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# Motor learning changes GABAergic terminals on spinal motoneurons in normal rats

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## Abstract

The role of spinal cord plasticity in motor learning is largely unknown. This study explored the effects of H-reflex operant conditioning, a simple model of motor learning, on GABAergic input to spinal motoneurons in rats. Soleus motoneurons were labeled by retrograde transport of a fluorescent tracer and GABAergic terminals on them were identified by glutamic acid decarboxylase $(GAD)_{67}$ immunoreactivity. Three groups were studied: (i) rats in which down-conditioning had reduced the H-reflex (successful HRdown rats); (ii) rats in which down-conditioning had not reduced the H-reflex (unsuccessful HRdown rats) and (iii) unconditioned (naive) rats. The number, size and GAD density of GABAergic terminals, and their coverage of the motoneuron, were significantly greater in successful HRdown rats than in unsuccessful HRdown or naive rats. It is likely that these differences are due to modifications in terminals from spinal interneurons in lamina VI–VII and that the increased terminal number, size, GAD density and coverage in successful HRdown rats reflect and convey a corticospinal tract influence that changes motoneuron firing threshold and thereby decreases the H-reflex. GABAergic terminals in spinal cord change after spinal cord transection. The present results demonstrate that such spinal cord plasticity also occurs in intact rats in the course of motor learning and suggest that this plasticity contributes to skill acquisition.

## Introduction

Activity-dependent spinal cord plasticity occurs in development, in response to trauma and disease and also during skill acquisition (Nielsen *et al.*, 1993; Perez & Nielsen, 2004; reviewed in Wolpaw & Tennissen, 2001). Nevertheless, studies of motor learning have traditionally focused on the cortex and other brain areas and ignored the spinal cord. As a result, the spinal cord's contributions to motor learning remain largely unknown and unstudied, despite its central role as the final common pathway for behavior. Operant conditioning of the H-reflex is a simple and powerful model for studying spinal cord plasticity during learning.

The H-reflex, the electrical analog of the spinal stretch reflex, is the simplest behavior of the vertebrate central nervous system. It is mediated primarily by a two-neuron, monosynaptic pathway comprised of the primary afferent fiber, its synapse on the alpha motoneuron and the motoneuron itself (Magladery *et al.*, 1951; Matthews, 1972; Baldissera *et al.*, 1981; Henneman & Mendell, 1981; Brown, 1984). As it is influenced by descending input from the brain, this pathway can be operantly conditioned. In response to an operant conditioning paradigm, monkeys, humans and rats can gradually decrease or increase the H-reflex or spinal stretch reflex (Wolpaw *et al.*, 1983; Wolpaw, 1987; Evatt *et al.*, 1989; Chen & Wolpaw, 1995; reviewed in Wolpaw, 1997, 2001 and Wolpaw & Tennissen, 2001). In terms of the definition of 'skill' as an adaptive behavior acquired through practice (Chen *et al.*, 2005), these changes, i.e. a smaller or larger H-reflex, constitute simple motor skills. Their acquisition depends on the corticospinal tract (CST) and is associated with multisite spinal cord plasticity that includes changes in the motoneuron, several different synaptic terminal populations on the motoneuron and probably also spinal interneurons and supraspinal regions (Wolpaw & Tennissen, 2001; Wolpaw, 2001).

Available data suggest that an operantly conditioned decrease in the H-reflex (i.e. down-conditioning) is due mainly to a positive shift in motoneuron firing threshold, while an increase (i.e. up-conditioning) is due largely to a decrease in non-reciprocal oligosynaptic group I inhibition of the motoneuron (Carp & Wolpaw, 1994, 1995). In monkeys, F-terminals (i.e. putative inhibitory terminals) on motoneuron cell bodies are larger and their active zone coverage is greater after down-conditioning than after up-conditioning (Feng-Chen & Wolpaw, 1996). This difference could reflect increased inhibition, in down-conditioned animals, from interneurons that convey the CST influence that modifies the motoneuron and/or decreased inhibition, in up-conditioned animals, from interneurons excited by the primary afferents responsible for the H-reflex.

F-terminals comprise about 60–65% of the synapses on motoneurons (Örnung *et al.*, 1996; Novikov *et al.*, 2000), come from spinal and supraspinal neurons and are largely GABAergic and/or glycinergic (Murphy *et al.*, 1996; Örnung *et al.*, 1996, 1998; Somogyi, 2002). Thus, the F-terminal effects of conditioning could arise in many ways. To elucidate their role in conditioning, we are delineating these effects more precisely by evaluating GABAergic terminals on motoneurons of naive rats and rats that have undergone conditioning of the soleus H-reflex. The present report describes the effects of down-conditioning. The data have been previously reported in abstract

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form (Wang *et al.*, 2003). A subsequent report will address the effects of up-conditioning.

The focus on GABAergic terminals was also motivated by evidence that these terminals change after spinal cord injury and can be further modified in spinalized animals by training (Tillakaratne *et al.*, 2000, 2002). We identified and evaluated GABAergic terminals by quantifying the immunoreactivity of glutamic acid decarboxylase $(GAD)_{67}$, one of the two GABA-synthesizing enzymes (Esclapez *et al.*, 1994; Soghomonian & Martin, 1998), in terminals on identified soleus motoneurons. The results give new insight into the spinal cord plasticity underlying the acquisition of a simple motor skill.

## Materials and methods

### *Subjects*

Subjects were 18 adult male Sprague-Dawley rats weighing 349 g (± 56 g SD) at the beginning of the study. Ten underwent electrode implantation and H-reflex down-conditioning and were then studied anatomically as described here. The other eight constituted a naive control group and were used only in the anatomical studies. All procedures satisfied the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, DC, 1996) and had been reviewed and approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. The H-reflex conditioning paradigm, which is fully described elsewhere (Wolpaw & Herchenroder, 1990; Chen & Wolpaw, 1995), is summarized below. Other procedures are described in detail.

