


Botulinum toxin in the masseter muscle: Lingering effects of denervation

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Abstract

Botulinum neurotoxins (BoNTs) are paralytic agents used to treat a variety of conditions in jaw muscles. Although their effect is considered temporary, there are reports of persistent functional changes. Using rabbits that received BoNT injection in one masseter muscle, the recovery of neuromuscular connection was investigated using nerve stimulation to evoke an electromyographic (EMG) response, and the recovery of muscle fibers was investigated using histological morphometry and bromodeoxyuridine (BrdU) immunohistochemistry. One month after treatment, evoked EMG was greatly reduced in both amplitude and duration, indicating that little reinnervation had taken place. Muscle fibers were atrophied and collagenous tissue was increased. Three months after treatment, evoked EMG duration was normal, indicating that at least some neuromuscular junctions were functional. Histologically, some muscle fibers were hypertrophied, some were still atrophied, and some appeared to have died. Fibrosis was still apparent amid slight increases in dividing cells and regenerating fibers. The histological effects of BoNT were evident although attenuated at a distance of about 1 cm from the injection level, but no regional differences could be discerned for the evoked EMGs. In conclusion, there were persistent muscular deficits seen 3 months after BoNT treatment that may have been caused by the failure of some affected muscle fibers to become reinnervated.

KEYWORDS

botulinum toxin, masseter muscle, motor innervation, rabbit

1 | INTRODUCTION

Botulinum toxins (BoNT) are used in muscles of mastication to relieve pain (Schwartz & Freund, 2002), unload the mandible (Canter, Kayikcioglu, Aksu, & Mavili, 2007), and cosmetically, to change a square face into an oval one (Yu, Chen, & Chen, 2007). It is widely believed that the effect of BoNT on nerves and muscles of the face and jaw is temporary (Naumann, Albanese, Heinen, Molenaers, &

Relja, 2016). Indeed, the prescribing information of the most-used product advises re-injection every 3–4 months for most applications (Allergan, 2020). The injected muscles do recover from paralysis, with some electromyographic (EMG) activity returning as early as the third week after injection into the rabbit masseter (Rafferty et al., 2012). However, EMG of rabbit masseters is still far from normal even at week 17 (Korfage, Wang, Lie, & Langenbach, 2012), and in human masseters low EMG has been observed for as long

as 12 months (Lee et al., 2007). Moreover, atrophy of the injected masseter persists for at least 12 weeks in rabbits (Rafferty et al., 2012) and 4–6 months in humans (Kim, Park, & Park, 2010). Histological observations of rabbit and human masseters 3–4 months postinjection suggest that not only do fiber dimensions and type remain abnormal, but fibers may degenerate (Kim, Chung, Park, & Park, 2005; Korfage et al., 2012). After a single injection, ultrastructural damage in human masseters is still severe at 6 months, although improved at 12 months (Ma, Zhai, Zhu, & Tang, 2018). With the cumulative effect of multiple injections (Kim et al., 2010), changes may become permanent.

Indications of long-term damage to muscle are not unique to branchiomeric muscles. Extraocular muscles of rats remained abnormal for at least 8 months after BoNT treatment (Kranjc, Sketelj, d'Albis, & Erzen, 2001). Mass, strength, and percent of contractile tissue all remained reduced 6 months after repetitive treatment of rabbit quadriceps (Fortuna, Horisberger, Vaz, & Herzog, 2013; Fortuna, Vaz, Rehan Youssef, Longino, & Herzog, 2011). Degenerated and abnormal fibers in the rat gastrocnemius persisted for a year after BoNT administration, as did abnormal motor axon branching and motor endplates (Duchen, 1970). Human volunteers also evinced neurogenic atrophy of gastrocnemius fibers 1 year after a single injection of toxin (Schroeder et al., 2009).

It is not clear to what extent long-term changes in BoNT-treated muscles arise directly from the failure of neuromuscular transmission, which is the toxin's main effect. Denervation alone produces damage that is not entirely reversed after reinnervation (Kääriäinen & Kauhanen, 2012; Pingel, Wienecke, Lorentzen, & Nielsen, 2016), but muscles treated by denervation plus BoNT showed less recovery than denervation alone (Akdeniz et al., 2015). Ultrastructural changes also suggest that the effects of BoNT go beyond denervation alone (Hassan, Jennekens, & Veldman, 1995). Unlike denervation, the toxin can diffuse by vascular or direct spread (Ramirez-Castaneda et al., 2013), thus affecting adjacent or even distant muscles. Furthermore, paralysis of one muscle changes motor patterns such as chewing, causing modifications in other muscles (Korfage et al., 2012; Tsai, Lin, Su, Yang, & Chiu, 2012). The situation is additionally complicated by the unknown extent of toxin effect. Large complex muscles such as the masseter have short fibers with many widely distributed motor endplates (Widmer, Klugman, & English, 1997), and it is likely that some fibers may escape the effects of BoNT even with multiple injection sites.

