Spatiotemporal Patterns of Dorsal Root-Evoked Network Activity in the Neonatal Rat Spinal Cord: Optical and Intracellular Recordings

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Running head: Optical and voltage recordings of spinal network excitation

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Abstract

Spatiotemporal patterns of dorsal root-evoked potentials were studied in transverse slices of the rat spinal cord by monitoring optical signals from a voltage-sensitive dye with multiple-photodiode optic camera. Typically, dorsal root stimulation generated two basic waveforms of voltage images: dual-component images consisting of fast, spike-like signal followed by a slow signal in the dorsal horn, and small, slow signals in the ventral horn. To qualitatively relate the optical signals to membrane potentials, whole-cell recordings were combined with measurements of light absorption in the area around the soma. The slow optical signals correlated closely with subthreshold postsynaptic potentials in all regions of the cord. The spike-like component was not associated with postsynaptic action potentials, suggesting that the fast signal was generated by presynaptic action potentials. Firing in a single neuron could not be detected optically, implying that local voltage images originated from synchronously activated neuronal ensembles. Blocking glutamatergic synaptic transmission inhibited excitatory postsynaptic potentials (EPSPs) and significantly reduced the slow optical signals, implying that they were mediated by glutamatergic synapses. Suppressing glycine-mediated inhibition increased the amplitude of both optical signals and EPSPs, while blocking GABA_A receptor-mediated synapses increased the amplitude and time course of EPSPs and prolonged the duration of voltage images in larger areas of the slice. The close correlation between evoked EPSPs and their respective local voltage
images demonstrates the advantage of the high temporal resolution optical system in measuring both the spatiotemporal dynamics of segmental network excitation and integrated potentials of neuronal ensembles at identified sites.

**Key words**

Voltage images, voltage-sensitive dye, dorsal root-evoked network excitation, rat spinal cord
Introduction

Intracellular and extracellular electrical recordings have provided high temporal resolution of activity in single neurons, but spatial resolution of network activity is limited by the restricted number of recording electrodes. A multiple-element photodiode array simultaneously recording voltage-related optical signals at numerous sites makes it possible to monitor real-time spatiotemporal patterns of transmembrane potential in neural circuits (reviewed by Cohen and Salzberg, 1978; Cohen and Lesher, 1986; Grinvald et al., 1988, Wu et al., 1993; Momose-Sato et al., 2001). Images of fast voltage-sensitive dyes have provided valuable information about the functional organization of neural networks in a wide variety of preparations ranging from the isolated invertebrate cultured neurons (e.g., Parsons et al., 1991) to in vivo imaging of neuronal activity in the rat barrel cortex (Petersen et al., 2003).

Multiple-site optical recordings have also proven useful in studies mapping the spatial extent of neuronal excitation in tissues that are inaccessible to conventional electrophysiological recordings (reviewed by Salzberg, 1983; Grinvald, 1985). Voltage images have been fundamental to our understanding of the ontogeny of electrophysiological function and network organization in embryonic chick brainstem and spinal cord, at stages when electrophysiological recordings are difficult, if not impossible (Arai et al., 1999; Mochida et al., 2001; Momose-Sato et al., 2001; Momose-Sato et al., 2003; Sato et al., 2002; reviewed by Momose-Sato et al., 2002, Sato et al.,
High temporal resolution optical measurements are advantageous in developing neuronal systems, because in the absence of myelin the translucent tissue provides relatively large optical signals. In embryonic chick spinal cord, sensory stimulation triggered voltage images consisting of fast and slow signals. Based on pharmacological manipulations it has been proposed that those were presynaptic action potentials and postsynaptic potentials, respectively. However, data were cautiously interpreted in the absence of quantitative comparison of membrane potentials and their respective optical signals. Combined electrophysiological and optical recordings revealed closely matched voltage images and potential changes in response to sensory and antidromic stimuli in chick spinal motoneurons (Wenner et al., 1996). Sophisticated combination of voltage-sensitive dye images and whole-cell potential recordings in the rat barrel cortex in vivo, demonstrated a strong correlation between sensory evoked subthreshold excitatory potentials and optical signals (Petersen et al., 2003).

