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RESEARCH ARTICLE

# Long-Term Effects of Botulinum Toxin Complex Type A Injection on Mechano- and Metabo-Sensitive Afferent Fibers Originating from *Gastrocnemius* Muscle

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## Abstract

The aim of the present study was to investigate long term effects of motor denervation by botulinum toxin complex type A (BoNT/A) from *Clostridium Botulinum*, on the afferent fibers originating from the *gastrocnemius* muscle of rats. Animals were divided in 2 experimental groups: 1) untreated animals acting as control and 2) treated animals in which the toxin was injected in the left muscle, the latter being itself divided into 3 subgroups according to their locomotor recovery with the help of a test based on footprint measurements of walking rats: i) no recovery (B0), ii) 50% recovery (B50) and iii) full recovery (B100). Then, muscle properties, metabosensitive afferent fiber responses to potassium chloride (KCl) and lactic acid injections and Electrically-Induced Fatigue (EIF), and mechanosensitive responses to tendon vibrations were measured. At the end of the experiment, rats were killed and the toxin injected muscles were weighted. After toxin injection, we observed a complete paralysis associated to a loss of force to muscle stimulation and a significant muscle atrophy, and a return to baseline when the animals recover. The response to fatigue was only decreased in the B0 group. The responses to KCl injections were only altered in the B100 groups while responses to lactic acid were altered in the 3 injected groups. Finally, our results indicated that neurotoxin altered the biphasic pattern of response of the mechanosensitive fiber to tendon vibrations in the B0 and B50 groups. These results indicated that neurotoxin injection induces muscle afferent activity alterations that persist and even worsen when the muscle has recovered his motor activity.

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## Introduction

*Botulinum* toxin complex type A (BoNT/A) is currently used to treat numerous medical conditions such as dystonia, neuromuscular disorders or pain. Its effects start between 2 and 5 days

after injections and are maintained between 3 and 6 months [1]. BoNT/A exerts its action by preventing the exocytosis of acetylcholine vesicles at the neuromuscular junction eliciting flaccid paralysis [2,3]. Furthermore, BoNT/A induces central alterations [4–6] such as inhibition of glutamate [7], substance P [8,9], calcitonin gene related peptide (CGRP) [9,10] and to a lesser extent gamma-aminobutyric acid (GABA) release [11]. Those central changes could be due to indirect action of the toxin (decrease of the postsynaptic element activation following the decrease in presynaptic element activation) or to the toxin retrograde transport to the spinal cord (SC) and transcytosis [12–14].

BoNT/A also prevents acetylcholine releases by  $\gamma$ -motor endings in intrafusal muscle fibers [15,16]. The lack of  $\gamma$ -motor endings discharge in the injected muscle induce an intrafusal muscle fiber relaxation and then a decrease of afferent (Ia and II) inputs originating from spindles. At the spinal level, these changes reduce the direct excitation of agonist motoneurons and the indirect inhibition of antagonist motoneurons leading to a larger relaxation of agonist muscle (BoNT/A treated) [17]. Thus, the BoNT/A alters the central adjustments by mechanosensitive (Ia and II from muscle spindle and Ib from Golgi tendon organ) [18] and metabosensitive (III and IV) muscle afferent fibers [19]. Ia afferents detect muscle length and velocity while II afferents are mainly sensitive to instantaneous changes in muscle length [18,20]. Ib afferents are sensitive to forces variations [21–23]. Muscle afferent fibers from groups III and IV detect change in muscle metabolism [24,25] and in intramuscular pressure [26]. They are selectively stimulated during and after muscle fatigue [27] or by different agents such as bradykinin, capsaicin [28], lactic acid,  $H^+$  [25,29], arachidonic acid, prostaglandin [25], thromboxane A2 [30] and potassium chloride [31,32].

In a recent paper, we show that BoNT/A induces alterations in mechano- and metabosensitive afferent fibers when the muscle is to its paralysis apogee [19]. However, data are missing when the toxin is degraded and during muscle recovery.

The main purpose of the present study was to measure, over recovery time, the effects of *gastrocnemius* BoNT/A injection on afferent fibers involved in the sensorimotor loop. The muscle afferent discharges from groups III and IV were recorded after direct electrical muscle stimulation inducing fatigue and intra-arterial injections of potassium chloride or lactic acid while the mechanosensitive afferents discharges were recorded after tendon vibrations. The muscle properties (muscle weight, tetanus threshold, twitch amplitude and Fatigue Index) were also measured.

## Materials and Methods

### 1. Animals

Twenty seven adult male Sprague Dawley rats, weighting 300–400g (Centre d'Élevage Roger Janvier<sup>®</sup>, Le Genest Saint Isle, France), were housed in smooth-bottomed plastic cages at 22°C with a 12-h light/dark cycle. Food (Safe<sup>®</sup>, Augy, France) and water were available *ad libitum*. An acclimation period of 1 week was allowed before the initiation of the experiment. Animals were randomized in 2 experimental groups: 1) untreated animals acting as control (Control, n = 6) and 2) treated animals in which the toxin was injected in the left muscle, the latter being itself divided into 3 subgroups according to their locomotor recovery with the help of a test based on footprint measurements of walking rats: i) no recovery (B0, n = 9, twelve days post-injection), ii) 50% recovery (B50, n = 7) and iii) full recovery (B100, n = 5).

### 2. Ethical approval

Anesthesia and surgery were performed according to the French law on animal care guidelines. The Animal Care Committees of *Aix-Marseille Université* (AMU) and *Centre National de la*

*Recherche Scientifique* (CNRS) approved our protocols. Individual conducting researches were listed in the authorized personnel section of the animal research protocol or added to a previously approved protocol (license n°A 13.013.06). Furthermore, experiments were performed following the recommendations provided in the *Guide for Care and Use of Laboratory Animals* (U.S. Department of Health and Human Services, National Institutes of Health) and in accordance with the European Community's council directive of 24 November 1986 (86/609/EEC).

Animals did not present clinical sign of pain or unpleasant sensation (i.e. screech, prostration, hyperactivity, anorexia) and no paw-eating behavior were observed through the study.

### 3. Toxin injection procedure

Animals from B0, B50 and B100 groups were anesthetized by an intra-muscular injection of solution containing a ternary mixture [5 ml of ketamine, (100 mg/kg, Virbac<sup>®</sup>, Carros, France); 2.5 ml of largactyl (1.2 mg/kg, Avensis<sup>®</sup>, Paris France); 2 ml of domitor (20 mg/kg, Novartis<sup>®</sup>, Mississauga, Canada); 0.1 ml/100 g of body weight, IM]. Lyophilized botulinum toxin complex type A (Dysport 500<sup>®</sup>, Beaufour Ipsen Pharma, Boulogne-Billancourt, France) was extemporary reconstituted in normal saline solution to obtain a 15 U.ml<sup>-1</sup> solution. BoNT/A solution was injected in the inferior and superior parts of the two left *gastrocnemius* muscle heads (i.e., 4 injections per rat = 2 injections in the lateral and 2 injections medial muscle heads). Each animal received a total of 15 U.kg<sup>-1</sup> in order to ensure a full neurotransmitter release blockage [33–35]. As previously described, effects of this toxin start between 2 and 5 days after injection and are maintained between 3 and 6 months [1]. Furthermore, it was also described that functional loss was still maximal at the twelfth day [19].

### 4. Functional assessment of hind limb loss

All the animals were familiarized to go through a walking track apparatus (150 cm long, 9 cm wide and 40 cm high) that was used to perform the functional tests during the pre-surgical week. Briefly, all the animals were conditioned to walk homogeneously into the recording apparatus three times per day and five days during the week before surgery. Heavy lighting was provided with two 500 W spots and a dark box was positioned at the chamber's end to promote walking. The Sciatic Functional Index (SFI) was calculated with the formula  $[-38.3(ePL-nPL)/nPL+109.5(eTS-nTS)/nTS+13.3(eIT-nIT)/nIT-8.8]$  adapted by Varejao et al., [36] from the Peroneal Functional Index [37–39] to evaluate the functional integrity of the sciatic nerve based on footprint measurements of walking rats. Footprint's length (PL, or longitudinal distance between the tip of the longest toe and the heel), total toes spreading (TS, or cross-sectional distance between the first and fifth toes) and intermediate toe spread (IT, distance between the second and fourth toes) are the main factors altered by blocking release of acetylcholine at the neuromuscular junction due to motor loss of the toe flexor, foot plantarflexor and everters. In order to do so, the animal hind feet were dipped into Chinese ink (Swop-Pads<sup>®</sup>, Trodat, France) and the footprints were recorded on paper track and manually analysed. The normal/uninjected (n, right) footprints and the contralateral experimental/injected (e, left) footprints were compared. The loss rate of the SFI was defined on a score of -100 to 0, where 0 (perfect symmetry) to -20 represents a normal function and -100 a total failure. Footprints were obtained on a daily, weekly or monthly basis and analysed from the injection day (before injection) to the day when the wished functional recovery was obtained (no recovery, 50% recovery and full recovery).

## 5. Electrophysiological recordings

At the end of the functional assessment period, rats were anesthetized with urethane ( $1.1 \text{ g}\cdot\text{kg}^{-1}$  i.p.), and atropine ( $1 \text{ mg}\cdot\text{kg}^{-1}$ , i.p.) was administered to reduce airway secretions. Animals were tracheotomized and artificially ventilated (Harvard<sup>®</sup> volumetric pump: rate  $40\text{--}60 \text{ min}^{-1}$ , tidal volume 2–4 ml; Southmatick, MA USA). Animal temperature was maintained between  $36\text{--}37^\circ\text{C}$  with a blanket controlled by rectal temperature probe. A polyethylene catheter was inserted into the right femoral artery from the non-injected hindlimb and pushed up to the fork of the abdominal aorta in order to transport supplemental dose of anaesthetic and chemicals (i.e., potassium chloride [KCl] and lactic acid [LA]) to the controlateral muscle. This catheter was positioned in order to let the blood flow freely to the left lower limb muscles. Animals were positioned in ventral *decubitus*. Dissection at the middle thigh level was carried out to expose the right common sciatic nerve. With microsurgical techniques and an operating microscope (x40, MZ75<sup>®</sup>, Leica, Heerbrugg, Switzerland), a longitudinal incision was made along the lateral thigh and upper leg. Then, the tibial nerve, a branch of the sciatic nerve, was dissected free from surrounding tissues over a 20 mm length and immersed in paraffin oil to avoid dehydration. Two tungsten stimulating electrodes (inter-electrode distance: 4–5 mm) were placed to the surface of the *gastrocnemius* muscle. The nerve was cut and pair of cuff electrodes was placed on the proximal tibial nerve end for stimulation or recordings. The ankle and the knee were firmly held by clamps on a horizontal support in order to avoid disturbing movements and to maintain the  $90^\circ$  knee and ankle joint angle during electrical nerve stimulations.