Alternatively, the long-term effects could arise indirectly from the toxin's primary effect on neurons (Baskaran & Thyagarajan, 2014) or secondary actions on the nervous system. Although the motor neuron cell

bodies and axons remain intact (Duchen & Strich, 1968), there is rampant sprouting to establish new sites of motor endplates (de Paiva, Meunier, Molgo, Aoki, & Dolly, 1999). Poisoned neuromuscular junctions may regain normal function as the sprouts retract (de Paiva et al., 1999), but abnormal morphology persists as long as 9 months (Duchen & Strich, 1968). Thus, reinnervation may fail, or competing reinnervation from different neurons and/or additional motor endplates may impair contraction (Favero, Busetto, & Cangiano, 2012). Fiber-type grouping after treatment implies that the original innervation was not restored even if fibers regained function (Minamoto, Suzuki, Bremner, Lieber, & Ward, 2015). Retrograde transport to the central nervous system has been demonstrated (Muñoz Lora, Del Bel Cury, Jabbari, & Lackovic, 2019) and may alter the sensorimotor loop (Caron et al., 2014) and aid in the analgesic effect of BoNT (Matak & Lackovic, 2014; Mazzocchio & Caleo, 2015; Muñoz Lora et al., 2019).

The goals of this study were (1) to assess atrophy, fibrosis, and cell replication in the rabbit masseter muscle after BoNT treatment; (2) to test the functional integrity of the neuromuscular junctions of the masseter in order to gauge the involvement of the nervous system; and (3) to evaluate whether the effect of BoNT is limited to the area of injection. We used an experimental protocol that previously produced a maximum functional deficit (EMG during mastication and tetanic muscle force) at 1–3 weeks posttreatment but almost full functional recovery by 11–12 weeks (Rafferty et al., 2012).

2 | MATERIALS AND METHODS

2.1 | Animals

All procedures were reviewed and approved by the University of Washington Institutional Animal Care and Use Committee under protocol #2331-20. Because of sex differences in New Zealand rabbit masseter physiology (English & Widmer, 2003), gender was restricted to avoid doubling sample size. Females were chosen by lot and represent a conservative choice, as they have a greater percentage of relatively toxin-resistant slow fibers (English, Eason, Schwartz, Shirley, & Carrasco, 1999). Animals were 5 months old when obtained from Western Oregon Rabbit Co. (Philomath, OR). Growth of the skull and mandible is essentially complete at this age (Masoud, Shapiro, & Moses, 1986). Sample size was determined using G*3, which uses Cohen's effect size (Faul, Erdfelder, Lang, & Buchner, 2007). The primary outcome measure of interest was muscle fiber least diameter; from a previous BoNT study on limb muscles (Chen et al., 2002) we calculated an effect size of 2.6, which suggested that a

sample size of 5 would lead to 95% power. We recognized that the physiological measures, which are typically more variable than anatomical measures, would likely be underpowered but still of interest for designing more definitive future work. Animals were maintained on a 12:12 light/dark schedule and received 225 g/day of rabbit pellets (16% Pellet, Albers, St. Louis, MO). Five control rabbits were not treated and were used for evoked electromyography (“evoked EMG”) soon after arrival. The remaining 10 animals received injections of 10 units of BoNT (Botox[®] Cosmetic, Actavis, Parsippany, NJ) in one randomly chosen masseter (Figure 1a). Five of these rabbits underwent evoked EMG 4 weeks later, and the remaining five 12 weeks later. Animals received IV injections of bromodeoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO, 40 mg/kg as a 10 mg/ml solution in phosphate-buffered saline) 1 week prior to their termination. After completion of the evoked EMG, still under anesthesia, animals were euthanized and perfused with glyoxal fixative (Prefer, Anatech Ltd., Battle Creek, MI). The masseter muscles from

most of these animals were removed, weighed, and used for histological assessment. The histological samples were supplemented with muscles derived from the previous study (Rafferty et al., 2012), in which similar rabbits had received unilateral masseter injections of either BoNT or saline. Rafferty et al. (2012) recorded EMG activity during spontaneous mastication and bite force produced by masseter stimulation in anesthetized animals. Saline-injected muscles did not differ from untreated muscles in either functional EMG or force production. Therefore, we considered sham injection and no injection to be equivalent. The resultant sample sizes and the use of shams versus uninjected muscles are detailed in the tables.

During the evoked EMG experiments and all the subsequent analyses, every investigator was blinded to the identity of the animal and the side injected.

2.2 | Evoked EMG

In evoked EMG, the nerve is stimulated to produce an EMG response from the muscle; lack of response implies that neuromuscular junctions are not functioning. Rabbits were mask-anesthetized with isoflurane. The procedures were carried out first on one masseter and then on the other. The masseteric nerve was exposed below the orbit. An insulated bipolar stimulating electrode was hooked around the nerve and the area was filled with mineral oil. The skin over the superficial masseter was marked with an array of 11 dots arranged in superior, middle, and inferior rows (Figure 1b), and a bipolar wire EMG electrode (0.05 mm nickel-chromium wire, 1 mm bared tips) was inserted at each dot. The EMG signals were amplified (Biopac Systems, Goleta, CA) and collected at 500 Hz (Acqknowledge III, Biopac). Nerve stimulation (Grass S48 with SIU5, Astro-Med, Warwick, RI) was delivered as 0.5 ms pulses at 2 Hz; voltage that was advanced gradually until evoked EMG signals plateaued, typically 3–7 V.

