mm diam})\). The labeling was usually strongest at the level of the dye application and got weaker rostrally and caudally. However, the rostrocaudal extent of the labeling always covered the entire length of the electrodes \((1, 3, \text{ or } 5 \text{ mm}, \text{ 1–6 segments}; \text{ Fig. 1F})\). Outside the electroporated area, only background fluorescence and fluorescent blood vessels could be seen, providing further evidence that the electric pulses are required to introduce the dye into the cells. Although there was some variation in the labeling pattern across experiments, typically we found that cells were labeled at all tissue depths from dorsal to ventral throughout the electroporated region (Fig. 1D). Labeling was found on both sides of the cord, although the side of the cord adjacent to the positive electrode was sometimes the most densely labeled (seen in 13 experiments). Cells around the central canal were always numerous and densely labeled and in most experiments \((27/31)\), labeled cells were also abundant in the dorsal horn. The frequency of labeled cells was most variable in the ventral region of the cord. In some experiments \((19/31)\), only a few ventral cells, recognizable as motoneurons, were labeled. In contrast, in the other experiments \((12/31)\), a large proportion of the motoneurons were labeled, forming well-defined motoneuron clusters (similar to Fig. 4B). Images taken at high power revealed that labeled cells had a typical neuronal morphology and that soma and processes were both labeled (Fig. 1C). Both the nucleus and the cytoplasm of electroporated cells were labeled uniformly, although the cytoplasmic labeling was often punctate. Visualization at high power also revealed the presence of many less densely labeled cells (Fig. 2, A and C).

To show that some of the electroporated cells were motoneurons, we retrogradely labeled motoneurons with Texas red applied to the ventral roots within the electroporated area \((n = 5)\). In these experiments, we first confirmed the presence of labeled motoneurons by optical imaging of antidromic signals and applied the Texas red to the appropriate roots. In all five experiments, after the tissue was fixed and sectioned, cells were found in which the electroporated calcium green and the retrogradely loaded Texas red were co-localized (Fig. 2, arrowheads), confirming that some of the electroporated cells were motoneurons. We found that \(~ 70\%\) of the motoneurons were labeled following electroporation (one-half strongly labeled, one-half weakly labeled). Some of the electroporated cells in the motoneuron region consisted of smaller cells that were not double-labeled (see Fig. 2).

**Labeling pattern following intravascular dye injection and electroporation**

In control experiments in which the dye was injected subdurally but not subsequently electroporated, we noticed that blood vessels often contained fluorescent dye (Fig. 1E). This was not autofluorescence, because in the absence of dye, no vascular fluorescence was seen. This observation raised the possibility that the dyes might be distributed within the cord, in part, through the vasculature.

To test this idea, we injected the dyes into the anterior spinal artery at the level of the rostral lumbar segments \((\sim L_1; \ n = 6)\). In four experiments, this was followed by electroporation (remote from the injection site, see **Methods**), and in two, no electroporation was performed. In the non-electroporated preparations, blood vessels were extensively labeled both centrally (Fig. 3A) and laterally (Fig. 3B), but no fluorescent cells were detected. When this type of injection was followed by electroporation \((24–33 \text{ V})\), in addition to the fluorescent vasculature, many cells were found labeled in the central part of the spinal cord where the vasculature is particularly dense (Fig. 3, C and E). Lateral cellular labeling was also apparent in three of four cords (Figs. 3D and 4, C and F).