### *Study protocol*

Under general anesthesia (ketamine HCl, 80 mg/kg and xylazine, 10 mg/kg, both i.p.), each rat was implanted with chronic stimulating and recording electrodes in the right hindleg. To elicit the H-reflex, a nerve-stimulating cuff was placed around the right posterior tibial nerve just proximal to the triceps surae branches. To record soleus EMG activity, fine-wire electrodes were inserted in the right soleus muscle. The Teflon-coated wires from the nerve cuff and muscle passed subcutaneously to a connector plug mounted on the skull.

Data collection started at least 20 days after implantation. During data collection, each rat lived in a standard rat cage with a flexible cable attached to the head plug. The cable, which allowed the rat to move freely about the cage, carried the wires from the electrodes to an electronic swivel above the cage, from which they passed to an EMG amplifier and a nerve-cuff stimulation unit. The rat had free access to water and food, except that, during H-reflex down-conditioning, it received food mainly by performing the task described below. Animal well-being was carefully checked several times each day and body weight was measured weekly. Laboratory lighting was reduced from 21:00 to 06:00 h each day.

### *H-reflex conditioning*

A computer system continuously monitored (24 h/day, 7 day/week) EMG from the soleus muscle and controlled the nerve-cuff stimulus. Whenever the absolute value (i.e. equivalent to the full-wave rectified value) of background (i.e. ongoing) EMG stayed within a defined range for a randomly varying 2.3–2.7-s period, a stimulus pulse (typically 0.5 ms in duration) was delivered by the nerve cuff. Pulse amplitude was initially set just above the M-response [i.e. direct muscle response (Brown, 1984)] threshold and then continuously and automatically adjusted to maintain M-response size unchanged throughout the entire period of data collection. Thus, the background EMG (reflecting soleus motoneuron tone at the time of H-reflex elicitation) and the M-response (reflecting the effective strength of the nerve-cuff stimulus) remained stable throughout data collection.

In the control mode, the computer simply measured the absolute value of soleus EMG for 50 ms following the stimulus. In the down-conditioning mode, it gave a food reward 200 ms after nerve stimulation if EMG amplitude in the H-reflex interval (i.e. typically 6.0–10.0 ms after stimulation) was below a criterion value. In the course of its daily activity, the animal usually satisfied the background EMG requirement, and thus received nerve-cuff stimulation, 3100–7500 times per day. H-reflex size was calculated as average EMG amplitude in the H-reflex interval minus average background EMG amplitude and was expressed in units of average background EMG amplitude. Each rat was first exposed to the control mode for 20 days to determine the control H-reflex size and then exposed to the down-conditioning mode for 50 days.

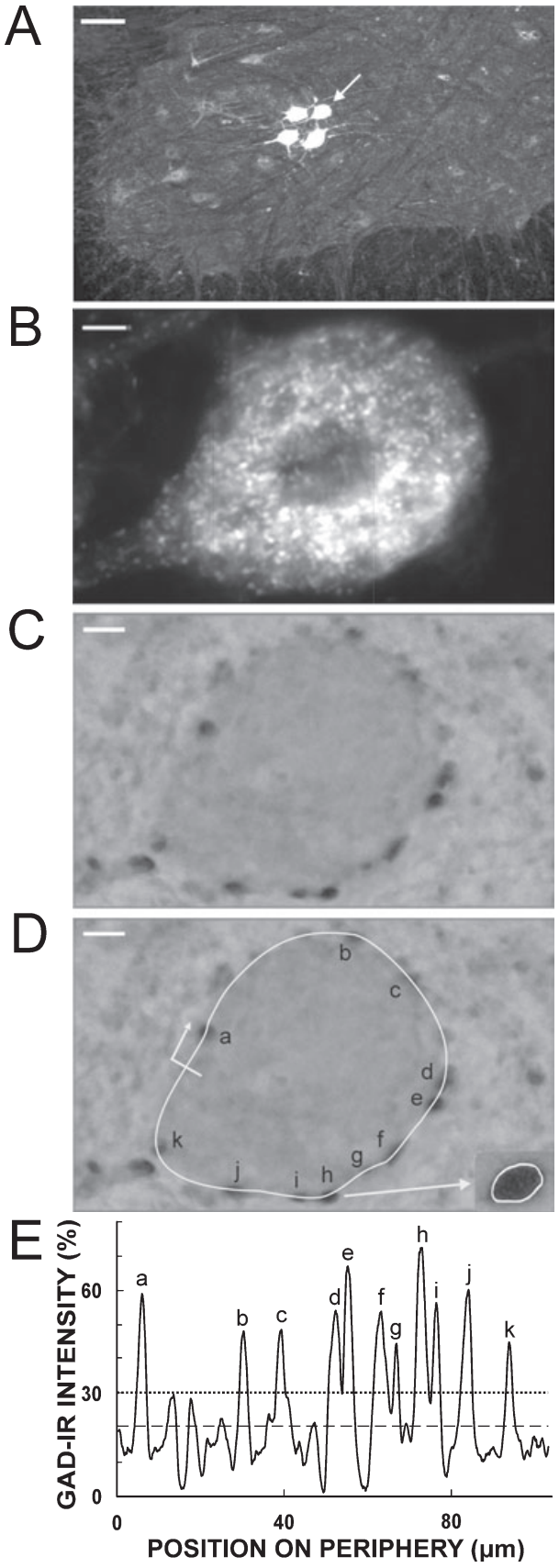
To determine the final effect on H-reflex size of exposure to the down-conditioning mode, average H-reflex size for the final 10 days of the 50-day exposure was calculated as percent of the control H-reflex size (i.e. the average of the final 10 control-mode days). Successful down-conditioning was defined as a decrease to ≤80% of control H-reflex size (Wolpaw *et al.*, 1993; Chen & Wolpaw, 1995).

