Plasticity of Spinal Cord Reflexes after a Complete Transection in Adult Rats: Relationship to Stepping Ability

Igor Lavrov\textsuperscript{a}, Yury P. Gerasimenko\textsuperscript{a,c}, Ronaldo M. Ichiyama\textsuperscript{a}, Gregoire Courtine\textsuperscript{a}, Hui Zhong\textsuperscript{a}, Roland R. Roy\textsuperscript{a,b} and V. Reggie Edgerton\textsuperscript{a,b}

\textsuperscript{a}Department of Physiological Science and \textsuperscript{b}Brain Research Institute, University of California, Los Angeles, California 90095; \textsuperscript{c}Pavlov Institute of Physiology, St. Petersburg, 199034, Russia

Running head: Spinal cord reflexes in adult spinal rats

Send all correspondence to: V. Reggie Edgerton, PhD., Department of Physiological Science, University of California, Los Angeles, 621 Charles E. Young Drive, Los Angeles, CA 90095-1527

Final version to the Journal of Neurophysiology on June 27, 2006
ABSTRACT

Changes in epidurally-induced (S1) spinal cord reflexes were studied as a function of the level of restoration of stepping ability following spinal cord transection (ST). Three types of responses were observed. The early response (ER) had a latency of 2.5 to 3 msec and resulted from direct stimulation of motoneurons. The middle response (MR) had a latency of 5 to 7 msec and was monosynaptic. The late response (LR) had a latency of 9 to 11 msec and was polysynaptic. After a complete mid-thoracic ST, the LR was abolished, whereas the MR was facilitated and progressively increased. The LR reappeared approximately three weeks after ST and increased during the following weeks. Restoration of stepping induced by epidural stimulation at 40 Hz coincided with changes in the LR. During the first two weeks post-ST, rats were unable to step and electrophysiological assessment failed to show any LR. Three weeks post-ST, epidural stimulation resulted in a few steps, and these coincided with reappearance of the LR. The ability of rats to step progressively improved from week three to week six post-ST. There was a continuously improved modulation of rhythmic EMG bursts that was correlated with restoration of the LR. These results suggest that restoration of polysynaptic spinal cord reflexes after complete ST coincides with restoration of stepping function when facilitated by epidural stimulation. Combined, these findings support the view that restoration of polysynaptic spinal cord reflexes induced epidurally may provide a measure of functional restoration of spinal cord locomotor networks after ST.

Key words: Locomotion, Central pattern generator, Functional electrical stimulation, Spinal cord transection, Rats.
INTRODUCTION

Numerous examples of clinical and electrophysiological changes after varying time periods following a spinal cord injury and a concomitant loss of supraspinal control have been reported. Flaccid paralysis and an absence of spinal reflexes appear immediately after trauma of the spinal cord, reflecting a period of spinal shock. A second phase after the trauma is often described by a facilitation of spinal cord reflexes and spastic paralysis (Dietz and Colombo, 2004; Ditunno et al. 2004). After this phase of spasticity, the excitability of the spinal reflexes has been reported to decrease after several months (Hiersemenzel et al. 2000). Despite the evolution of these clinical phases, it has been difficult to identify corresponding in vivo electrophysiological characteristics that provide insight into the mechanisms of these clinical phases. In humans, the H-reflex is facilitated during the spinal shock phase and can remain stable over several months, a period when the clinical signs are progressively changing (Hiersemenzel et al. 2000). Some correlations between electrophysiological and clinical evaluation, however, have been reported, e.g., the flexor reflex is abolished during spinal shock and then reappears during the spastic phase (Dietz and Muller 2004).

Different explanations have been proposed for the alterations observed in the spinal cord after trauma, e.g., γ-motoneuron depression that could explain the loss of the tendon reflex during spinal shock (Weaver et al. 1963), and α–motoneuron hyperexcitability due to the abolishment of supraspinal inhibitory control (Ashby et al. 1974). It appears that the restoration of spinal cord activity after injury most likely encompasses a range of reorganization strategies involving interneuronal circuits. It has been suggested that a loss of supraspinal control of interneurons may be one reason for the observed changes in the polysynaptic flexor reflexes after injury (Hiersemenzel et al. 2000). Flexor reflex abolishment during spinal shock may reflect the inhibi-
tion of interneuronal activity (Lundberg 1979). Based on these types of observations, one could reason that the re-organization of spinal circuits after an injury could lead to the restoration of basic spinal cord reflex properties, including interlimb reflexes that could be related to the ability to generate locomotor activity. In fact, an important role of polysynaptic spinal cord reflexes has been emphasized previously in the functional organization of the locomotor pattern in rats (Schouenborg 2002) and cats (Janskowska et al. 1995).

