Title: CORTICAL STIMULATION CAUSES LONG-TERM CHANGES IN H-REFLEXES AND SPINAL MOTONEURON GABA RECEPTORS

Authors: Yu Wang
Yi Chen
Lu Chen
Jonathan R. Wolpaw
Xiang Yang Chen

Address: Laboratory of Neural Injury and Repair, Wadsworth Center, New York State Department of Health and State University of New York, Albany, New York 12201-0509

Author Contributions:

Y. Wang was directly responsible for the tissue processing, the immunohistochemical analyses, the anatomical data processing, and preparation of the manuscript.

Y. Chen participated extensively in the collection of the long-term behavioral data.

L. Chen participated in animal surgery, in long-term oversight during data collection, and in tissue processing.

J.R. Wolpaw planned the study with X.Y. Chen, participated in data analysis, and was primarily responsible for formulating the present manuscript.

X.Y. Chen planned the study with J.R. Wolpaw, was directly responsible for animal surgery, long-term oversight during data collection, analysis of behavioral data, and preparation of the manuscript.

Contact Information: Xiang Yang Chen
Laboratory of Neuronal Injury and Repair
Wadsworth Center, New York State Dept. of Health
P.O. Box 509, Albany, New York 12201-0509
Voice: 518-486-4916, 518-473-3631; Fax: 518-486-4910
E-mail: chenx@wadsworth.org

Running Head: Cortical stimulation and long-term change in the spinal cord

Number of Figures and Tables: 5 figures; no tables
Number of Words: Abstract 120; Introduction 445; Discussion 2069

Copyright © 2012 by the American Physiological Society.
The cortex gradually modifies the spinal cord during development, throughout later life, and in response to trauma or disease. The mechanisms of this essential function are not well understood. In this study, weak electrical stimulation of rat sensorimotor cortex increased the soleus H-reflex, increased the numbers and sizes of GABAergic spinal interneurons and GABAergic terminals on soleus motoneurons, and decreased GABA\(_A\) and GABA\(_B\) receptor labeling in these motoneurons. Several months after the stimulation ended, the interneuron and terminal increases had disappeared, but the H-reflex increase and the receptor decreases remained. The changes in GABAergic terminals and GABA\(_B\) receptors accurately predicted the changes in H-reflex size. The results reveal a new long-term dimension to cortical-spinal interactions and raise new therapeutic possibilities.

Key words: brain stimulation; spinal cord plasticity; GABAergic terminal, interneuron, and receptor; learning and memory
INTRODUCTION

The immediate effects of cortical neuronal activity on the spinal cord are widely studied (e.g., Alstermark and Isa, 2012), but its long-term effects have received much less attention. Nevertheless, during development and throughout life the cortex gradually changes spinal pathways to support the acquisition and maintenance of skills such as locomotion; and impairments in this regulation contribute to the disabilities produced by strokes, spinal cord injuries, and other disorders (Knikou 2010; Wolpaw 2010). The mechanisms of this long-term regulation are poorly understood. We are using long-term cortical regulation of the H-reflex, the electrical analog of the spinal stretch reflex (SSR), as a model for studying this important cortical function. The H-reflex, the simplest behavior of the vertebrate CNS, is produced by a wholly spinal and largely monosynaptic pathway consisting of the primary afferent neuron, the motoneuron, and the synapse between them.

In primates and rodents, an operant conditioning protocol can change sensorimotor cortex (SMC) activity and thereby gradually increase or decrease H-reflex size (Wolpaw 2010; Wolpaw and Chen 2009). These larger or smaller reflexes, which persist after conditioning ends, are simple motor skills (i.e., adaptive behaviors acquired through practice (Compact OED 1993)). The reflex changes are produced and maintained by a complex hierarchy of plasticity at both spinal and supraspinal sites (Wolpaw and Chen 2006, and Wolpaw 2010 for review). While much remains to be learned about this hierarchy and the sites and mechanisms of plasticity, it is clear that SMC activity modifies the H-reflex by producing changes in the motoneuron (e.g., in firing threshold), as well as elsewhere in the spinal cord. Recent studies showing that operantly conditioned H-reflex decreases are accompanied by marked changes in GABAergic interneurons in the ventral horn and in GABAergic terminals on spinal motoneurons suggest that changes in GABAergic function may play a key role in producing the motoneuron plasticity directly underlying H-reflex conditioning (Wang et al. 2006a, 2009).

Another recent study revealed that weak electrical stimulation of SMC, like the operant conditioning protocol, can change (i.e., increase) H-reflex size (Chen et al. 2007a). Motivated by this finding and by the data suggesting that change in GABAergic function contributes to H-reflex conditioning, the present study asked three questions. First, is the H-reflex change produced by SMC stimulation also accompanied by changes in GABAergic spinal interneurons, terminals on the motoneurons, or receptors in the
motoneurons? Second, do the GABAergic changes that occur persist after stimulation ends? Three, do they correlate with the persistence of the H-reflex change? The results are clear and surprising. They demonstrate that transient cortical stimulation can produce lasting changes in spinal cord GABA receptors and motor function; and they provide insight into how these changes occur.

MATERIALS AND METHODS

Subjects were 31 young adult Sprague-Dawley rats (male, <6 months old, 546(±90SD) g (range 405-716 g) at the time of perfusion). The Experimental Group of 18 rats (584(±78SD) g) underwent electrode implantation, H-reflex monitoring, SMC stimulation, perfusion, and anatomical study. The Control Group of 13 rats (511(±64SD) g) underwent perfusion and anatomical study. All procedures satisfied the "Guide for the Care and Use of Laboratory Animals" (National Academy Press, Washington, D.C. 2011) and had been approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. The procedures for electrode implantation, H-reflex monitoring, SMC stimulation, motoneuron labeling, and immunohistochemical processing have been described previously (Chen et al. 2007a; Wang et al. 2006a, 2009; Pillai et al. 2008) and are summarized here. GABA receptor labeling methods are described in detail.

Electrode implantation, H-reflex elicitation, and SMC stimulation

Under general anesthesia, each Experimental Group rat was implanted with recording and stimulating electrodes. To record soleus electromyographic (EMG) activity, a pair of fine-wire EMG recording electrodes was implanted in the right (n=7) or both soleus muscles (n=11). To elicit the soleus H-reflex, a nerve cuff containing a pair of fine-wire electrodes was implanted on the right or both posterior tibial nerves just above the triceps surae branches. To stimulate SMC, a pair of stimulating electrodes (0.5 mm-diameter stainless steel screws) was placed in the skull above the dura over the leg area of the left SMC (i.e., 1.0 mm and 3.0 mm caudal to bregma and 2.8 mm lateral to the midline). To record the descending spinal cord volley produced by SMC stimulation, a pair of fine-wire electrodes was placed above the dura over the dorsal midline of the spinal cord at T12 (Chen et al. 2007a for detail). The Teflon-coated wires from all electrodes passed subcutaneously to a connector plug on the skull.