**Labeling pattern following central canal dye injection and electroporation**

We also observed that a subdural injection of the dye often resulted in dye entry into the central canal. This occurred even though the electrode was restricted to the subdural layer and did not penetrate the cord. This finding suggested another potential route for dye distribution. To investigate this possibility, we injected the dye directly into the central canal and electroporated the cord one to two segments caudal to the injection site \((n = 4)\). In one non-electroporated cord, the dye remained restricted to the central canal, and no cellular labeling was seen (Fig. 4A). Some blood vessels, in particular the central branch of the anterior spinal artery, were often labeled (Fig. 4A, arrowhead) as seen in control cords injected subdurally (Fig. 1E). Electroporation \((21–27 \text{ V})\) systematically induced a characteristic pattern of cellular labeling comprising two groups of laterally located cells: one in the ventral horn and another cluster of small cells in the lateral intermediate gray matter (Fig. 4B). Although more extensive, this labeling was similar to the one observed with ventral artery injection (cf. Figs. 4, B and C, D and F, and 3D and 4H). Particularly striking was the massive labeling of ventrally located cluster of cells, presumably motoneurons, occurring with this type of injection procedure (Fig. 4, D, G, and H). In these experiments, high power views of the central canal area revealed that some
cells lining the central canal (clearly seen at the top of Fig. 3E) were making contact with blood vessels, often the central branch of the anterior spinal artery, through end feet projections (Fig. 4E).

Viability of electroporated cords

To establish if electroporation compromised the viability or function of the cord, we used ventral root electrical recordings to determine if reflex and motor behaviors were preserved after electroporation. In addition, we determined if the dye-loaded cells were capable of producing optical signals in response to antidromic stimulation, reflex, and motor behaviors.

Effects of electroporation on reflex function

We first tested the effects of electroporation on mono- and polysynaptic ventral root potentials evoked by dorsal root stimulation. Previous work has shown that a suprathreshold stimulus applied to a lumbar dorsal root evokes a monosynaptic reflex on the homonymous ventral root and a polysynaptic reflex on the homonymous and adjacent ventral roots. Therefore we stimulated and recorded from one dorsal/ventral root pair outside the electroporated area (L₁) and one within the electroporated region (L₆; electroporation electrodes spanning L₅ to S₂; Fig. 5D) and monitored the responses before and after electroporation. In this way, it was possible to establish if the effect of electroporation on spinal cord function was restricted to the tissue between the electrodes.

In 7/11 experiments, the effects of electroporation on reflex function were studied in the absence of dye application. Electroporations performed between 5 and 9V (3 experiments, 7 electroporations) did not abolish monosynaptic or polysynaptic components of the reflex responses within the electroporated area (data not shown). Electroporation performed at a voltage of 9 –21 V (5 experiments, 7 electroporations) caused a temporary and local abolition of the monosynaptic and polysynaptic reflex followed by a partial or complete recovery (Fig. 5, A and B). The monosynaptic component of the dorsal root–evoked reflex recovered less completely than the polysynaptic reflex followed by a partial or complete recovery (Fig. 5, A and B). The monosynaptic component of the dorsal root–evoked reflex recovered less completely than the polysynaptic component (Fig. 5B). In five experiments, the monosynaptic reflex evoked by stimulation of dorsal root L₆ and recorded from ventral root L₆ (within the electroporated area), recovered to 54.8 ± 19.2% of its control value (data measured 62–143 min after electroporation). In contrast, over the same period, the L₆ polysynaptic dorsal root reflexes recovered to 117.3 ± 43.2% of their control value (5 experiments, Fig. 5B). In these same experiments, reflex function was also measured in L₁, outside the electroporated area. We found no depression of
either monosynaptic or polysynaptic reflexes evoked by stimulation of the L₁ dorsal root and recorded in L₁ ventral root. Instead, the reflex amplitudes were potentiated. The amplitudes of the monosynaptic and polysynaptic components of the reflex were increased to 172.8 ± 36.6 and 110.1 ± 22.3% of their respective control values.

Above 30 V (2 experiments, 2 electroporations), the reflexes evoked and recorded within the electroporated area were abolished and did not recover for the duration of the experiment (≤4 h after electroporation). However, the polysynaptic reflex evoked in L₆ ventral root by the stimulation of the L₁ dorsal root did partially recover, showing that the L₆ area is still functional at these voltages and that the dorsal roots within the electrodes are likely to be damaged by the electroporation.

Effects of electroporation on locomotor-like activity

We also examined the effects of electroporation on rhythmic locomotor-like activity generated by a train of stimuli applied to a coccygeal ganglion (n = 11; 9 electroporation alone and 2 electroporation and sub-dural dye injection). This activity com-
prises rhythmic bursts that alternate between the left and right sides and between rostral (L1) and caudal lumbar (L6) ventral roots (Whelan et al. 2000).