**Twitch contraction and tetanus threshold measurements.** The contractile response of the *gastrocnemius* to nerve or muscle stimulation (twitch contraction, which is a reflection of the tension generated in the muscle) was obtained with a neurostimulator (Grass S88K<sup>®</sup>, Grass Technologies, Natus Neurology Inc., Warwick, Rhode Island, USA) delivering single rectangular pulses (duration: 0.1 ms, frequency 0.5 Hz) through an isolation unit and measured with an isometric strain gauge (Micromanometer 7001<sup>®</sup>, Ugo Basile SRL, Comerio VA, Italy) fixed to the distal part of the *gastrocnemius* tendon (calcaneum tendon). The intensity of the stimulation was increased until the maximal response was found. Twitch contraction was recorded with Biopac MP150<sup>®</sup> system (sampled at 2000 Hz, filtered with Low Pass at 150 Hz) and analyzed (AcqKnowledge<sup>®</sup> 3.7.3 software) in terms of peak amplitude ( $A$  in Newton, N).

Then, the tetanus threshold, defined as the frequency from which we observed a sustained contraction with no relaxation between twitches, was recorded. After determining a threshold able to elicit a twitch, pulse train intensity was set to a supramaximal level. Tetanic threshold was obtained by increasing frequency by 5 Hz steps. The voltage was 20% higher than the voltage evoking a maximal twitch. The duration of stimulus trains was 500 ms, and trains were repeated each second to produce a series of contractions. Pulse duration was 2 ms and five single stimulations were delivered in each 500 ms train (10 Hz).

**Electrically-induced fatigue.** Half an hour of rest after the last stimulations used to evoke twitch and tetanus contractions, a 3-min electrically-induced muscle fatigue (EIF) was performed. For this purpose, rhythmic muscle contractions were produced by the neurostimulator (Grass S88K<sup>®</sup>) delivering rectangular pulse trains to the pair of electrodes placed on the muscle surface (pulse duration: 0.1 ms; frequency: 10 Hz, i.e., 5 shocks in each 500 ms train; duty cycle: 500/1000 ms, voltage range: 5 to 8 volts). The voltage was supramaximal, i.e., +20% higher than that used to elicit a maximal twitch contraction. Muscle strength was recorded from the beginning to the end of muscle electrical stimulation with the isometric strain gauge (Micromanometer 7001<sup>®</sup>) and fatigue was assessed from the decay of force throughout the 3-min EIF period. Thus, the fatigue index (FI), defined conventionally as the percentage of the force lost at the end of the 3-min EIF trial, was calculated [40,41].

**Metabosensitive afferent recordings.** Furthermore, during repetitive muscle stimulation, the response of muscle afferent fibers from groups III and IV was also recorded (Biopac MP150<sup>®</sup> and AcqKnowledge<sup>®</sup> software). The neural signals were amplified (10 to 100 K) and filtered (filtered with Low Pass at 150 Hz) with a differential amplifier (P2MP<sup>®</sup> SARL, Marseille, France) and referred to a ground electrode implanted in a nearby muscle. Signals were fed into pulse window discriminators (P2MP<sup>®</sup> SARL, Marseille, France) which simultaneously analyzed afferent spikes. The output of these discriminators provided noise-free tracings (discriminated units) which were computed using data analysis system (Biopac AcqKnowledge<sup>®</sup> software). Before applying stimulus known to activate the afferent fibers from group III and IV, a baseline recording ( $F_{\text{impluses}} \cdot \text{s}^{-1}$ ) was achieved to ensure that the discharge rate remained stable. The recording was considered suitable only if the fluctuation of baseline impulse activity ranged between 100–103%. Consequently, changes in firing rate were related only to the stimuli applied and not to environmental conditions. The discharge rate was averaged over a 1-min period preceding EIF (baseline activity), and its change was measured during the first minute period following stimulation. Thus, changes were expressed in percentage of the corresponding baseline discharge rate; i.e. baseline discharges corresponded to 100%.

After a period of rest of one hour, KCl (0.5 ml; 1, 5, 10, and 20 mM) and LA (0.1 ml; 0.5, 1, 2, and 3 mM) were randomly injected into the contralateral artery while nerve discharge was continuously recorded. The injections, which required 5–10 s to be completed, were washed with 0.1 ml of normal saline. Baseline afferent activity was averaged on 1 min long period pre- and post-injection. The post-stimulus discharge firing rate was compared to the corresponding baseline discharge rate and variations were expressed in percentage of the corresponding baseline discharge firing rate. There was a 10 min delay between each injection in order to let the afferent activity go back to its baseline activity. Thus, like in a previous study [19,27] the dose/response curves to KCl and LA from femoral nerve were drawn.

**Mechanosensitive afferent recordings.** Muscle mechanosensitive fibers are activated by tendon vibration without activating group muscle metabosensitive afferents [42] in a range of 10–100 Hz [27,43], depending on the animal species and also on application through the skin or directly on the muscle tendon. Static spindle afferents (type II) and also Golgi tendons organs (type Ib, 50% are discharging with no volunteer contraction) are activated by low-frequency vibrations, whereas dynamic spindle afferents (type Ia) are activated by high-frequency vibrations [44]. Rectangular shocks were delivered perpendicularly to the longitudinal muscle axis on calcaneal tendon by a mechanical vibrator (Ling Dynamic System<sup>®</sup>, LDS group, Herfordshire, U.K) connected to a frequency generator (GenTrad Function Generator GF763AF, ELC<sup>®</sup>, Annecy, France). Vibrations were applied for 5-sec periods and frequency was increased step-by-step from 10 to 100 Hz, with 5-sec rest between each step, while the discharge of afferent units was recorded. The maximal mechanosensitive afferents discharge rate elicited by tendon vibrations was considered as the reference discharge rate (100%). The discharge rate induced by the others frequencies of vibrations were expressed in percentage of the corresponding reference discharge rate.

## 6. Muscle atrophy

At the end of the electrophysiological recordings, all rats were killed by a 3 ml intra-arterial overdose of pentobarbital sodium solution (Pentobarbital Sodique<sup>®</sup>, 0.6 g.kg<sup>-1</sup> Sanofi Santé Animal, Sanofi France, Paris France). Left *gastrocnemius* muscles were harvested and immediately weighted on a precision scale (Navigator<sup>™</sup> N30330 model, OHAUS Corp., Parsippany, NJ, USA). Muscle mass was measured using a muscle weight/body weight ratio and atrophy was evaluated by comparing to the Control group ratio.



## 7. Statistics

Data processing was performed using a software program (SigmaStat<sup>®</sup> 2.03, Statistical software, San Jose, CA, USA). Data were expressed as mean  $\pm$  Standard Error of the Mean (SEM).

For functional assessments, data concerning each animal were individually identified, in order to allow follow-up over time. Differences were tested by two-way repeated analysis of variance (ANOVA test, factors: group x timing) completed by a Student-Newman-Keuls *post-hoc* test to compare the SFI score, the effect of EIF and drug injections on metabosensitive afferent discharges, mechanosensitive afferent discharges to tendon vibrations and the muscle properties in the different groups. Finally, the intergroup comparison of the percentage of mechanosensitive fibers responding to mechanical vibration was examined with a Chi square test ( $\chi^2$ ).

Results were considered statistically significant if the p-value fell below 0.05.

## Results

### 1. Functional assessment of hindlimb locomotor property

In the B0 group, measurement of the SFI indicated that functional loss started the first day post-injection, reached a minimal value the third day and remained at this minimum until the twelfth day (when electrophysiological recordings were performed). As previously shown [19], this functional score was significantly lower ( $p < 0.001$ ) than those recorded before injection or in Control group. No difference was observed with the B50 and B100 groups. In the B50 and B100, a functional recovery was observed at the 60<sup>th</sup> day post-injection. In the B50 group, animals reached a mean score of  $-47.47 \pm 0.57$  after  $128.43 \pm 7.43$  days. In the B100 group, animals reached a mean score of  $-18.78 \pm 3.23$  after  $371.83 \pm 24.82$  days.

### 2. Muscle properties

Intergroup comparison indicated that there is a significant differences ( $p < 0.001$ ) in twitch amplitude, tetanus threshold, fatigue index (FI), muscle weight and muscle weight/body weight ratio.

In the B0 (twelve days after the injection), the electrical stimulation applied on tibial nerve did not elicit a muscle contraction (in a voltage range known to induce one). However, muscle stimulation induced a twitch with an amplitude significantly lower ( $p < 0.001$ ) than that of Control group. The tetanus threshold was also lower ( $p < 0.05$ ) than that of Control group. The FI obtained during the 3-min EIF (10 Hz) was not different than the Control group but significantly higher than the FI obtained in the B50 ( $p < 0.001$ ) and B100 ( $p < 0.01$ ) groups. Finally, the left muscle weight and the muscle weight/body weight ratio were significantly lower ( $p < 0.001$ ) than the Control group, indicating a muscle atrophy.

In the B50 group, muscle stimulation elicited a muscle contraction with a lower amplitude ( $p < 0.01$ ) than that of the Control group. The FI was significantly lower than that of the Control ( $p < 0.01$ ) and B0 ( $p < 0.001$ ) groups. Finally, the left muscle weight and the muscle weight/body weight ratio were significantly lower than that of the Control ( $p < 0.001$ ). The ratio was also significantly lower ( $p < 0.01$ ) than that of the B0 group.

In the B100 group, muscle stimulation elicited a twitch similar to Control group and significantly higher ( $p < 0.01$ ) than that of the B0 and B50 groups. The tetanus threshold was only different than that of the B0 ( $p < 0.05$ ) group. The FI was significantly lower ( $p < 0.05$ ) than that of the Control and B0 groups. Finally, the left muscle weight was significantly higher ( $p < 0.001$ ) than that of the B0 and B50 groups. However, the muscle weight/body weight ratio was always

significantly lower ( $p < 0.001$ ) than that of the Control group but became higher ( $p < 0.001$ ) than that of the B50 group indicating a slight muscle mass recovery.

Numerical data concerning these parameters are presented in [Table 1](#).

