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TETANUS TOXIN REDUCES LOCAL AND DESCENDING REGULATION OF THE H-REFLEX

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Abstract

Introduction—Skeletal muscles that are under the influence of tetanus toxin show an exaggerated reflex response to stretch. We examined which changes in the stretch reflex may underlie the exaggerated response.

Methods—H-reflexes were obtained from the tibialis anterior (TA) and flexor digitorum brevis (FDB) muscles in rats 7 days after intramuscular injection of tetanus toxin into the TA.

Results—We found effects of the toxin on the threshold, amplitude, and duration of H-waves from the TA. The toxin inhibited rate-dependent depression in the FDB between the stimulation frequencies of 0.5–50 HZ and when a conditioning magnetic stimulus applied to the brain preceded a test electrical stimulus delivered to the plantar nerve.

Conclusions—Tetanus toxin increased the amplitude of the H-wave and reduced the normal depression of H-wave amplitude that is associated with closely timed stimuli, two phenomena that could contribute to hyperactivity of the stretch reflex.

Keywords

H-reflex; rate-dependent depression; presynaptic inhibition; rat; stretch reflex; transcranial magnetic stimulation; tetanus toxin

The unique ways in which tetanus toxin is handled by the nervous system and its enzymatic activity account for its ability to selectively increase motor neuron activity. At the neuromuscular junction, tetanus toxin binds to motor neuron terminals, which are enriched with receptors for the toxin.¹ The toxin is then internalized into membrane-bound endosomes, which are trafficked by retrograde fast axonal transport to the motor neuron cell body in the spinal cord. Once the endosomes reach the cell body, they likely fuse with the plasma membrane, resulting in release of the toxin into the extracellular space, where it

binds to presynaptic terminals in the vicinity of motor neurons.² The toxin then undergoes a second round of endocytosis followed by liberation of the proteolytic light chain portion into the cytosol of the second-order presynaptic terminals. Within the presynaptic terminals of spinal interneurons, the light chain cleaves vesicle associated membrane protein (predominantly the VAMP2 isoform), blocking fusion of synaptic vesicles.³ The result is failure of neurotransmitter release, primarily at inhibitory synapses in the vicinity of motor neurons.

The degree of toxin spread from the site of its elaboration likely accounts for whether many muscles, or just one muscle, become affected. Widespread intoxication through the systemic circulation results in the continuous involuntary muscle contractions that characterize clinical generalized tetanus, while spatially restricted uptake of the toxin results in a localized state of muscle hyperexcitability.

Tetanus toxin produces repetitive segmental motor neuron discharges in response to muscle stretch.^{4,5} The resulting increase in limb rigidity is suggestive of some form of reflex hyperactivity, but there is conflicting evidence regarding the effect of the toxin on the stretch reflex. Early as well as more recent reports indicated that the toxin leaves the reflex unchanged,^{6–9} enhanced,^{4,5,10,11} or depressed.¹² In part, the different outcomes can be attributed to a broad range of toxin dosages, injection locations, and timing of observations. This study used the Hoffman reflex, or “H-reflex,” to examine aspects of the muscle stretch reflex that may be affected by tetanus toxin. The neural pathway that produces the H-reflex does not rely on active inputs from stretch receptors. Consequently the H-reflex offers an incomplete view of the active circuit that controls the stretch reflex. Nonetheless, the H-reflex has become a useful tool in assessing spinal motor reflex function.

Six features of H-waves from animals with unilateral hind limb tetanus were examined by peripheral nerve stimulation: (i) threshold, (ii) maximal wave amplitude, (iii) latency, (iv) duration, (v) gain (estimated from the slopes of recruitment curves), and (vi) rate-dependent depression. To examine whether tetanus toxin can modulate the effects of descending inputs to the local stretch reflex, we also studied depression of H-reflex amplitude using a twin pulse paradigm where conditioning activation of descending pathways by magnetic stimulation of the brain/upper spinal cord was followed by a test pulse delivered with conventional electrodes to a peripheral nerve.