Stepping in spinal animals can be improved by step training (Edgerton et al. 2001; Fouad et al. 2000), by epidural spinal cord stimulation (Ichiyama et al. 2005), and/or by application of neuropharmacological agents that mimic supraspinal excitatory drive (Fong et al. 2005; Douglas et al. 1993; Feraboli-Lohnherr et al. 1999) or reduce inhibition (de Leon et al. 1999). To understand the importance of these interventions requires the identification of the changes that occur in the spinal cord after the injury and the relationship between these changes and the restoration of stepping ability. In the present study, we tested the hypothesis that the restoration of spinal cord reflexes would provide a measure of the functional restoration of the spinal cord networks responsible for generating stepping after a complete spinal cord transection (ST) in adult rats.

METHODS

Nine adult female Sprague Dawley rats (270-300 g body weight) were used in this study. The experimental procedures comply with the guidelines of National Institute of Health Guide for the Care and Use of Laboratory Animals and are conducted in accordance with protocol approved by the Animal Care Committee at the University California, Los Angeles, CA.
Surgical procedures

The rats were anesthetized deeply by a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) administered i.p. and maintained at a surgical level with supplemental doses of ketamine as needed. All surgeries were performed under aseptic conditions.

Headplug

A small skin incision was made at the midline of the skull. The muscles and fascia were retracted laterally, small grooves were made in the skull with a scalpel, and the skull was thoroughly dried. Two 12-pin Omnetic circular connectors (Omnetics, Minneapolis, MN, USA) with Teflon-coated stainless steel wires (AS632, Cooner Wire, Chatsworth, CA, US) were securely attached to the skull with screws and dental cement as previously described (Ichiyama et al. 2005; Roy et al. 1991).

EMG implants

Skin and fascial incisions were made to expose the bellies of the vastus lateralis (VL), semitendinosus (St), medial gastrocnemius (MG) and tibialis anterior (TA) muscles bilaterally. Using hemostats, the EMG wires were routed subcutaneously from the back incision to the appropriate locations in the hindlimb. Bipolar intramuscular EMG electrodes were inserted into the muscles as described previously (Roy et al. 1991). The EMG wires were coiled near each implant site to provide stress relief. Stimulation through the headplug was used to verify the proper placement of the electrodes in each muscle.

Spinal cord transection and epidural stimulation electrode implants

A mid-dorsal skin incision was made between T6 and L4 and the paravertebral muscles retracted as needed. A partial laminectomy was performed at the T8-T9 level and the dura was opened longitudinally. Lidocaine was applied locally and the spinal cord was completely tran-
sected as described previously (Talmadge et al. 2002). A partial laminectomy then was performed at the L2 vertebral level (S1 spinal cord level) and one wire was affixed to the dura at the midline for epidural stimulation using 9.0 suture as described previously (Ichiyama et al. 2005). A small notch made in the Teflon-coating (~0.5 to 1.0 mm) of the wire used for epidural stimulation was placed towards the spinal cord and served as the stimulating electrode. The wire was coiled in the back region to provide stress relief. The Teflon-coating (~1 cm) was stripped from the distal cm of one wire that was inserted subcutaneously in the back region and served as a common ground.

All incision areas were irrigated liberally with warm, sterile saline and closed in layers, i.e., investing fascia and then the skin. All closed incision sites were cleaned thoroughly with saline solution. Analgesia was provided by buprenex (0.5 to 1.0 mg/kg s.c., bid). The analgesics were initiated prior to completion of the surgery and continued for a minimum of 2 days. Baytril, a general antibiotic, was added (2 ml/125 ml H₂O) into their water bottle for 5 days. The rats were allowed to fully recover from anesthesia in an incubator. The rats were housed individually and the bladders of the spinal rats were expressed manually three times per day for the first two weeks after surgery and twice per day thereafter. The hindlimbs of the spinal rats were moved passively through a full range of motion once per day to maintain joint mobility.

Stimulation and recording procedures

Testing procedures were started one week after surgery and were performed at 1, 3, 4, and 6 weeks post-ST (Fig. 1). Electrophysiological assessments were performed in 4 control rats. Subsequently, the spinal cords of two of those rats were transected and electrophysiological assessments were made after the transection. In 5 other rats electrophysiological testing was per-
formed only after ST. Therefore, these analyses were performed on 4 rats in the control condition and on 7 rats after ST. All testing was performed when the rats were fully awake, except for the terminal experiment (see below). The EMG signals were recorded (2000 Hz), amplified, and filtered (10 to 1,000 Hz band-pass). Stimulation was performed using a Grass S88 Stimulator (Grass Instruments) and a stimulus isolation unit (Grass SIU5, Grass Instruments). Epidural stimulation at S1 was performed via single stimuli (duration of 0.5 ms). Spinal cord reflexes were tested initially by stimulation at 0.2 Hz to determine the stimulus intensity-response amplitude curve. Averages of 7 responses were collected at stimulation intensities between 0.5 to 10 V. The average responses at 0.2 Hz were compared with those at 1, 3, and 5 Hz. Changes in the reflex responses at the different frequencies of stimulation are expressed as a percent of the response at 0.2 Hz.