### 3. Metabosensitive afferent responses

Muscle afferent fibers identified as metabosensitive fibers have spontaneous tonic low frequency baseline activity (4–10 Hz) under our experimental conditions. After potassium chloride (KCl) or lactic acid (LA) injections, or electrically-induced fatigue (EIF) an increase of the baseline tonic activity was observed.

**Responses to electrically-induced fatigue (EIF) (S1 File).** The metabolites release by the repetitive stimulation in the muscle interstitium activated muscle free afferent endings. Thus, animals from all groups exhibited a significant ( $p < 0.001$ ) increase in afferent discharge frequency after a 3-min stimulation of the *gastrocnemius* muscle. The activation of muscle afferents was immediate when the stimulation stopped, and persisted during 3 min with a maximal response in the 2 first minutes. Intergroup comparison indicated that there is a significant ( $p < 0.001$ ) difference between groups. Data indicate that the mean discharge rate was significantly ( $p < 0.001$ ) lower in B0 group compared to the 3 other groups ([Fig 1](#)).

**Responses to chemical injections (S2 File).** The pattern of responses of metabosensitive afferent fibers from groups III and IV to chemical stimuli consisted of a burst of discharge beginning within 5–10 s after the bolus injection and a return to baseline values within the 3 next minutes. In a previous experiment performed in the *Tibialis anterior* muscle of Sprague-Dawley rats, we showed there was a relationship between the doses of KCl and the change in afferent discharge rate, whereas the activation of muscle afferents by LA culminated for the 1 mM concentration, and then declined [[27](#)]. Here, we showed that the *gastrocnemius* afferent fibers patterns of response were similar when KCl ([Fig 2A](#)) and LA ([Fig 2B](#)) were injected in the blood vessel irrigating the muscle. Significant ( $p < 0.01$  and  $p < 0.001$ ) increases in afferent discharge frequency, as compared to baseline recording, were observed in the Control and BoNT/A injected animals from B0 and B50 groups at all concentrations of KCl or LA solution tested.

Intergroup comparison indicated that there is a significant difference ( $p < 0.05$ ) in the KCl curve dose response between groups. In the B100 group, KCl injections did not induce a significant discharge rate increase compared to baseline. Compared to the respective concentrations of the other groups, the responses were significantly ( $p < 0.05$ ) lower for 1 mM, 5 mM, 10 mM and 20 mM.

Intergroup comparison indicated that there is a significant difference ( $p < 0.05$ ) in the LA curve dose response between groups. At the dose of 1 mM, the afferent response was significantly lower ( $p < 0.05$ ) in the B0 and B50 groups compared to Control group. Furthermore, the B100 group exhibited a lowered response for the 1 mM compared to the Control ( $p < 0.001$ ), B0 ( $p < 0.01$ ) and B50 ( $p < 0.05$ ) groups.

### 4. Mechanosensitive afferent responses

*Calcaneal* tendon vibrations resulted in an abrupt increase in the mechanosensitive afferent discharge frequency, which persisted throughout the tendon vibration period. In Control group, the rate of firing units was non-linearly related to the frequency of vibration, i.e., changes in afferent discharge evoked by vibration were bimodal with peaks measured at 40 and 80 Hz ([S3 File](#)). Thus, as previously described for the *tibialis anterior* muscle [[27](#)], two populations of muscle afferents were identified in our Control group with respect to the frequency of vibration giving an optimal activation; 66% and 34% of the units responding below and over 50



Table 1. Gastrocnemius muscle properties.

		Control group	BoNT/A injected groups			Comparison
			B0 group	B50 group	B100 group	
<b>Muscle Stimulation</b>	Twitch Amplitude (mN)	222.34±8.94	92.17±16.01	125.20±27.08	203.80±5.97	p<0.001
			***	**	## \$ \$	
<b>Tetanus Contraction</b> (5 Hz stepwise increase frequency)	Tetanus Threshold (Hz)	33.75±2.26	24.95±2.29	29.29±1.70	36.25±1.25	p<0.001
			*		#	
<b>Electrically Induced Fatigue (10 Hz)</b>	Fatigue Index (Δ%)	75.92±5.47	80.07±7.37	43.07±1.87	38.64±7.78	p<0.001
				** ###	** ##	
<b>Muscle Properties</b>	Muscle Weight (g)	2.25±0.04	1.07±0.04	1.07±0.07	2.09±0.17	p<0.001
			***	***	### \$ \$ \$	
		Muscle Weight/ Body Weight ratio	0.48±0.01	0.25±0.02	0.16±0.01	
			***	***	***	
				##	\$ \$ \$	

Comparison were performed versus Control (\*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001), B0 (##, p<0.01; ###, p<0.001) or B50 (\$, p<0.05; \$ \$ \$, p<0.001) group.

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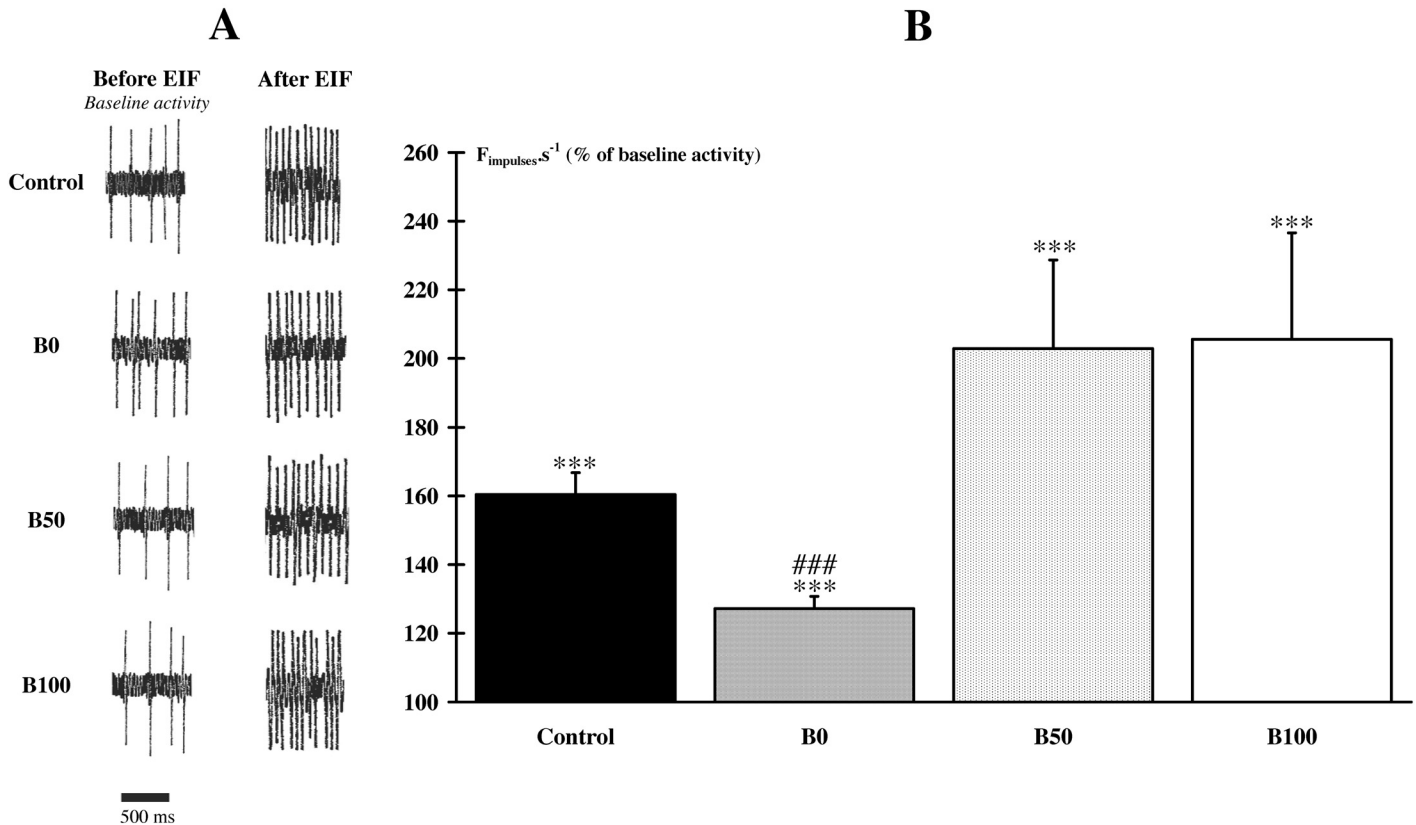
Hz, respectively. In the B0 group, these proportions were 22% and 78% while in B50 and B100 groups they were equally distributed below and above 50 Hz. When we consider the response to vibration frequencies only lower than 50 Hz, the maximal response was observed at 40 Hz (Control, B50 and B100 groups) and 50 Hz (B0 group). This maximal response was at 70 Hz (B0 and B50 groups) and 80 Hz (Control and B100 groups) for units responding maximally at frequencies higher than 50 Hz (Fig 3).

## Discussion

Previously, we demonstrated that responses of mechano- and metabosensitive afferent fibers were rapidly altered after full motor denervation, occurring within 12 days after a single injection of botulinum toxin complex type A from *Clostridium Botulinum* [19]. The experiment described in the present paper was a continuation of this previous work, and was designed to study if alterations of the mechano- and metabosensitive afferent fibers persist after toxin recovery, when locomotor function was partially and fully restored with a follow-up over many months.

Recording of afferent activities at rest and after use of specific activators indicate that neurotoxin induce long lasting changes in mechano- and metabosensitive responses despite paralysis recovery. Indeed, after half recovery (B50 group), the mechanosensitive response to tendon vibrations was still altered and the metabosensitive response was not recovered for KCl and LA injections but was exaggerated for EIF. Furthermore, in the B100 group, in which we observed a full locomotor and muscle mass recovery, the metabosensitive responses to KCl and LA were even more degraded than those of the B0 and B50 groups, and the response to EIF was still excessive. Thus, our results point out that efferent recovery is not always associated with afferent recovery.