When the electroporation voltage was ≤9 V (3 experiments, 7 electroporations), we found little effect on this type of rhythmic activity. In contrast, when the electroporation voltage was between 9 and 21 V (7 experiments, 9 electroporations), we found that locomotor-like activity was transiently but fully depressed after electroporation (Fig. 5C). Early after electroporation, not even reflex responses to individual stimuli could
be seen. Typically, the ability to generate any kind of activity first recovered in L₁, several segments away from the rostral limit of the electroporated area (L₅). The sequence of recovery usually exhibited the following pattern: no response (1–10 min), reflex responses to individual stimuli of the train (5–15 min), sustained tonic depolarization (10–20 min), tonic bursting (15–20 min), and the first rhythmic discharges (20–30 min; Fig. 5C). A similar sequence of recovery occurred within the electroporated region (L₅) but with a 10- to 30-min delay. After rhythmic discharges had appeared on both roots, the quality of the rhythm kept improving, and between 1 and 2 h after electroporation, the proper alternation between the rostral and caudal roots was observed (Fig. 5C, right). Finally, for voltages >30 V (2 experiments, 2 electroporations), only tonic bursting
and rare nonalternating discharges could be recorded even 3–4 h after electroporation.

Optical signals from electroporated cords

It might be argued that the recovery of function after electroporation depended on cells that were not themselves electroporated or loaded with dye. This could occur if the electroporated cells were silenced by damage or if their function was compromised by an unusually high dye concentration. To test these ideas and to establish if loaded cells could produce optical transients, we monitored fluorescence changes during antidromic and reflex activity. In previous experiments in the chick, mouse, and rat spinal cord, we have suggested that the calcium transients evoked during antidromic stimulation or rhythmic behaviors are generated primarily by action potentials (Bonnot et al. 2002b; Lev-Tov and O’Donovan 1995; O’Donovan et al. 1994). As a result, the presence of optical signals synchronized with the electrical activity provides good evidence that dye-labeled cells are capable of generating action potentials and of participating in network activity.

In 25 electroporated preparations (9 calcium green dextran, 16 calcium green salt), optical signals were successfully recorded in response to stimulation of ventral or dorsal roots. Most recordings were made using conventional epifluorescence microscopy (Figs. 6, A–C, and 7, A–C). However, four electroporated preparations were also images using two-photon microscopy (Figs. 6, F–I, and 7, D–F) allowing cellular resolution as deep as 350 μm into the cord.

With epifluorescence microscopy, the labeling seen through the lateral white matter (after 1–2 h of wash-out following electroporation) consisted mostly of a diffuse fluorescence spanning the electroporated area (Fig. 6A). Dorsal and ventral columnar areas of stronger labeling could be delineated at low magnification, and blurred cellular profiles could sometimes be detected (especially with the dextran). As a consequence, when optical signals were recorded, they comprised diffuse population activity, and cellular signals were rarely seen. We detected optical signals following electroporation of both dextran and the salt, although signals were about one-half the size with the dextran compared with the salt. For this reason, the data described below are restricted to the electroporation experiments using the salt (16 experiments).

Antidromic stimulation of motoneurons

We first present the results of antidromic stimulation of motoneurons (Fig. 6). In 11/16 experiments, stimulation of any ventral root within the electroporated area resulted in optical signals from neurons spanning approximately one segment and located in the ventral third of the cord when viewed through the lateral white matter (Fig. 6, A and B). This region corresponds to the location of motoneurons. We examined the frequency dependence of the antidromic signal in six experiments (9 ventral roots). As shown in Fig. 6C, we found that the optical signal increased linearly with the stimulation frequency from 2.6 ± 0.7% at 5 Hz to 6.6 ± 2.0% at 50 Hz, and the optical response to a single shock could be resolved in each case (2.1 ± 0.9%; Fig. 6C). In the five remaining experiments, in which only a few motoneurons were observed in fixed sections, antidromic optical responses were weak (peak ΔF/F < 2.7%, 50 Hz train).