After filtering at 60 Hz and rectifying, the mean amplitude and duration of each evoked response were recorded for 10–15 consecutive pulses. Integrated EMG was calculated as the product of amplitude and duration. For comparison with muscle horizontal section levels (see below and Figure 1), sites 1–4 (superior row, approximately 1 cm superior to the level of BoNT injection) were averaged to correspond with the superior histological section. BoNT injection level coincided roughly with the border between middle and inferior rows, and so the remaining sites (5–11) were averaged to correspond with the injection-level histological section (Figure 1).

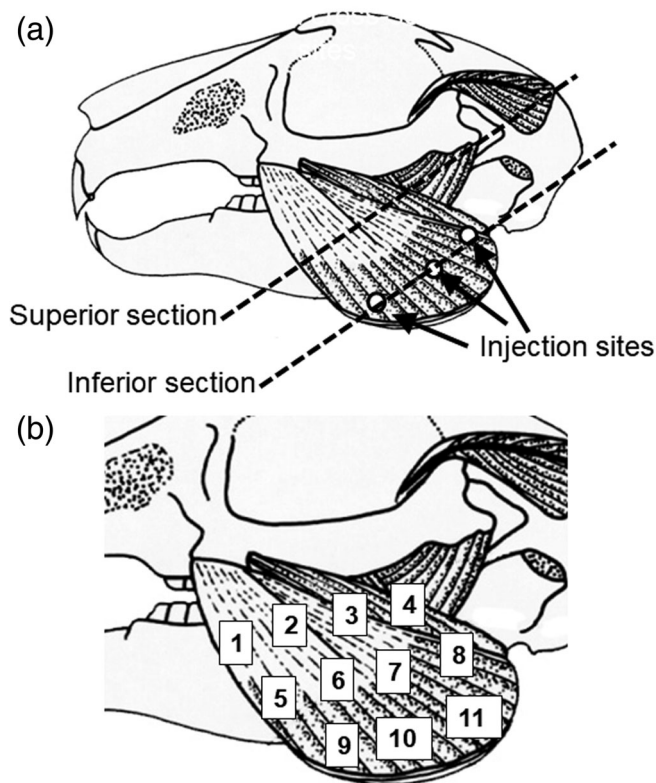


FIGURE 1 Rabbit masseter muscle. (a) The open circles show the injection locations for BoNT or (in sham animals) saline. The dashed lines indicate the superior and inferior (injection) horizontal levels for histological sectioning. (b) Electrode insertion sites (#1–#11) for recording evoked electromyography. Locations #1–#4 were averaged as the superior row, #5–#8 as the middle row, and #9–#11 as the inferior row

2.3 | Muscle morphometry

The masseters were cut into superior, middle, and inferior horizontal blocks as shown in Figure 1a, embedded in paraffin, sectioned at 7 μm , and stained with trichrome or reserved for BrdU immunohistochemistry. From each muscle, one trichrome section was selected from the junction of the middle and inferior thirds, approximating the level of BoNT injection, and a second section was selected from the junction of upper and middle thirds. The sections were used for tabulation of unusual features, measurements of fiber dimensions, and analysis of tissue composition.

Least diameter was used to measure fiber size in order to avoid overestimates caused by obliquely sectioned fibers. However, fiber area gave very similar results. Each muscle section was divided into six parts (anterior, middle, and posterior thirds, each separated into an inner and an outer part, Figure 2aA). Images (20 \times) were acquired at the center of each part. Large tendons and torn muscle fibers were avoided. A grid containing 20 intersections was superimposed over each of the six magnified images in Photoshop (Adobe, San Jose, CA) (Figure 2aB, see caption for details of methodology). Muscle fibers at or nearest the 20 intersections were measured. The least diameter was defined as the caliper width perpendicular to the longest chord (Figure 2aC) and was measured using MetaVue software (Molecular Devices, Sunnyvale, CA). For the 120 muscle fibers per section, specimen averages were calculated, and the minimum and maximum values per section were recorded.

Tissue composition was analyzed using a grid method (Figure 2b). Overlapping images were acquired at 1 \times and merged in Photoshop. A grid was superimposed over the whole section and tissue types were identified at each intersection: muscle, collagenous tissue, or interstitial space. The number of “hits” for each tissue type was counted and expressed as a percentage of the total number of intersections over the section.