## Electrically Induced-Fatigue (EIF)

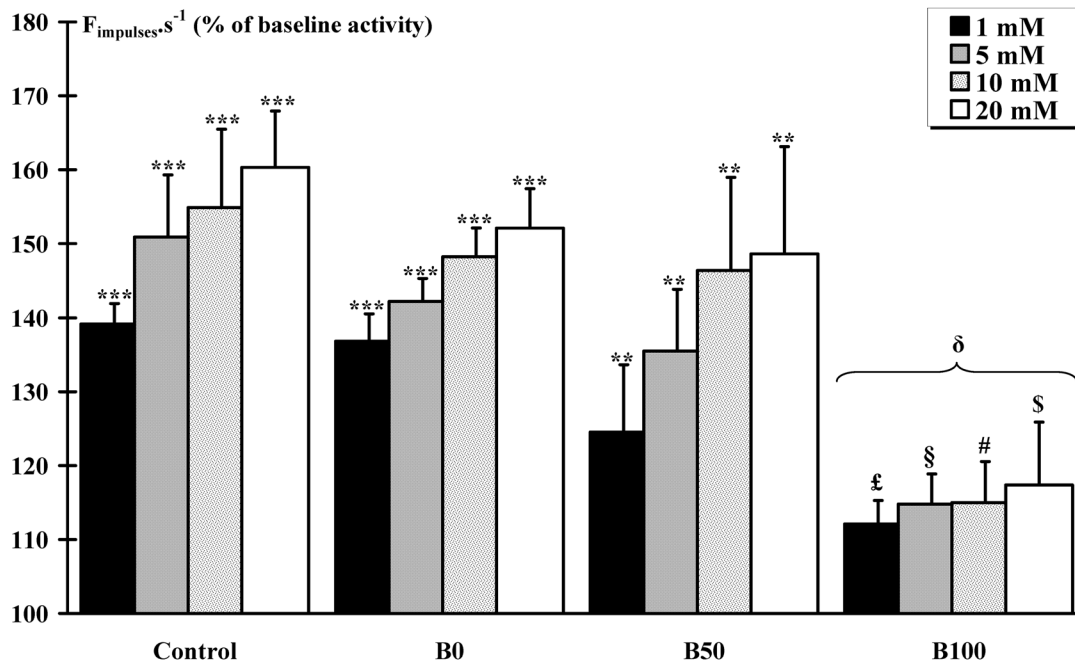


**Fig 1. Response of the metabosensitive fibers to electrically induced fatigue (EIF).** Animals from all groups exhibited a significant (\*\*\*,  $p < 0.001$ ) increase in afferent discharge frequency after a 3-min stimulation of the *gastrocnemius* muscle. **A.** Examples of recordings before (baseline activity) and after EIF. **B.** Comparison between the post-EIF changes indicate that the mean discharge rate was significantly (###,  $p < 0.001$ ) lower in the B0 group compared to the 3 other groups.

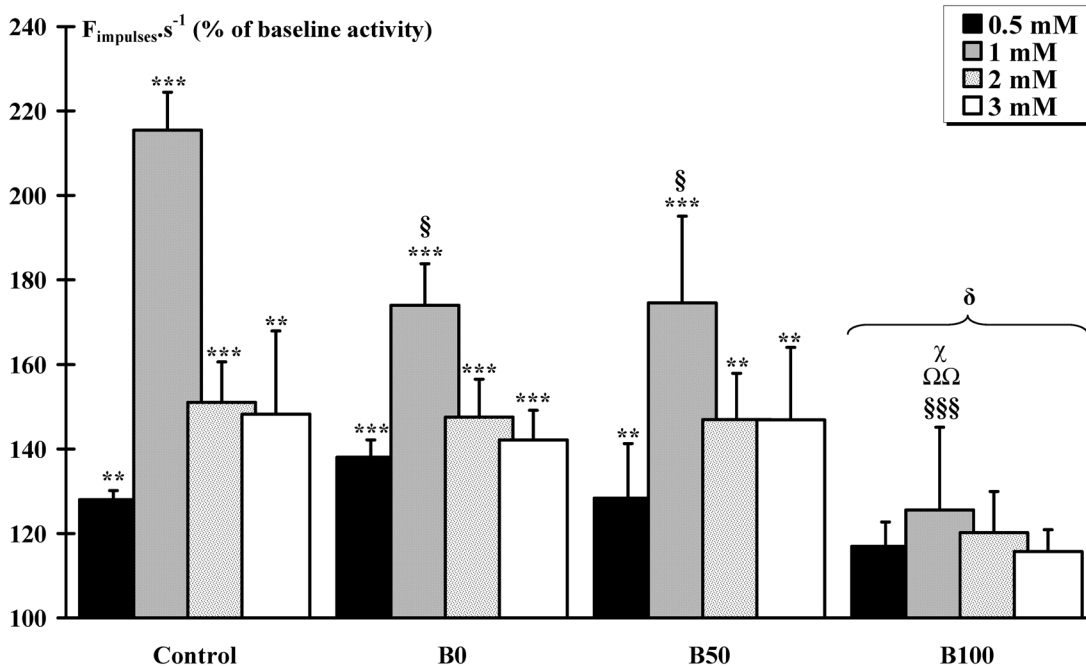
doi:10.1371/journal.pone.0140439.g001

In our study, the half and full recovery occurred at  $128.43 \pm 7.43$  and  $371.83 \pm 24.82$  days after a  $15 \text{ U.kg}^{-1}$  BoNT/A injection, respectively. This recovery time is consistent with data reported in the literature. Indeed, Sloop et al. studied the human muscle paralysis resulting from intramuscular injections of BoNT/A by measuring the *extensor digitorum brevis* M-wave amplitude after injection with 17 different doses of toxin (from 1.25 to 480 units) in healthy volunteers [45]. Two weeks post-injection, the maximal paralysis was 70 to 80% with 7.5 to 10U and at 57 weeks post-injection 22% of the original muscle paralysis was still present. In a rat model, Ma et al. reported that muscle mass, motor evoked potential and muscle force were significantly reduced during 1–2 weeks after injection of BoNT/A into the *gastrocnemius* muscle at the dose of 6 units/kg body weight but returned to nearly normal at 6 months post-injection and that the neuromuscular junction morphometry normalized at 1 year [46]. Finally, Billante et al. reported that the percentage of neuromuscular transmission was around 35% at the 200<sup>th</sup> day after injection of a dose of 10U in the *gastrocnemius* muscle of rat [35]. Duration of the toxin action is mainly determined by the life-time of the toxin's light chain in the cytosol. In the present experiment, the presence of the toxin in the cytosol of terminals innervating the *gastrocnemius* muscle appears to be at least 1 year.

### A - Potassium Chloride (KCl) Injections



### B - Lactic Acid (LA) Injections



**Fig 2. Response of the metabosensitive fibers to chemicals.** Responses ( $F_{\text{impulses}\cdot\text{s}^{-1}}$ ) of tonically active muscle afferents during stepwise increase in potassium chloride (A) and lactic acid (B) concentration in injected solutions were recorded. Significant (\*\*,  $p < 0.01$  and \*\*\*,  $p < 0.001$ ) increases in afferent discharge frequency, as compared to baseline recording, were observed in the Control and BoNT/A injected animals from B0 and B50 groups at all concentrations of KCl or LA solution tested. **A.** Intergroup comparison indicated that there is a significant difference ( $\delta$ ,  $p < 0.05$ ) in the KCl curve dose response for B100 group compared to other groups. Responses to KCl were significantly decreased in the B100 group for all concentrations. Compared to the respective concentrations of the other groups, the responses were significantly lower for 1 mM ( $\text{£}$ ,  $p < 0.05$ ), 5 mM ( $\text{§}$ ,  $p < 0.05$ ), 10 mM ( $\text{\#}$ ,  $p < 0.05$ ) and 20

mM (\$,  $p < 0.05$ ). **B.** Intergroup comparison indicated that there is a significant difference ( $\delta$ ,  $p < 0.05$ ) in the LA curve dose response for B100 group compared to other groups. Responses to LA were significantly decreased for the 1 mM concentration in all BoNT/A groups (B0: \$,  $p < 0.05$ ; B50: \$,  $p < 0.05$  and B100: \$\$\$,  $p < 0.001$ ) compared to the respective concentration of the Control group. Moreover, at the concentration of 1 mM, the B100 group also exhibited significant differences with B0 ( $\Omega\Omega$ ,  $p < 0.01$ ) and B50 ( $\chi$ ,  $p < 0.05$ ) groups.