In a separate series of experiments, we established how many action potentials occurred in lumbar motoneurons following a single antidromic stimulus applied to the ventral root. Whole cell recordings obtained from lumbar motoneurons (15 cells, 10 preparations) showed that a single action potential was evoked by a single ventral root stimulus (Fig. 6, D and E). Ten of 15 motoneurons revealed a prominent afterdepolarization (ADP; Fig. 6D), which is known to be accompanied by calcium entry through voltage-dependent calcium channels (Kobayashi et al. 1997; Viana et al. 1993; Walton and Fulton 1986). This observation indicates that calcium transients detected in electroporated motoneurons in response to a single ventral root stimulus correspond to a single action potential. In Fig. 6E, we show the time course of the antidromic action potential together with the normalized and averaged calcium transients from electroporated and retrogradely loaded motoneurons. The peak of the calcium transients coincided with the end of the ADP, and the decay kinetics of the transients were similar for electroporated and retrogradely labeled motoneurons. The interpolated duration of the transient at 50% amplitude was 9.1 ± 1.5 frames (~100 ± 50 ms; n = 5 preparations) for the electroporated motoneurons and 10 ± 5.4 frames (330 ± 178 ms; n = 4 preparations) for the retrogradely loaded motoneurons.

Because of the poor cellular resolution obtained with conventional epifluorescence microscopy, we performed additional experiments using two-photon confocal imaging. In three of four experiments, fluorescently labeled motoneurons were detected deep within the spinal cord tissue (60–170 μm; Fig. 6, F and G), and cellular signals could be resolved in individual motoneurons (Fig. 6, H and I). The absence of signal in the background extracellular region (region of interest 1) confirms the cellular nature of these signals. The fractional change in fluorescence measured during antidromic stimulation at 20 Hz was 40.6 ± 18.8% (11 cells in 3 experiments). In the

FIG. 6. Calcium transients recorded from electroporated cells in response to antidromic stimulation of the ventral roots. A: epifluorescence images of the L5–L6 area following subdural application of the dye (same experiment as in Fig. 2) and viewed through the lateral white matter, as shown in the inset. B: difference image of the dotted area in A, obtained by subtracting the control fluorescence (image A) from an average generated during antidromic stimulation of the L5 ventral root at 20 Hz. C: fluorescent transients generated in the ventral area (black box in B) in response to ventral root stimulation at different frequencies. Single shock responses are arrows. D: whole cell recording from a motoneuron in the L5 segment of the neonatal mouse spinal cord showing that a single action potential is evoked in response to a single stimulus applied to the ventral root. Note the prominent afterdepolarization (ADP), which is known to be accompanied by calcium entry through voltage-dependent calcium channels. E: comparison of averaged time course of the antidromic action potential recorded from 7 lumbar motoneurons in 6 preparations (red traces, ±SD) with the calcium transients recorded from lumbar motoneurons filled with either the calcium green salt by electroporation (averaged from 5 preparations; green trace, ±SD) or with calcium green dextran by retrograde loading (averaged from 4 preparations; blue trace, ±SD). Intracellular recordings were made in a different set of experiments from optical recordings, and cells were not filled with calcium dyes. F: a single optical scan 100 μm under the lateral surface of an electroporated cord obtained using 2-photon microscopy. Ventral area outlined by the red box is shown at higher magnification in G: average image generated over a 2-s period (frame scan: 180 ms) in the absence of activity. H: active difference image obtained by subtracting the control fluorescence (image G) from an average generated during 20 Hz stimulation of the L6 ventral root. I: calcium transients corresponding to the 5 regions of interest drawn in H (4 neurons and 1 background).
same three experiments, 20 Hz antidromic optical signals were also measured using epifluorescence and averaged 5.86 ± 6.5%.