H-reflex data collection began at least 10 days later. During data collection, the animal lived in a standard rat cage with a 40-cm flexible cable attached to the skull plug 24 hr/day. The cable, which allowed
the animal to move freely in the cage, conveyed the wires to a commutator above the cage, which
connected to amplifiers and stimulation units. The rat had free access to food and water throughout. Animal
well-being was carefully checked several times/day, and weight was measured weekly. Laboratory lights
were dimmed from 2100 to 0600 daily.

A computer-based system continuously (24 hr/day, 7 day/wk) monitored EMG from the soleus
muscles. Whenever the absolute value (i.e., full-wave rectified value for each successive 50-ms period) of
background (i.e., ongoing) EMG in each soleus muscle stayed within a defined range (i.e., about 1-2% of
maximum M wave) for a randomly varying 2.3-2.7 s period, a stimulus pulse (typically 0.5 ms in duration)
was delivered to each nerve cuff. Pulse amplitude was initially set for each side to produce a maximum
soleus H-reflex and a small M wave (typically just above M-wave threshold), and then automatically
adjusted after each trial to maintain M wave size (i.e., average EMG amplitude in the M-wave interval
(typically 2.0-4.5 ms after stimulation)) in each leg unchanged throughout data collection. H-reflex size was
defined as average EMG amplitude in the H-reflex interval (typically 6-10 ms after stimulation) minus
average background EMG amplitude. Background EMG amplitude was calculated as average EMG
amplitude just prior to the nerve stimulation.

For each rat, soleus H-reflex data were collected for 12-24 weeks. For the first 10-20 days (the initial
baseline period), no SMC stimulation was delivered. Then, each rat was exposed to SMC stimulation (i.e., a
1-s train of 25 1-ms biphasic pulses (25-Hz pulse rate) through the left SMC electrode pair every 10 s). This
stimulation continued for 40 days (two 20-day periods separated by 20 non-stimulation days (as in Chen et
al. (2007) (11 rats) or for one 30-day period (7 rats).

SMC stimulation amplitude (typically ~30 μA) was initially set to produce a small spinal cord volley at
the T12 epidural electrodes (3.5-5.5 ms after stimulus onset) and a small EMG response from the right
soleus muscle (7.5-11.0 ms after stimulus onset). From then on, the stimulus amplitude was automatically
adjusted after each stimulus to maintain the same spinal cord volley and/or right soleus EMG response
throughout the days of stimulation (Chen et al. 2007a). This SMC stimulation produced no visible movement
and caused no apparent distress. The rats appeared to ignore the stimulus, and they continued to thrive
throughout. They remained normally active and gained weight as expected. H-reflex data collection
continued throughout the baseline, stimulation, and post-stimulation periods.
**Soleus motoneuron labeling and rat perfusion**

Thirteen Experimental Group rats were anesthetized at the end of SMC stimulation and injected in the right (n=7) or both (n=6) soleus muscles with 50 μg (in 50 μl distilled water) of cholera toxin subunit B conjugated Alexa fluor 488 (CTB-Fluor 488; Molecular Probes, Eugene OR) to label the soleus motoneurons (Wang et al. 2006a; Pillai et al. 2008). The other five Experimental Group rats were similarly injected 60, 62, 65, 66, or 109 days after SMC stimulation ended. The rats injected at the end of stimulation are referred to as SMC\textsubscript{EARLY} rats and those injected 60-109 days after stimulation ended are referred as SMC\textsubscript{LATE} rats. The 13 Control Group rats were similarly injected in the right soleus muscle with CTB-Fluor 488.

Three days after CTB-Fluor 488 injection, each rat was injected with Heparin (for anti-coagulation, 5mg/kg, i.p.), euthanized by an overdose of sodium pentobarbital (120 mg/kg, i.p.), and perfused intracardially with 0.05 M PBS buffered saline (freshly prepared, 20°C, quantity (ml) = ½ body weight (g), over 5 min) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) (freshly prepared, 20°C, quantity (ml) = 2 x body weight (g), over 30 min). The lumbosacral spinal cord was removed, postfixed in the same fixative for 2 h, washed with 0.05 M phosphate buffer containing 137 mM NaCl (PBS, pH 7.4), and infiltrated with 30% sucrose for 24 hours. The spinal cord segment containing the soleus motoneuron pool (i.e., lumbar segments 4 and 5) was blocked, embedded in OCT compound (Tissue-Tek), and frozen on dry ice. Transverse 25-μm frozen sections were cut with a cryostat, mounted onto precoated glass slides (Superfrost; Fisher), and examined by fluorescence microscopy to identify CTB-Fluor 488 retrograde-labeled soleus motoneurons (e.g., Fig. 1A&B). All labeled soleus motoneurons were photographed with a digital camera (Olympus Magnafire\textsuperscript{TM} SP, IKH027801). Their fluorescent images were saved for analysis and all sections were stored at -80°C until immunohistochemical processing.

**Immunohistochemical processing**

The standard Avidin-biotin complex (ABC)-peroxidase system (ABC Elite; Vector Laboratories, Burlingame, CA) was used to assess: glutamic acid decarboxylase 67 immunoreactivity (GAD\textsubscript{67}-IR) in spinal interneurons and in synaptic terminals on soleus motoneurons (Wang et al. 2006a, 2009; Pillai et al. 2008); and GABA\textsubscript{A} (i.e., GABA\textsubscript{A} β\textsubscript{2}β\textsubscript{3} subunit) and GABA\textsubscript{B} (i.e., GABA\textsubscript{B1} subunit) receptor immunoreactivities in soleus motoneurons (GABA\textsubscript{A}-IR and GABA\textsubscript{B}-IR, respectively). Out of every four serial sections from each of
the Experimental or Control group rats, one was processed for GAD\textsubscript{67}-IR, one for GABA\textsubscript{A}-IR, one for GABA\textsubscript{B}-IR, and one for other studies (e.g., KCC2-IR (see below)). Sections from Experimental and Control rats were processed together.