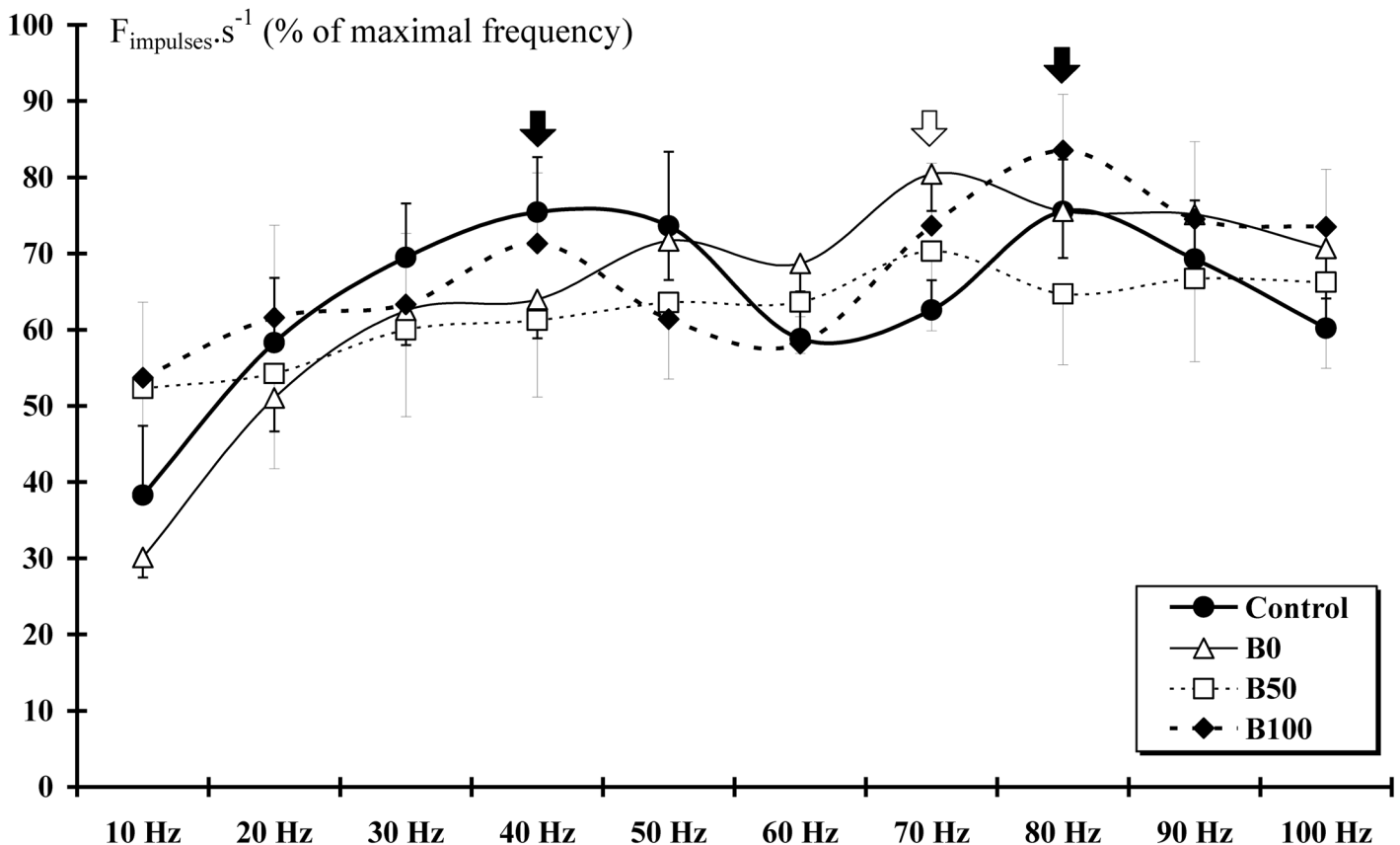
doi:10.1371/journal.pone.0140439.g002

### 1. Muscle properties

It was previously reported that toxin recovery depend of different factors such as the rate of toxin degradation within poisoned nerve terminals, the rate of repair of the poisoned nerve terminals, the rate of new (non-poisoned) nerve terminal growth, recruitment of resting nerve terminals that escaped poisoning and a return of normal muscle mass [47]. In the B100 group, only the muscle weight/animal weight ratio remain lower than the Control group indicating that muscle mass recovery was not linearly related to the animal weight gain over time, i.e., the animal weight increasing faster than the injected muscle.

The muscle fibers of the *gastrocnemius* are mostly type II fast-twitch fibers that have an anaerobic metabolism used to create short bursts of strength and are prone to rapid fatigue [48,49]. The fall of the FI values over time should indicated a phenotype switch toward a less

## Calcaneal Tendon Vibrations



**Fig 3. Response of the mechanosensitive fibers to calcaneal tendon vibrations.** In all groups, a response ( $F_{\text{impulses}\cdot\text{s}^{-1}}$ ) persisting throughout the tendon vibration period was recorded for each vibration frequency. In Control and B100 groups, the changes in afferent discharge evoked by vibration are bimodal with peaks measured at 40 and 80 Hz (black arrows). In the B0 and B50 groups, only one peak is measured at 70 Hz (white arrow).

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fast phenotype. However, the tetanus threshold and strength decreased for B50 and returned to value similar to Control when the recovery was full. These results are in accordance with previous studies showing a switch from IIB to IIA/x muscle fibers (i.e. a slight slowing of muscle contraction) associated with muscle atrophy and a reduction of force output after BoNT/A injection [50,51]. Because a higher oxidative activity for IIA/x compared to I muscle fibers was previously shown in the rat [52], this switch induces both a greater strength and fatigue resistance. BoNT/A injection impaired acetylcholine release at the neuromuscular junction leading to partial or full muscle paralysis that induces changes in contractile material and decrease in muscle cell cross sectional area. As previously suggested, changes in muscle phenotype and decrease in motor inputs (and motor units recruitment) lead to reduce muscle strength [53].

All explanations based on aging process should be excluded because the literature indicates that muscles reach their maturity by 12 months of age then decline after 18 months of age in rat [54–56].

## 2. Evoked metabosensitive activities

Group III myelinated and group IV unmyelinated afferents act primarily as mechano- and metabosensory nerve endings respectively. However, some group III fibers respond to metabolic stimuli and some group IV fibers respond to mechanical stimuli [57]. As previously demonstrated, potassium is a specific activator of metabosensitive afferent fibers and not a non-specific stimulus acting by depolarizing all fibers [27,58,59]. Furthermore, arterial KCl injections increase the potassium concentration in the muscular interstitium to levels similar to those evoked by static contractions known to activate metabosensitive afferents in rats [60], rabbits [61], cats [62] and dogs [31,63]. Lactic acid (LA) is also known to activate metabosensitive afferents via acid-sensing (proton-gated) ion channel 3 (ASIC3) and/or transient receptor potential vanilloide 1 (TRPV1) receptors [64]. Previous works [27,31] also reported that the discharge rate of metabosensitive fibers in response to increased [KCl] was concentration-dependent or culminated at 1 mM for LA. In Control and in BoNT/A injected groups, we confirmed not only this observation but also that the response of metabosensitive afferent fibers to KCl and LA injections is not (KCl) or slightly ([1 mM LA]) altered in the B0 and B50 groups. The altered response to LA injections could be explained by a reduced expression of the TRPV1 receptor into the afferent terminals which is not due to transcriptional downregulation but to the inhibition of the TRPV1 trafficking to the plasma membrane and proteasome-mediated degradation in the cytoplasm [65,66].

The unaltered responses to KCl injections in the B0 and B50 groups should be explained by the fact that ASIC3 and TRPV1 receptors are not involved in the activation of metabosensitive fibers by KCl. Unexpectedly, we observed altered responses in the B100 group with a full motor recovery after KCl and LA injections. Two explanations should be proposed. First, this mismatch between fall in metabosensitive activity and increase in motor activity indicate that there is no link between the recovery of the two pathways. Second, the switch in muscle typology, as suggested above, should be responsible of this alteration, i.e., the afferents innervating a IIA/x phenotype muscle being less sensitive to KCl and LA or the discharge rate of these afferents was already near maximal at rest with no possible further activation by KCl and LA. Furthermore, we observed that EIF still induced a response in the B50 and B100 groups contrary to the B0 group. In the B0 group, the slight response of the III and IV afferent fibers should be explained by either a strong muscular atrophy altering metabolic production and then a lesser afferent activation, either by the decrease of the neurogenic inflammation (the BoNT/A impairing the substance P and CGRP release) participating to the production of inflammatory products known to activate the afferent fibers [67–69]. In the B50 and B100 groups, we can suggest

that the altered metabosensitive response from group IV afferents was over compensated by group III afferents during repetitive contractions as suggested by Smith et al. [70,71] when metabosensitive afferent fibers from group IV were silent. We can conclude that the alteration observed in the B100 group is due to the group IV metabosensitive afferent fibers. This alteration could probably impact the exercise pressor reflex (EPR) adjusting the heart rate, ventilation and blood pressure during the performance of normal, daily tasks and ambulation and then indirectly the muscle mass. As previously described, skeletal muscles seem to be innervated by autonomic nervous system [72–74] and it was observed the presence of 1) adrenergic  $\alpha_1$ - and  $\alpha_2$ -receptor phenotypes expressed in higher proportion in muscles that are highly vascularized [75] or  $\alpha_1$ -receptor subtype in atrophied slow muscles of hypokalemic rats [76] and 2) adrenergic  $\beta_1$  and  $\beta_2$ -receptor phenotypes in slow and fast-twitch muscles with an abundance of the  $\beta_2$  subtype on slow muscles [72,77–81]. Adrenergic  $\alpha$ -receptor phenotypes seem to be implicated in vasoconstriction [82] while activation of adrenergic  $\beta$ -receptors induce muscle hypertrophy [83–86], vasodilatation [87] and are thought responsible for skeletal muscle apoptosis [88,89]. Furthermore, recently, it was shown that EPR mediated by III and IV afferent fibers [90] induces adrenergic activation [91]. In our experiment, because responses of III and IV afferents were changed after *gastrocnemius* BoNT/A injection, we cannot exclude an EPR down regulation in the B0 group and then a diminution of catecholamine release also contributing to muscle atrophy. In the B50 and B100 groups, the exaggerated response to EIF that could up-regulate the EPR recovery and then participate to the muscle mass recovery.

### 3. Evoked mechanosensitive activities

It is well known that muscle vibrations induce several effects on tonic and phasic reflexes [92]. Indeed, vibrations induce the muscle to slowly develop tension (tonic contraction) due to activation of anterior horn cells by the resulting afferent spindle discharge, which reaches  $\alpha$ -motoneurons *via* monosynaptic pathways [93,94]. The literature describes that muscle spindle discharges to vibrations, and its sensibility is increased by stimulation of both types of fusimotor fibers [43]. However, mechanical vibrations were unable to activate metabosensitive afferent fibers from groups III and IV [42]. In the present study, the B0 and B50 groups presented an altered response to vibrations. This alteration was observed for frequencies of 50 and 80 Hz known to induce the greatest response in the control animals [19]. In the B0 and B50 groups, the incomplete  $\alpha$ - and  $\gamma$ -motoneurons acetylcholine release induced by BoNT/A impairs the muscle spindle adjustments and then Ia and II afferents response. This decrease of influx transmission to the spinal level should, in turn, decrease the  $\alpha$ - and  $\gamma$ -motoneurons discharge and so on, and then increasing the muscle paralysis. This vicious cycle, which is the opposite of the vicious cycle inducing hyperexcitability should delay the recovery.

Several explanations can be advanced to explain the alterations observed in the response of mechanosensitive afferents. Indeed, the literature reported that motor denervation by botulinum toxin such as with nerve injury induces a decrease in M-wave amplitude [45] and a depolarization of the resting membrane potential in the denervated muscle [95–98]. This resting membrane depolarization is associated to a reduced quantal content of subthreshold end-plate potentials and an increase in muscle fatigue [99]. The recovery is associated by a slow recovery of the quantal release and decrease of acetylcholine receptor sensitivity. More recently, Paterson et al. hypothesized about the cellular mechanisms that could be responsible for the altered mechanotransduction after BoNT/A injection [100]. Indeed, in cultured rodent primary sensory neurons, they reported a decreased in the proportion of neurons expressing slowly adapting mechanically gated currents linked to mechanical pain transduction [101]. They suggested that TRPA1 channel is required for the generation of a slowly adapting current in a subset of



peptidergic dorsal root ganglion neurons [102] because its blockade reduces action potential firing in response to noxious peripheral mechanical stimulation [103] but TRPA1 channel is not sufficient alone for mechanotransduction [104]. They also suggested that there is probably no direct pharmacological action on mechanosensitive channels but an effect of BoNT-A on the trafficking of mechanosensitive channels, i.e., the tetanus toxin suppress the mechanically gated currents by blocking vesicle trafficking [105]. Vesicular trafficking plays also a role in the transmission of nerve influx at the terminals of the mechanosensitive afferent fibers [106]. Indeed, it is involved in the insertion of ion channels into the cell membrane and transport of proteins such as proteins of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex [107]. Thus, the alteration of the vesicular trafficking by the toxin could affect the genesis of nerve impulses at the nerve endings of mechanosensitive neurons.