H-reflex conditioning was successful in six of the 10 down-conditioned rats. This success rate is similar to the typical success rate of 75% found in previous studies (Chen & Wolpaw, 1995, 1997, 2002; Chen *et al.*, 2002, 2003). In the six successful down-conditioned rats, final H-reflex size averaged 51% (± 3% SEM) of its control value. In each of the four unsuccessful rats, final H-reflex size was within 20% of its control value and the average value for the four rats was 105% (± 7% SEM) of control. For all 10 rats, background EMG and M-response remained stable throughout data collection.

### *Soleus motoneuron labeling and rat perfusion*

At the end of the down-conditioning period, the rat was anesthetized with ketamine and xylazine as described above and injected in the right soleus muscle with 50 μg (in 50 μL distilled water) of cholera toxin subunit B-conjugated Alexa Fluor488 (Molecular Probes, Eugene, OR, USA). A 100-μL Hamilton syringe with a 33-gauge needle was used. The right soleus muscle was exposed through a small incision in the skin of the lateral aspect of the calf. Under a dissection microscope, the needle was inserted near the distal tendon and advanced carefully to the middle of the muscle. The dye was injected at three points near the middle. Injection was very slow (over 3 min) to prevent leakage along the needle track. After the injection, the needle was left *in situ* for 2–3 min and then slowly withdrawn. The area was rinsed thoroughly with saline for 5 min to remove any leakage and then the wound was sutured. Eight naive unimplanted control rats of similar weight were also anesthetized and injected.

Three days later, the rat was killed with an overdose of sodium pentobarbital and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The lumbosacral spinal cord was removed and postfixed in the same fixative for 2 h, washed with 0.05 M phosphate-buffered saline (PBS) containing 137 mM NaCl (pH 7.4) and infiltrated with 30% sucrose for 24 h. The spinal cord containing the soleus motoneuron pool 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 fluorescent microscopy to identify cholera toxin subunit B-conjugated Alexa Fluor488 retrograde-labeled soleus motoneurons. All labeled soleus motoneurons were photographed with a digital camera (Olympus Magnafire$^{TM}$ SP, IKH027801). The slides were then stored in a low-temperature freezer (at −80 °C) before further immunohistochemical processing. Figure 1A shows a cluster of labeled soleus motoneurons.

### *Immunohistochemical processing*

The standard avidin–biotin complex-peroxidase system (ABC Elite; Vector Laboratories, Burlingame, CA, USA) was used to assess $GAD_{67}$ immunoreactivity (GAD-IR) in every other 25-μm section through the portion of the spinal cord containing labeled soleus motoneurons. The sections were washed three times (10 min each) with 0.05 M PBS containing 0.1% Triton X-100 (pH 7.4), incubated for 30 min with 5% normal goat serum and then incubated for 18–20 h in a humid chamber at room temperature (22°C) in 0.05 M PBS containing 0.1% Triton X-100 containing 3% bovine serum albumin with rabbit anti-$GAD_{67}$ polyclonal antibody (K2 antibody, 1 : 2000 dilution; Chemicon, Temecula, CA, USA) as the primary antibody. The sections were again washed with 0.05 M PBS containing 0.1% Triton X-100 and the biotinylated secondary antibody (goat anti-rabbit, 1 : 200 in PBS) was applied for 1.5 h. After endogenous peroxidase activity was quenched by 0.3% $H_2O_2$ for 15 min, the sections were reacted for 1.5 h with the avidin–biotin complex (1 : 100 in PBS). Then, 0.05 M Tris-HCl buffer (Tris-buffered saline, pH 7.6) was used to wash the sections and adjust pH to 7.6 before color development. Finally, the sections were reacted with 0.04% DAB solution and 0.006% $H_2O_2$ for 8 min to optimize the signal-to-noise ratio.

Sections processed without primary antibody or with $GAD_{67}$ antibody diluted 1 : 10 000 did not show any GAD-IR in the ventral horn of the spinal cord (although the diluted antibody still showed strong GAD-IR in the superficial laminae of the dorsal horn). The $GAD_{67}$ specificity of the present study was also confirmed by concomitant processing of cerebellar sections, which showed the expected (Oertel *et al.*, 1981) densely labeled $GAD_{67}$ terminals on the cell bodies and proximal dendrites of the Purkinje cells and in glomeruli in the granular layer.

### *Image analysis preparation and protocol*

In the $GAD_{67}$-labeled sections, soleus motoneurons were identified by matching them to their cholera toxin subunit B-conjugated Alexa Fluor488-labeled images using location, somatic features and other

FIG. 1. Assessment of glutamic acid decarboxylase$_{67}$ immunoreactivity (GAD-IR). (A) A low-magnification picture showing four soleus motoneurons labeled by Alexa Fluor488 located in the ventral horn of the spinal cord in a rat. The arrow indicates the motoneuron shown in B–D. (B) Single soleus motoneuron. (C) The same motoneuron showing GAD-IR. (D) Tracing of the perimeter of the same motoneuron with identified GABAergic terminals indicated. Dotted line and small arrow indicate starting point and direction of perimeter luminance measurement. Inset shows tracing of one of these terminals for measurement of area and diameter. Bar, 65 μm in A, 5 μm in B–D, 1 μm in D inset. (E) Luminance vs. position on the perimeter of this same motoneuron (0%, luminance of a totally clear slide and 100%, luminance 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.

landmarks (e.g. Fig. 1B). Analysis was confined to those soleus motoneurons in the $GAD_{67}$-labeled sections that had a nucleus and a clearly defined somatic border. Sixty-eight percent of the cholera toxin subunit B-conjugated Alexa Fluor488-labeled motoneurons satisfied these criteria. [The remainder, which were either invisible in the $GAD_{67}$-labeled sections (12%), lacked a clear somatic border (12%) or lacked a nucleus (8%), did not differ significantly in frequency among the three rat groups defined in the Data analysis section below.]