METHODS

Creating Focal Tetanus

Adult female Sprague-Dawley rats (225–250 g) were given an intramuscular injection of tetanus toxin in a 5- μ l volume by 28-gauge Hamilton syringe into one TA muscle. The contralateral control TA received 5 μ l of 0.9% saline. In preliminary experiments, we found that injection of <10 ng of tetanus toxin into the TA produced no signs of generalized tetanus (no difference in body weight gain, spinal posture, or breathing compared with normal rats), and the forelimbs and contralateral leg were not noticeably affected. Injection of 2 ng of toxin into the TA produced ipsilateral limb stiffness, with upper and lower leg extension and moderate plantar flexion, within 5 days. The effect of tetanus toxin was

evident during wakefulness, sleep, and ketamine (50–100 mg/kg) anesthesia. Limb stiffness was attenuated strongly by ketamine–xylazine (40–10 mg/kg), pentobarbital (50 mg/kg), or isoflurane (2%) anesthesia.

H-Reflex Recording

M- and H-waves were recorded using platinum 30-gauge recording electrodes (Astro-Med) under ketamine anesthesia 7 days after toxin injection. Unlike anesthetics that directly modulate γ -aminobutyric acid A (GABA A) receptor-mediated inhibitory transmission, ketamine is an N-methyl-D-aspartate receptor antagonist and is not believed to interfere substantially with the H-reflex.¹³ In preliminary experiments, we determined that the H-wave was often detectable minimally from control TAs. Accordingly, bilateral recordings were also made from the FDB muscles. The minimum voltage required to initiate M- or H-waves (threshold), maximum wave amplitudes, delay between nerve stimulation and initiation of the waves (latency), and duration of the waves were recorded by giving a 300- μ s constant voltage stimulation pulse at the sciatic notch for the TA or the medial plantar nerve for the FDB. Recruitment curves of the M- and H-waves for the FDB were produced by plotting the peak-to-peak wave amplitude relative to Mmax versus stimulation intensity relative to threshold intensity.¹⁴ Intensity was increased in the range of 75–200% threshold of the M-wave, which was sufficient to produce 3–5 data points inclusive of the relatively linear steep rising phase of the curves for both waves. Slopes of the recruitment curves were determined by least squares regression of the data points. Rate (i.e., stimulation frequency) - dependent depression of the H-wave from FDB muscles was examined by delivering 10 pulses at 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 25, and 50 Hz to the medial plantar nerve. Each step was preceded by a 2-min rest period. At the stimulation rate of 0.1–0.2 Hz, there was no attenuation of H-wave amplitude in a volley of 10 pulses. Observations from preliminary experiments indicated that the early H-waves (e.g., the second wave) of higher frequency volleys showed stable amplitudes, while later H-waves in the same volley were of inconsistent amplitudes. Accordingly, the 6th–10th H-waves recorded at each frequency were averaged to obtain a composite representation of the later H-waves.

Magnetic Stimulation

The possible effects of tetanus toxin on descending inputs to the H-reflex from the FDB muscle were examined by transcranial magnetic stimulation (TMS) (Magstim Rapid2) under ketamine anesthesia (50 mg/kg). A conditioning TMS pulse was delivered by a 70-mm circular coil centered at the base of the rostrum. The TMS pulse strength was adjusted to reduce involuntary movement while still producing 250 μ V evoked potentials in both FDBs. The TMS pulse was followed with delays from 10 to 100 ms by stimulation of the medial plantar nerves, with a 2-min recovery between sets of pulses. All procedures were approved by the Office of Animal Welfare Assurance at the University of Maryland, Baltimore.

Statistical Analysis

The Wilcoxon matched-pairs test was used to compute statistical significance for the wave parameters shown in Table 1, the Hmax/Mmax ratios, and the normalized FDB data shown in the figures. All data are presented as mean $\pm$ SD.

RESULTS

H-Reflex After Unilateral Focal Tetanus

The possibility that tetanus toxin produces hyperactivity of the stretch reflex was tested by comparing the H-waves of TAs injected with saline or tetanus toxin. Our efforts at recording the H-reflex from the TA confirm the previously reported difficulty with obtaining H-waves from many rat hind limb muscles. In particular, the TA and other flexors in rats and humans show relatively weak stretch reflexes and H-waves.^{15–17} Only 6 of 11 TAs injected with saline produced an H-wave compared with all 11 TAs injected with tetanus toxin (Table 1). In TA muscles, the threshold was reduced, and the maximum amplitude and duration of the H-waves were increased, by the toxin.