2.4 | BrdU immunohistochemistry

BrdU is a thymidine analog and labels cells in the S-phase of mitosis, enabling an estimate of cell division. From each available masseter block of the evoked EMG rabbits, one section was processed with a BrdU staining kit (Becton-Dickinson, Franklin Lakes, NJ) and a methyl green counterstain. Negative controls were run with each batch by omitting the primary antibody. The total number of labeled nuclei per section was counted, with annotations as to whether the labeled cell was associated with

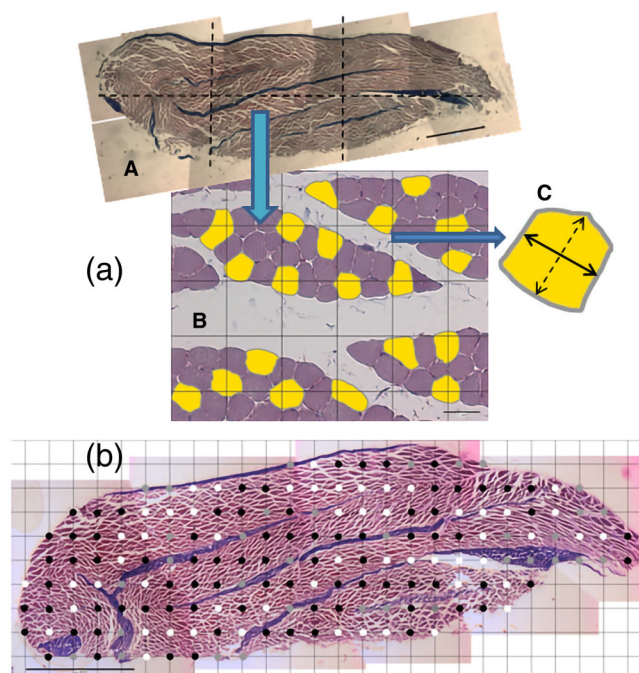


FIGURE 2 Methods for muscle morphometry. (a) For fiber size, masseter sections were geometrically divided into six parts (A). Images (20 \times) were acquired at the center of each of the six parts. Each image was overlaid by a grid (B). Muscle fibers closest to each of the 20 intersections (yellow) were selected; because selected muscle fibers could not be adjacent (a thresholding limitation), sometimes the next closest to the intersection was used instead. This resulted in measurement of 120 fibers/muscle. The least diameter of each selected fiber (C) was defined as the caliper width of the object (solid line) perpendicular to the longest cord (dashed line). (b) For tissue composition the compound image was overlaid by a grid and each intersection was classified as muscle (black dots), collagen (gray dots), or empty space, which could represent interstitial space or dissolved fat (white dots). The frequency of each component was divided by the total number of intersections to calculate the percent tissue composition. Muscle sections are oriented with anterior to the left and lateral to the top. The horizontal bars represent 5 mm for a(A) and b and 50 μm for a(B)

connective tissue or muscle fibers. Data from the three sections per muscle were averaged.

2.5 | Statistical analysis

Nonparametric statistics were used to evaluate tissue composition outcomes. Where appropriate, fiber measurements were explored with paired or two-sample Welch *t*-tests with Bonferroni correction for multiple tests. These analyses were carried out using Microsoft Excel 2010, IBM SPSS 19, GraphPad and QuickCalcs. The statistical environment R (version 3.6.2) was used to identify 12-week least fiber diameter distribution shape

statistics and to perform repeated measures analyses to assess treatment reliabilities (<https://www.R-project.org>). Equality of BoNT fiber least diameter distributions was evaluated using the two-sample Kolmogorov–Smirnov (K-S) test (ks.test in R). As the K-S test has moderate sensitivity to the location and shape characteristics of the empirical distribution functions of two samples, we also evaluated skewness and kurtosis for each distribution. These shape statistics were expressed as point and 95% confidence intervals (CI) obtained from 9,999 bootstrap replications from each sample of $n = 1920$ fiber diameters from the BoNT injected-side and $n = 1920$ diameters from the uninjected-side (confintr package, v. 0.1.1).

The 12-week fiber diameter outcomes were compared between conditions using the linear mixed model for repeated measures (lme4 package, v.1.1-21). The model incorporated a random intercept, and the fixed effects were treatment, injection location and the treatment \times location interaction. Least fiber diameters were log-transformed to satisfy the normality assumption for

model residuals. Residuals for the BoNT condition exhibited greater variance than did the uninjected condition, but mixed model regression is robust to heteroscedasticity (Verbeke & Molenberghs, 1997). Mixed model 12-week fiber diameter results are presented as geometric means and 95% CIs obtained by exponentiation of model coefficients. Statistical significance was established at $p < .05$, two-tailed. In some analyses, 95% CIs convey the likely range of point estimates, and non-overlapping 95% CIs denote significance.

3 | RESULTS

As in the previous study (Rafferty et al., 2012), unilateral masseter paralysis had no identifiable effect on rabbit mastication or well-being. Body mass was similar in all groups. Means and 95% CIs were as follows: untreated controls (4.20 kg, 3.95–4.45 kg); 4-week BoNT (4.10 kg, 3.93–4.26 kg); 12-week shams (4.37 kg, 3.89–4.85 kg); 12-week BoNT (4.17 kg, 3.96–4.38 kg).