Relatively little is known about the spatiotemporal pattern of sensory evoked network activity in the rodent spinal cord. We have examined the spatiotemporal dynamics of spontaneous depolarizations in transverse slices of embryonic and newborn rat spinal cords using a voltage-sensitive dye with a 464-element photodiode-fiber optic camera (Demir et al., 2002). Real-time fluorescence images revealed coordinated rhythmic activity with spontaneous voltage oscillations alternating between mirror locations in the right and left sides of the cord. Extracellular potentials confirmed that these were representatives of local voltage changes.
The objectives of this study were: (1) to characterize the spatiotemporal pattern of dorsal root-evoked network excitation using images from voltage-sensitive absorption dye, (2) to examine the correlation between whole-cell potentials and simultaneously recorded local voltage images originated from neuronal ensembles in the area around the soma, and (3) to study the contribution of excitatory and inhibitory synapses to the spatiotemporal dynamics of evoked network activity.

A preliminary report of this study was published in an abstract form (Ziskind-Conhaim and Redman, 2003).
Methods

Spinal Cord Slices

Lumbar segments of the spinal cord were isolated from 1-4-day old postnatal Sprague-Dawley rats (P1-4). The procedure for dissecting out the spinal cord was similar to that described previously (Gao and Ziskind-Conhaim 1998). Postnatal rats were anesthetized by hypothermia, and the lumbar region of the spinal cord was removed and placed in oxygenated cold dissection solution. The isolated spinal cord was embedded in agar (2% in extracellular solution), and 400-600 μm transverse slices with dorsal roots intact were cut with a Leica VT1000S vibratome. Slices were incubated in extracellular solution at room temperature for 30-60 mins.

Voltage-sensitive dye staining

Slices were stained for 30-40 min in extracellular solution containing the voltage-sensitive oxonol dye, RH-155 (0.2 mg/ml, Molecular Probes, Eugene, OR). The excess dye was washed away with dye-free extracellular solution. For comparison, in a few experiments slices were stained with RH-482 (0.2 - 0.3 mg/ml, NK3630, Hayashibara Biochemical Laboratory, Japan). RH-155 was the dye of choice in our experiments for two main reasons. First, slices incubated in RH-482 seemed to degenerate more rapidly, possibly because RH-482-containing solution foamed when aerated, making it difficult
to adequately equilibrate it with 95% O₂ and 5% CO₂. Second, RH-155 had larger signal-to-noise ratio than RH-482 (also Momose-Sato, 1999), although this might be partly attributed to its staining of glial cells (Kojima et al., 1999). Phototoxicity could not be detected with either dye (Chang and Jackson, 2003). The disadvantage of staining with RH-155 was its relatively rapid wash out during long-term recordings. One hour superfusion in extracellular solution resulted in approximately 20% signal attenuation compared to < 10% reduction in slices stained with RH-482. Similar dye characteristics were described previously during long-term recordings in hippocampal slices (Momose-Sato et al., 1999).

Voltage-sensitive dye imaging

Stained slices were transferred to a recording chamber mounted on the stage of an Olympus microscope (BX50WI), and were held with a nylon mesh in a glass-covered recording chamber. The slices were superfused with extracellular solution at a rate of 1 - 2 ml/min at a temperature of 29-31° C (Demir et al., 2002). Dorsal roots were stimulated using tight suction electrodes or fine tungsten wires insulated to about 10-20 μm from the tip. In most slices square pulses of 300-800 μA/0.3-0.5 ms triggered optical responses in the ventral horn. Experiments were discontinued if stimuli did not produce optical signals in the ventral horn.

Light from a 100 W halogen bulb powered by a regulated power supply (Kepco, Inc. Flushing, NY) was passed through an interference filter with a transmission
maximum at $710 \pm 10$ nm (Omega Optical, Brattleboro, VT). The filter was placed between the light source and the recording chamber (Fig. 1). The experimental approach was to detect changes in the intensity of transmitted light, which is inversely related to changes in light absorption. The light was focused onto a photodiode-fiber optic camera with 464 hexagonally arranged detectors (RedShirtImaging, Fairfield, CT), and voltage-sensitive dye signals were recorded with either 10X (N.A. 0.4) or 20X (N.A. 0.95) objectives. The diameter of the optical field of each photodiode was 30 µm at 20X. The amplitude of optical signals was measured as peak change in absorption normalized to the resting light from each photodiode ($\Delta I/I$).

**Figure 1:** Schematic drawing of voltage imaging and electrophysiological recording systems. For optical recordings, stained slices were illuminated through a $710 \pm 10$ nm interference filter and light changes were monitored with a 464 hexagonally arranged detectors. To visualize neurons for whole-cell voltage recording, the interference filter was removed and the light was directed to the camera. During simultaneous optical and whole-cell voltage recording, the filter was reinserted and the light was redirected to the detectors.
Amplified photodiode signals were high-pass filtered with a 0.2 sec time constant, and read into a Pentium computer using multiplexers and an A/D conversion board (Microstar, Bellevue, WA). The program Neuroplex (RedShirtImaging, Fairfield, CT) was used to read in the data at full frame acquisition intervals of 0.62 msec. Analysis was performed with Neuroplex, and with additional programs written in IDL (Research Systems, Inc. Boulder, CO).