The ratio Hmax/Mmax gives some indication of the share of motor axons stimulated by the IA reflex arc relative to the total motor axon pool.¹⁸ Stimulation of the sciatic nerve at 0.1 Hz produced Hmax/Mmax ratios of 0.05 ± 0.02 and 0.36 ± 0.10 in saline- and toxin-injected TAs respectively, when the 5 nonresponding TAs were omitted. The lack of response from many of the saline-injected TAs severely biased statistical comparisons, and the remaining experiments and analyses focused on the H-reflex from ipsilateral–contralateral pairs of FDB muscles. With stimulation of the plantar nerve, FDBs from the saline- and toxin-injected sides had similar Hmax/Mmax ratios of 0.40 ± 0.15 and 0.47 ± 0.14 , respectively; both were within the range of ratios obtained from normal rat muscles at low stimulation rates.^{13,19–21}

The effect of tetanus toxin on the freedom with which excitatory drives of increasing intensity can activate the pool of neuromuscular junctions (M-wave gain) or spinal motor neurons (H-wave gain) was estimated using recruitment curves (Fig. 1). The recruitment curves displayed a rapidly rising component generally in the range between 1 and 1.5 times threshold stimulus intensity for the M-waves, and 0.8–1.25 times threshold stimulus intensity for the H-waves. Asymptotes for both waves were maintained to at least 1.5–2 times the threshold stimulus intensity. The average slope of the recruitment curves for the M-waves were the same whether the FDB was from saline- or toxin-treated sides. Similarly, there was no difference in the average slopes of the H-waves between the two sides, suggesting that tetanus toxin did not change the gain of the reflex.

Changes in H-Reflex Amplitude with Repetitive Stimulation

The possibility that tetanus toxin induces resistance to decay of H-wave amplitude with repetitive stimulation would suggest a potential basis for the stretch reflex hyperactivity previously attributed to the toxin. A comparison of the effect of tetanus toxin with stimulation at 0.2 Hz and 20 Hz from a representative animal is shown in Figure 2A. Tetanus toxin appeared to decrease rate-dependent depression of the H-wave, an effect

visible in previous recordings made with 4–8 Hz stimulation.²² The effect of the toxin on rate-dependent depression of the H-wave was examined in greater detail by stimulating the medial plantar nerve with a train of 10 pulses delivered at increasing frequencies. At 0.2 Hz, there appeared to be some tendency of the second H-waves to be depressed on the saline-treated sides, so quantitative comparisons were made using 0.1 Hz as maximal amplitude. Increases in stimulation rate above 0.2 Hz quickly brought about depression of the second H-wave in FDBs from the saline-treated sides (Fig. 2B). After saline injection, the second H-wave was depressed to approximately 50% of maximal amplitude at around 1 Hz, with a further decrease to approximately 20% of maximal amplitude by 5 Hz. In contrast, the toxin prevented the second H-wave from decreasing much below 60% maximal amplitude up to at least 50 Hz. Differences in susceptibility to rate-dependent depression were also evident with the later H-waves in a train. The averaged 6th–10th H-waves showed both groups reaching 50% amplitude in the 0.5–1 Hz range, but there were significant differences in the amplitudes of the 6th–10th H-waves between groups at most frequencies; the toxin-treated sides showed less susceptibility to H-wave depression (Fig. 2C). A diminished effect of tetanus toxin on the later H-waves in a volley with high frequency stimuli has also been observed in cats.^{23,24}

Conditioning Stimulation of Descending Pathways

The effect of central nervous system (CNS) stimulation on the H-reflex was examined using TMS to evoke a conditioning stimulus traveling down the spinal cord to the sacral plexus, where the medial plantar nerve originates. The conditioning pulse was followed by a test pulse delivered with conventional stimulating electrodes to the medial plantar nerve. A possible effect of tetanus toxin on the H-wave was then studied by varying the delay between the central conditioning pulse and the peripheral test pulse. As shown in Figure 3, the amplitude of the FDB H-waves from the two sides exhibited a relatively parallel linear relation with interstimulus intervals of 100 and 50 ms. With a further decrease in the delay to 20 ms between stimuli, H-waves from the saline-injected sides showed marked depression of relative amplitude, while tetanus toxin prevented the depression. With 10 ms between stimuli, the H-waves were considerably diminished, presumably the result of motor neuron refractoriness due to some combination of coincident arrival of descending, retrograde motor, and/or sensory inputs.