**Dorsal root-induced optical signals**

In 13/16 experiments, using the same lateral visualization, large optical signals were recorded in dorsal populations of neurons during reflex responses to a single shock or a train applied to the ipsilateral L6 dorsal root (Fig. 7A and B). The peak fractional change in fluorescence measured in response to a single dorsal root stimulus averaged 9.7 ± 2.0% (SD; range, 7.2–12.7%, n = 11). The onset of the optical signal coincided with the onset of the simultaneously recorded ventral root electrical response. Dorsal root–activated optical signals were also observed in the ventral region, although they were smaller (<50%) than those in the dorsal region (P < 0.05, n = 7). In the remaining three experiments, the optical signals accompanying dorsal root stimulation were small (peak ΔF/F < 5%, single stimuli or 20 Hz train). Subsequent analysis of fixed sections obtained from two of these preparations revealed weak labeling in the dorsal area.

Confirmation of the cellular origin of these dorsal-root evoked signals was obtained with two-photon microscopy. As shown in Fig. 7D, cellular profiles were easily resolved, and signals were obtained from individual neurons. The fractional change in fluorescence during a train of dorsal root stimuli at 20 Hz averaged 41.8 ± 26.7% (16 cells in 3 experiments). In the same three experiments, the dorsal root evoked signals measured with epifluorescence averaged 10.5 ± 5.2%.

**Optical imaging of locomotor-like rhythmicity**

Locomotor-like activity is one of the most organized behaviors generated by the isolated mouse spinal cord. In the previous section, we established that this type of activity is initially depressed but recovers following electroporation. In the next set of experiments, we investigated if neurons participating in this behavior can be labeled by electroporation and can generate optical signals. This was particularly important because standard methods for labeling locomotor-related interneurons have significant limitations.

In 10/25 optically recorded preparations, trains of coccygeal stimulation successfully evoked alternating discharges on rostral and caudal ventral roots, characteristic of locomotor-like behavior (Whelan et al. 2000). Such trains gave rise to a tonic optical activation sustained for the whole duration of the train (10 s) with an average peak intensity of 7.4 ± 3.6% (range, 3.3–12.0%, n = 9). The activity was concentrated in the ventral and intermediate parts of the spinal cord (Fig. 8B), with
significantly less activity in the dorsal portion of the cord (≤50% of that in the ventral region, $P < 0.05, n = 8$). In three cases where the locomotor-like rhythm was robust and the labeling strong, we observed a 1–2% rhythmic variation of the fluorescence signal superimposed on a tonic increase of fluorescence (Fig. 8C). These rhythmic fluorescence variations were strictly coupled to the rhythmic discharge recorded from the ventral roots and were observed only in the most ventral column of cells, shown to be mostly motoneurons. Similar rhythmic signals have been recorded previously from retrogradely labeled motoneurons (Bonnot et al. 2002b). In contrast to the ventral recordings, signals from the intermediate region displayed a stable level of tonic activation for the whole duration of the coccygeal train.

**DISCUSSION**

We have shown that electroporation can be used to rapidly load calcium-sensitive dyes into neurons over several segments of the spinal cord, that the electroporated cord can generate electrical activity, and that loaded cells can generate optical signals during antidromic, reflex, and locomotor-like activity. The extent of labeling throughout the three-dimensional volume of the cord and the diversity of cells labeled are unmatched by any of the currently available methods for introducing calcium dyes into neurons. In combination with confocal or two-photon microscopy, this new technique allows neuronal and network function to be visualized in relatively intact tissues in a manner not possible before. In the spinal cord, for example, it will now be feasible to analyze the dynamics of the interneuronal networks and neurons responsible for locomotion. To date, this has only been possible using intracellular techniques that are limited to recording from one or two cells simultaneously (Butt and Kiehn 2003). In addition, because of both the growing use of electroporation for the introduction of genes in vivo and the increased depth of optical resolution obtained with two-photon confocal imaging, we...
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 anticipate the extension of this approach to in vivo imaging of network function. We believe that in vivo studies will have an inherent advantage over in vitro studies because the electroporation can be performed well before the recordings, allowing adequate time for washout of extracellular dye.