To test for locomotor ability, the ST rats were secured in an upper body harness support system and the hindlimbs were placed on a moving treadmill as described previously (Ichiyama et al. 2005). Spinal cord stimulation at S1 was induced by continuous epidural electrical stimulation at 40 Hz (25 ms pulse interval) and a pulse duration of 0.2 ms. The automated body weight support system was used to provide the amount of body weight support necessary to enable walking. EMG activity was collected during stepping using a custom-made LabView program. As a measure of locomotor capability, the duration of continuous stepping was recorded under optimal epidural stimulation conditions.

**Terminal experiment**

After six weeks of evaluation (Fig. 1), four ST rats were tested for the classical H-reflex/M-response ratio. The rats were anesthetized deeply with ketamine (100 mg/kg) and xy-
lazine (5 mg/kg) i.p. A skin incision was made on the lateral aspect of the left leg to expose the soleus muscle and a pair of wires was implanted for EMG recordings as described above. A second skin incision was made on the dorsal surface of the left leg from mid-thigh to slightly below the popliteal fossa. The fascial sheath separating the St and biceps femoris muscles was incised longitudinally to expose the sciatic nerve and its two main branches, i.e., the tibial and peroneal nerves. Using metal pins in the lower lumbar vertebral column and at the malleoli, the left hindlimb was positioned such that partial flexion and extension was possible at the hip, knee, and ankle joints. Silver hook electrodes were placed on the sciatic nerve immediately above its subdivision into the tibial and peroneal nerves and single stimuli (pulse duration of 0.2 ms) were delivered. The cathode was oriented proximal to the anode. The average responses at stimulation frequencies of 1.0, 3.0, 5.0, and 10 Hz and at a pulse duration of 0.5 ms were compared with responses at 0.2 Hz. A 5-min rest interval was allowed between the testing at each stimulation frequency. During testing, the body temperature of the rat was maintained at 36±1°C using a circulating water heating pad and the exposed muscles and nerves were kept moist with warm saline.

**Insert Figure 1 near here**

**Data analysis**

All recordings were collected between a range of 0.5 to 10 V. To identify the latencies and amplitudes of a given response, the responses at the full range of voltages were examined simultaneously. The latencies were determined at the threshold voltage at which each type of response appeared. Comparisons of the responses at different voltages were used to identify the onset and amplitude of the early response (ER), middle response (MR), and late response (LR)
(Gerasimenko et al., 2005). In each test session, it could be determined that these three responses were modulated differentially, were clearly voltage dependent, and the onset of each of response could be determined consistently (Fig. 2). All electrophysiological recordings from the muscles were analyzed within a 14 ms interval from the stimulus artifact and divided into three windows based on the onset latencies of the three responses, i.e., 1.5 to 6.5 ms for the ER, 6.5 to 10.5 ms for the MR, and 10.5 to 13.5 ms for the LR. The maximum peak-to-peak amplitude of each response was calculated as the average of seven responses and reported as a percentage of the control value. The peak-to-peak amplitude was on the positive slope of the response for the ER and on the negative slope for the MR and LR (Fig. 2). The absolute values of the ER were used for comparisons across time. The control value for the MR was based on the response amplitude before ST for the 2 rats that were evaluated before and after surgery, and the response amplitude at one week post-surgery for the other 7 rats. The Lrmax/Ermax ratio was used for evaluation of the LR.

**Statistical analyses.**

All data are reported as mean±SEM. Statistically significant differences were determined using a one-way repeated measures analysis of variance (ANOVA). Values that were not normally distributed were analyzed using the nonparametric Kruskal-Wallis rank test for overall changes and the Wilcoxon sign rank test to determine differences between the first and last test. The criterion level for statistical significance was set at P< 0.05 for all analyses.
RESULTS

All control rats were trained to stand quietly in a bipedal position in a body weight support system. A detailed analysis of the responses produced by epidural electrical stimulation in control rats has been reported previously (Gerasimenko et al. 2005). Briefly, when the rats were in a bipedal standing position stimulation of S1 produced ERs with latencies of 2 to 3 ms, MRs with latencies of 5 to 7 ms, and LRs with latencies of 8.5 to 11 ms (Figs. 2A and 3A). The MRs and LRs appeared at the lower voltages (< 4 V) of stimulation and were inhibited with an increase in stimulation intensity (up to 6 V). The ER appeared at the higher intensities of stimulation (> 4 V), with the amplitude increasing with increased stimulus strength (Fig. 2A).

**Insert Figure 2 near here**

The evoked responses to S1 stimulation in ST rats were evaluated at 1, 3, 4, and 6 weeks after surgery. After ST, the latencies of the three responses were similar to those observed before ST, and were significantly different from each other (Fig. 3A and B). However, the amplitude of the three responses induced by S1 stimulation differed from the control condition. For example, in contrast to the control condition (see above), the MR was facilitated and the LR appeared only at the higher stimulation intensities at six weeks post-ST (Fig. 2B).