Figure 2: The spatiotemporal pattern of dorsal root-evoked voltage-sensitive dye images. A. Transverse slice (500 µm) with dorsal root intact from segment L3 of a P3 rat spinal cord. The root was stimulated with a fine tungsten wire. Ventral horn is up. The diagonal lines are part of the nylon mesh that held the slice in the recording chamber. The photograph was taken with a 10X objective, showing mostly one side of the cord. B. Color maps demonstrating the high temporal resolution of dorsal root-evoked voltage images along the dorso-ventral axis with absorption encoded as color.
An increase in light absorption (red) corresponded to positive signal (transmembrane depolarization). Frames are shown at 1.2 ms intervals. The sequence starts from left to right in the upper row and continuing down by the rows. C. Optical traces from 464 locations in a transverse slice using 10X and 20X objectives. Traces were overlaid on a sketch of the slice (grey area) from which the recording was made. The noisy signals at the periphery outline the edges of the slice. Asterisks mark the traces recorded at the same locations using the 10X and 20X objectives, and shown in Fig. 3. In this and all other figures, ΔI/I is the peak change in absorption normalized to the resting light from each detector.

*Whole-cell voltage recording*

To identify the photodiode corresponding to the site of whole-cell voltage recording, digital images of the slice were first taken with a 10X objective, and those were overlaid on the image composed by the optic camera (Fig. 2A and C). At the end of the experiment, a digital image was taken again with the recording pipette in place. Whole-cell voltage recordings corresponded to optical signals detected by the photodiode in the center of the 464 hexagonally arranged detectors (Fig. 2C, 20X). Neurons at specific locations in the transverse slice were visualized for whole-cell recordings using infrared differential interference contrast (DIC)-videomicroscopy (Ziskind-Conhaim et al., 2003). The interference filter was removed and the light was directed to the camera (Fig. 1). After the whole-cell current clamp configuration was established the filter was reinserted for optical measurements.
Patch pipettes were pulled to tip resistances of 3-5 MΩ using a multi-stage puller (Sutter Instruments, Novato, CA). Intracellular potentials were recorded with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). Potentials were filtered at 3 kHz and digitized at 10-20 kHz. Membrane potentials were corrected offline for a 10 mV liquid junction potential (Gao et al., 2001).

**Solutions**

Dissection solution contained (in mM): 113 NaCl, 3 KCl, 1 CaCl₂, 6 MgCl₂, 25 NaHCO₃, 1 NaH₂PO₄, and 11 glucose. The pH was 7.2-7.3 and the osmolarity was 300-310 mOsm. The extracellular solution was composed of (in mM): 113 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1 NaH₂PO₄, and 11 glucose. The solution was equilibrated with 95% O₂ and 5% CO₂ (pH 7.2 at 20-22 °C). Patch pipettes were filled with solution containing (in mM): 140 K-Gluconate, 9 KCl, 10 HEPES, 0.2 EGTA, 1 Mg-ATP, and 0.1 GTP. The solution was adjusted to pH 7.2 using KOH, and the osmolarity was 290 mOsm.

Receptor antagonists used in our study included: D-2-Amino-5-phosphonovaleric acid (D-APV, 20 µM) and 6-cyano-7-nitroquinoxaline (CNQX, 10 µM), NMDA and non-NMDA receptor antagonists, respectively; strychnine (0.5 µM), glycine receptor antagonist, and picrotoxin (10 µM), bicuculline (5 µM) or SR-95531 (1 µM), all GABAₐ receptor antagonists.
**Results**

*Characteristics of dorsal root-evoked voltage images*

Typically, dorsal root stimulation evoked optical signals that propagated throughout the slice (Figs. 2B and C). In most experiments, initial voltage images were captured with a 10X objective, because at this magnification the 464-photodiode fiber optic camera detected voltage-dependent light changes from one entire side of the cord (Figs. 2A-B). After confirming that dorsal root stimulation produced optical responses in the ventral horn, the 10X objective was replaced with a 20X objective, to visualize neurons for whole-cell recordings. The spatial resolution was higher with the 20X objective, because at this magnification each photodiode recorded voltage-dependent light changes from one quarter of the area recorded using a 10X objective. However, under our experimental conditions, signals with a similar time course were recorded with either objective (Figs. 2C and 3). This might imply that dorsal root stimulation produced a relatively homogeneous response in local neuronal ensembles.