Finally, we observed a complete recovery in the B100 group indicating normal muscle spindle adjustments and then Ia and II afferents response, and suggesting a recovery in vesicle trafficking and muscle membrane conduction. However, although we observed a complete recovery in the B100 group at  $371.83 \pm 24.82$  days after BoNT/A injection we can not claim that there was no trace of toxin in the nerve endings. Following administration, only a minority of the toxin enters nerve cells. The remaining portion remains in the extracellular compartment. When the toxin enters in the terminals, it undergoes an immediate metabolic transformation. During its translocation in the cytosol, the disulfide bridge linking the heavy and light chains is broken, releasing the light chain to express its catalytic activity in the cytosol [108,109]. This process marks the end of the intracellular existence of the intact and biologically molecule. Even if the light chain or heavy chain were to be exported from the cell, only the holotoxin possesses the ability to process through the multiple steps that culminate in blockade of transmission [110]. To date, although several mechanisms of metabolism and elimination of the toxin have been hypothesized, the literature does not reported a full understanding of these mechanisms in either the extracellular or intracellular compartments [110]. A pharmacokinetic study indicated that the half-life of the toxin is about 255 min in the blood of rats [111]. However some active toxin can be detected in serum 25 days after the onset of botulism in human [110]. It seems that the persistence of BoNT intoxication can be influenced both by the ability of the toxin protease or its cleaved SNARE protein substrate to resist turnover. Finally, Shoemaker and Oyler explained the several reasons why it is not practically possible to measure the remarkable persistence BoNT/A intoxication which results from retention of active BoNT/A protease within the terminals [112].

#### 4. Conclusion

In the present experiment, we showed that injection of BoNT/A in a *gastrocnemius* muscle induced an early decrease of the metabosensitive afferent response that is aggravated or exaggerated even when locomotor activity and muscle mass recovered contrary to the mechanosensitive afferent response that seemed to recover over time. This pattern of response should be due to a down regulation of afferents from group IV associated with an up regulation of afferents from group III, as suggested in other pathology [71]. This change of operating mode could interfere with the functioning of the sensorimotor loop and then adjustment of muscle contractions and physiological reflexes during motor activities leading to an early fatigue. As previously described, an EPR overactivity is associated to a chronic hypertension that induces a pathological hypertrophic cardiac remodeling leading to heart failure [113]. Furthermore, the toxin transports along motor (retrograde) and sensory (anterograde) axons [114,115] could induced a direct alteration of the glutamate, substance P or GABA transmission in the central neurons [11,116,117] that could contribute further to disturb the spinal networks involved in

sensorimotor activity and also to reduce pain sensation [118] and neurogenic inflammation [69].

Motor denervation with *botulinum* toxin in patients suffering of movement disorders or other pathologies, and in whom it was injected neurotoxin for cosmetic purposes, may still present proprioception and physiological alterations several months after the motor effects of the toxin have disappeared. Clinicians should keep in mind that the effects of *botulinum* toxin are sustainable and it induces alterations that are still present even after a complete motor recovery.

## Supporting Information

### S1 File.

(XLS)

### S2 File.

(XLS)

### S3 File.

(XLS)

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## Author Contributions

Conceived and designed the experiments: GC TM PD. Performed the experiments: GC TM PD. Analyzed the data: GC TM PD. Contributed reagents/materials/analysis tools: GC TM PD. Wrote the paper: GC TM PD.

## References

1. Dressler D, Rothwell JC (2000) Electromyographic quantification of the paralyzing effect of botulinum toxin in the sternocleidomastoid muscle. *Eur Neurol* 43: 13–16. PMID: [10798896](#)
2. Rizo J, Sudhof TC (2002) Snares and Munc18 in synaptic vesicle fusion. *Nat Rev Neurosci* 3: 641–653. PMID: [12154365](#)
3. Hallett M, Glocker FX, Deuschl G (1994) Mechanism of action of botulinum toxin. *Ann Neurol* 36: 449–450. PMID: [8080256](#)
4. Curra A, Trompetto C, Abbruzzese G, Berardelli A (2004) Central effects of botulinum toxin type A: evidence and supposition. *Mov Disord* 19 Suppl 8: S60–64. PMID: [15027056](#)
5. Abbruzzese G, Berardelli A (2006) Neurophysiological effects of botulinum toxin type A. *Neurotox Res* 9: 109–114. PMID: [16785106](#)
6. Caleo M, Schiavo G (2009) Central effects of tetanus and botulinum neurotoxins. *Toxicon* 54: 593–599. doi: [10.1016/j.toxicon.2008.12.026](#) PMID: [19264088](#)
7. Cui M, Khanijou S, Rubino J, Aoki KR (2004) Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. *Pain* 107: 125–133. PMID: [14715398](#)
8. Welch MJ, Purkiss JR, Foster KA (2000) Sensitivity of embryonic rat dorsal root ganglia neurons to *Clostridium botulinum* neurotoxins. *Toxicon* 38: 245–258. PMID: [10665805](#)
9. Lucioni A, Bales GT, Lotan TL, McGehee DS, Cook SP, et al. (2008) Botulinum toxin type A inhibits sensory neuropeptide release in rat bladder models of acute injury and chronic inflammation. *BJU Int* 101: 366–370. doi: [10.1111/j.1464-410X.2007.07312.x](#) PMID: [18184328](#)
10. Meng J, Wang J, Lawrence G, Dolly JO (2007) Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and inhibition by botulinum toxins reflects their anti-nociceptive potential. *J Cell Sci* 120: 2864–2874. PMID: [17666428](#)

11. Verderio C, Grumelli C, Raiteri L, Coco S, Paluzzi S, et al. (2007) Traffic of botulinum toxins A and E in excitatory and inhibitory neurons. *Traffic* 8: 142–153. PMID: [17241445](#)
12. Wiegand H, Erdmann G, Wellhoner HH (1976) 125I-labelled botulinum A neurotoxin: pharmacokinetics in cats after intramuscular injection. *Naunyn Schmiedebergs Arch Pharmacol* 292: 161–165. PMID: [59905](#)
13. Kim DY, Oh BM, Paik NJ (2006) Central effect of botulinum toxin type A in humans. *Int J Neurosci* 116: 667–680. PMID: [16753894](#)
14. Antonucci F, Rossi C, Gianfranceschi L, Rossetto O, Caleo M (2008) Long-distance retrograde effects of botulinum neurotoxin A. *J Neurosci* 28: 3689–3696. doi: [10.1523/JNEUROSCI.0375-08.2008](#) PMID: [18385327](#)
15. Filippi GM, Errico P, Santarelli R, Bagolini B, Manni E (1993) Botulinum A toxin effects on rat jaw muscle spindles. *Acta Otolaryngol* 113: 400–404. PMID: [8390772](#)
16. Rosales RL, Arimura K, Takenaga S, Osame M (1996) Extrafusal and intrafusal muscle effects in experimental botulinum toxin-A injection. *Muscle Nerve* 19: 488–496. PMID: [8622728](#)
17. Priori A, Berardelli A, Mercuri B, Manfredi M (1995) Physiological effects produced by botulinum toxin treatment of upper limb dystonia. Changes in reciprocal inhibition between forearm muscles. *Brain* 118 (Pt 3): 801–807. PMID: [7600096](#)
18. Rosales RL, Dressler D (2010) On muscle spindles, dystonia and botulinum toxin. *Eur J Neurol* 17 Suppl 1: 71–80. doi: [10.1111/j.1468-1331.2010.03056.x](#) PMID: [20590812](#)
19. Caron G, Rouzi T, Grelot L, Magalon G, Marqueste T, et al. (2014) Mechano- and metabosensitive alterations after injection of botulinum toxin into gastrocnemius muscle. *J Neurosci Res* 92: 904–914. doi: [10.1002/jnr.23370](#) PMID: [24615939](#)
20. Pierrot-Deseilligny E, Burke D (2005) *The circuit of the Human Spinal Cord: Its Role in Motor Control and Movement Disorders*. New York: Cambridge University Press.
21. Brown MC, Engberg I, Matthews PB (1967) The relative sensitivity to vibration of muscle receptors of the cat. *J Physiol* 192: 773–800. PMID: [4293790](#)
22. Brown AG, Iggo A (1967) A quantitative study of cutaneous receptors and afferent fibres in the cat and rabbit. *J Physiol* 193: 707–733. PMID: [16992307](#)
23. Brown AG, Iggo A, Miller S (1967) Myelinated afferent nerve fibers from the skin of the rabbit ear. *Exp Neurol* 18: 338–349. PMID: [6028148](#)
24. Kaufman MP, Rybicki KJ (1987) Discharge properties of group III and IV muscle afferents: their responses to mechanical and metabolic stimuli. *Circ Res* 61: 160–65. PMID: [3652404](#)
25. Rotto DM, Kaufman MP (1988) Effect of metabolic products of muscular contraction on discharge of group III and IV afferents. *J Appl Physiol* 64: 2306–2313. PMID: [3136123](#)
26. Ge W, Khalsa PS (2003) Encoding of compressive stress during indentation by group III and IV muscle mechano-nociceptors in rat gracilis muscle. *J Neurophysiol* 89: 785–792. PMID: [12574456](#)
27. Decherchi P, Darques JL, Jammes Y (1998) Modifications of afferent activities from Tibialis anterior muscle in rat by tendon vibrations, increase of interstitial potassium or lactate concentration and electrically-induced fatigue. *J Peripher Nerv Syst* 3: 267–276. PMID: [10970127](#)
28. Kaufman MP, Iwamoto GA, Longhurst JC, Mitchell JH (1982) Effects of capsaicin and bradykinin on afferent fibers with ending in skeletal muscle. *Circ Res* 50: 133–139. PMID: [7053873](#)
29. Victor RG, Bertocci LA, Pryor SL, Nunnally RL (1988) Sympathetic nerve discharge is coupled to muscle cell pH during exercise in humans. *J Clin Invest* 82: 1301–1305. PMID: [3170747](#)
30. Kenagy J, VanCleave J, Pazdernik L, Orr JA (1997) Stimulation of group III and IV afferent nerves from the hindlimb by thromboxane A2. *Brain Res* 744: 175–178. PMID: [9030430](#)
31. Rybicki KJ, Waldrop TG, Kaufman MP (1985) Increasing gracilis muscle interstitial potassium concentrations stimulate group III and IV afferents. *J Appl Physiol* 58: 936–941. PMID: [2984167](#)
32. Decherchi P, Vuillon-Cacciatolo G, Darques JL, Jammes Y (2001) Changes in afferent activities from tibialis anterior muscle after nerve repair by self-anastomosis. *Muscle Nerve* 24: 59–68. PMID: [11150967](#)
33. Holds JB, Fogg SG, Anderson RL (1990) Botulinum A toxin injection. Failures in clinical practice and a biomechanical system for the study of toxin-induced paralysis. *Ophthal Plast Reconstr Surg* 6: 252–259. PMID: [2271481](#)
34. Hott JS, Dalakas MC, Sung C, Hallett M, Youle RJ (1998) Skeletal muscle-specific immunotoxin for the treatment of focal muscle spasm. *Neurology* 50: 485–491. PMID: [9484377](#)
35. Billante CR, Zeale DL, Billante M, Reyes JH, Sant'Anna G, et al. (2002) Comparison of neuromuscular blockade and recovery with botulinum toxins A and F. *Muscle Nerve* 26: 395–403. PMID: [12210370](#)