**Insert Figure 3 near here**

*Restoration of spinal cord reflexes after ST*

To compare the three responses before and after ST, two of the seven rats that were spinalized were evaluated prior to and during the six weeks after surgery. The perturbation of the
ER was not tested at > 6 V due to the apparent discomfort to the rat. An example of the changes in the MR from the period prior to ST and up to six weeks post-ST are shown for one rat in Figure 4A. Compared to control values, the MR was facilitated in the distal (> 2-fold), but not proximal muscles, one week after ST and continued to increase thereafter. The MR of the distal muscles was facilitated (3- to 4-fold) at six weeks after ST. The LR initially was depressed after surgery, but reappeared about 3 to 5 weeks post-ST (Fig. 4B). Although the LR increased after its reappearance, the amplitude remained lower than control even six weeks post-ST (30-60% of pre-ST values).

The amplitudes of the ER, MR, and LR gradually increased post-ST in all seven rats tested (Fig. 5). The ER gradually increased following ST. At six weeks post-ST, the mean amplitude of the ER was increased by 100, 34, 67, and 75% for the MG, VL, TA, and St, respectively, compared to the values at one week post-ST. For the same comparison, the mean amplitude of the MR was increased by 95% for the MG, 135% for the VL, 149% for the TA (P> 0.05), and 193±% for the St (P> 0.05) (Fig. 5B). The amplitude of the LR, estimated by the relationship between LRmax/ERmax, was increased by ~6-, 7-, 11-, and 7-fold for the MG, VL, TA, and St, respectively, compared to 1 week post-ST (Fig. 5C).

Rate-sensitive depression
When normalized to the values at 0.2 Hz, there was a progressive decrease in the MR amplitude with increasing frequency of stimulation (Fig. 6B). At 5 Hz, the MR amplitudes for the MG, TA, VL, and St were 29, 33, 28, and 50% of the value at 0.2 Hz. The LR also showed a progressive decrease in amplitude with increased frequencies of stimulation such that the values at 3 and 5 Hz were < 10% of those at 0.2 Hz for all muscles (Fig. 6C). In contrast, the ER was not significantly affected with increased rates of stimulation.

Insert Figure 6 near here

During the terminal experiment the rats were under ketamine anesthesia. Under these conditions the MR was present, but decreased with increased frequencies of stimulation (Fig. 7A). At 5 Hz stimulation, the mean MR amplitude was ~40, 41, 27, and 33% of the amplitude at 0.2 Hz stimulation for the MG, TA VL and St, respectively. In contrast, the ER in all muscles tested was unaffected, and the LR was absent in the TA, MG, and VL at all stimulation frequencies. A very small response was observed in the St in 2 out of the rats tested. Figure 7B shows the effects of increased stimulation frequency on the responses in the TA muscle in an anesthetized rat.

Insert Figure 7 near here

H-reflex/M-wave testing

To compare the responses produced by S1 epidural stimulation with the classical monosynaptic H-reflex and the direct M-wave, experiments were conducted on 4 rats 43 days post-ST
The rats were anesthetized (ketamine, 100 mg/kg body weight), the sciatic nerve stimulated with single pulses, and the motor responses monitored in the soleus muscle (see Methods). Sciatic nerve stimulation produced two motor responses: a M-wave with a latency of 1-3 ms and a H-reflex with a latency of 5-7 ms (Fig. 8B). The amplitude of the M-wave was unaffected by varying stimulation frequencies (Fig. 8B), whereas the amplitude of the H-reflex progressively decreased at stimulation frequencies from 0.2 to 10 Hz (Fig. 8A). The H-reflex amplitude at 3, 5, and 10 Hz was 72, 50, and 30% of the amplitude at 0.2 and 1 Hz.

Insert Figure 8 near here

Restoration of stepping after ST

All rats were tested for their ability to step with epidural stimulation once per week post-ST. Stimulation of S1 at 40 Hz failed to produce any rhythmic activity during the first two weeks post-ST (Fig. 9A). After three weeks, S1 stimulation evoked a few steps, but the EMG signals were erratic (Figs. 9A, 10A). The mean duration of this rhythmic activity was 8±5 sec. After four weeks, the rhythmic activity between the flexors and extensors within a limb and between the left and right hindlimbs was more coordinated than after 3 weeks. In addition, the mean duration of the bouts of stepping increased to 23±2 sec. Stepping performance and the duration of the stepping bouts progressively increased during the next two weeks, such that the EMG bursts reflected a well-organized stepping pattern and the mean duration of the bouts of stepping was increased to 70±17 sec at 42 days post-lesion (Fig. 9A). During all evaluation periods, the stepping was characterized as short periods of robust rhythmic activity. It should be noted, however, that despite the robust EMG bursts, and clear swing and stance phases observed within 6 weeks after
surgery, a high level of weight support (85-95% of body weight) was required to generate these stepping patterns.

**Insert Figures 9 and 10 near here**

The restoration of stepping using epidural stimulation at S1 coincided with the restoration of the LR for all rats tested. One week after surgery a single stimulus failed to evoke a LR. After 3 weeks, the appearance of a small LR coincided with the generation of a few steps on the treadmill when being stimulated epidurally at 40 Hz (Figs. 9 and 10). Furthermore, the restoration of the LR amplitude between 4 and 6 weeks post-ST corresponded to the restoration of a coordinated stepping pattern with improved EMG activity.