Voltage images consist of extrinsic and intrinsic components. Extrinsic signals depend on dye-mediated light absorption, while intrinsic signals are independent of dye properties, and are related to intrinsic light-scattering changes (e.g., swelling, Sato et al., 1997) that might be associated with electrical activity in neural cells (Grinvald et al., 1982; reviewed by Cohen, 1973). To determine whether dye-independent signals contributed to voltage image waveforms, recordings were performed at 710 and 580 nm.
In the action spectrum of the dye RH-155, the transmitted light intensity was expected to decrease (absorption increased) at 710 nm and increase (absorption decreased) at 580 nm. Indeed, similar to previous reports (e.g., Momose-Sato et al., 2001), changing the light wavelength from 710 to 580 nm reversed the signals and decreased their amplitude ($n = 3$, not shown). Moreover, dorsal root-evoked optical signals were not evident in unstained slices ($n = 4$), suggesting that images recorded in the stained slice reflected voltage-related changes in dye absorption. Voltage images presented in our study were averages of 3-5 stimuli at 0.1- 0.2 Hz. The signal ($\Delta I/I$) was of the order of $10^{-3}$.

Distinct optical waveforms were recorded along the dorsoventral axis of the slice. Typically, at stimulus intensities that evoked ventral horn responses, a spike-like signal followed by a large, slow component that decayed over several tens of milliseconds was recorded in the dorsal horn (Fig. 3, $n = 52$). Similar dual-component images were also apparent in the intermediate areas in approximately half the slices ($n = 24/52$), but these were smaller than signals recorded in the dorsal horn. In contrast, only small, slow optical responses with latencies of 4-7 ms were recorded in the ventral horn of all slices. Dual-component voltage images have been recorded in the spinal cord of chick embryos (Arai et al., 1999; Mochida et al., 2001), and based on pharmacological manipulations it has been suggested that the spike-like component corresponded to presynaptic action potentials, and the slow response originated from synaptic potentials. To characterize the spatial distribution of dorsal root afferents, transverse slices were stained with anti-
vesicular glutamate transporter1 (VGLUT1) antibody that is expressed in glutamatergic projections from cutaneous and muscle mechanoreceptors in the rat spinal cord (Alvarez et al., 2004). The spatial architecture of VGLUT1 immunoreactivity varied in different lumbar segments, but similar to the observations in the rat spinal cord, in all segments the highest density was evident in the dorsal horn and the lateral intermediate area (n = 3, not shown), where spike-like signals were frequently recorded.

**Figure 3:** Distinct optical waveforms were recorded in the dorsal and ventral horns in a spinal cord slice of a P3 rat. Dorsal root-evoked voltage images consisted of a spike-like component followed by a large, prolonged component in the dorsal horn. With the 20X objective, the values of ΔI/I were ~ 0.006 and 0.0035 for the fast and slow components, respectively. Smaller dual-component images (ΔI/I of 0.0025 and 0.0001) were detected in the intermediate area. Only small signals (< 0.001) were measured in the ventral horn. Signals were recorded with 10X and 20X objectives at the locations marked by the asterisks in Fig. 2C.
Close correlation between dorsal root-evoked voltage images and subthreshold postsynaptic potentials

The interpretation that spike-like optical signals represented presynaptic action potentials and the slow signals were related to postsynaptic potentials remained speculative in the absence of direct measurements quantitatively relating voltage-sensitive dye signals to neuronal membrane potentials. To relate local voltage images to neuronal membrane potentials, simultaneous whole-cell recordings were performed at targeted sites. The presence of the whole-cell recording pipette did not distort the optical signals, because similar waveforms were measured in the presence and absence of the pipette. Similar resting membrane potentials (more negative than -50 mV) and action potential waveforms were recorded in stained and unstained slices, indicating that the dye did not significantly affect membrane properties. It is reasonable to assume that the dye did not change synaptic transmission, because the frequency of spontaneous synaptic potentials (0.4 to 1.7 Hz) was similar to that recorded in our previous studies (e.g., Gao et al., 1998).