DISCUSSION

After intramuscular injection of tetanus toxin, an effect on M-wave amplitude was absent up to the maximum frequency examined (50 Hz), confirming that unlike large doses of the toxin and botulinum toxin serotype A, tetanus toxin at low doses does not interfere with neuromuscular transmission.^{25,26} Nerve conduction velocities were not measured directly by the recording procedure, but the latency between the stimulation artifact and the initiation of the M- or H-waves showed no differences after injection of the toxin, similar to studies using analogous human peripheral nerves.^{27,28} The total duration of the M- and H-waves, which might be altered if tetanus toxin interfered directly with the intrinsic excitability of α -motor neurons²⁹ or neuromuscular transmission,^{30,31} was similarly unaffected by the toxin. The slopes of the recruitment curves for the M- and H-waves, which

have been interpreted to reflect changes in the gain of excitability of the stretch reflex,³² were different between the TA and FDB, but there appeared to be no change in gain due to tetanus toxin in either muscle. Consequently the effects of tetanus toxin on the H-reflex appear to be restricted to the efficacy of transmission between IA terminals and α -motor neurons.

Dual Effects of the Toxin on the H-Reflex

Experiments using intramuscular or intraspinal injections to produce focal tetanus have provided conflicting evidence regarding the effect of tetanus toxin on the actual muscle stretch reflex. That prompted our use of the H-reflex to investigate the relationship between the stretch reflex and tetanus toxin. However, measurements of H-wave amplitude obtained at rest or under general anesthesia presuppose direct relevance to the stretch reflex, and that is not always the case. Electrical stimulation of a nerve, while selective for the largest diameter afferents, activates not only Ia but also Ib afferents that overlap in diameter, creating a different pattern of α motor neuron activation between the H-reflex and the stretch reflex. During locomotion, sensory inputs from plantar cutaneous nerves produce facilitation of the stretch reflex over a broad range of conditioning-test intervals during which the H-reflex is inhibited.³³ Additional modulation of the reflex occurs as a result of supraspinal influences. While the data in Figure 3 captured one view of that influence, it did not simulate the cyclic modulation of the reflex that occurs as a result of descending inputs during locomotion. As a consequence, it is not possible to claim with certainty that results obtained using the H-reflex accurately reflect the relationship between the stretch reflex and tetanus toxin. Instead, our results indicate potential sources for previous inconsistent results.

The differences in how the amplitudes of the H-waves recorded from the FDB and TA, both fast-twitch lower limb flexors, were affected by tetanus toxin may be related to their unique physiologic functions during locomotion. The toxin did not produce an increase in H-wave amplitude in the FDB, which shows a naturally robust H-wave in rats and humans.³⁴ The FDB causes plantar flexion of the middle and proximal phalanges, one of the final acts of forward propulsion during locomotion. IA afferents emanating from FDB spindles may be under relatively weak spinal inhibitory control, freeing spindle activity to synchronize motor output and enhance muscle stiffness just before contraction, as reportedly occurs for the soleus muscle during the stance phase in running.³⁵ If the H-reflex is already relatively disinhibited in the FDB, the action of tetanus toxin might not be expected to produce a further increase in H-wave amplitude. The TA normally produces a weak H-wave in rats and humans,¹⁶ and the effectiveness of afferent stimulation was strongly amplified by the toxin. The TA dorsiflexes the ankle in preparation for heel strike, a process that occurs over the relatively long duration of the swing phase of locomotion. For the TA to work effectively, there is less need for the tight synchrony of motor neuron firing required for explosive force development as occurs in the FDB. Consequently, strict inhibitory control of the stretch reflex of the TA is maintained. A reduction by the toxin of the inhibitory influences on the reflex greatly expands the proportion of TA motor neurons that are activated by stimulation of IA afferents, resulting in an increase in the amplitude of the H-wave in that muscle.