36. Varejao AS, Cabrita AM, Patricio JA, Bulas-Cruz J, Gabriel RC, et al. (2001) Functional assessment of peripheral nerve recovery in the rat: gait kinematics. *Microsurgery* 21: 383–388. PMID: [11757066](#)
37. Bain JR, Mackinnon SE, Hunter DA (1989) Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. *Plast Reconstr Surg* 83: 129–138. PMID: [2909054](#)
38. de Medinaceli L, Freed WJ, Wyatt RJ (1982) An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. *Exp Neurol* 77: 634–643. PMID: [7117467](#)
39. Hruska RE, Kennedy S, Silbergeld EK (1979) Quantitative aspects of normal locomotion in rats. *Life Sci* 25: 171–179. PMID: [491843](#)
40. Kostyukov AI, Hellstrom F, Korchak OE, Radovanovic S, Ljubicavljivic M, et al. (2000) Fatigue effects in the cat gastrocnemius during frequency-modulated efferent stimulation. *Neuroscience* 97: 789–799. PMID: [10842025](#)
41. Lopez-Guajardo A, Sutherland H, Jarvis JC, Salmons S (2001) Induction of a fatigue-resistant phenotype in rabbit fast muscle by small daily amounts of stimulation. *J Appl Physiol* (1985) 90: 1909–1918.
42. Kaufman MP, Waldrop TG, Rybicki KJ, Ordway GA, Mitchell JH (1984) Effects of static and rhythmic twitch contractions on the discharge of group III and IV muscle afferents. *Cardiovasc Res* 18: 663–668. PMID: [6498873](#)
43. Burke D, Schiller HH (1976) Discharge pattern of single motor units in the tonic vibration reflex of human triceps surae. *J Neurol Neurosurg Psychiatry* 39: 729–741. PMID: [956859](#)
44. Roll JP, Vedel JP, Ribot E (1989) Alteration of proprioceptive messages induced by tendon vibration in man: a microneurographic study. *Exp Brain Res* 76: 213–222. PMID: [2753103](#)
45. Sloop RR, Cole BA, Escutin RO (1997) Human response to botulinum toxin injection: type B compared with type A. *Neurology* 49: 189–194. PMID: [9222189](#)
46. Ma J, Elsaidi GA, Smith TL, Walker FO, Tan KH, et al. (2004) Time course of recovery of juvenile skeletal muscle after botulinum toxin A injection: an animal model study. *Am J Phys Med Rehabil* 83: 774–780; quiz 781–773. PMID: [15385786](#)
47. Keller JE (2006) Recovery from botulinum neurotoxin poisoning in vivo. *Neuroscience* 139: 629–637. PMID: [16490322](#)
48. Duchen LW (1971) Changes in the electron microscopic structure of slow and fast skeletal muscle fibres of the mouse after the local injection of botulinum toxin. *J Neurol Sci* 14: 61–74. PMID: [5119452](#)
49. Duchen LW (1971) An electron microscopic study of the changes induced by botulinum toxin in the motor end-plates of slow and fast skeletal muscle fibres of the mouse. *J Neurol Sci* 14: 47–60. PMID: [5119451](#)
50. Dodd SL, Selsby J, Payne A, Judge A, Dott C (2005) Botulinum neurotoxin type A causes shifts in myosin heavy chain composition in muscle. *Toxicon* 46: 196–203. PMID: [15975617](#)
51. Legerlotz K, Matthews KG, McMahon CD, Smith HK (2009) Botulinum toxin-induced paralysis leads to slower myosin heavy chain isoform composition and reduced titin content in juvenile rat gastrocnemius muscle. *Muscle Nerve* 39: 472–479. doi: [10.1002/mus.21247](#) PMID: [19260067](#)
52. Delp MD, Duan C (1996) Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. *J Appl Physiol* (1985) 80: 261–270.
53. Duchateau J, Hainaut K (1990) Effects of immobilization on contractile properties, recruitment and firing rates of human motor units. *J Physiol* 422: 55–65. PMID: [2352193](#)
54. Yu BP, Masoro EJ, Murata I, Bertrand HA, Lynd FT (1982) Life span study of SPF Fischer 344 male rats fed ad libitum or restricted diets: longevity, growth, lean body mass and disease. *J Gerontol* 37: 130–141. PMID: [7056998](#)
55. Cartee GD (1994) Aging skeletal muscle: response to exercise. *Exerc Sport Sci Rev* 22: 91–120. PMID: [7925554](#)
56. Chiu CS, Weber H, Adamski S, Rauch A, Gentile MA, et al. (2011) Non-invasive muscle contraction assay to study rodent models of sarcopenia. *BMC Musculoskelet Disord* 12: 246. doi: [10.1186/1471-2474-12-246](#) PMID: [22035016](#)
57. Kaufman MP, Forster H (1996) Reflexes controlling circulatory, ventilatory and airway responses to exercise.; Rowell LB, Shepherd JT, editors. New York: Oxford University Press.
58. Marqueste T, Decherchi P, Dousset E, Berthelin F, Jammes Y (2002) Effect of muscle electrostimulation on afferent activities from tibialis anterior muscle after nerve repair by self-anastomosis. *Neuroscience* 113: 257–271. PMID: [12127084](#)
59. Dousset E, Marqueste T, Decherchi P, Jammes Y, Grelot L (2004) Effects of neonatal capsaicin deafferentation on neuromuscular adjustments, performance, and afferent activities from adult tibialis anterior muscle during exercise. *J Neurosci Res* 76: 734–741. PMID: [15139032](#)

60. Thimm F, Baum K (1987) Response of chemosensitive nerve fibers of group III and IV to metabolic changes in rat muscles. *Pflugers Arch* 410: 143–152. PMID: [3684503](#)
61. Darques JL, Jammes Y (1997) Fatigue-induced changes in group IV muscle afferent activity: differences between high- and low-frequency electrically induced fatigues. *Brain Res* 750: 147–154. PMID: [9098539](#)
62. Mense S, Prabhakar NR (1986) Spinal termination of nociceptive afferent fibres from deep tissues in the cat. *Neurosci Lett* 66: 169–174. PMID: [3725183](#)
63. Hnik P, Holas M, Krekule I, Kuriz N, Mejsnar J, et al. (1976) Work-induced potassium changes in skeletal muscle and effluent venous blood assessed by liquid ion-exchanger microelectrodes. *Pflugers Arch* 362: 85–94. PMID: [943782](#)
64. Gao Z, Henig O, Kehoe V, Sinoway LI, Li J (2006) Vanilloid type 1 receptor and the acid-sensing ion channel mediate acid phosphate activation of muscle afferent nerves in rats. *J Appl Physiol* (1985) 100: 421–426.
65. Apostolidis A, Popat R, Yiangou Y, Cockayne D, Ford AP, et al. (2005) Decreased sensory receptors P2X3 and TRPV1 in suburothelial nerve fibers following intradetrusor injections of botulinum toxin for human detrusor overactivity. *J Urol* 174: 977–982; discussion 982–973. PMID: [16094018](#)
66. Shimizu T, Shibata M, Toriumi H, Iwashita T, Funakubo M, et al. (2012) Reduction of TRPV1 expression in the trigeminal system by botulinum neurotoxin type-A. *Neurobiol Dis* 48: 367–378. doi: [10.1016/j.nbd.2012.07.010](#) PMID: [22820141](#)
67. Darques JL, Decherchi P, Jammes Y (1998) Mechanisms of fatigue-induced activation of group IV muscle afferents: the roles played by lactic acid and inflammatory mediators. *Neurosci Lett* 257: 109–112. PMID: [9865939](#)
68. Marqueste T, Decherchi P, Messan F, Kipson N, Grelot L, et al. (2004) Eccentric exercise alters muscle sensory motor control through the release of inflammatory mediators. *Brain Res* 1023: 222–230. PMID: [15374748](#)
69. Carmichael NM, Dostrovsky JO, Charlton MP (2010) Peptide-mediated transdermal delivery of botulinum neurotoxin type A reduces neurogenic inflammation in the skin. *Pain* 149: 316–324. doi: [10.1016/j.pain.2010.02.024](#) PMID: [20223589](#)
70. Smith SA, Mitchell JH, Garry MG (2006) The mammalian exercise pressor reflex in health and disease. *Exp Physiol* 91: 89–102. PMID: [16282366](#)
71. Smith SA, Mitchell JH, Naseem RH, Garry MG (2005) Mechanoreflex mediates the exaggerated exercise pressor reflex in heart failure. *Circulation* 112: 2293–2300. PMID: [16216976](#)
72. Lynch GS, Ryall JG (2008) Role of beta-adrenoceptor signaling in skeletal muscle: implications for muscle wasting and disease. *Physiol Rev* 88: 729–767. doi: [10.1152/physrev.00028.2007](#) PMID: [18391178](#)
73. Caswell AH, Baker SP, Boyd H, Potter LT, Garcia M (1978) beta-adrenergic receptor and adenylate cyclase in transverse tubules of skeletal muscle. *J Biol Chem* 253: 3049–3054. PMID: [205539](#)
74. Barker D, Saito M (1981) Autonomic innervation of receptors and muscle fibres in cat skeletal muscle. *Proc R Soc Lond B Biol Sci* 212: 317–332. PMID: [6115396](#)
75. Rattigan S, Appleby GJ, Edwards SJ, McKinstry WJ, Colquhoun EQ, et al. (1986) Alpha-adrenergic receptors in rat skeletal muscle. *Biochem Biophys Res Commun* 136: 1071–1077. PMID: [3013164](#)
76. Akaike N, Hirata A, Kiyohara T, Oyama Y (1983) Neural regulation on the active sodium-potassium transport in hypokalaemic rat skeletal muscles. *J Physiol* 341: 245–255. PMID: [6137559](#)
77. Kim YS, Sainz RD, Molenaar P, Summers RJ (1991) Characterization of beta 1- and beta 2-adrenoceptors in rat skeletal muscles. *Biochem Pharmacol* 42: 1783–1789. PMID: [1681810](#)
78. Williams RS, Caron MG, Daniel K (1984) Skeletal muscle beta-adrenergic receptors: variations due to fiber type and training. *Am J Physiol* 246: E160–167. PMID: [6320672](#)
79. Ryall JG, Gregorevic P, Plant DR, Sillence MN, Lynch GS (2002) Beta 2-agonist fenoterol has greater effects on contractile function of rat skeletal muscles than clenbuterol. *Am J Physiol Regul Integr Comp Physiol* 283: R1386–1394. PMID: [12388476](#)
80. Ryall JG, Plant DR, Gregorevic P, Sillence MN, Lynch GS (2004) Beta 2-agonist administration reverses muscle wasting and improves muscle function in aged rats. *J Physiol* 555: 175–188. PMID: [14617677](#)
81. Martin WH 3rd, Murphree SS, Saffitz JE (1989) Beta-adrenergic receptor distribution among muscle fiber types and resistance arterioles of white, red, and intermediate skeletal muscle. *Circ Res* 64: 1096–1105. PMID: [2541942](#)
82. Buckwalter JB, Clifford PS (2001) The paradox of sympathetic vasoconstriction in exercising skeletal muscle. *Exerc Sport Sci Rev* 29: 159–163. PMID: [11688788](#)