Recordings were performed with a 20X objective and because of its large dimensions, measurements were restricted to sites at least 100 μm away from the stimulating electrode. Therefore, in the majority of the experiments, recordings did not include neurons in laminae I and II. Most recordings in the ventral horn were
performed in lamina VII interneurons and did not include neurons in laminae VIII and IX (motor nuclei) because of the noisy optical signals near the edge of the slice (Fig. 2). Optical signals primarily originated from neurons in focus (superficial), but presumably potential changes in neurons in deeper layers also contributed to the signals. The 20X objective had a 30 μm-diameter optical field, and with a cell diameter of 20-25 μm, it was estimated that in the 500 μm-thick slice each diode received light from at least 20 cell bodies, and an unknown number of dendrites and axons.

Dorsal root stimulation at intensities that generated optical responses in the ventral horn, produced postsynaptic potentials in neurons located in most regions of the cord (n = 24). Although voltage images around the soma presumably resulted from potential changes in a set of neurons, the time course of optical responses with duration > 100 ms (from peak response to its return to baseline) was closely correlated with the time course of subthreshold synaptic potentials (Fig. 4, n = 18).
Figure 4: Similar time course of local dorsal root-evoked optical responses and synaptic potentials recorded in the intermediate area (A) and the ventral horn (B). ΔI/I of 0.0017 and 0.0012 were measured in photodiodes in the intermediate area and ventral horn, respectively. These corresponded to synaptic potentials of ~ 7 mV with delays of 5.9 ms (A) and 4.7 ms (B). The duration of voltage images and synaptic potentials were > 100 ms. Traces are averages of 5 trials at 0.1 Hz. Recordings in the intermediate area and ventral horn were from different slices of P2 rats.

Differences between optical and potential waveforms became apparent when dorsal root stimulation generated postsynaptic action potentials. Action potentials were not reflected in the voltage images, and were not correlated with spike-like optical signals (Fig. 5A, n = 3). This might indicate that dorsal root stimulation did not produce synchronous firing in the majority of neurons in the optical field. Differences between local voltage images and whole-cell potentials were also evident in dorsal horn
recordings, in which spike-like optical signals were not associated with postsynaptic action potentials (Fig. 5B, n = 5).

**Figure 5:** Differences between local voltage images and simultaneously recorded whole-cell potentials. A. Postsynaptic action potentials were not reflected in the optical signals. Averaged optical signals and intracellular potentials recorded in the intermediate area of the spinal cord of a P2 rat. The delay of the synaptic potential was 6.2 ms. The averaged action potential did not exceed zero potential, because only 3/5 stimuli generated action potentials. B. Spike-like optical signal in the dorsal horn was not associated with a postsynaptic action potential. The amplitude of the averaged postsynaptic potential was ~ 4 mV and the synaptic delay was 4.6 ms.
Our data suggested that action potentials in a single neuron cannot be measured optically when recording local voltage images of neuronal ensembles. To directly test this hypothesis, single action potentials or a train of action potentials were evoked by intracellular current injection, and optical signals were monitored at the location corresponding to whole-cell recording. To increase the signal-to-noise ratio of voltage images, 100-200 trials were averaged. Spike triggered averaging of 200 voltage images in the area around the soma could not detect light changes generated by 200 action potentials (Fig. 6). Similarly, repetitive firing generated by intracellular injection of a prolonged depolarizing current could not be detected optically (Fig. 7A). These findings indicated that changes in light absorption generated in a single neuron could not be detected when averaged with activity of other neurons in the optical field.

**Figure 6:** Spike triggered averaging of 200 action potentials could not be detected optically. Action potentials were produced by intracellular injection of 20 pA/60 ms depolarizing current at 0.5 Hz. Membrane potential was ~ -60 mV. The average latency was 32.3 ± 0.7 ms (the onset of current injection is not shown). The figure shows an averaged action potential generated by 200 trials. Traces are images of the center photodiode and 6 surrounding diodes. Note that ΔI/I is ~ 10-fold smaller than in all other figures.
To determine whether responses to repetitive stimuli could be measured optically, dorsal roots were stimulated at 5-10 Hz (Fig. 7B, n = 3). Optical signals with the same frequency as dorsal root stimuli were recorded in many regions of the cord, suggesting that light originating from repetitive potential changes in synchronously activated neuronal ensembles can be detected optically. The amplitude of dorsal root-evoked signals decreased at stimulus frequencies > 1 Hz, similar to the attenuated excitatory postsynaptic potentials (EPSPs) recorded in response to repetitive stimuli in motoneurons of newborn rats (Ziskind-Conhaim, 1990; Seebach and Ziskind-Conhaim, 1994).