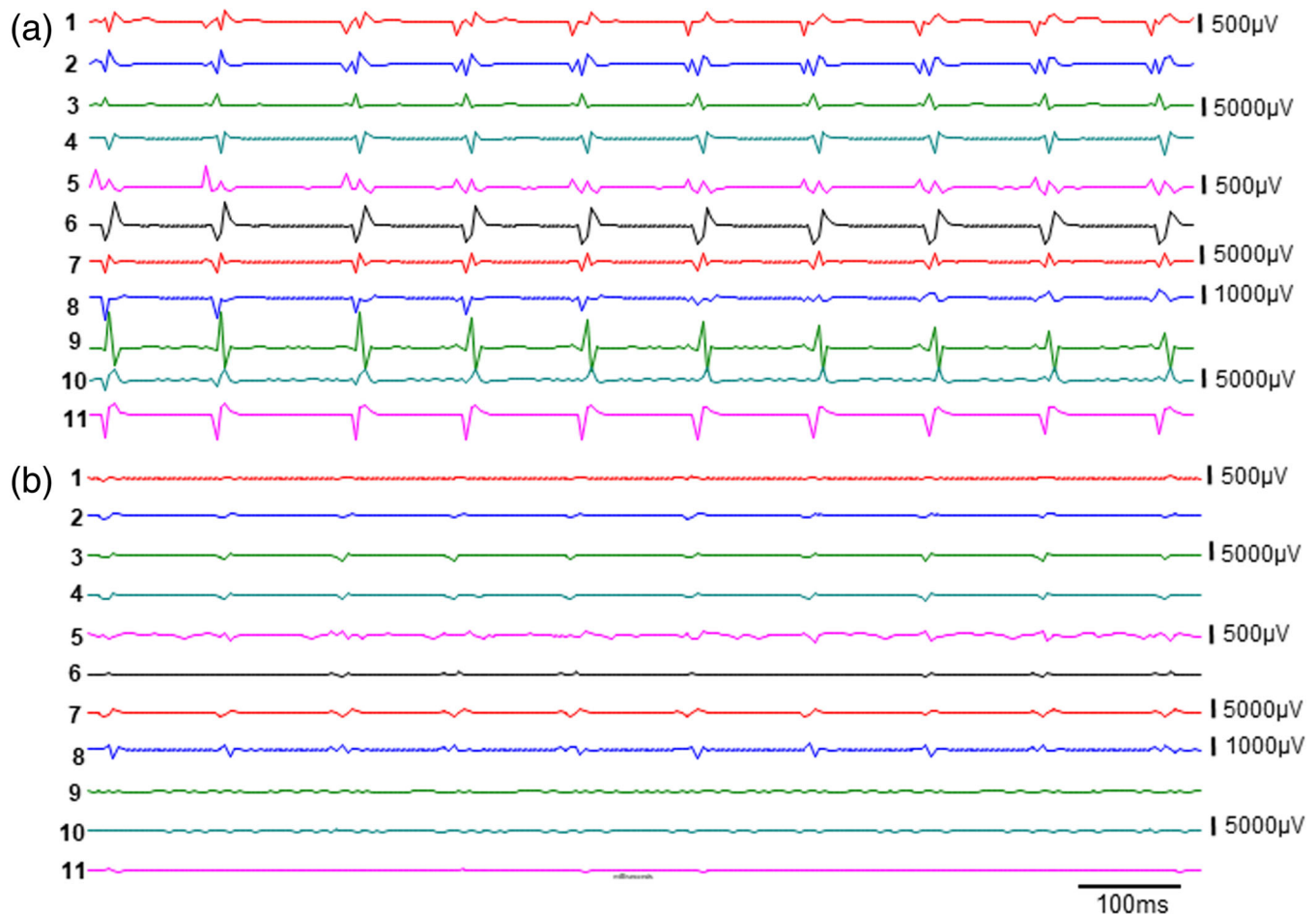


FIGURE 3 Comparisons of evoked electromyographic responses from (a) the uninjected masseter and (b) the BoNT-injected masseter of the same rabbit 12 weeks after injection, indicating the great reduction of amplitude in all 11 sites

TABLE 1 Average muscle evoked electromyographic amplitude and duration (mean \pm SD) over all 11 sites/muscle, $n = 5$ per group

	Injected side	Uninjected side (paired <i>t</i> -test)
Amplitude ($\mu\text{V} \pm \text{SD}$)		
Control (no injection, average of right and left sides)	5,837 \pm 1,193 ($p < .0001$ for all comparisons, two-sample <i>t</i> -tests)	
4-weeks postinjection	252 \pm 139	1,215 \pm 312 ($p < .01$)
12-weeks postinjection	217 \pm 101	742 \pm 883 ($p = .22$)
Duration (ms \pm SD)		
Control (no injection, average of right and left sides)	3.95 \pm 0.19 ($p = .001$ vs. 4-week injected side, two-sample <i>t</i> -test; other comparisons not significant after Bonferroni correction)	
4-weeks postinjection	2.55 \pm 0.59	3.63 \pm 0.26 ($p = .03$)
12-weeks postinjection	3.90 \pm 1.02	4.42 \pm 0.76 ($p = .28$)

3.1 | Evoked EMG

Evoked action potentials were identified in all sites of control animal masseters and the uninjected masseters of experimental rabbits (Figure 3a), but were sometimes absent in BoNT-injected muscles (Figure 3b), especially at the 4-week endpoint. Average amplitude values over the whole masseter ranged up to almost 6,000 μV in control muscles, although variation among individuals was great (Table 1). Evoked EMG amplitudes of the control animals were strikingly greater than those of experimental animals, even their uninjected sides, a surprising finding addressed in Section 4.2. In the experimental groups, BoNT-injected masseters showed smaller amplitudes compared to the uninjected side, but this was only statistically significant at the 4-week time period owing to individual variation of the uninjected sides of the 12-week postinjection group (range 84–2,230 μV).