**DISCUSSION**

The ability to assess the efficacy of identifiable spinal pathways in vivo during normal behavior has been limited largely to the H-reflex, an electrically-induced version of a monosynaptic reflex. Furthermore, the study of this reflex has been largely limited to distal muscles such as the soleus and interosseous muscles. Recently, we reported the ability to measure monosynaptic and polysynaptic responses from multiple motor pools simultaneously in normal and ST awake rats during standing and stepping (Gerasimenko et al. 2005; Lavrov et al. 2005). These results showed clear and consistent modulation of these responses with epidural stimulation of the L2 or S1 segments of the spinal cord that was related to specific phases of the step cycle.

In the present study, we report the responses to similar modes of epidural stimulation before and up to 6 weeks after a complete mid-thoracic ST. We evaluated the long-term perturba-
tion of the spinal cord reflexes, and determined the relationship between the restoration of the ability to step produced by epidural stimulation and the restoration of the spinal cord responses. We found new evidence that provides further insight into the nature of the responses produced by epidural spinal cord stimulation. The major changes observed after ST include the following: a gradual increase of the ER, a facilitation of the MR at all time points studied, an initial abolishment of the polysynaptic LR during the early stages, followed by a gradual recovery beginning about three weeks post-ST. Furthermore, the restoration of the ability to step in response to epidural stimulation about three weeks after surgery coincided with the restoration of the LR. Perhaps the re-appearance of these electrically-induced polysynaptic reflexes after a spinal cord injury may signify the presence of functional locomotor networks.

The nature of the responses produced by spinal cord epidural stimulation

ER. The characteristics of the ER are consistent with the direct activation of motor fibers or motoneurons. This response has a short latency (2.5 to 3.0 ms) and shows a maximum amplitude with supra-maximal stimulation. The short latency of the ER, theoretically, includes only the neuromuscular synaptic delay and the conduction time of the stimulus to the muscle, and involves no synaptic delay within the spinal cord. (Note that a synaptic delay in rats has been estimated to be ~2 to 3 ms (Meinck 1976). This interpretation would be consistent with the observation that direct spinal cord stimulation at the lumbar level by magnetic field produces an M-wave with a latency of ~1.5 ms (Chiba et al. 2003). The ER was the only response that was not affected by varying the frequency of stimulation, but was increased with increasing stimulus intensity.
The MR reflects the behavior of one or more synaptic delays. The MR has a latency of ~5 to 6 ms and is inhibited by increasing either the intensity or the frequency of stimulation. In normal rats the MR was inhibited by Achilles tendon vibration and during double pulse testing (Gerasimenko et al. 2005). The MR was facilitated at all time points after ST, similar to the behavior of the H-reflex in spinal animals (Reese et al. 2005; Valero-Cabre et al. 2004; Skinner et al. 1996). The latency of the MR that we observed was similar to that of the monosynaptic response to magnetic stimulation of the lumbar enlargement in normal and spinally contused rats (Chiba et al. 2003). In the present study, stimulation at 5 Hz after six weeks of ST inhibited the MR by 50 to 70% of control values. Reduction in frequency-dependent depression has been described for the monosynaptic response in ST (Skinner at al. 1996; Reese et al. 2005) and contused rats (Thompson et al. 1992; Lee et al. 2005). With stimulation at 10 Hz, the H-reflex was almost completely inhibited (7% of control) in control rats, but was maintained at a significantly higher level (56% of control) in ST rats (Skinner et al. 1996). The lower level of inhibition of the H-reflex after ST may be related to a decrease in presynaptic inhibition on Ia afferents after degeneration of descending tracts (Thompson et al. 1992; Delwaide 1973).

To determine if the main responses produced by S1 stimulation were related to the M-wave and H-reflex, we performed acute terminal experiments under ketamine anesthesia. Single pulse stimulation of the sciatic nerve produced two responses in the soleus muscle. The latencies of the first (direct M-wave) and second (monosynaptic H-reflex) were ~2 to 3 ms and ~5 to 7 ms, respectively, and correspond to the latencies for the M-wave and H-reflex in the classical testing of the H-reflex in the soleus muscle (Wolpaw and Chen 2001; Chen, Wolpaw 1995). The MR was inhibited by increasing the stimulus frequency from 1 to 5 Hz at 6 weeks post-ST in response to epidural stimulation with the same rats awake or under ketamine anesthesia. The level
of inhibition of the H-reflex in response to sciatic nerve stimulation under ketamine anesthesia was similar to the inhibition of the MR noted above. The similar percent of inhibition for the MR with S1 stimulation and for the H-reflex with sciatic nerve stimulation suggests that there are some common components of these two responses.

**LR.** The LR has the longest latency of the three responses (9 to 11 ms) and represents a polysynaptic event. The latency difference between the ER and LR was ~6.5 to 8 ms and may correspond to as many as 3 synaptic delays. With increased stimulus intensity the amplitude of the LR was initially increased and then was completely inhibited at high stimulus intensities. Similar to the MR, the LR was inhibited by Achilles tendon vibration and during double pulse testing (Gerasimenko et al. 2005). The LR was completely inhibited by stimulation at 3 Hz, whereas the MR was only partially inhibited by stimulation at 5 Hz. These observations may reflect the inhibition of different neural circuits as suggested by Schindler-Ivens and Shields (2000).