**Figure 7**
Figure 7: Local optical responses to repetitive firing in a single neuron and in response to repetitive dorsal root stimuli. A. Intracellular injection of depolarizing current (20 pA/500 ms) produced repetitive firing at 38 Hz in a neuron in the intermediate area. Light changes generated by repetitive firing in a single neuron could not be detected by any of the center photodiodes (circle). B. Dorsal root stimuli at 5 and 10 Hz/1 sec produced repetitive optical responses in the dorsal horn, intermediate area and ventral horn. Successive responses attenuated at both frequencies. Partial recovery was evident after 800 ms (5 Hz) and 600 ms (10 Hz). Images shown are single trials.

Modulation of dorsal root-evoked optical responses by excitatory and inhibitory synapses

To determine the contributions of excitatory and inhibitory synaptic transmission to voltage image waveforms, synapses were blocked with specific receptor antagonists. Blocking glutamate receptors by D-APV (20 µM) and CNQX (10 µM), NMDA and non-NMDA receptor antagonists, significantly reduced the amplitude of the large, slow component in the dorsal horn and intermediate area and completely blocked the small signal in the ventral horn (Fig. 8, n = 9). Blocking glutamatergic synaptic transmission inhibited dorsal root-evoked EPSPs in all regions of the cord (n = 5, not shown). These findings suggested that a major component of the slow optical signals was generated by
glutamate-mediated EPSPs. Glutamate receptor antagonists did not affect the amplitude of the spike-like component in the dorsal horn, but reduced it in 2/5 recordings in the intermediate area (Fig. 8). It is conceivable that the fraction of D-APV- and CNQX-sensitive spike-like component in the intermediate region corresponded to postsynaptic action potentials.

The finding that a major component of the spike-like signal was independent of glutamatergic transmission further supported the hypothesis that the fast signal was reflective of action potentials in presynaptic axons. To determine whether voltage-gated Na\(^+\) currents contributed to D-APV- and CNQX-resistant fast and slow components, TTX (1 \(\mu\)M) was added to the mixture of glutamate receptor antagonists. TTX blocked D-APV- and CNQX-resistant spike-like signal and the slow component (Fig. 8, n = 5), suggesting that these were mediated by Na-dependent action potentials.

**Figure 8**

![Figure 8](image-url)
Figure 8: Blocking glutamatergic synaptic transmission reduced to varying degrees the slow voltage images in the three regions of the cord. Glutamate receptor antagonists, D-APV (20 μM) and CNQX (10 μM) significantly reduced the amplitude of the slow optical component in the dorsal horn (37% of control) and in the intermediate area (39% of control), and completely blocked the signal in the ventral horn. The amplitude of the fast, spike-like signal was reduced in the intermediate area (76% of control), but not in the dorsal horn. TTX (1 μM) blocked the fast and slow D-APV- and CNQX-resistant signals in all recorded regions.

The contribution of inhibitory synapses to the properties of dorsal root-evoked voltage images and whole-cell potentials was examined by blocking glycinergic and GABAergic synaptic transmission using specific concentrations of glycine and GABA<sub>A</sub> receptor antagonists (Gao et al., 2001; Hinckley et al., 2005b). Exposure to strychnine (0.5 μM), induced an average increase of 47% in the amplitude of the optical responses (Fig. 9A, n = 11). The increase in optical signals was closely correlated with an increase in EPSP amplitude (n = 4). In one neuron, the larger EPSP reached threshold for action potential (Fig. 9A), but as shown in Fig 5A, the action potential was not correlated with a spike-like optical signal.
Figure 9: Effects of blocking synaptic inhibition on local voltage images and synaptic potentials. A. Strychnine (0.5 μM) increased the amplitude of optical signals recorded in the ventral horn of a P3 rat. Similarly, it increased EPSP amplitude, which exceeded action potential threshold in 3/5 trials. B. Picrotoxin (10 μM) and SR-95531 (1 μM) increased the amplitude and prolonged the duration of optical signals and EPSPS recorded in the intermediate area (picrotoxin) and dorsal horn (SR-95331). Dorsal root stimulation generated postsynaptic action potentials in 2/4 trials in the dorsal horn neuron.
Exposure to picrotoxin (10 μM), bicuculline (5μM) or SR-95531 (1-2 μM), all GABA_A receptor antagonists, increased the rate of spontaneous firing. Blocking GABAergic inhibition increased the amplitude and duration of voltage images (n = 16) in a manner correlated with the EPSPs (Fig. 9B, n = 6). The distinct effect of blocking GABA_A synaptic transmission on network excitation can be clearly demonstrated with color maps of the spatiotemporal pattern of optical responses (Fig. 10). Exposure to bicuculline but not strychnine prolonged the duration of the voltage images, and increased the spread of neuronal excitation in all regions of the cord. Similar actions of bicuculline on optical signals and subthreshold EPSPs have been reported in neurons in layer 2/3 of the rat somatosensory barrel cortex (Petersen and Sakmann, 2001).