83. Apseloff G, Girten B, Walker M, Shepard DR, Krecic ME, et al. (1993) Aminohydroxybutane bisphosphonate and clenbuterol prevent bone changes and retard muscle atrophy respectively in tail-suspended rats. *J Pharmacol Exp Ther* 264: 1071–1078. PMID: [8450451](#)
84. Hinkle RT, Hodge KM, Cody DB, Sheldon RJ, Kobiika BK, et al. (2002) Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the beta2-adrenergic receptor. *Muscle Nerve* 25: 729–734. PMID: [11994968](#)
85. Mersmann HJ (1998) Overview of the effects of beta-adrenergic receptor agonists on animal growth including mechanisms of action. *J Anim Sci* 76: 160–172. PMID: [9464897](#)
86. Sato S, Shirato K, Tachiyashiki K, Imaizumi K (2011) Muscle plasticity and beta(2)-adrenergic receptors: adaptive responses of beta(2)-adrenergic receptor expression to muscle hypertrophy and atrophy. *J Biomed Biotechnol* 2011: 729598. doi: [10.1155/2011/729598](#) PMID: [22190857](#)
87. Berecek KH, Brody MJ (1982) Evidence for a neurotransmitter role for epinephrine derived from the adrenal medulla. *Am J Physiol* 242: H593–601. PMID: [6278965](#)
88. Burniston JG, Tan LB, Goldspink DF (2005) beta2-Adrenergic receptor stimulation in vivo induces apoptosis in the rat heart and soleus muscle. *J Appl Physiol* (1985) 98: 1379–1386.
89. Burniston JG, Clark WA, Tan LB, Goldspink DF (2006) Dose-dependent separation of the hypertrophic and myotoxic effects of the beta(2)-adrenergic receptor agonist clenbuterol in rat striated muscles. *Muscle Nerve* 33: 655–663. PMID: [16411205](#)
90. McCloskey DI, Mitchell JH (1972) Reflex cardiovascular and respiratory responses originating in exercising muscle. *J Physiol* 224: 173–186. PMID: [5039977](#)
91. Kaur J, Spranger MD, Hammond RL, Krishnan AC, Alvarez A, et al. (2014) Muscle metaboreflex activation during dynamic exercise evokes epinephrine release resulting in beta2-mediated vasodilation. *Am J Physiol Heart Circ Physiol*: ajpheart 00648 02014.
92. Fromm C, Haase J, Wolf E (1977) Depression of the recurrent inhibition of extensor motoneurons by the action of group II afferents. *Brain Res* 120: 459–468. PMID: [832135](#)
93. Hagbarth KE, Eklund G (1966) Tonic vibration reflexes (TVR) in spasticity. *Brain Res* 2: 201–203. PMID: [5968925](#)
94. De Gail P, Lance JW, Neilson PD (1966) Differential effects on tonic and phasic reflex mechanisms produced by vibration of muscles in man. *J Neurol Neurosurg Psychiatry* 29: 1–11. PMID: [5910574](#)
95. Albuquerque EX, Thesleff S (1968) A comparative study of membrane properties of innervated and chronically denervated fast and slow skeletal muscles of the rat. *Acta Physiol Scand* 73: 471–480. PMID: [5708174](#)
96. Albuquerque EX, Schuh FT, Kauffman FC (1971) Early membrane depolarization of the fast mammalian muscle after denervation. *Pflugers Arch* 328: 36–50. PMID: [4331161](#)
97. Lewis GE (1981) *Biomedical aspects of botulism*. London: Academic Press Inc.
98. Sellin LC, Thesleff S (1981) Pre- and post-synaptic actions of botulinum toxin at the rat neuromuscular junction. *J Physiol* 317: 487–495. PMID: [6273549](#)
99. Tonge DA (1974) Chronic effects of botulinum toxin on neuromuscular transmission and sensitivity to acetylcholine in slow and fast skeletal muscle of the mouse. *J Physiol* 241: 127–139. PMID: [4371301](#)
100. Paterson K, Lollignier S, Wood JN, McMahon SB, Bennett DL (2014) Botulinum toxin-A treatment reduces human mechanical pain sensitivity and mechanotransduction. *Ann Neurol* 75: 591–596. doi: [10.1002/ana.24122](#) PMID: [24550077](#)
101. Drew LJ, Rugiero F, Cesare P, Gale JE, Abrahamsen B, et al. (2007) High-threshold mechanosensitive ion channels blocked by a novel conopeptide mediate pressure-evoked pain. *PLoS One* 2: e515. PMID: [17565368](#)
102. Vilceanu D, Stucky CL (2010) TRPA1 mediates mechanical currents in the plasma membrane of mouse sensory neurons. *PLoS One* 5: e12177. doi: [10.1371/journal.pone.0012177](#) PMID: [20808441](#)
103. Kerstein PC, del Camino D, Moran MM, Stucky CL (2009) Pharmacological blockade of TRPA1 inhibits mechanical firing in nociceptors. *Mol Pain* 5: 19. doi: [10.1186/1744-8069-5-19](#) PMID: [19383149](#)
104. Rugiero F, Wood JN (2009) The mechanosensitive cell line ND-C does not express functional thermoTRP channels. *Neuropharmacology* 56: 1138–1146. doi: [10.1016/j.neuropharm.2009.03.012](#) PMID: [19348834](#)
105. Di Castro A, Drew LJ, Wood JN, Cesare P (2006) Modulation of sensory neuron mechanotransduction by PKC- and nerve growth factor-dependent pathways. *Proc Natl Acad Sci U S A* 103: 4699–4704. PMID: [16537426](#)
106. Bewick GS, Banks RW (2015) Mechanotransduction in the muscle spindle. *Pflugers Arch* 467: 175–190. doi: [10.1007/s00424-014-1536-9](#) PMID: [24888691](#)



107. Aguado F, Majo G, Ruiz-Montasell B, Llorens J, Marsal J, et al. (1999) Syntaxin 1A and 1B display distinct distribution patterns in the rat peripheral nervous system. *Neuroscience* 88: 437–446. PMID: [10197765](#)
108. Koriazova LK, Montal M (2003) Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat Struct Biol* 10: 13–18. PMID: [12459720](#)
109. Fischer A, Montal M (2007) Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. *J Biol Chem* 282: 29604–29611. PMID: [17666397](#)
110. Simpson L (2013) The life history of a botulinum toxin molecule. *Toxicon* 68: 40–59. doi: [10.1016/j.toxicon.2013.02.014](#) PMID: [23518040](#)
111. Ravichandran E, Gong Y, Al Saleem FH, Ancharski DM, Joshi SG, et al. (2006) An initial assessment of the systemic pharmacokinetics of botulinum toxin. *J Pharmacol Exp Ther* 318: 1343–1351. PMID: [16782822](#)
112. Shoemaker CB, Oyler GA (2013) Persistence of Botulinum neurotoxin inactivation of nerve function. *Curr Top Microbiol Immunol* 364: 179–196. doi: [10.1007/978-3-642-33570-9\\_9](#) PMID: [23239354](#)
113. Takimoto E, Champion HC, Li M, Belardi D, Ren S, et al. (2005) Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med* 11: 214–222. PMID: [15665834](#)
114. Akaike N, Shin MC, Wakita M, Torii Y, Harakawa T, et al. (2013) Transsynaptic inhibition of spinal transmission by A2 botulinum toxin. *J Physiol* 591: 1031–1043. doi: [10.1113/jphysiol.2012.242131](#) PMID: [23109108](#)
115. Koizumi H, Goto S, Okita S, Morigaki R, Akaike N, et al. (2014) Spinal Central Effects of Peripherally Applied Botulinum Neurotoxin A in Comparison between Its Subtypes A1 and A2. *Front Neurol* 5: 98. doi: [10.3389/fneur.2014.00098](#) PMID: [25002857](#)
116. Purkiss JR, Welch MJ, Doward S, Foster KA (1997) Capsaicin stimulates release of substance P from dorsal root ganglion neurons via two distinct mechanisms. *Biochem Soc Trans* 25: 542S. PMID: [9388756](#)
117. Purkiss J, Welch M, Doward S, Foster K (2000) Capsaicin-stimulated release of substance P from cultured dorsal root ganglion neurons: involvement of two distinct mechanisms. *Biochem Pharmacol* 59: 1403–1406. PMID: [10751549](#)
118. Aoki KR (2005) Review of a proposed mechanism for the antinociceptive action of botulinum toxin type A. *Neurotoxicology* 26: 785–793. PMID: [16002144](#)