The soleus motoneurons found suitable for analysis were photographed with an Olympus BH2-RFCA microscope (500× magnification, fixed illumination) equipped with an Olympus DP12 digital camera (magnification 10×, standard illumination). The resulting images were then renamed with codes by a person who was not otherwise involved in this study, so that all measurements could be performed in a blinded fashion. The GAD-IR of each image was then assessed twice, once by each of two independent raters who did not know whether the image came from a naive rat or a successful or unsuccessful HRdown rat.

### *Quantification of glutamic acid decarboxylase$_{67}$ immunoreactivity*

Each rater measured the soma size and GAD-IR of the soma and its perimeter as follows. With the image magnified 5000×, the Image J program (NIH, version 1.29x) was used to trace the perimeter of each motoneuron (excluding proximal dendrites, which differ to some extent from the soma in terminal coverage) (Starr & Wolpaw, 1994; Rose & Neuber-Hess, 1991, Örnung *et al.*, 1996). The program then calculated: soma diameter (i.e. Feret's diameter, which is the long axis of the soma cross-section) and area; the average luminance of the entire soma excluding the perimeter; the average luminance of the perimeter and a plot of perimeter luminance vs. location on the perimeter. No corrections were made for tissue shrinkage during processing.

To determine GAD-IR, each luminance value was converted into percent, with the luminance of a totally clear slide defined as 0% and the luminance of a totally opaque slide defined as 100%. A soleus motoneuron with its perimeter traced and the corresponding plot of GAD-IR vs. perimeter location are shown in Fig. 1C–E.

### *Identification and measurements of GABAergic terminals on soleus motoneurons*

Each rater assessed the numbers, sizes and GAD density of the GABAergic terminals on each motoneuron as follows. GABAergic terminals were identified as punctate or bead-like densities (Border & Mihailoff, 1985) that were on the perimeter of the motoneuron soma. These identifications were verified by confirming that each terminal was evident on the plot of GAD-IR vs. perimeter location as a abrupt peak with a maximum GAD-IR value that exceeded the average GAD-IR of the perimeter by at least 10% [of the total range from 0% (the value of a clear slide) to 100% (the value of an opaque slide) as described above]. Each confirmed terminal was then magnified 10 000× and the Image J program was used as described above to determine its: Feret's diameter (i.e. the long axis of the terminal); area; average diameter [i.e. two times square root of (area/$\pi$)] and GAD density (i.e. GAD-IR value of the terminal divided by its average diameter to correct for terminal thickness). It also calculated the terminal coverage of each motoneuron (i.e. sum of Feret's diameters of all the terminals on the motoneuron expressed as percent of the motoneuron's perimeter).

### *Data analysis*

The analysed images were decoded and their data sorted by rat and motoneuron. The data of the two independent raters were found to be very similar, differing by only 1–4% across the different measurements made. These minor differences were resolved by joint evaluation and discussion (i.e. for differences in terminal identification) or by averaging the two raters' values (i.e. for quantitative measurements).

The data were collated and evaluated statistically for the three experimental groups of rats: successful HRdown rats [down-conditioning successful (DS) rats; $n = 6$]; unsuccessful (i.e. failed) HRdown rats [down-conditioning failed (DF) rats; $n = 4$] and naive control (NC) rats ($n = 8$).

For each measure, the three groups were compared by one-way ANOVA. When a difference was detected with $P < 0.01$, it was then confirmed by nested ANOVA, with terminals nested in cells, cells nested in rats and rats nested in groups. Significant ($P < 0.01$) differences between specific groups were detected by the Tukey-Kramer HSD test.

## Results

Data were gathered for 416 soleus motoneurons and 1642 GABAergic terminals from the DS, DF and NC rat groups. These data are summarized in Table 1 and measures for which the DS group differed significantly from the NC group are shown in Fig. 2. The DF and NC groups did not differ significantly from each other in any measure.

### *Soleus motoneurons*

The average number of soleus motoneurons found per rat was consistent with estimates of the size of the population (Ishihara *et al.*, 1987; Westerga & Gramsbergen, 1992; Gramsbergen *et al.*, 2000; Bose *et al.*, 2005). The groups did not differ in the number of labeled soleus motoneurons per rat found suitable for analysis and nor did the motoneurons of the groups differ in diameter, perimeter, area or