The sections were washed in 0.05-M phosphate-buffered saline containing 0.1% Triton X-100 (PBST, pH 7.4) three times (10 min each), blocked with 5% normal goat serum and 3% bovine serum albumin for 1.5-2.0 hr, and then incubated with primary antibody (i.e., rabbit anti-GAD\textsubscript{67} (Chemicon, Temecula, CA; 1:2000 dilution), mouse anti-GABA\textsubscript{A}\beta\textsubscript{2}\beta\textsubscript{3} (Millipore, clone 62-3G1, 1:100 in PBST), or rabbit anti-GABA\textsubscript{B} (Santa Cruz Biotechnology Inc., CA, 1: 250 dilution)) for 18-20 hr in a humid chamber at 4°C. They were washed with PBST and reacted with the biotinylated secondary antibody (goat anti-mouse or goat anti-rabbit, 1:200 in PBST) for 1.5 hr. Endogenous peroxidase activity was quenched by 0.3% H\textsubscript{2}O\textsubscript{2} for 30 min, and the sections were reacted for 1.5 hr with the avidin-biotin complex (1:100 in PBS). Finally, they were washed with 0.05 M TRIS-HCl buffer (TBS, pH 7.6) and reacted with 0.04% DAB solution and 0.006% H\textsubscript{2}O\textsubscript{2} (pH 7.6) for 8 min (for GAD\textsubscript{67}-IR labeling) or 15 min (for GABA\textsubscript{A}-IR or GABA\textsubscript{B}-IR labeling) to optimize the signal-to-noise ratio.

The spinal cords of the SMC\textsubscript{LATE} rats were processed exactly as described above except that, for most of them, mouse anti-GAD\textsubscript{67} (Chemicon, Temecula, CA; 1:1000 dilution) (instead of rabbit anti-GAD\textsubscript{67}) was used as primary antibody and goat anti-mouse (instead of goat anti-rabbit) was used as secondary antibody because K2 antibody (i.e., rabbit anti-GAD\textsubscript{67}) was no longer available. To provide proper controls for these rats, 4 Control rats were processed at the same time with the same primary and secondary antibodies, and the interneuron and terminal data from these SMC rats were compared to the data from these 4 Control rats. (The equivalence of the results provided by these two different primary/secondary antibody combinations was confirmed by data from three test rats. For each of these rats, one set of sections were processed by one combination and another set were processed by the other combination. The data were expressed in percent of their respective Control rat data and compared. The results provided by the two combinations did not differ significantly.)

Because changes in GABA-mediated function can be associated with changes in membrane potassium-chloride cotransporter 2 (KCC2) metabolism (e.g., after spinal cord injury (Boulenguez et al. 2010), in 5 SMC\textsubscript{EARLY}, 5 SMC\textsubscript{LATE}, and 5 Control rats we also assessed KCC2 immunoreactivity (KCC2-IR)
in the soleus motoneurons using indirect fluorescent immunostaining. One of every four serial sections (see above) was briefly rinsed in PBST and then immersed in 0.5% sodium borohydride diluted in PBS containing 0.01% sodium azide (pH 7.4) for 30 min to quench PFA autofluorescence, blocked with PBS containing 5% BSA and 4%-normal goat serum for 2 hrs, and incubated with rabbit anti-KCC2 primary antibody (abcan, 1:200) for 20 hrs at 4°C in a humid chamber. The sections were rinsed and washed in PBST 3 times (10 min each), incubated with goat anti-rabbit secondary antibody conjugated to Alexa fluor 594 (Invitrogen 1:2000 in PBS) for 80 min, washed with PBST again, and mounted with Prolong® Gold anti-fade reagent.

To verify the specificity of GAD67-IR, GABA_A-IR, and KCC2 labeling during each processing, four sections from a Control rat were processed as negative controls by simply omitting the primary GAD67, GABA_Aβ2β3, or KCC2 antibody, or by replacing it with purified normal rabbit or mouse IgG. In these negative controls, no positive GAD67-IR, GABA_Aβ2β3-IR, or KCC2 labeling was found in the ventral horn of the spinal cord.

Two control procedures verified the specificity of GABA_B-IR labeling. The first omitted the primary antibody or replaced it with purified normal rabbit IgG. In neither case was GABA_B-IR labeling found in the ventral horn. The second procedure pre-absorbed the primary antibody with human GABA_B antigen (Abnova Co.). Once again, no positive GABA_B-IR labeling was found in the ventral horn. These control procedures verified the specificity of our GAD67-IR, GABA_A-IR, KCC2-IR, and GABA_B-IR labeling methodologies.

**Image preparation**

All images were photographed in an identical manner across the groups. As described in Wang et al. (2006a), soleus motoneurons were identified in GAD67-IR sections by matching these sections to the CTB-Fluor 488-labeled fluorescence photomicrographs using location, shape, and other landmarks. GAD67-IR analysis was confined to those soleus motoneurons that had a nucleus and a clearly defined somatic border. Seventy-two percent of the CTB-Fluor 488-labeled motoneurons satisfied these criteria. The remainder, which were either invisible in the GAD67-labeled sections (8%), lacked a clear somatic border (11%), or lacked a nucleus (9%), were excluded from further analysis. GAD67-labeled sections containing soleus motoneurons that qualified for analysis were photographed with an Olympus BH2-RFCA bright field
microscope (500X magnification with a 40X objective lens, fixed illumination) and an Olympus DP70 digital camera (magnification 10X, fixed illumination).

Soleus motoneurons were identified in GABA_A and GABA_B-labeled sections by matching their photomicrographs with those of the CTB-Fluor 488-labeled sections described above. Each identified soleus motoneuron was photographed at high magnification (500X, 40X objective, fixed illumination). In addition, to assess other effects of stimulation on GABAergic function, spinal laminae I-III and IV-VI in GABA_A and GABA_B-labeled sections were photographed with an Olympus BX61 microscope (100X, 10X objective, fixed illumination) through a coupled CCD digital camera (Model C8484-03G02, Hamamatsu Photonics K.K.).

**Quantifying GAD_{67}-immunoreactivity in motoneurons and interneurons and identifying GABAergic synaptic terminals**

The images were relabeled with codes by a person not otherwise involved in the study so that all measurements could be made in a blinded fashion. Each image was then assessed by each of two independent raters who did not know whether the image came from an SMC_{EARLY}, an SMC_{LATE}, or a Control rat. No corrections were made for tissue shrinkage.

The protocol for quantification of GAD_{67}-immunoreactivity (GAD_{67}-IR) on soleus motoneurons and for identification of GABAergic terminals on these motoneurons is detailed in Wang et al. (2006a). Briefly, with the image magnified 5,000X, soma size and the GAD_{67}-IR of the soma and its perimeter were measured by tracing the perimeter of each motoneuron soma (excluding proximal dendrites) using the Image J program (NIH, version 1.39o). The program calculated the Feret’s diameter of the soma (i.e., the long axis of the soma), the soma area, the average GAD_{67}-IR labeling intensity of the entire soma excluding the perimeter, and the average intensity of the perimeter; and it also plotted the perimeter intensity vs. location on the perimeter. Labeling intensity was expressed in percent, with the intensity of a totally clear slide defined as 0% (i.e., no detectable IR) and the intensity of a totally opaque slide defined as 100% (i.e., highest IR).