Although we have focused on neuronal labeling in this paper, it is very likely that glial cells were also labeled by this method. Indeed, our observation of loaded central canal cells with end feet on blood vessels supports this idea. Unfortunately, nothing is known about glial calcium transients in the spinal cord at the ages we have studied. However, we note that the calcium transients we have recorded in response to antidromic stimulation and locomotor-like activity have the same time course as those recorded earlier from retrogradely filled neurons, a case in which glial cells were not labeled. Furthermore, using two-photon microscopy, we have been able to monitor the calcium transients of individual neurons. Nevertheless, in future experiments, it will be important to confirm glial labeling directly by immunocytochemistry and to establish if any of the optical signals we have recorded during antidromic, reflex, or locomotor-like activity contain contributions from labeled glial cells.

Mechanism of dye distribution throughout the cord

Our experiments have established that cellular loading is caused by electroporation and not by some other mechanism (e.g., endocytosis). Control cords that were exposed to the dye but were not electroporated or were electroporated at low voltages (<15 V) exhibited no labeled cells in fixed sections and did not produce optical signals during electrical activity. Second, when the dye was injected subdurally or spread widely throughout the cord following intravascular or central canal injections, the labeled cells were co-extensive with the electroporation electrodes, and no labeling was found outside this region.

Following subdural dye injection (at the level of the ventral commissure), we found that electroporation resulted in neuronal labeling throughout the three-dimensional extent of the cord. Although neuronal labeling was sometimes stronger on the side of the cord adjacent to the cathode, in many cases labeling was of similar extent and intensity on both sides. This is in contrast to electroporation following DNA injection into a closed cavity such as the developing neural tube of the chick embryo (Ogura 2002) or the brain ventricles of the Xenopus tadpole (Haas et al. 2002) and mouse (Saito and Nakatsuji 2001) or into the central canal of the mouse spinal cord (Saba et al. 2003). It is unlikely that the labeled result is the label of lateral spread of the dye by the glial cell membrane. This observation has demonstrated that the dye is driven into the extracellular space by the electroporation pulses.

Electroporation-induced depression of neuronal activity

To assess the effects of electroporation on neuronal function, we monitored mono- and polysynaptic dorsal root-evoked responses in motoneurons and the capacity of the cord to generate locomotor-like rhythmic behavior. These experiments were generally done without dye injections or the low calcium/high magnesium solutions we used during electroporation of the dyes. While it is likely that the low calcium/high magnesium solution offered some tissue protection during electroporation, we could not use it because it would have depressed neural function during the period we were monitoring its recovery.

Following electroporation, we observed recovery of polysynaptic reflex and rhythmic activity as long as the electroporation voltage was between 5 and 25 V, with a recovery time course strictly dependent on the applied voltage. While the polysynaptic dorsal root reflexes recovered to control values within 20–40 min (Fig. 5B), the monosynaptic dorsal root reflexes regained only 50–60% of their control level by 2 h after the electroporation. The reasons for this incomplete recovery are unknown, but we note that it did not compromise the ability of the cord to produce complex locomotor-like rhythmic behavior. One possibility is that the cut dorsal roots might have been damaged by their proximity to the electroporation electrodes. Typically, the electroporation electrodes were positioned <1 mm from the cord edges. As a result, the cut dorsal roots sometimes touched the electrodes during electroporation, possibly sustaining some damage. The fact that the polysynaptic dorsal-root-evoked responses did recover com-
completely may reflect network homeostatic behavior that has been identified in the developing chick spinal cord (Chub and O’Donovan 1998).

The source of the electroporation-induced depression of function is unknown, but presumably it involves the exchange of the intracellular and extracellular solutions during the electroporation process (Weaver 1993). However, the fact that recovery could occur after the electroporation-induced depression indicates that cellular homeostatic mechanisms can restore cellular function under our experimental conditions.

Finally, we observed a potentiation of the monosynaptic reflex in L₁, remote from the electroporated area that might be explained by a decreased presynaptic inhibition exerted in L₁ by electroporated caudal primary afferents.