TABLE 1. Motoneuron and GABAergic terminal numbers and properties for each experimental group

| | Experimental group | | |
|---|---|---|---|
| | DS | DF | NC |
| Rats ($n$) | 6 | 4 | 8 |
| Motoneurons ($n$) | 137 | 91 | 188 |
| Motoneurons per rat | 23 ± 3 | 23 ± 5 | 24 ± 3 |
| Feret's diameter (μm) | 44 ± 1 | 44 ± 1 | 44 ± 1 |
| Perimeter (μm) | 119 ± 2 | 115 ± 3 | 117 ± 2 |
| Area (μm$^2$) | 915 ± 21 | 856 ± 26 | 852 ± 18 |
| Somatic GAD-IR (%) | 20.1 ± 0.5 | 18.6 ± 0.7 | 17.0 ± 0.5 |
| Perimeter GAD-IR (%) | 23.1 ± 0.4*** | 19.4 ± 0.7 | 17.6 ± 0.5 |
| GABAergic terminals ($n$) | 710 | 323 | 607 |
| Terminals per cell | 5.1 ± 0.2*** | 3.1 ± 0.3 | 3.3 ± 0.2 |
| Feret's diameter (μm) | 2.12 ± 0.03** | 2.07 ± 0.05 | 1.97 ± 0.03 |
| Area (μm$^2$) | 2.12 ± 0.05* | 2.11 ± 0.09 | 1.92 ± 0.06 |
| GAD density (GAD-IR/diameter) | 26.1 ± 0.3*** | 24.3 ± 0.5 | 24.2 ± 0.3 |
| Coverage of soma (%) | 9.6 ± 0.4*** | 6.3 ± 0.5 | 6.0 ± 0.3 |

Data are presented as means ± SEM. ***$P < 0.0001$, **$P < 0.001$, *$P < 0.01$ compared with the naive control rats (NC) group. Glutamic acid decarboxylase immunoreactivity (GAD-IR) is in percent of maximum possible GAD-IR (i.e. GAD-IR of an opaque slide; see text). Terminal GAD density and coverage are determined as described in the text. DS, successful down-conditioned rats; DF, failed down-conditioned rats.

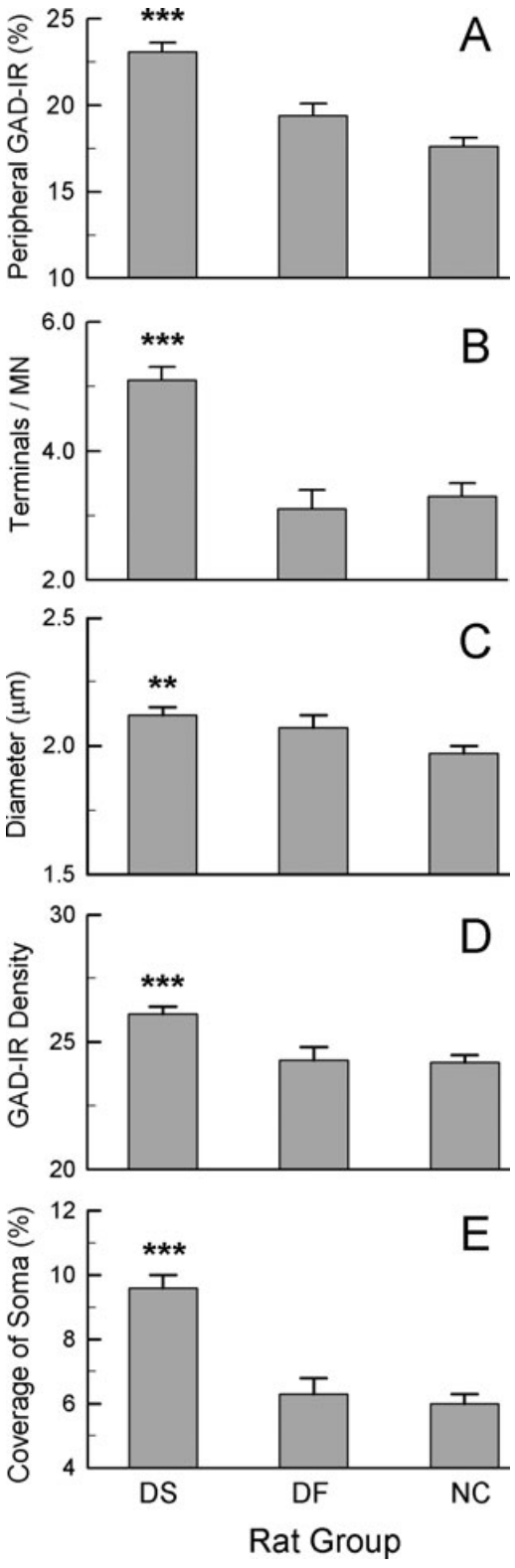



FIG. 2. Average (± SEM) values for: down-conditioning successful (DS), down-conditioning failed (DF) and naive control (NC) rat groups for measures that differed significantly among the groups. (A) Motoneuron perimeter glutamic acid decarboxylase immunoreactivity (GAD-IR) intensity. (B) Number of GABAergic terminals per motoneuron (MN). (C) Feret's diameter of GABAergic terminals. (D) Terminal GAD density (i.e. GAD-IR/diameter, as described in the text). (E) GABAergic terminal coverage of soma (expressed as percent of perimeter, see text). ***$P < 0.0001$, **$P < 0.001$ compared with the NC group.

somatic GAD-IR. Motoneuron sizes were similar to those found using quantitative enzyme histochemistry methods (Ishihara *et al.*, 2001).

The groups did differ in perimeter GAD-IR. This measure was significantly greater in DS rats than in NC or DF rats ($P < 0.0001$ for each) (Fig. 2A). Perimeter GAD-IR was increased only by successful down-conditioning and was not affected by unsuccessful down-conditioning.

### *GABAergic terminals*

The DS rats exceeded NC and DF rats ($P < 0.0001$ for each) in the number of GABAergic terminals found per cell (Fig. 2B). This difference was quite large as DS motoneurons averaged 55% more GABAergic terminals than NC motoneurons. The difference was not attributable to a difference in somatic GAD-IR, which did not differ significantly among the groups. Furthermore, additional analysis showed that there was little or no correlation between the number of GABAergic terminals on a motoneuron and its somatic GAD-IR ($r^2 < 0.07$ for each of the three rat groups).