Involvement of Spinal Inhibitory Neurons

Tetanus toxin may induce hyper-reflexia by decreasing output of spinal GABAergic neurons. A trisynaptic pathway that involves presynaptic inhibition of Ia afferents is believed to contribute to depression of H-wave amplitude with rapid stimulation, a phenomenon known as frequency- or rate-dependent depression.³⁶ Tetanus toxin could interfere with rate-dependent depression by lowering the efficacy of inhibitory interneurons that synapse with Ia afferent terminals.³⁷

As an unintended consequence of stimulating whole nerve, antidromic stimulation can produce substantial effects on the H-reflex. Antidromic stimulation may be the cause for declining H-wave amplitude with supramaximal intensity stimulation,¹³ a factor we attempted to minimize by stimulating with an intensity that produced maximum amplitude H-waves, rather than some arbitrary positive integer multiple of that intensity. As expected, our preparation showed some evidence of decreased motor neuron excitability with high frequency stimulation. In Figure 2 at 20 Hz, the M-wave amplitudes at the end of a volley (6th–10th traces) are of slightly lower amplitude than at the beginning of the volley (first and second traces) for both the TA and FDB, regardless of whether they were from the saline- or toxin-injected side. Even though the inhibitory effects of Renshaw cells on motor neurons can be suppressed by tetanus toxin,^{38,39} if tetanus toxin had inhibited the activity of the Renshaw cells, there should have been less of a decrease in M-wave amplitude on the toxin-treated sides.

Evidence regarding the synaptic basis of rate-dependent depression of the H-wave also weakens the likelihood of a role for Renshaw cells. The interneurons that produce primary afferent depolarization operate in the apparent absence of substantial Renshaw-Ia synapses in adults.⁴⁰ In addition, Renshaw cell inhibition is particularly tuned to 10 Hz stimulation,⁴¹ yet we found no amplitude minimum around 10 Hz in saline (control) muscles. Instead, the H-wave amplitude declined steadily (on a semi-log scale) between 0.5 Hz and 25 Hz. The selective loss of Renshaw cell inhibition is also closely associated with development of tremor,⁴¹ but tremors were not observed in affected hind limbs at doses both lower and higher than the eventual working dose of 2 ng.

Rather than shifting between states of excitation and relaxation as in a tremor, the appearance was that of a sustained isometric contraction of affected muscles that resembled extensor spastic paralysis. In otherwise normal spinal segments, disinhibition by tetanus toxin of inhibitory synapses on Ia terminals could support continuous motor neuron excitation by Ia afferents that are also tonically active,⁴² leading to widespread, continuous excitation of extrafusal fibers.

Disinhibition of Descending Control by the Toxin

Most of the limb stiffness that was observed in conscious rats was also evident under ketamine anesthesia, and ketamine was found to be permissive for stimulation of motoneurons by TMS, as previously noted in monkeys.⁴³ This combination of factors made it possible to examine whether the effects of supraspinal inputs to the H-reflex can be modulated by tetanus toxin.

Similar to the aforementioned peripheral mechanism, descending inputs are believed to activate primary afferent depolarizing neurons that decrease Ia terminal activation, affecting the stretch reflex in anesthetized animals⁴⁴ and during standing and movement in humans.^{45–47}

This results confirm that activation of a proximal spinal pathway(s) can condition depression of the H-wave by a subsequent test pulse applied to a peripheral nerve.^{48–51} As well as increasing reflex activation by a purely peripheral effect on the monosynaptic reflex, tetanus toxin also appeared to decrease inhibition of the H-wave resulting from activation of the descending pathway. The effect likely took place at a local level, because such a small amount of toxin injected intramuscularly would be expected to remain almost exclusively within the spinal segments that innervate the injected muscle rather than ascend the spinal cord.^{22,52}

The effect of inhibiting rate-dependent depression could contribute to the apparent reflex hyper-activity that occurs when a muscle exposed to tetanus toxin is stretched,²² but it leaves open the question of whether dysregulation of the stretch reflex contributes to the marked increase in muscle tone produced by the toxin even when the limb is at rest. Local toxin disinhibition of both an oscillatory descending motor drive and the stretch reflex could set up a reinforcing cycle of stretch-induced contractions between opposing muscles, eventually resulting in fused contractions in both flexors and extensors. Recordings from awake animals, comparing spontaneous EMG activity in tetanized muscles before and after unilateral rhizotomy would seem to be a direct way of approaching the question.