Evoked EMG amplitudes were averaged by horizontal level to compare the superior row of electrodes to the injection level, represented by the middle and inferior rows. Results are shown in Table 2. There were no statistically significant differences, but all rows showed the same pattern of control > uninjected side > injected side as seen for the muscle as a whole. Table 2 also suggests a tendency in control/uninjected side muscles for the superior row to have lower EMG amplitudes than the middle and inferior rows. In contrast, the superior row had the highest row values on the BoNT-injected masseters.

In addition to its amplitude, the duration of the evoked EMG response was reduced by BoNT (Figure 3). Quantitative results for duration of the response are presented in Table 1 for the muscle as a whole and Table 2 for the separate rows. Four weeks after BoNT injection, the average duration of the response was reduced in the injected muscles in comparison to the uninjected side

and in comparison to control values. However, by 12 weeks postinjection durations were normalized. The uninjected sides of experimental animals did not differ significantly from controls in contraction duration at either time period. The breakdown by row suggested that a slight tendency for longer durations inferiorly was not altered by injection.

Although amplitude and duration showed somewhat similar patterns, these variables were not correlated ($p = .12$ – $.90$) except when the 4-week injected and uninjected masseters were considered alone ($r = .695$, $p = .04$), reflecting the lack of variation in duration except for the reduction in 4-week injected muscles.

3.2 | Muscle morphometry

Before histological processing, masseter muscles were weighed (Table 3). BoNT-injected muscles differed significantly from the uninjected side only at 4 weeks, and this difference was due to enlargement of the uninjected side, which was also heavier than the control muscles and marginally heavier than the uninjected masseters of 12-week animals. The BoNT-injected masseters were similar in mass at both timepoints and also similar to those of control masseters. The histological appearance of the saline-injected side of sham animals was the same as the uninjected side; there was neither indication of trauma from the injection nor of any effect of the saline.

However, BoNT-injected muscles showed a variety of unusual characteristics compared to saline-injected and uninjected muscles, which did not differ among groups. Four weeks after BoNT injection, in addition to obviously smaller muscle fibers surrounded by an empty endomysial perimeter, collagenous tissue, blood vessels, nerve bundles, and muscle spindles were all more

TABLE 2 Evoked electromyographic average amplitude and duration (mean \pm SD) by horizontal row (see Figure 1b), $n = 5$ per group

	Control (average of left and right)	4-week injected side	4-week Uninjected side	12-week injected side	12-week Uninjected side
Amplitude ($\mu\text{V} \pm \text{SD}$)					
Superior row sites 1–4	5,016 \pm 1,293	255 \pm 395	883 \pm 401	249 \pm 147	574 \pm 640
Middle row Sites 5–8	5,299 \pm 1735	190 \pm 129	1,517 \pm 196	218 \pm 137	712 \pm 713
Inferior row Sites 9–11	6,804 \pm 1,628	167 \pm 84	1,277 \pm 737	111 \pm 65	1,087 \pm 1,508
Duration (ms \pm SD)					
Superior row sites 1–4	3.64 \pm 0.24	2.34 \pm 0.47	3.70 \pm 0.38	3.81 \pm 0.77	4.06 \pm 0.43
Middle row sites 5–8	3.96 \pm 0.23	2.67 \pm 0.71	3.56 \pm 0.30	3.97 \pm 1.13	4.30 \pm 0.93
Inferior row locations 9–11	4.56 \pm 0.75	2.59 \pm 0.98	3.65 \pm 0.30	4.40 \pm 1.46	4.90 \pm 1.10

prominent (Figure 4). Centrally located nuclei were seen more frequently in BoNT-injected masseters than in uninjected muscles. Empty profiles suggested either fat accumulation (adipose tissue would have dissolved during preparation) or fiber dissolution. At 12 weeks, fiber sizes of the BoNT-injected masseters were highly irregular, with some fibers still small and others appearing larger than normal (Figure 5). Collagenous connective tissue and empty profiles were more prominent than at 4 weeks. Using $20\times$ images from 12-week muscles, a count was made of fibers with centrally located nuclei, resulting in averages of 29 (SD 23) on the injected side and 5 (SD 3) on the uninjected side ($p < .02$, $n = 8$ per group). Similar but less emphasized differences were seen between injected and uninjected muscles at the superior histological level (Figure 5, bottom row). For example, superior-section fibers with central nuclei averaged 21 (SD 14) in the injected masseter versus 3 (SD 3) in the uninjected masseter ($p < .03$, $n = 8$ per group).