The DS rats also exceeded NC rats in terminal Feret's diameter ($P < 0.001$) (Fig. 2C) and area ($P < 0.01$). For all three groups, the relationships between diameter and area indicated that, if the terminals were assumed to be ellipsoid, their minor diameters were about 60% of their major diameters.

The DS rats exceeded NC and DF rats ($P < 0.0001$ for each) in GAD density (Fig. 2D). Thus, the terminals of DS rats were larger than those of NC rats and also had higher GAD density.

The DS rats greatly exceeded NC and DF rats ($P < 0.0001$ for each) in terminal coverage of the somatic membrane (i.e. the percentage of the perimeter occupied by GABAergic terminals) (Fig. 2E). This difference was attributable mainly to the much greater number of GABAergic terminals on DS motoneurons and also to the greater size of the DS terminals.

## Discussion

### *Data set*

Spurred by current hypotheses (see Introduction) as to the mechanisms of H-reflex conditioning (see above) (Wolpaw & Tennissen, 2001), and also by evidence that conditioning affects F-terminals (Feng-Chen & Wolpaw, 1996) and that spinal cord transection and subsequent training affect spinal GABAergic terminals (Tillakaratne *et al.*, 2000, 2002), this study set out to determine the effects of down-conditioning of the rat soleus H-reflex on GABAergic terminals contacting soleus motoneurons. To do this, we identified soleus motoneurons by injecting a fluorescent retrograde tracer into the muscle and identified their GABAergic terminals by their GAD-IR. $GAD_{67}$ is one of the two principal forms of the GABA-synthesizing enzyme GAD and is the one more evident in terminals on motoneurons (in contrast to $GAD_{65}$, which is more evident in terminals presynaptic to primary afferent terminals on motoneurons) (McLaughlin *et al.*, 1975; Mackie *et al.*, 2003; Riddell *et al.*, 2003). We measured GAD-IR, rather than immunoreactivity for GABA itself, because the latter would have required electron microscopic examination, which would have severely limited the number of motoneurons and rats that could be studied and thereby prevented effective statistical analysis. That we achieved robust and specific labeling of soleus motoneurons and their GABAergic terminals is indicated by the numbers and locations of the soleus motoneurons identified (Ishihara *et al.*, 1987; Westerga & Gramsbergen, 1992; Starr & Wolpaw, 1994; Gramsbergen *et al.*, 2000; Bose *et al.*, 2005) and by the specificity of GAD-IR labeling

demonstrated in the control studies described in Materials and methods.

The study focused on comparison of data from three rat groups: successful down-conditioned rats (DS rats), naive control rats (NC rats) and unsuccessful (failed) down-conditioned rats (DF rats). The last group was a byproduct of the fact that H-reflex conditioning is successful in most (i.e. about 75%), but not all, animals (Chen & Wolpaw, 1995, 1996; Wolpaw, 1997). Furthermore, successful and unsuccessful animals (whether rats or monkeys) do not appear to represent a continuum of success but rather fall into two distinct groups (e.g. Wolpaw *et al.*, 1993). This is evident in the present study, where final H-reflex values in the six DS rats ranged from 43 to 63% of control and averaged 51% (± 8% SD), while final values in the four DF rats ranged from 89 to 119% and averaged 105% (± 15% SD). Thus, the DF rats provided a distinct additional data set. This set was particularly valuable as a control for non-specific effects of the implantation and long-term data collection. A set of DF monkeys provided a similarly valuable control in an earlier study of the effects of down-conditioning on motoneuron physiological properties (Carp & Wolpaw, 1994).

### *Group comparisons*

The three rat groups and their GAD-IR data were compared in terms of their soleus motoneurons and the GABAergic terminals on these motoneurons. The basic properties of the motoneurons (i.e. number/-rat, diameter and area) did not differ among groups. Somatic GAD-IR also did not differ among them. The only motoneuron measure that did differ was perimeter GAD-IR, which was clearly greater in DS rats than in the other two rat groups.

While this measure indicates that the GAD-IR of terminals on soleus motoneurons was greater in DS rats, it does not by itself indicate whether the difference was due to differences in the number, size and/or GAD density of GABAergic terminals. Nevertheless, it provides the most objective, and thus most incontrovertible, evidence that GABAergic terminals were affected by successful down-conditioning. In contrast, the specific measures of individual terminals rest inevitably on their initial identification and thus involve some element of rater subjectivity. At the same time, the use of two independent raters who were blinded as to rat group reduced the dangers of conscious or unconscious rater biases.

### *Effects of successful down-conditioning on GABAergic terminals*

Glutamic acid decarboxylase$_{67}$ is the dominant form of GAD in terminals that contact motoneurons whereas GAD$_{65}$ dominates in presynaptic terminals (McLaughlin *et al.*, 1975; Mackie *et al.*, 2003; Riddell *et al.*, 2003). Terminals that receive presynaptic contacts (i.e. primary afferent terminals) are absent or rare on the motoneuron soma (Holstege & Calkoen, 1990; d'Incamps *et al.*, 1998; Watson & Bazzaz, 2001). Thus, while GABAergic terminals inhibit motoneurons both postsynaptically and presynaptically (Rudomín *et al.*, 1987; Rudomín, 1990; Maxwell & Riddell, 1999), we are confident that the GABAergic terminals identified in this study were largely or wholly on the motoneuron rather than presynaptic to other terminals. [Furthermore, although electron microscopic evaluation would be needed to visualize the terminal contacts on motoneurons, the uniformly close apposition of the GABAergic terminals and the motoneuron membrane (e.g. Fig. 1C) and the lack of similar terminals nearby indicate that these terminals were on the motoneuron.]