Implications for Clinical Rehabilitation

CNS traumatic injury or stroke can create not only spasticity, but also under-activation of skeletal muscle.⁵³ The reduced population of surviving cortical motor neurons after stroke and other forms of brain injury generates a reduced voluntary motor signal, resulting in minimal activation of the target spinal motor neurons and inadequate muscle contraction. Decreasing rate-dependent depression could decrease hyperpolarization of motor neurons. That situation might decrease the amount of descending excitatory activity necessary to activate a muscle and augment motor output under conditions where CNS traumatic injury or stroke has lessened descending excitatory inputs. These results suggest that the toxin can increase the amplitude of the H-wave in the TA and reduce the influence of local and descending inputs that generate rate-dependent depression of the H-wave in the FDB. Those observations support the hypothesis that tetanus toxin could be used to produce a state of overactivity in a selected population of motor neurons and support the need for clinical study of this biologic toxin with a unique mechanism of action.

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Abbreviations

CNS	central nervous system
EMG	electromyogram
FDB	flexor digitorum brevis
GABA	γ -aminobutyric acid
TA	tibialis anterior
TMS	transcranial magnetic stimulation
VAMP	vesicle associated membrane protein

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FIGURE 1.

M- and H-wave recruitment curves from the flexor digitorum brevis (FDB) muscle. **(A)** Representative data from 1 animal. Dashed lines represent M-waves, solid lines H-waves. Empty circles are from the saline-injected side, filled circles from the toxin-injected side. In the lower panel of **(A)**, least squares regression lines of points from the upper panel were used to determine slope. **(B)** Effect of tetanus toxin on the mean slopes of the M- or H-waves. Data are from the 11 animals in Table 1.

FIGURE 2.

Changes in H-reflex rate-dependent depression in the flexor digitorum brevis (FDB) muscle. (A) M- and H-waves at low- (0.2 Hz) and high-frequency (20 Hz) stimulation, 1 week after injection of saline (left traces) or tetanus toxin (right traces). Shown are the first, second, and averaged 6th–10th waves from a volley of 10 pulses. Data are from a representative animal. (B) Amplitude data from the second H-wave in a volley of 10 pulses. Empty circles are from the saline-injected side, filled circles from the toxin-injected side. (C) Data averaged from the 6th–10th waves in the same volley. Calibration bars are 1 mV and 2 ms. $*P < 0.01$

FIGURE 3.

Effect of upper central nervous system stimulation on flexor digitorum brevis (FDB) H-wave amplitude. **(A)** Magnetic stimulation on the skull followed with long- or short-delay by peripheral nerve stimulation. **(B)** Effect on H-wave amplitude in FDBs from the sides treated with tetanus toxin (filled circles) compared with those from the saline-treated sides (empty circles). Calibration bars are 1 mV and 25 ms. * $P < 0.05$

Table 1

Characteristics of M- and H-waves at 0.1 Hz, 1 week after intramuscular injection.

Parameter	N	Threshold, V	Amplitude, max. mV	Latency, ms	Duration, ms
M-wave					
TA saline	11	4.7 ± 1.8	11.7 ± 3.1	1.2 ± 0.4	4.9 ± 1.5
TA tetanus	11	4.8 ± 2.5	9.3 ± 2.1	1.4 ± 0.2	5.0 ± 1.2
FDB saline	11	6.8 ± 2.7	6.7 ± 1.9	1.6 ± 0.4	4.6 ± 1.0
FDB tetanus	11	7.6 ± 4.8	6.1 ± 1.5	1.7 ± 0.3	4.2 ± 0.8
H-wave					
TA saline	6	10.0 ± 2.0	0.5 ± 0.2	6.3 ± 1.7	2.2 ± 0.6
TA tetanus	11	4.2 ± 2.5*	3.1 ± 0.4*	5.9 ± 0.8	4.6 ± 1.2*
FDB saline	11	7.2 ± 2.7	2.4 ± 0.2	7.0 ± 0.6	3.0 ± 0.9
FDB tetanus	11	8.4 ± 3.3	2.7 ± 0.4	6.8 ± 0.7	3.3 ± 1.2

Values are mean ± SD. Threshold was the stimulus voltage when the wave amplitude reached twice background. Latency and duration were determined at the shown maximum amplitude. Latencies for the H-wave are minus the terminal motor delay.

* $P < 0.01$ vs. saline control.