The LR was abolished in most of the muscles when the animals were tested under ketamine anesthesia. The ability of ketamine, a non-competitive NMDA antagonist, to inhibit NMDA receptors via NMDA channel block and to depress the polysynaptic reflexes is well known. In adult rats, ketamine inhibits NMDA receptors, but has a sparing effect on kainate and AMPA receptors (Farkas and Ono 1995). A significant effect of ketamine on NMDA receptors of dorsal horn neurons at a concentration of 5 mg/kg (administered iv) has been reported (Chizh et al. 1997) and would be expected to have been present at the anesthetic dosages used in the present study. Inhibition of the LR by ketamine suggests a polysynaptic NMDA dependent origin of the LR. An effect of ketamine on monosynaptic reflexes, however, also should be noted (Farkas and Ono 1995).
The question is how does the polysynaptic response as induced epidurally relate to the more classically defined reflexes, such as the flexor and crossed extensor reflexes. The flexor reflex pathways contain 2 to 3 synapses and are mediated by ipsilateral and contralateral cutaneous afferents from pain and temperature endings (C), touch, temperature and pain endings (A\(\epsilon\)), pacinian corpuscles (vibration), touch receptors (A\(\beta\)), and joint receptors (A\(\alpha\)). The last two axonal types, i.e., the A\(\beta\) and A\(\alpha\), are thick fibers with diameters between 12 to 20 and 5 to 12 \(\mu\)m, respectively, have a low threshold of activation, and may have responded to the low intensities of stimulation in the present study. Group II and III afferents from the muscle spindles and pressure-pain endings also may be considered as afferents which could have mediated the LR as discussed previously (Gerasimenko et al. 2005). Another possible explanation for the LR is a direct activation of interneurons, but due to the high thresholds required to activate these neurons, this seems unlikely. Regardless, it is clear that the LR produced by spinal cord epidural stimulation has a polysynaptic origin. At this time, however, the identification of the polysynaptic chains and their role in spinal cord synaptic organization are unclear.

Changes in the spinal cord responses after ST

The three electrically-induced responses were affected differentially post-ST. We found continuously increasing amplitudes of the ER, facilitation of the MR, and an initial disappearance and then a gradual reappearance of the LR beginning at about three weeks post-ST.

ER. Inhibition of a direct response after spinal cord injury has been described in paraplegic humans (Hiersemenzel et al. 2000) and in spinal rats (Valero-Cabre et al. 2004). These adaptations can be explained by reversible changes in axonal properties after injury (LoPachin et al. 1999, Agrawal and Fehlings 1996) that correspond to inhibition of the M-wave in paraplegic, but
not in quadriplegic patients (Hiersemenzel et al. 2000). Due to the high intensity stimulation required to evoke a direct motor response, we could not evaluate the ER in normal awake rats. However, based on the previous observations noted above, we interpret the increasing ER post-ST as a restoration of the direct response.

**MR.** In humans and non-human primates there is an inhibition of the H-reflex for a few days after spinal cord injury (Hiersemenzel et al. 2000). In other species this period has been reported to be shorter, e.g., the inhibition of the H-reflex was about 6 days after spinal cord contusion in rats (Thompson et al. 1993). In addition, facilitation of the H-reflex within one hour after a complete ST (T9) in rats without a period of inhibition has been reported (Valero-Cabre et al. 2004). This variability, however, may be related to differences in the type of injury and experimental designs. In our study of complete mid-thoracic adult spinal rats, the MR was facilitated one week post-ST, being up to 2- to 3-fold higher than in control rats. Facilitation of the mono-synaptic reflexes (corresponding to the MR) after a spinal cord injury has been reported in spinal rats (Malmsten 1983, Chen et al 2001), cats (Hultborn and Malmsten 1983), and humans (Leis et al. 1996, Hiersemenzel et al. 2000). H-reflex facilitation after a spinal cord injury could be attributed to morpho-functional changes in motoneurons, and/or changes at the pre-motoneuron level due to disinhibition. The first type of change could include α-motoneuron hyperexcitability due to changes in the intrinsic properties of the motoneurons (Taylor et al. 1984), changes in motoneuron morphology, and/or synaptic growth. The second type of change might be attributable to a decrease in presynaptic inhibition (Calancie et al 1993; Faist et al 1999), and/or a decrease in post-activation depression of transmission from Ia fibers (Nelson et al. 1979). In rats, the peak facilitation of the H-reflex was observed at 45 days post-ST, after which the amplitude of the H-reflex decreased (Valero-Cabre et al. 2004). There have been no long-term investigations, how-
ever, to determine whether there is an eventual complete restoration of the H-reflex after a spinal cord injury.