**Optical signals from electroporated cells**

One concern with the technique, which we have been able to disprove, is that the electroporated cells would be incapable of producing optical signals. This could occur if they were irreversibly damaged by the electroporation or alternatively because the dye might become compartmentalized within the cell. Although we did observe some evidence of compartmentalization in the sectioned material (e.g., Fig. 1C), electroporated cells were capable of generating optical signals during antidromic, reflex, and locomotor behaviors. In addition, we showed that these various sources of stimulation induce different and recognizable patterns of activation among the labeled population of cells.

These observations suggest that the dye concentration achieved during electroporation does not compromise network function. Although we did not estimate the dye concentration directly in electroporated neurons, it was our impression (based on the intensity of the fluorescence) that the concentration achieved with electroporation was similar or lower than that achieved with retrograde loading (Bonnot et al. 2002b). Consistent with this conclusion, we found that the decay kinetics of the calcium transients accompanying a single antidromic action potential were similar for electroporated and retrogradely loaded motoneurons (Fig. 6E). In a recent study employing retrograde loading of calcium dyes into spinal neurons, the final dye concentration was estimated to be ~10 μM (Viana di Prisco and Alford 2004). Because retrograde loading has not been shown to alter network behavior (Bonnot et al. 2002b; Nakayama et al. 2002; O’Donovan et al. 1994; Ritter et al. 2001; Viana di Prisco and Alford 2004), it seems reasonable to conclude that the dye concentration achieved with electroporation is also unlikely to significantly perturb neuronal function.

When whole cords loaded by electroporation were visualized through the lateral or ventral white matter using epifluorescence microscopy, the fluorescent labeling was usually diffuse. Cellular profiles were more apparent with the dextran form of calcium green dextran than with the salt, but in either case, the cellular resolution was not as sharp as that seen with retrograde filling, and smaller optical signals were obtained (see Bonnot et al. 2002b). Epifluorescent optical signals obtained during antidromic stimulation of motoneurons following electroporation of the calcium green salt were about four to six times smaller than similar signals obtained from retrogradely filled preparations of the neonatal mouse or rat (Bonnot et al. 2002b; Lev-Tov and O’Donovan 1995). This could be due to several factors. First, in contrast to retrograde loading, with electroporation there is a significant amount of extracellular dye that degrades cellular contrast. Consistent with this idea, we noticed a reduction of background fluorescence and an improvement of cellular resolution, as the extracellular dye washed out during the experiment. Because extracellular dye is not reactive to intracellular calcium changes, it will reduce the activity-dependent changes of fluorescence that are normalized to the resting level of fluorescence. In addition, some of the dye was compartmentalized in the electroporated cells and was therefore not available to cytoplasmic calcium (Malgaroli et al. 1987). Second, as discussed above, the intracellular concentration of dye reached with electroporation may have been lower than with retrograde loading, making it difficult to detect cells through the white matter with conventional epifluorescence microscopy and probably contributed to the smaller size of the optical signals. Finally, retrograde filling labels a specific subgroup of neuronal somata and their associated processes, whereas electroporation tends to label many more cells and processes, again degrading our ability to resolve somatic profiles with epifluorescence. We do not, however, consider cellular resolution and signal size to be limiting factors with this technique because the optical signals exhibited a good signal to noise ratio and, using two-photon microscopy, much larger cellular signals could be resolved from neurons located 60–350 μm from the white matter surface.

**Concluding remarks**

Electroporation has allowed us to label and to visualize the activity of a greater diversity of spinal neurons, throughout a greater extent of the dorso-ventral and medio-lateral axis of the cord and over more segments than any currently available method. In the future, this will allow us to address questions that have hitherto been inaccessible. For example, the limitations of other loading methods have prevented us from visualizing the cellular and spatiotemporal organization of spinal interneuronal activity during reflex or locomotor-like behavior in the isolated spinal cord.

The coupling of two-photon microscopy with a spatially controlled delivery of calcium indicators to extended cell populations should allow calcium imaging to become even more informative in many different parts of the nervous systems. Ultimately, we expect to expand the use of this approach to optical imaging in vivo of the brain or spinal cord of adult rodents. Furthermore, in future experiments it may be possible to combine calcium imaging with gene delivery, a capability that only this method of loading can offer.

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**References**


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