The terminal measurements shown in Table 1 indicate the reasons for the higher perimeter GAD-IR found for DS motoneurons. DS motoneurons had many more GABAergic terminals and these terminals were bigger and had higher GAD density. As the GAD density shown in Table 1 is corrected for terminal thickness (i.e. it is calculated by dividing the terminal GAD-IR value by the terminal diameter), it should reflect actual GAD density within the terminal. (It is conceivable, although unlikely, that the higher GAD density contributed significantly to the larger terminal area by causing the raters to trace slightly larger terminals.) In summary, terminal number and GAD density, and probably terminal size, were increased by successful down-conditioning. As a result, the coverage of the motoneuron somatic membrane by GABAergic terminals was 60% greater in DS than NC rats.

Furthermore, the fact that none of the changes noted in DS rats was detected in DF rats indicates that these changes were not a non-specific effect of the down-conditioning protocol but rather were associated with successful down-conditioning. This clear association suggests that the terminal changes had a key role in the H-reflex decrease.

F-terminals comprise 60–65% of the terminals on motoneuron cell bodies (Örnung *et al.*, 1996; Novikov *et al.*, 2000). Their major transmitters are GABA and/or glycine (Murphy *et al.*, 1996; Örnung *et al.*, 1996, 1998; Somogyi, 2002). Furthermore, a substantial fraction contain more than one transmitter (Örnung *et al.*, 1996; Jonas *et al.*, 1998; Nicoll & Malenka, 1998; Somogyi, 2002). Thus, the increased terminal number, size and GAD density found in DS rats may have reflected an increase in the fraction of F-terminals that were GABAergic and in the average size and GAD density of these terminals. Alternatively, the extra GABAergic terminals could have been newly sprouted. However, this possibility appears to be less likely, particularly in the light of the primate data showing that down-conditioning is associated with larger, but not more, F-terminals (Feng-Chen & Wolpaw, 1996).

It should be noted that the present data are anatomical, rather than functional. While they show that GABAergic input on motoneurons was greater in DS rats, they only imply that actual GABA release to motoneurons was correspondingly greater.

### *Origin of the modified GABAergic terminals in DS rats*

Glycine and GABA are the main inhibitory transmitters in the mammalian spinal cord and most F-terminals on motoneurons contain one or both (Magoul *et al.*, 1987; Murphy *et al.*, 1996; Örnung *et al.*, 1996; Martin & Tobin, 2000; Todd & Maxwell, 2000). Descending GABAergic input to motoneurons in the ventral horn comes from ventromedullary reticular formation via the ipsilateral dorsolateral funiculus (Holstege, 1991). However, transection of the entire ipsilateral lateral column, including the dorsolateral funiculus, does not impair acquisition or maintenance of H-reflex down-conditioning (Chen & Wolpaw, 1997, 2002). Furthermore, the CST, the only descending tract essential for down-conditioning, is only excitatory and almost certainly does not reach motoneurons in the rat lumbosacral spinal cord (Gemma *et al.*, 1987; Kuang & Kalil, 1990; Yang & Lemon, 2003; Alstermark *et al.*, 2004). These facts, and the fact that the changes in GABAergic terminals noted here occurred only in DS and not DF rats, suggest that the modified GABAergic terminals of DS rats came from spinal interneurons rather than from descending projections.

Inhibitory projections to motoneurons arise from the intermediate zone (Rexed's laminae VI–VII) where group Ia, Ib and II inhibitory interneurons are located (Jankowska, 1992, 2001; Rudomín *et al.*,

1987; Edgley, 2001), commissural interneurons in ventral lamina VII–VIII (Eide *et al.*, 1999; Butt *et al.*, 2002; Kiehn & Butt, 2003) and Renshaw cells in lamina VIII–IX (Fyffe, 1991). Renshaw cells project mainly to motoneuron dendrites (Fyffe, 1991; Schneider & Fyffe, 1992; Maltenfort *et al.*, 2004). Thus, they probably did not account for the changes in somatic GABAergic terminals described here. As discussed further below, interneurons in the intermediate zone were the most likely origin of the modified GABAergic terminals in DS rats.

### *Role of the modified GABAergic terminals in down-conditioning*

The increases in GABAergic terminal number, size and GAD density in DS rats are consistent with the F-terminal data from down-conditioned monkeys (Feng-Chen & Wolpaw, 1996). The fact that these increases were found in successful, but not unsuccessful, down-conditioned rats indicates that they were not non-specific effects of the implantation or the conditioning protocol and suggests that they contributed to making the H-reflex smaller. Whether similar changes occur in glycinergic terminals [many central synapses colocalize and release both glycine and GABA (Jonas *et al.*, 1998; O'Brien & Berger, 1999; Gao *et al.*, 2001; González-Forero & Alvarez, 2005)] remains to be determined. The simplest possibility, that this increased GABA input strengthens background postsynaptic inhibition and thus reduces motoneuron excitation by the nerve-cuff stimulus, is ruled out by the fact that the H-reflex decrease occurs without change in the level of background EMG. [The conditioning protocol ensures that the overall level of excitation of the soleus motoneuron population at the time of H-reflex elicitation does not change from its original control-mode value (see Materials and methods).] Furthermore, evaluation of the relationship between the change in number of GABAergic terminals and motoneuron size did not support the possibility of subtle changes in motoneuron recruitment order.