LR. After ST, the LR was abolished for two weeks. At the beginning of the third week the LR reappeared as a low-amplitude, unstable response and showed a tendency to be restored during the following evaluation periods. The timing of the restoration of the LR in our studies corresponds with the reappearance of polysynaptic reflexes in ST rats. For example, flexor reflexes were inhibited immediately after ST and then restored 2 to 3 weeks post-ST (Valero-Cabre et al. 2004). These authors described different patterns of restoration of the polysynaptic reflexes on the ipsilateral and contralateral sides of the lesion. All ipsilateral and crossed reflex components were abolished immediately after injury except for the ipsilateral reflex components mediated by the thick sensory Aα and Aα/Aβ fibers. Over the next 6 weeks post-ST, all polysynaptic reflexes mediated ipsilaterally by all axonal types tested increased toward normal. However, the ipsilateral Aα- and Aα/Aβ-mediated reflexes increased up to 3-fold greater than normal. Since we used direct stimulation at the midline of the spinal cord, we most likely activated both ipsilateral and crossed polysynaptic reflex pathways.

Spinal cord reorganization following injury and restoration of stepping ability

After a spinal cord injury in humans, the restoration of polysynaptic flexor reflexes has been reported to coincide with the appearance of the “spastic” phase of recovery, and appears to be related to the recovery of α–motoneuron excitability and/or adaptation of interneuronal activity. The interneuronal hypothesis is supported by the fact that there is a lower threshold for flexor reflex activation in injured than non-injured individuals (Hiersemenzel 2000; Dietz, Muller 2004). The recovery of complex spinal cord reflexes such as the flexor reflex after an injury may
provide a functional basis for the restoration of spinal cord circuits responsible for locomotion. In fact, some of the spinal cord networks that control stepping seem to share the same interneurons that are involved in flexion reflexes, i.e., the spinal circuits involved with the flexor reflex have been suggested to be part of the spinal networks responsible for locomotion (Dietz 2002; Parise et al 1997). Thus several findings support the view that the same spinal pathways are involved in generating the flexor reflex and the swing phase of stepping (Jankowska et al. 1967; Lundberg 1979; Bussel et al. 1989; Nicol et al. 1995). In the present study, the reappearance of the LR post-ST may reflect the restoration of polysynaptic networks responsible for flexor and cross-extension reflexes that are involved in locomotor circuits. Consistent with this supposition are the results of a recent long-term study reporting the restoration of flexor reflexes in ST rats, most likely reflecting some reorganization of the same spinal pathways (Valero-Cabre et al. 2004). From this perspective, the stepping patterns in response to epidural stimulation presumably can be attributed to the stimulation of the same afferents as the evoked responses studied in the present paper.

Conclusions

In the present study, the electrophysiological evaluation of the functional chronic state of the spinal cord for six weeks after a complete, mid-thoracic ST and of the restoration of epidurally-induced locomotor activity was performed. The results show that the restoration of stepping when facilitated by epidural stimulation of the spinal cord coincided with the restoration of late polysynaptic responses. Therefore, the electrophysiological assessment of spinal cord reflexes induced by epidural stimulation to facilitate stepping appears to be a useful tool for investigating spinal cord reorganization after a spinal cord injury.
Acknowledgments

This research was supported, in part, by the Christopher Reeve Foundation, California Roman Reed Fund, and the National Institute of Health Grant NS16333. We thank Maynor Herrera for providing excellent animal care, Sharon Zdunowski, Edward Lan, Rebekah Molyneux and Stephanie Enriquez for their technical assistance and constructive comments.
REFERENCES


Jankowska E, Jukes MG, Lund S, and Lundberg A. The effect of DOPA on the spinal cord.


FIGURE LEGENDS

**Figure 1.** Schematic of the experimental design. Electrophysiological data (control test) were collected on 4 rats 10 days prior to the spinal cord transection (ST) surgery. A complete ST (T7-8) was performed on 7 rats (including 2 rats tested prior to the lesion) and these rats were evaluated electrophysiologically at 7, 21, 28, and 42 days post-ST. Four of these rats (including 2 rats tested prior to the lesion) were tested in a terminal experiment (43 days post-ST). d, days; Imp, day of implant of the EMG and epidural electrodes.

**Figure 2.** S1 epidural stimulation produces three responses before (A) and 6 weeks after a complete ST (B) as shown for the tibialis anterior (TA): a direct motor early response (ER) with a short latency, a middle monosynaptic response (MR), and a late response (LR) with polysynaptic origin. Each trace is the average of 7 records. Stimulation intensity varied from 4 to 5.5 V. The traces shown in this and all subsequent Figures begin immediately following the stimulation artefact (0.5 ms duration, recorded on a separate channel). The windows for each of the three responses are delineated with dashed lines (see Methods for further details) in this and subsequent Figures. The peak amplitude (vertical lines with small arrowheads) of the ER was calculated on the positive slope for the ER and on the negative slope for the MR and LR. This was done consistently for all rats. Since the maximum amplitudes were elicited at varying voltages and these voltages differed between control and ST rats, this Figure does not show maximum amplitudes for all responses under the conditions studied in the present paper. Also note that although the responses after ST suggest a shorter latency, this was not the case in that the latencies were defined
at the voltage threshold at which they first appeared, i.e., at voltages lower than shown in this Fig.