The number of GAD_{67}-labeled terminals on the motoneuron perimeter was determined first with a magnification of 250X (with a 20X objective) and then with a magnification of 500X (with a 40X objective). The two magnifications consistently yielded the same number of terminals. GABAergic terminals were identified as punctate or bead-like densities (Border and Mihailoff 1985) on the perimeter of the soma (Fig.
1C&D). They were further confirmed by the GAD$_{67}$-IR vs. perimeter location plot, in which each terminal was
evident as a sharp peak with a maximum GAD$_{67}$-IR value that exceeded the average GAD$_{67}$-IR of the
perimeter by at least 10% (Fig. 1E). Using the DP Controller Program of the camera, each motoneuron
image (magnification 500X) was analyzed at a focal plane at which all the terminals on the perimeter of the
soma could be seen. Each confirmed terminal was then magnified 10,000X and measured with the Image J
program to determine its Feret's diameter (i.e., the long axis of the terminal), area, GAD$_{67}$-IR labeling
intensity, and GAD$_{67}$-IR density (i.e., GAD$_{67}$-IR intensity of the terminal divided by its Feret's diameter to
correct for terminal thickness).

To assess the validity of our identification of those GABAergic terminals that contacted the
motoneuron soma, sections from several additional control rats were fluorescence double-labeled for
GAD$_{67}$-IR labeled terminals and CTB-IR labeled soleus motoneurons (labeled with CTB-biotin conjugate).
By focusing down through these double-labeled sections, we were able to determine whether each GAD$_{67}$-
positive punctum that appeared to contact the soma membrane actually did so. In 20 motoneurons studied
in this way, 84% of the GAD$_{67}$-IR terminals that appeared to touch the motoneuron membrane by our
standard method actually did touch the membrane. Given that sections from SMC rats and Control rats
were always processed and evaluated concurrently using the same methodology, and in a blinded fashion,
this ancillary test indicates that the results provided by our standard method are reliable indicators of the
numbers of GAD$_{67}$-IR terminals, and thus are suitable for detecting any differences in these numbers
between Experimental and Control rats.

The protocol for quantification of GAD$_{67}$-labeled interneurons is detailed in Wang et al. (2009). Briefly,
in the ventral horns of GAD$_{67}$-labeled sections GAD$_{67}$-positive neurons that had a clearly defined somatic
border, a nucleus and/or at least one dendritic process, an average soma diameter (average of the long and
short somatic axes) under 25 μm, and an average somatic area under 400 μm$^2$ were identified as
GABAergic interneurons and assigned a laminar location (Barber et al. 1982; Molander et al. 1984). For
each of these putative GABAergic interneurons, soma diameter (i.e., Feret's diameter, equivalent to the
long axis of the soma), soma area, and the average GAD$_{67}$-IR labeling intensity of the entire soma were
calculated using the Image J program (NIH, version 1.39o).

Quantifying GABA$_A$-IR, GABA$_B$-IR, and KCC2-IR
Soleus motoneuron GABA\textsubscript{A}-IR and GABA\textsubscript{B}-IR were quantified in the right (i.e., contralateral to SMC stimulation) side of the spinal cord with the image magnified 5,000X. The soma perimeter (excluding dendrites) was traced using the Image J program (NIH, version 1.39o) (e.g., Fig. 1F&G). The program calculated GABA\textsubscript{A} or GABA\textsubscript{B} intensity as average intensity of the soma perimeter. GABA\textsubscript{B} intensity was also calculated as average intensity of the entire soma. Intensity was expressed in percent, with that of a clear slide defined as 0% (i.e., no detectable IR) and that of a totally opaque slide defined as 100% (i.e., highest IR). KCC2-IR was quantified in the same fashion, except that the intensity of a totally black field (i.e., gray value <20) was defined as 0% (i.e., no detectable IR) and the intensity of a totally white field (i.e., gray value=256) was defined as 100% (i.e., highest IR). We also calculated the average GABA\textsubscript{A}-IR and GABA\textsubscript{B}-IR intensities of spinal laminae I-III and IV-VI (identified according to Molander et al. (1984)).

**Data analysis**

The two independent blinded raters agreed 97% of the time. Their infrequent differences were resolved by joint evaluation and discussion. The images were then decoded, and the data were sorted by rat and by group (i.e., SMC\textsubscript{EARLY}, SMC\textsubscript{LATE}, or Control). For each measure, the three groups were compared by one-way ANOVA. When a difference was detected with p<0.01, differences between groups were tested by least mean contrast.

Using interneuron or terminal numbers, sizes, and labeling, we estimated for each rat GABAergic interneuron strength as [average number of interneurons per section] times [average interneuron area] times [average interneuron labeling intensity], and GABAergic terminal strength as [average number of terminals per motoneuron] times [average terminal area] times [average terminal labeling density]. These calculations assumed that labeling intensity (interneurons) or density (terminals) reflected GABA (or more precisely, GAD\textsubscript{67}) concentration per unit area; and thus that the total amount of GABA in the interneuron (or present at the terminal contact on the motoneuron) was proportional to this number multiplied by interneuron (or terminal) area and number. Strengths were expressed in percent of average Control Group strengths.

Finally, to assess the variance in H-reflex size accounted for by metabotropic GABAergic changes, we performed a multiple regression analysis in which the independent variables were terminal strength, GABA\textsubscript{B} receptor strength (i.e., average GABA\textsubscript{B} receptor intensity of the soleus motoneurons), and their interaction,
and the dependent variable was H-reflex size. The focus on metabotropic GABAergic function was based on the strong inverse correlation between GABAB receptor intensity and H-reflex size (see Results) and on the evidence that changes in metabotropic GABAergic function may produce the changes in motoneuron properties underlying operant conditioning of the H-reflex (see Discussion and Wang et al. (2006a), Wolpaw and Chen (2009), Wolpaw (2010)).

RESULTS

Animal well-being and postmortem examination

All SMC rats remained healthy and active and gained weight throughout the study. Right and left soleus muscle weights (in percent of body weights) did not differ significantly from each other or from those of Control (i.e., unimplanted and unstimulated) rats. In all rats, the EMG electrodes, the nerve cuffs, the T12 spinal cord epidural electrodes, and the cranial screw electrodes were found to be located appropriately. The nerve cuffs showed the expected connective tissue sheath and good preservation of the nerve within the sheath. That the tibial nerves of both sides remained intact structurally and functionally was further indicated by the normal muscle weights and their bilateral symmetry, and also by the fact that the average daily amplitude of the nerve cuff stimulus needed to elicit the target M wave remained stable throughout. The spinal cord under the epidural electrodes appeared to be in good condition. The meninges and cortex under the epidural screw electrodes appeared normal, except that in some rats the cortical surface under the screw was slightly indented, as seen previously (Chen et al. 2007a). (See Chen et al. (2007a) for description of the minimal cortical histological change (i.e., slight gliosis with no apparent loss of neurons).)