Another possibility is that this increased GABAergic input conveys stronger oligosynaptic inhibition from the group I afferents excited by the nerve cuff stimulus. As such inhibition can reach motoneurons quickly enough to affect the H-reflex (Baldissera *et al.*, 1981; Jankowska, 1992; Stephens & Yang, 1996), its increase could potentially account for an H-reflex decrease. This mechanism would presumably decrease the latter part of the H-reflex more than the earlier part and would thereby decrease the mean latency, as well as the size, of the reflex. However, successful down-conditioning is not accompanied by a detectable change in mean latency (Wolpaw & Chen, 2001). Thus, increased group I inhibition is probably not the mechanism of H-reflex down-conditioning.

A more promising possibility is that these modified GABAergic terminals convey to the motoneuron the CST influence that is essential for the acquisition and maintenance of a smaller H-reflex (Chen & Wolpaw, 1997, 2002). The CST projects strongly to the intermediate zone (lamina VI–VII) (Kuang & Kalil, 1990; Liang *et al.*, 1991; Brösamle & Schwab, 1997; Yang & Lemon, 2003) which contains inhibitory interneuron populations (Jankowska, 1992, 2001) that, in turn, project to motoneurons. The CST projections are excitatory and largely glutamatergic and GABAergic interneurons (as well as glycinergic and GABA- and glycine-coreleasing interneurons) in the intermediate zone show AMPA-type glutamate receptor immunoreactivity (Tachibana *et al.*, 1994; Spike *et al.*, 1998; Nagy *et al.*, 2004). Furthermore, these interneurons depend on motor cortex activity for synaptogenesis (Clowry *et al.*, 2004) and glutamatergic inputs to them appear to regulate synapse density in spinal cord ventral horn (Rosato-Siri *et al.*, 2002). Thus, they could constitute the path through which the CST activity essential for down-conditioning reaches and changes the motoneuron.

The H-reflex decrease produced by the down-conditioning protocol appears to be largely attributable to a positive shift in motoneuron firing threshold (Carp & Wolpaw, 1994). Furthermore, the decrease in motoneuron axonal conduction velocity noted with successful down-conditioning in both monkeys and rats (Carp & Wolpaw, 1994; Carp *et al.*, 2001a,b) implies that this threshold change is present throughout the entire motoneuron and a modelling study suggests that it results from a positive shift in the activation voltage of sodium channels in the motoneuron membrane (Halter *et al.*, 1995). While the mechanism of this shift remains unknown, involvement of protein kinases A and/or C is plausible (Carp & Wolpaw, 1994), given their powerful modulatory control over sodium channel function (Lotan *et al.*, 1990; Numann *et al.*, 1991; Li *et al.*, 1992; Cantrell *et al.*, 1996; Smith & Goldin, 1996) and their involvement in associative learning (Selcher *et al.*, 2002). However, activation of these protein kinases to phosphorylate sodium channels is typically associated with decreased sodium channel availability and changes in the time course of activation and inactivation, rather than with change in the voltage dependence of activation. Alternatively, altered activation of metabotropic GABA-B receptors by GABAergic interneurons that are driven by the CST could modify GABA-B receptor-linked G-protein activation. G-protein activation could change the voltage dependence of sodium channel activation and inactivation (Ma *et al.*, 1994, 1997) or affect transcription factors (Barthel *et al.*, 1996; White *et al.*, 2000) that modify sodium channel properties. Each of these alternatives might account for the positive shift in motoneuron firing threshold that appears to underlie successful down-conditioning.

### *Spinal GABAergic plasticity in other situations*

Spinal cord GABAergic function is affected by peripheral and central lesions of various kinds (Dumoulin *et al.*, 1996; Lindå *et al.*, 2000; Tillakaratne *et al.*, 2000; Moore *et al.*, 2002). Furthermore, in cats with spinal cord transections, locomotor training changes GABAergic activity around lumbosacral motoneurons (Tillakaratne *et al.*, 2002). This change is apparently driven by input from the periphery. The GABAergic plasticity described in the present study differs from that after spinal cord transection in that it occurs in intact rats and appears to be initiated and guided by descending activity (i.e. in the CST). Nevertheless, it is likely that the spinal cord mechanisms and consequences of both these examples of spinal cord GABAergic plasticity may overlap to some degree.

Initial analyses of GABAergic terminals from up-conditioned rats (Wang *et al.*, 2004) suggest that up-conditioning has effects distinct from, but not opposite to, those of down-conditioning. These data presumably reflect the heterogeneity in function and origin of GABAergic terminals on motoneurons (Holstege, 1991; Örnung *et al.*, 1996, 1998; Rekling *et al.*, 2000; Somogyi, 2002; Raiteri *et al.*, 2005) and they are consistent with earlier data indicating that H-reflex down-conditioning and up-conditioning are not mirror images of each other but rather have different mechanisms (Wolpaw *et al.*, 1986; Carp & Wolpaw, 1994, 1995; Wolpaw & Chen, 2001).

### *Conclusions*

Down-conditioning of the H-reflex, a simple model of motor learning, markedly changes GABAergic terminals on spinal motoneurons. The most striking effects are the substantial increases in GABAergic

terminal number, size and GAD density. These modified GABAergic terminals probably arise from interneurons in spinal cord lamina VI–VII and may convey the CST influence that modifies motoneuron firing threshold and thereby decreases the H-reflex. The results indicate that activity-driven plasticity in spinal GABAergic terminals accompanies, and is likely to contribute to, motor learning in intact rats. Thus, they illustrate the occurrence and potential importance of spinal cord plasticity in motor learning.

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## Abbreviations

CST, corticospinal tract; DF, down-conditioning failed; DS, down-conditioning successful; GAD, glutamic acid decarboxylase; GAD-IR, glutamic acid decarboxylase$_{67}$ immunoreactivity; NC, naive control; PBS, phosphate-buffered saline.

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