Fiber least diameter (henceforth “fiber diameter”) is summarized quantitatively in Table 3. Control and uninjected masseters averaged 30–40 μm in fiber diameter. Four weeks after BoNT treatment, average fiber diameter of the injected masseters was reduced to 23 μm ; this reduction did not affect every fiber, because the largest fibers (mean maximum fiber diameter, Table 3) remained similar to those of the uninjected masseters (52 vs. 56 μm). Twelve weeks after treatment, average fiber diameter in the injected muscles was much improved (29 μm), albeit still less than the opposite, uninjected side. Surprisingly, this improvement did not involve the smallest fibers (mean minimum fiber diameter, Table 3), which at the injection level were even smaller than at 4 weeks. Rather, the increase in mean diameter at week

12 was a function of an increase in the size of the largest fibers, most notably at the distant-from-injection superior level (maximum fiber diameter, Table 3). This difference between superior and injection level 12-week maximum fiber diameter (89 vs. 66 μm) was the only statistically significant difference due to position within the masseter in this analysis.

A more complete picture of 12-week muscles emerged from the analysis of diameter distributions. Figure 6 demonstrates the marked alteration in BoNT-injected muscles compared to the uninjected sides. In BoNT-treated masseter muscles, regardless of level, mean and median diameters were shifted to lower values while at the same time long positively skewed tails were created, indicative of a subset of unusually large fibers. The two-sample K-S test comparing BoNT to saline injection overall was highly significant ($D = 0.33$, $p < .0001$). Bootstrap resampling confirmed strikingly more positive skewness in BoNT (0.89) compared to uninjected sides (0.18), with unambiguously non-overlapping 95% CIs ([0.74, 1.22] vs. [0.07, 0.29]). Kurtosis was also greater in the BoNT (4.31) compared to uninjected sides (3.17), again with nonoverlapping 95% CIs ([3.48, 6.89] vs. [2.98, 3.42]).

While uninjected muscles showed similar fiber diameter distributions at both levels examined, the superior level for BoNT muscles evinced a modest but significant right-shift compared to the injection level (Figure 6; K-S test, $D = 0.09$, $p < .001$). Skewness was greater in the BoNT-superior level (0.93, 95% CI [0.74, 1.37]) compared to the BoNT-injection level (0.60, 95% CI [0.47, 0.76]). Similarly, kurtosis was greater for BoNT-superior level compared to BoNT-injection level (4.38, 95% CI [3.38, 6.95] vs. 2.93, 95% CI [2.60, 3.58]).

TABLE 3 Muscle mass and least fiber diameters (mean \pm SD) for rabbit masseter muscles

	Muscle mass (g) ^a	Least fiber diameter (μ m, 120 fibers/muscle)			
			Minimum	Mean	Maximum
Control ($n = 3$) plus 4-week sham ($n = 1$) ^b	6.25 \pm 0.65	Injection level	17.5 \pm 4.3	30.7 \pm 5.3	44.3 \pm 4.8
		Superior level	16.7 \pm 4.1	30.8 \pm 5.0	47.7 \pm 6.8
4-week injected side ($n = 7$)	6.27 \pm 0.50 ^{c**}	Injection level	10.6 \pm 2.1 ^{c**,d,e**}	22.6 \pm 7.9 ^{c**}	52.4 \pm 12.7 ^d
		Superior level	9.6 \pm 1.9 ^{c*,e**}	21.7 \pm 9.0 ^{c*}	64.6 \pm 23.3 ^d
4-week uninjected side ($n = 5$)	7.56 \pm 0.13 ^{d,e*}	Injection level	14.1 \pm 2.1	35.7 \pm 8.0	56.2 \pm 4.5 ^{e**}
		Superior level	16.9 \pm 6.5	37.3 \pm 7.9	56.0 \pm 7.9
12-week sham ($n = 4$)	nd	Injection level	23.1 \pm 4.1	40.7 \pm 7.8	63.0 \pm 12.0
		Superior level	18.2 \pm 6.5	40.6 \pm 8.7	63.1 \pm 11.7
12-week injected side ($n = 8$)	6.78 \pm 0.93	Injection level	8.1 \pm 1.6 ^{c****,d,e***}	29.4 \pm 12.1	66.5 \pm 9.7 ^{c**,d,f}
		Superior level	8.3 \pm 2.9 ^{c***,e**}	32.4 \pm 14.7	89.3 \pm 18.3 ^{c***,d,e*,f}
12-week uninjected side ($n = 8$)	6.97 \pm 0.54 ^d	Injection level	17.1 \pm 3.0 ^{e*}	36.4 \pm 7.7	55.8 \pm 4.6
		Superior level	15.8 \pm 3.7	35.8 \pm 8.2	57.7 \pm 6.1

Abbreviation: nd, no data.

^aMuscle mass data include only rabbits from the current study, not the supplemental muscles (controls $n = 3$, sides averaged; 4-week $n = 5$; 12-week $n = 4$ as one sample was lost).

^bThe sham samples were the uninjected masseters of saline controls from an earlier study which included muscle mass but not histological data (Rafferty et al., 2012).

^cDifference between injected vs. uninjected masseters at the same level (2-sample t -tests, * $p < .05$, ** $p < .01$, *** $p < .001$, and **** $p < .0001$, respectively). For clarity, superscripts are shown only for the injected masseters.

^dDifference between 4- and 12-week groups for the same side at the same level (2-sample t -tests, $p < .05$).

^eDifference between muscles of BoNT animals and those of the time-matched sham/control group (2-sample t -tests, * $p < .05$, ** $p < .01$, *** $p < .001$ respectively). For clarity, superscripts are shown only for the BoNT animals (both sides).