**Figure 3.** Mean (± SEM) latencies of the ER, MR, and LR produced by S1 epidural stimulation before (n = 4) (A) and 7, 21, 28, and 42 days after ST (n = 7) (B). Note the consistency of the latencies of each response across the four muscles studied and the similarity in the latencies of each response for up to 42 days post-ST. For each muscle, the mean latencies for the ER, MR, and LR were significantly different from each other at each time point (p< 0.05). MG, medial gastrocnemius; VL, vastus lateralis, and St, semitendinosus. Other abbreviations, same as in Fig. 2.

**Figure 4.** Changes in the amplitudes of the MR (A) and LR (B) for the MG, TA, VL, and St muscles from 1 to 6 weeks post-ST expressed as a percentage of pre-ST values for one representative rat. In general, there was a progressive increase in the MR and a decrease in the LR post-ST. Abbreviations, same as in Figs. 2 and 3.

**Figure 5.** Changes in the mean (± SEM) amplitudes of the ER (A), MR (B), and LR (C) for the MG, TA, VL, and St muscles from 1 to 6 weeks after ST. Each data point represents the average of 7 recordings from 7 rats. The ER amplitudes (A) are shown as absolute values. The MR (B) and LR (C) values are expressed as a percentage of maximum MR values at week one post-ST and of maximum ER at each time point, respectively. There a significant increase in the amplitude of each response in all muscles over time post-ST, except for the MR in the St and TA. Abbreviations, same as in Figs. 2 and 3.
**Figure 6.** Changes in amplitude of MR and LR as a function of the frequency of stimulation. Representative traces for the TA muscle showing the ER, MR, and LR elicited at different stimulation frequencies are shown in (A). Changes in the mean (± SEM) amplitudes of the MR (B) and LR (C) for the MG, TA, VL, and St muscles as a function of the frequency of stimulation expressed as a percentage of the amplitude at 0.2 Hz. Each data point represents the average of 7 recordings from 7 rats on the last day of testing (42 days post-ST). There was a general trend for a decrease in the amplitude of each response in all muscles with an increase in stimulation frequency. The MR and LR values at each stimulation frequency are significantly lower than the values at 0.2 Hz (P < 0.05), except for the LR at 1 Hz. Abbreviations, same as in Figs. 2 and 3.

**Figure 7.** Changes in the mean (± SEM) amplitudes of the MR for the MG, TA, VL, and St muscles as a function of the frequency of stimulation expressed as a percentage of the amplitude at 0.2 Hz with the rat anesthetized with ketamine (100 mg/kg body weight) (A). Each data point represents the average of 7 recordings from 4 rats. There was a general trend for a decrease in the amplitude of each response in all muscles with an increase in stimulation frequency. The MR is significantly lower at 5 than 0.2 Hz (P< 0.05). A representative trace for the TA muscle at each stimulation frequency is shown in (B). Note that the MR decreases with an increase in stimulation rate, whereas the ER is unaffected. No LR was observed under these conditions. Abbreviations, same as in Figs. 2 and 3.
Figure 8. Changes in the mean (± SEM) amplitudes of the H-reflex for the soleus muscle as a function of the frequency of stimulation expressed as a percentage of the amplitude at 0.2 Hz (A). Each data point represents the average of 7 recordings from 4 rats. There was a general trend for a decrease in the H-reflex amplitude with an increase in stimulation frequency. The H-reflex is significantly lower at 10 than 0.2 Hz (P< 0.05). A representative trace of the H-reflex and M-wave at each stimulation frequency is shown in (B). Note that the M-wave and H-reflex as defined in the anesthetized preparation when directly stimulating the sciatic nerve are thought to be equivalent to the ER and MR when elicited by epidural stimulation.

Figure 9. Changes in the mean (± SEM) duration of stepping at 1 to 6 weeks post-ST (0 on abscissa is the day of the ST surgery) (A). Each data point represents the average from 4 rats. Note that the duration of the stepping bouts progressively increases from week 3 to week 6. The relationship between the LR amplitude expressed as a percent of the maximum ER and the duration of stepping induced by epidural stimulation is shown in (B). Individual data from 4 rats are plotted in each graph. All points overlap at week 1 when the spinal rats could not step and there was no LR. Note that there is a progressive increase in the LR amplitude in each muscle that is associated with an increase in stepping duration from week 1 to week 6.

Figure 10. EMG patterns of the MG, TA, VL and St muscles at 3, 4 and 6 weeks post-ST during stepping induced by epidural stimulation (40 Hz) at S1 (A). Note the more robust and consistent EMG burst patterns at 6 compared to 3 and 4 weeks. Representative ER, MR, and LR from the
TA muscle at 4.5 V stimulation 3, 4, and 6 weeks after ST are shown in (B). Note that the LR is minimal at 3 weeks and progressively increases from 4 to 6 weeks.