H-reflex size in SMCEARLY and SMCLATE rats

Right soleus H-reflex size rose steadily over the days of left SMC stimulation, reaching 174(±9SEM)% of its initial (i.e., pre-stimulation) value by the end of stimulation (p<0.001 vs. initial value; ANOVA followed by Tukey test). This increase persisted. In the SMCLATE rats, H-reflex size at the time of perfusion (i.e., 60-109 days after stimulation ended) averaged 200(±20)% of its initial value (p<0.01). Figure 2E summarizes and illustrates the H-reflex increase and its persistence. E1 shows average H-reflex size (in % of initial) for all SMC rats at the end of stimulation and for the SMCLATE rats just before perfusion. E2-E4 show average post-stimulus EMG (absolute value) from one SMCLATE rat for a day prior to SMC stimulation.
(E2), a day at the end of SMC stimulation (E3), and a day 61 days later, just before perfusion (E4).

**GABAergic interneurons, terminals, and receptors in SMC<sub>EARLY</sub> rats**

In rats perfused immediately at the end of SMC stimulation (SMC<sub>EARLY</sub> rats), GABAergic interneurons in the ventral horn contralateral to SMC stimulation were much more numerous and considerably larger than in Control rats. Figure 2A summarizes and illustrates these effects; and Figure 3 shows their laminar and lateral specificity and their significance. Most of the GABAergic interneurons were found in lamina VII, due primarily to its greater area. After SMC stimulation, the number of interneurons was increased in contralateral laminae VII and VIII and to a lesser extent in ipsilateral lamina VII. Interneuron area was increased in laminae VI-IX bilaterally. Interneuron labeling intensity was increased primarily in contralateral laminae VII and VIII and in ipsilateral lamina VII.

The GABAergic interneuron effects were paralleled by equally dramatic effects on GABAergic terminals on contralateral soleus motoneurons. These terminals were much more numerous and substantially larger than in Control rats (p<0.0001 for each). Figure 2B summarizes and illustrates these effects. Similar but lesser terminal changes were found ipsilateral to the SMC stimulation.

In sharp contrast to the increases in GABAergic interneurons and terminals, GABA<sub>A</sub> and GABA<sub>B</sub> receptor labeling (i.e., calculated as the intensity of the somatic perimeter) of the soleus motoneurons of SMC<sub>EARLY</sub> rats was much weaker than in Control rats (p<0.0001 for each). Figures 2C&D summarize and illustrate these receptor effects. (The GABA<sub>B</sub> receptor results were very similar when labeling was calculated as the intensity of the entire soma.) At the same time, SMC stimulation had no detectable effect on KCC2 labeling in the soleus motoneurons.

In addition to its effects on motoneuron GABA<sub>A</sub> and GABA<sub>B</sub> receptors, SMC stimulation significantly reduced these receptors elsewhere in the contralateral (and to a lesser extent the ipsilateral) spinal cord. GABA<sub>A</sub> intensity in contralateral (i.e., right) laminae I-III and IV-VI averaged 59.4(±1.2SEM)% and 44.0(±1.0)%, respectively, in Control rats, and 50.1(±1.0)% and 35.0(±1.0)%, respectively, in SMC<sub>EARLY</sub> rats (p<0.001 for each region). GABA<sub>B</sub> intensity in contralateral laminae I-III and IV-VI averaged 50.7(±1.2)% and 39.1(±1.1)%, respectively, in Control rats, and 44.6(±1.2)% and 34.5(±1.0)%, respectively, in SMC<sub>EARLY</sub> rats (p<0.001 for each region). Figure 4 shows GABA<sub>A</sub> (A) and GABA<sub>B</sub> (B) labeling in representative spinal cord sections from a Control rat (A1, B1) and an SMC<sub>EARLY</sub> rat (A2, B2). GABA<sub>A</sub>-IR and GABA<sub>B</sub>-IR labeling
was greatly reduced in SMC rats compared to Control rats, and the reduction was greater in the contralateral spinal cord. Note that light to moderate GABA$_A$-IR and GABA$_B$-IR labeling were also present in the white matter as reported by Margeta-Mitrovic et al. (1999).

**GABAergic interneurons, terminals, and receptors in SMC$_{LATE}$ rats**

In rats perfused 60-109 days after the end of SMC stimulation (SMC$_{LATE}$ rats), the numbers of GABAergic interneurons and terminals had decreased back to Control levels, while their sizes and the interneuron labeling intensity had fallen below Control levels (p<0.0001 for each). Figures 2A&B summarize and illustrate these results.

In contrast to the disappearance of the increases in GABAergic interneurons and terminals, GABA$_B$ and (to a lesser degree) GABA$_A$ receptor labeling in soleus motoneurons remained significantly depressed (p<0.0001 for each) in SMC$_{LATE}$ rats. Figures 2C&D summarize and illustrate this persistence. (As for the SMC$_{EARLY}$ rats, the GABA$_B$ receptor results for the SMC$_{LATE}$ rats were very similar when labeling was calculated as the intensity of the entire soma.) As in SMC$_{EARLY}$ rats, no difference from Control rats in soleus motoneuron KCC2 labeling was found.

While the laminae I-III GABA$_B$ receptor decreases found in SMC$_{EARLY}$ rats were not present in SMC$_{LATE}$ rats, the laminae IV-VI decreases were still evident (Fig. 4B3).

**Correlation between GABAergic interneuron and terminal changes**

To evaluate the likelihood that the lamina VII GABAergic interneurons provided the GABAergic terminals on the motoneurons, we used the interneuron and terminal number, area, and labeling data to estimate interneuron strength and terminal strength for each rat (see Methods). Across all SMC rats, interneuron strength and terminal strength showed a very strong positive within-rat correlation ($r^2=0.75$, p<0.001) (Fig. 5A). This finding supports the hypothesis that the identified GABAergic interneurons provided many of the identified GABAergic terminals.

**Correlation of GABAergic changes and H-reflex changes**

Across all SMC rats, GABA$_B$ receptor strength (i.e., labeling intensity) and H-reflex size displayed a significant inverse correlation ($r^2=0.42$, p=0.005) (Fig. 5B). In contrast, GABA$_A$ receptor strength did not correlate significantly with H-reflex size ($r^2=0.03$, p=0.63). GABAergic terminal strength appeared to be inversely correlated with H-reflex size ($r^2=0.23$, p=0.06).
To further evaluate the possible link between the GABAergic changes and the H-reflex changes in the SMC rats, we used a multiple regression analysis (Methods) to determine the extent to which motoneuron GABAergic terminal strength, GABA_B receptor strength, and their interaction predicted H-reflex size. The $r^2$ value was 0.76 ($p<0.01$) (Fig. 5C). Thus, each rat’s GABAergic terminal and GABA_B receptor strengths in combination predicted its H-reflex size with a high degree of accuracy. This strong relationship is shown in Figure 5C. It supports the hypothesis that the long-term decrease in GABA_B receptor strength contributed importantly to the long-term increase in the H-reflex.