**Figure 10:** Color maps show the spatiotemporal patterns of dorsal root-evoked optical responses during the inhibition of glycinergic and GABAergic synaptic transmission. Glycinergic and GABAergic synapses were blocked by strychnine (0.5 μM) and bicuculline (5 μM), respectively. The maps demonstrate the spread of dorsal root-
evoked optical signals along the dorso-ventral axis in one side of the cord (10X objective, see Fig. 2). Amplitude of membrane depolarizations (red) increased in the presence of strychnine. It lasted longer and occupied larger areas in the presence of bicuculline. Maps are shown at 3.2 ms intervals for a total period of 80 ms. The sequence starts from left to right in the upper row. Dorsal is down.

Voltage-sensitive dye images monitored with multiple-photodiode device provided real-time records of the spatiotemporal dynamics of potential changes in sensory-motor networks. Direct comparison of optical signals and postsynaptic potentials suggested that dorsal root-evoked voltage images in the dorsal horn consisted of presynaptic action potentials and primarily glutamate-mediated EPSPs. Only small signals, reflecting EPSPs, were recorded in the ventral horn. The well matched time course of local voltage images and EPSPs makes the optical recording a valuable experimental technique for measuring pre- and postsynaptic potentials in targeted set of neurons.
**Discussion**

The characteristics and spatial propagation of dorsal root-evoked neuronal excitation in the postnatal rat spinal cord was measured by recording optical signals from voltage-sensitive absorption dye. Previous studies have employed similar high temporal resolution voltage imaging techniques to monitor spontaneous and evoked network activity in chick and rodent spinal cords, but this is the first study to combine optical and whole-cell recordings and to quantitatively compare voltage images and synaptic potentials in different regions of the spinal cord. The strong correlation between local voltage images and dorsal root-evoked EPSPs indicates that voltage-sensitive dye imaging can reconstruct with a single-cell resolution the synchronous subthreshold activity of set of neurons in the optical field.

*Optical signals from voltage-sensitive dye in the rat spinal cord*

Voltage images of spontaneous and evoked activity in the embryonic chick spinal cord have provided valuable information regarding the ontogeny of neuronal excitation with relation to functional network organization and properties of newly formed neural networks (Arai et al., 1999; Ikeda et al., 1998; Mochida et al., 2001). Only recently fast, voltage-sensitive dyes have been employed to monitor electrical activity in the postnatal rodent spinal cord. A complex spatiotemporal pattern of spontaneous oscillations was
apparent in transverse sections of the neonatal rat spinal cord with depolarizations alternating between mirror locations in the right and left sides of the cord (Demir et al., 2002). Voltage images have also been used in an attempt to characterize the patterns of network activity during neurochemically induced locomotor-like rhythms in the neonatal mouse spinal cord (Arai et al., 2002, 2003). The spatiotemporal pattern of induced rhythms could not be clearly resolved in the cord except for the motor nuclei, in which rhythmic optical signals were closely correlated with ventral root alternating rhythms. It is reasonable to assume that rhythmic voltage images in the motor nuclei are reflective of the synchronous excitation of clustered motoneurons. The inability to detect rhythmic optical signals in other regions of the cord raises the intriguing possibility that interneurons constituting part of the rhythm-generating networks do not aggregate in large clusters (Hinckley et al., 2005a).

Functional interpretation of optical signals can be complicated in the absence of direct comparison with transmembrane potentials, because voltage images consist of extrinsic, dye-dependent signals and intrinsic, dye-independent signals. Under our experimental conditions, as in the neonatal mouse spinal cord (Arai et al., 2002), dorsal root-evoked images were dye-dependent, reflecting voltage-related changes in dye absorption. Interestingly, Arai and colleagues have shown that in the same preparation, induced locomotor-like optical signals recorded with voltage-sensitive absorption dye are dye-independent and therefore are not related to changes in transmembrane potentials. Close correspondence has been shown between optical and electrical responses to dorsal
and ventral root stimuli in chick spinal motoneurons antidromically labeled with voltage-sensitive dye (Wenner et al., 1996). Similarly, we have demonstrated that the spontaneous optical oscillations that alternate between the left/right sides of the rat spinal are closely correlated with extracellular recordings (Demir et al., 2002).