DISCUSSION

The brain’s long-term regulation of spinal cord function during development, throughout later life, and in response to trauma or disease is drawing increasing attention both for its theoretical significance and for its practical importance as the basis for new rehabilitation methods (Wolpaw and Tennissen 2001 and Wolpaw 2010 for review). In a previous study, we found in rats that low-level stimulation of sensorimotor cortex (SMC) over several weeks produced a persistent increase in the H-reflex, a simple measure of spinal cord function (Chen et al. 2007a). The present study further explored this long-term effect and its underlying mechanisms by studying spinal GABAergic interneurons and motoneuron GABAergic terminals and receptors. This focus on spinal GABAergic function was based on the evidence that operant down-conditioning of the H-reflex, which is driven by SMC and its corticospinal tract (CST) output (Chen and Wolpaw 1997, 2002; Chen et al. 2002, 2003, 2006a), is associated with large changes in GABAergic interneurons in the ventral horn and GABAergic terminals on spinal motoneurons; (Wang et al. 2006a, 2009; Pillai et al. 2008). These studies suggested that the CST acts through GABAergic interneurons and their terminals on motoneurons to produce the motoneuron plasticity underlying H-reflex down-conditioning, and thereby motivated the present investigation of GABAergic involvement in the effect of SMC stimulation on the H-reflex.

The results show that SMC stimulation does have marked spinal GABAergic effects: it increases GABAergic interneurons in lamina VII particularly and GABAergic terminals on motoneurons, and it decreases GABA_A and GABA_B receptors in motoneurons and elsewhere in the spinal cord. Furthermore and most notably, while the interneuron and terminal increases disappear within two months, the receptor
decreases and the H-reflex increase remain, and the H-reflex increase is strongly correlated with the 
GABA$_B$ receptor decrease. Even though the stimulation was relatively weak (judging from the small 
descending volley and the minimal muscle contraction), it nevertheless has striking trans-synaptic impact on 
spinal cord neurons and synapses. While no gross changes were noted in motor behavior, the possibility of 
more subtle changes was not formally explored; and GABAergic changes comparable to those found here 
are associated with changes in locomotion (Tillakaratne et al. 2002; Chen et al. 2005; Wang et al. 2006a, 
2009). By indicating the widespread and persistent spinal plasticity caused by modest SMC stimulation, the 
results provide insight into the mechanisms of chronic disorders of brain/spinal cord interactions such as 
cerebral palsy and spinal cord injuries; and they suggest new approaches to restoring motor function (e.g., 
Chen et al. 2006b).

This discussion focuses on four questions. First, are the quantitative immunohistochemical methods 
valid and are their results credible? Second, how does SMC stimulation produce these anatomical changes 
and how do they relate to each other? Third, are these changes related to, or perhaps even responsible for, 
the H-reflex increase produced by SMC stimulation? Fourth, how do these changes affect behavior?

Methods

The physiological, anatomical, and immunohistochemical methods used here for quantifying 
GABAergic terminals and interneurons are based largely on our previous work (Chen et al. 2007a; Wang et 
al. 2006a, 2009; Pillai et al. 2008). These earlier papers discuss the characteristics and potential limitations 
of these methods in detail. Quantitative immunohistochemistry is clearly challenging. Even for a well-
characterized antibody, the results depend on the quality of fixation and on the processing parameters (e.g., 
temperature, incubation time, reagent concentrations). Minor differences in these parameters can have 
major effects on outcome.

We took the following steps to minimize or eliminate the impact of these factors. First, we made 
great efforts to ensure that perfusion and tissue fixation procedures were consistent across the animals 
(see Methods). Second, sections from SMC rats and Control rats were always processed together, and all 
images were photographed in exactly the same manner. Third, the specificities of the GAD67-IR, GABA$_A$- 
IR, GABA$_B$-IR, and KCC2-IR labeling methods were verified by control procedures as described. Fourth, a 
large body of data was analyzed. A total of 2304 spinal cord sections, 2021 soleus motoneurons, 8576
GABAergic terminals, and 6087 GABAergic interneurons from 31 control and SMC rats were studied. Finally, all measurements were performed in a blinded fashion by two independent raters. Based on these methodological precautions, we are confident that the results are valid.

The origin of the spinal GABAergic plasticity

SMC stimulation might affect the spinal cord directly through the CST and/or indirectly through other descending pathways (Brösamle and Schwab 1997; Kuang and Kalil 1990; Liang et al. 1991; Holstege 1991; Rudomin and Schmidt 1999; Yang and Lemon 2003). Since CST axons synapse on lamina VII interneurons (Jankowska 1992, 2001; Jankowska and Stecina 2007; Chakrabarty et al. 2009), the fact that the GABAergic interneuron increase is most prominent in contralateral spinal lamina VII suggests that SMC stimulation may affect the spinal cord through the CST. The lesser ipsilateral interneuron effects might be mediated by the ipsilateral CST projection (Brösamle and Schwab, 1997) or via commissural connections that affect the right SMC.

The marked increase in identifiable GABAergic interneurons probably reflects increased GABA expression by interneurons that previously did not contain enough GABA to be identified as GABAergic and/or new GABA expression by interneurons that previously did not express GABA (e.g., purely glycinergic interneurons), rather than the generation of new interneurons. Several considerations suggest that these increased numbers of GABAergic interneurons give rise to the increased numbers of GABAergic terminals: (1) the interneuron and terminal increases are found with both SMC stimulation and successful down-conditioning (Wang et al. 2006a, 2009); (2) lamina VII (and VI) GABAergic interneurons do synapse on motoneurons (Jankowska 1992, 2001); (3) the interneuron and terminal strengths found here are strongly correlated (i.e., \( r^2 = 0.75, \ P < 0.001 \)) (Fig. 5A); and (4) the interneuron increase and the terminal increase both disappeared 2-3 months after SMC stimulation ended. At the same time, it is possible that axons that descend from GABAergic neurons in the ventromedullary reticular formation via the ipsilateral dorsolateral funiculus and synapse on spinal motoneurons contribute to the increased numbers of terminals (Holstege 1991).

The decreases in GABA\(_A\) and GABA\(_B\) receptors imply that the motoneurons were effectively desensitized to GABAergic inputs that act through these receptors. These receptor decreases are surprising. Repeated exposure to GABA\(_A\) or GABA\(_B\) agonists does not reduce GABA\(_B\) receptors in intact
animals, nor does it induce endocytosis of GABA$_B$ receptors in vitro (Lehmann et al. 2003; Grampp et al. 2007; Vargas et al. 2008; Terumuma et al. 2010). Furthermore, H-reflex down-conditioning, which also increases motoneuron GABAergic terminals, does not reduce GABA$_B$ receptors (Wang et al. unpublished data). The reason for the difference in receptor effect between SMC stimulation and down-conditioning may relate to the nature of the descending activity (e.g., synchronous vs. asynchronous for SMC vs. down-conditioning, respectively) and/or to the fact that SMC stimulation affects the motoneurons of many muscles, while the impact of the operant conditioning protocol is much more focused, affecting mainly soleus motoneurons (Wolpaw et al. 1983; Wolpaw et al. 1993; Chen et al. 2005).

As noted in the Introduction, the changes in GABAergic function associated with operant conditioning of the H-reflex are part of a complex pattern of plasticity at spinal and supraspinal sites (Wang et al. 2006a, 2009; Wolpaw and Chen 2006, Wolpaw 2010 for review). The effects of SMC stimulation may be similarly complex. A variety of studies suggest that the GABA receptor depression described here might be secondary to changes produced by the SMC stimulation in excitatory glutaminergic inputs (Stelzer et al. 1987; Lu et al. 2000; Kuramoto et al. 2007; Martikainen et al. 2004; Vargas et al. 2008). This possibility is consistent with preliminary results (Wang et al. 2006b) indicating that SMC stimulation increases vesicular glutamate transporter 2 (VGLUT2) labeled terminals and increases glutamate receptors on soleus motoneurons. The fact that the receptor depression persists months beyond the end of stimulation despite the disappearance of the GABAergic interneuron and terminal increases implies that it may reflect a change in gene expression.

**The relationship between spinal GABAergic plasticity and H-reflex change**

The present results add to the growing evidence of close association between spinal GABAergic plasticity and functional changes. This evidence includes the GABAergic changes associated with the locomotor effects of treadmill training in spinal cord-transected animals (Tillakaratne et al. 2002) and with H-reflex down-conditioning in normal animals (Wang et al. 2006a, 2009). Successful down-conditioning of the rat soleus H-reflex is associated with increases in lamina VII GABAergic interneurons and motoneuron terminals similar to those found here with SMC stimulation. Given the dependence of down-conditioning on the CST (Chen and Wolpaw 1997, 2002) and the shift in motoneuron firing threshold that largely accounts for the H-reflex decrease (Carp and Wolpaw 1994; Halter et al. 1995), we hypothesize that the critical CST
influence reaches the motoneuron through these GABAergic interneurons and terminals, and that it may act through metabotropic GABA_B receptors to produce a protein kinase C-mediated shift in sodium channel activation voltage that shifts firing threshold positively and thereby reduces H-reflex size (Carp and Wolpaw 1994; Halter et al. 1995; Wang et al. 2006a, 2009).

Both SMC stimulation and H-reflex down-conditioning increase GABAergic interneurons and terminals, but the first increases the H-reflex while the latter decreases it. The reason for this difference appears to be that, although both increase GABAergic interneurons and terminals, SMC stimulation decreases GABA_B receptors in the motoneuron (Fig. 2), while H-reflex down-conditioning does not do so (Wang et al. unpublished data). The significant correlation between GABA_B receptor decrease and H-reflex (Fig. 5B) increase strengthens the hypothesis that these metabotropic GABA receptors in the motoneuron are a key link in the pathway leading from cortical activity to H-reflex change (Wolpaw 2010; Chen and Wolpaw 2009). In contrast, the GABA_A receptor decrease was not significantly correlated with H-reflex size, and KCC2 intensity was not significantly affected.

The importance of GABA_B receptors in determining H-reflex size is further indicated by the multiple regression analysis in which GABAergic terminal strength and GABA_B receptor strength (i.e., labeling intensity) accounted for 76% of the variance in H-reflex size. As Figure 5C shows, an SMC rat’s GABAergic terminal and GABA_B receptor strengths predicted the size of its H-reflex quite accurately. In SMC_EARLY rats, the receptor decrease apparently obviated the impact of the terminal increase so that the H-reflex increased; and in SMC_LATE rats, the receptor decrease and the disappearance of the terminal increase combined to produce an even greater increase in the H-reflex. GABA_B receptors have a wide range of synaptic and other effects on neuronal function (White et al. 2000; Chalifoux and Carter 2011). In the present case, their decrease may affect the activation voltage of sodium channels in the motoneuron membrane (e.g., Halter et al. 1995).

**The behavioral effects of SMC stimulation**

The long-term behavioral impact of SMC stimulation, like that of H-reflex operant conditioning, is almost certainly not limited to its effect on the H-reflex: any behavior that uses the same pathway is likely to be affected (Chen et al. 2005; Wolpaw 2010). In normal rats that undergo H-reflex operant conditioning, such effects appear to trigger compensatory plasticity elsewhere that ensures the preservation of normal
locomotion (Chen et al. 2011). In rats with spinal cord injuries, these effects can improve defective
locomotion (Chen et al. 2006b). The fact that SMC stimulation produced persistent H-reflex change and
persistent GABAergic changes elsewhere in the spinal cord suggests that it probably induced widespread
compensatory plasticity to preserve a variety of important behaviors. The impact of training protocols in
spinal cord-transected rats provides additional evidence of the association between spinal GABAergic
changes and behavioral changes (Tillakaratne et al. 2002; Khristy et al. 2009). Furthermore, a preliminary
study of the effect of SMC stimulation in rats with a locomotor asymmetry due to spinal cord injury found
that the H-reflex increase caused by the stimulation was accompanied by an increase in the soleus
locomotor burst that eliminated the locomotor asymmetry (Chen et al. 2007b). Thus, appropriately focused
cortical stimulation protocols might prove useful therapeutically, helping to restore more effective spinal
function after partial spinal cord injuries or in other disorders (Wolpaw 2010).

Conclusions

The results show that cortical stimulation can produce changes in spinal cord GABAergic receptors
and reflex function that persist long after the stimulation ends. These changes are probably mediated at
least in part by the corticospinal tract and spinal GABAergic interneurons. They are likely to reflect changes
in gene expression and to alter the neural activity underlying many important behaviors. This study exposes
a new long-term dimension to cortical-spinal interactions and suggests new therapeutic possibilities.
ACKNOWLEDGMENTS

We thank Mr. Richard Cole (Wadsworth Advanced Light Microscopy Core), Ms. Rongliang Liu, and Drs. Shreejith Pillai, Dennis J. McFarland, and Gerwin Schalk for technical assistance and advice, and Drs. Jonathan S. Carp, Arthur W. English, and Elizabeth Winter Wolpaw for comments on the manuscript. We are grateful to Dr Niranjala J. K. Tillakaratne of the University of California, Los Angeles, for her advice and for providing the K2 antibody. This work was supported by NIH (HD36020 (XYC), NS22189 (JRW), NS061823 (XYC&JRW), and HD32571(AWE)), and The NYS Spinal Cord Injury Research Board (XYC).
REFERENCES


Chen XY, Wolpaw JR. Dorsal column but not lateral column transection prevents down conditioning of H-


Wolpaw JR. What can the spinal cord teach us about learning and memory? The Neuroscientist, 16:532-549, 2010.