Properties of dorsal root-evoked voltage images

Dorsal root-evoked optical responses consisting of dual-component fast and slow voltage images and single-component slow signals were similar to those recorded in the chick spinal cord. The latencies of the slow voltage images in the intermediate area and the ventral horn varied from 4 to 7 ms. Although it is likely that these slow images corresponded to polysynaptic EPSPs, we cannot rule out the possibility that some were caused by monosynaptic EPSPs. Nerve- and dorsal root-evoked monosynaptic EPSPs with latencies ranging from 3 to 5 msec were recorded in spinal motoneurons of the neonatal rat (Ziskind-Conhaim, 1990, Seebach and Ziskind-Conhaim, 1994). It has been suggested that the spike-like signal was reflective of action potentials in afferent axons, while the slow optical signal was related to postsynaptic potentials (Arai et al., 1999; Mochida et al., 2001, reviewed by Momose-Sato, 2001). Our finding supports this assumption, because the spike-like optical component was not correlated with postsynaptic action potential. Based on our preliminary immunohistochemical observations that the highest density of afferent projections was in the dorsal horn and
the lateral intermediate area, it is reasonable to assume that spike-like signals in those areas originated from synchronous firing in dorsal root afferents.

The time course of slow optical signals was remarkably similar to that of whole-cell subthreshold membrane potentials. This is somewhat surprising because voltage images originated from membrane potential changes in numerous cell bodies, dendrites and axons in the area around the soma. Strong correlation between sensory-evoked optical signals and subthreshold synaptic potentials in single neurons has also been shown in layer 2/3 pyramidal neurons in the rat somatosensory barrel cortex in vitro and in vivo (Petersen and Sakmann, 2001; Petersen et al., 2003). These studies indicated that the high temporal resolution of voltage-sensitive dye imaging can reflect with a single-cell time course the synchronous subthreshold excitation of group of neurons in the optical field.

Blocking glutamatergic synaptic transmission suppressed both synaptic potentials and the slow images in the ventral horn, indicating that those were glutamate-mediated potentials. However, inhibiting glutamatergic synapses only partially blocked the large, slow optical response that followed the spike-like component in the dorsal horn and intermediate area. We made no attempt to characterize the D-APV- and CNQX-resistant component. It is conceivable that this signal corresponded to a slow Ca-dependent afterdepolarizing potential similar to that described in motoneurons in the postnatal rat spinal cord (Walton and Fulton, 1986). An alternative possibility is that the component is reflective of glial depolarization (Konnerth and Orkand, 1986; Lev-
Ram and Grinvald, 1986; Kojima et al., 1999). Optical signals from parallel fibers in the cerebellum of the skate consisted of a spike-like component and a larger, slow component that lasted for more than 400 ms (Konnerth et al., 1987). The slow component that was recorded when using the voltage-sensitive absorption dye RH-155 but not RH-482 was attributed to accumulation of extracellular potassium and glial membrane depolarization. We cannot rule out the possibility that the slow component recorded in our experiments originated from glial depolarization. However, the close correspondence of changes in amplitude and time course of EPSPs with changes in optical signals suggest that a significant fraction of that signal originated from subthreshold excitatory postsynaptic potentials.

GABAergic and glycinergic synapses regulated in a similar manner the amplitude and time course of both EPSPs and voltage images. Disinhibition increased the amplitude of both optical signals and EPSPs, and blocking GABAergic synaptic transmission also prolonged their duration. The prolonged duration might be attributed to the slow decay time constant of GABA$_A$ receptor-mediated synaptic currents (Jonas et al., 1998; Gao et al., 2001). Similar to the distinct actions of glycinergic and GABAergic inhibition on dorsal root-evoked voltage images and EPSPs, we have recently shown that exposure to glycine and GABA$_A$ receptor antagonists affect differently neurochemically induced locomotor-like rhythms in the mouse spinal cord (Hinckley et al., 2005b). Blocking GABA$_A$ receptor-mediated synapses synchronized
the onset and prolonged the duration of rhythmic discharges, while blocking glycine receptors increased tonic discharges and reduced the phase correlation between the alternating rhythms.

Our study demonstrated that dorsal root-evoked local voltage images are closely correlated with EPSPs, indicating that the imaging technique can be effectively used to monitor subthreshold membrane potentials in targeted areas in the postnatal rodent spinal cord. Moreover, the temporal resolution of optical signals from voltage-sensitive dyes provide valuable information regarding the sites of synchronous firing in presynaptic neurons.
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