Wolpaw JR, Herchenroder PA, Carp JS. Operant conditioning of the primate H-reflex: factors affecting the


**FIGURE LEGENDS**

1: Methods for assessing GAD$_{67}$-immunoreactivity (GAD$_{67}$-IR) and GABA receptor immunoreactivity.

A: Three Alexa Fluor-488 labeled soleus motoneurons in lamina IX of the ventral horn of rat lumbar spinal cord. B: A higher magnification picture of the motoneuron indicated by the arrow in A. C: The same motoneuron showing GAD$_{67}$-IR. D: The same motoneuron with the perimeter traced and identified GABAergic terminals indicated (a-k). The white line and small arrow show the starting point and direction of perimeter intensity measurement. The inset shows tracing of one of these terminals for measurement of diameter, area, and labeling intensity. (Scale bars: 100 µm in A; 5 µm in B-D; 1 µm in D inset.) E: Labeling intensity versus position on the perimeter of this same motoneuron (0% = intensity of a totally clear slide and 100% = intensity of a totally opaque slide). The peaks corresponding to the terminals identified in D are indicated. The dashed line is the average value of the perimeter and the dotted line is 10% above this average. F: Image of a soleus motoneuron with GABA$_A$ immunoreactivity (top), and with the perimeter traced for quantifying GABA$_A$ receptor immunoreactivity (i.e., intensity) (bottom). G: Image of a soleus motoneuron with GABA$_B$ immunoreactivity (top), and with the perimeter traced for assessing GABA$_B$ receptor immunoreactivity (i.e., intensity) (bottom). (Scale bar: 20 µm in F and G.) (See text for details.)

2: Effects of SMC stimulation on GABAergic interneurons (A), terminals (B), GABA$_A$ receptors (C), GABA$_B$ receptors (D), and soleus H-reflexes (E). A1-D1 show (in % of Control rat average): average (±SEM) interneuron number, area, and labeling intensity; average terminal number, area, and labeling density; and average GABA$_A$ and GABA$_B$ receptor intensities for Control, SMC$_{EARLY}$, and SMC$_{LATE}$ rats. The other A-D panels show representative examples of interneurons, terminals, and receptor-labeled soleus motoneurons from Control (A2-D2), SMC$_{EARLY}$ (A3-D3), and SMC$_{LATE}$ (A4-D4) rats, respectively. E1 shows average H-reflex size (in % of each rat’s initial size) at the end of SMC stimulation, and 60-109 days later after SMC stimulation ended just before perfusion. E2-4 show average post-stimulus EMG (absolute value), with M waves and H-reflexes indicated, from an SMC$_{LATE}$ rat for a day before SMC stimulation (E2, average of 5615 trials), a day at the end of SMC stimulation (E3, average of 5963 trials), and the 61st day after SMC stimulation ended just before perfusion (E4, average of 6657 trials). At the end of stimulation (e.g., A3-E3), the interneurons and terminals are increased in number and size, the receptors are decreased, and the H-
reflex is larger. Two-three months later (e.g., A4-E4), the interneuron and terminal increases in number and size have disappeared, but the receptors remain decreased, and the H-reflex remains larger. Background EMG level (i.e., level at time 0 when the nerve-cuff stimulus occurs) and M-wave do not change. (*: p<0.01, **: p<0.001, ***: p<0.0001 vs. Control rats.) (#: number; sect: section; MN: motoneuron.) The scale bar in A2 applies to all the histological panels.

3: Effects of SMC stimulation on GABAergic interneurons. Average (±SEM) number/section, area, and GAD$_{67}$-IR labeling intensity of GABAergic interneurons (INs) in laminae VI-IX of: right lumbar spinal cord segments 4 and 5 in Control rats; and the contralateral (i.e., right) and the ipsilateral (i.e., left) sides of lumbar segments 4 and 5 in SMC$_{EARLY}$ rats. Inset (top right) shows a line drawing of the spinal cord gray matter illustrating the laminae from which the measurements were taken. (**: p<0.001, *: p<0.01 vs. Control rats.)

4: Effects of SMC stimulation on GABA$_A$-IR and GABA$_B$-IR throughout the spinal cord. A: GABA$_A$ labeling (GABA$_A$-IR) in representative spinal cord sections from a Control rat (A1), an SMC$_{EARLY}$ rat (A2), and an SMC$_{LATE}$ rat (A3). GABA$_A$-IR labeling in the SMC$_{EARLY}$ and SMC$_{LATE}$ rats is much weaker than that in the Control rat throughout the spinal cord, and the effect is slightly greater on the side contralateral to stimulation (i.e., the right side). B: GABA$_B$ labeling (GABA$_B$-IR) in representative spinal cord sections from a Control rat (B1), an SMC$_{EARLY}$ rat (B2), and an SMC$_{LATE}$ rat (B3). GABA$_B$-IR labeling in the SMC$_{EARLY}$ and SMC$_{LATE}$ rats is much weaker than that in the Control rat throughout the spinal cord, and the effect is clearly greater on the side contralateral to stimulation (i.e., on the right side). Scale bars: 500 μm.

5: Correlations between interneuron and terminal strengths and between actual H-reflex sizes and H-reflex sizes predicted by terminal and GABA$_B$ receptor strengths. A: GABAergic terminal strengths (Methods) versus lamina VII GABAergic interneuron strengths for SMC rats. The high correlation ($r^2$=0.75, p<0.001) supports the hypothesis that these interneurons supply these terminals. B: Average H-reflex sizes (in % of initial size) for the last 5 days of data collection before perfusion for SMC rats versus GABA$_B$ receptor strength (i.e., labeling intensity). The significant correlation ($r^2$=0.42, p<0.01) supports the hypothesis that the GABA$_B$ receptor decrease contributed to the H-reflex increase. C: Actual average H-reflex sizes (in % of initial size) for the last 5 days of data collection before perfusion for all SMC rats with both GABAergic terminal and GABA$_B$ receptor data versus the H-reflex sizes predicted by a multiple
regression analysis in which the independent variables were GABAergic terminal strength, motoneuron GABA$_B$ receptor strength, and their interaction. The strong correlation ($r^2=0.76$, $p<0.01$) further supports the hypothesis that the GABAergic changes contributed substantially to the H-reflex increase.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5