^fDifference between superior and injection levels in the same masseter (paired t -test, $p = .01$).

Mixed model analysis further confirmed the overall patterns, revealing a highly significant main effect of BoNT treatment on log-transformed least fiber diameters ($p < .0001$). The main effect of level was not significant ($p = .23$), but the BoNT \times level interaction was highly significant ($p = .0003$). At the level of the injection, the BoNT muscle geometric mean fiber diameter was 9.0 μ m smaller than the 35.4 μ m value for uninjected muscles ($p < .0001$; 95% CI [-10.09, -7.88]). At the superior level, the BoNT mean was 6.4 μ m smaller than the uninjected value of 34.7 μ m ($p < .0001$; 95% CI [-7.50, -5.14]). For the BoNT-injected muscles, the mean diameter of 26.4 μ m at the injection level was 1.9 μ m smaller than at the superior level ($p < .0001$; 95% CI [0.76, 3.13]). Thus, on average for the 12-week rabbits, BoNT treatment reduced fiber diameters by approximately 25% at the injection level site and by approximately 18% at the superior level, while at the same time creating a subgroup of anomalously large-diameter fibers, more accentuated at the superior level.

An attempt was made to investigate whether fiber diameter was correlated with the strength (i.e., integrated values) of the evoked EMG response in the BoNT-

injected muscles. Four weeks postinjection, there was no correlation, primarily because of very low evoked EMG at all locations. At 12 weeks a moderate positive correlation was seen at injection level ($r^2 = .36$, Pearson correlation) and a stronger one was seen at the superior level ($r^2 = .76$).

3.3 | Tissue composition

As shown in Table 4, the cross-sectional area of the muscle was well tracked by counting grid intersections ($r^2 = .99$), so the grid method was considered adequate for tissue proportions within the muscle. Both measures suggested that at 4 weeks after BoNT administration, the injected muscle cross-section was smaller than controls, and the uninjected opposite masseter was larger than controls. At 12 weeks postinjection, the cross-sectional area for neither injected nor uninjected masseter differed from the shams, although they remained different from each other. As shown in Table 4, muscle tissue was by far the most prominent component of the sections, accounting for 58–68% of the total area. While the muscle tissue

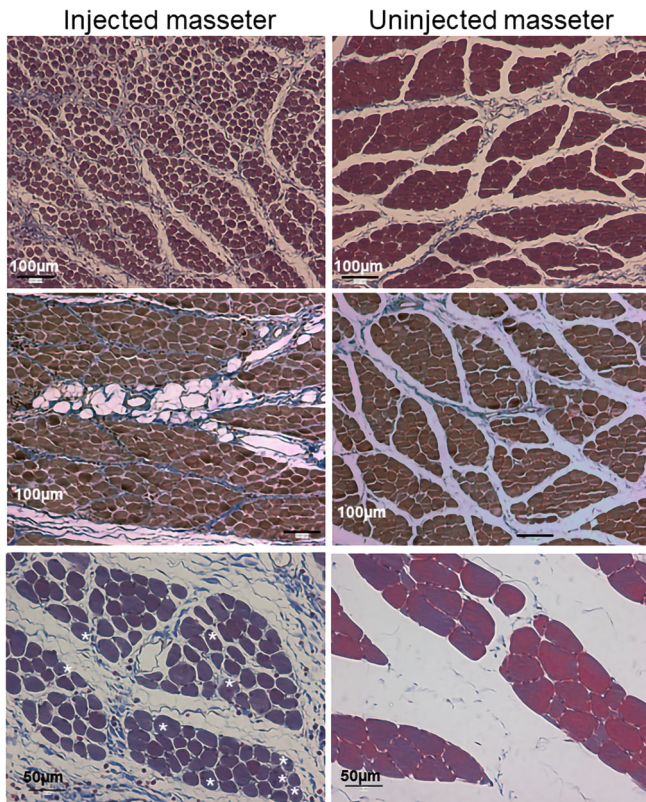


FIGURE 4 Exemplar masseter pairs from 4-week BoNT animals (trichrome stain). On the injected side, fiber diameters are reduced and fibers are not close-packed. Perimysial collagen is prominent (blue). An apparent accumulation of fat is seen in the injected masseter shown in the center row. Asterisks in the injected masseter in the bottom row indicate fibers with central nuclei. The noninjected sides look like the masseters of no-injection controls

percentages tended to be lower for BoNT-injected masseters, the differences did not approach statistical significance. Collagenous tissue constituted 17–33% of total area and was increased in the injected masseters compared to the opposite uninjected masseters. As the proportion of collagen increased in the injected muscles, the proportion of interstitial space decreased relative to the uninjected side. Examination of the sections at 20 \times showed that both collagen and interstitial space were predominantly perimysial rather than endomysial.

3.4 | Cell proliferation

Only a few cells in these adult masseter muscles were positive for BrdU, a marker for replication. None of the six masseters from the three control rabbits had any BrdU label. However, in the BoNT-injected rabbits, positive cells were seen bilaterally at both timepoints, with increased label in the injected muscle. At 4 weeks the

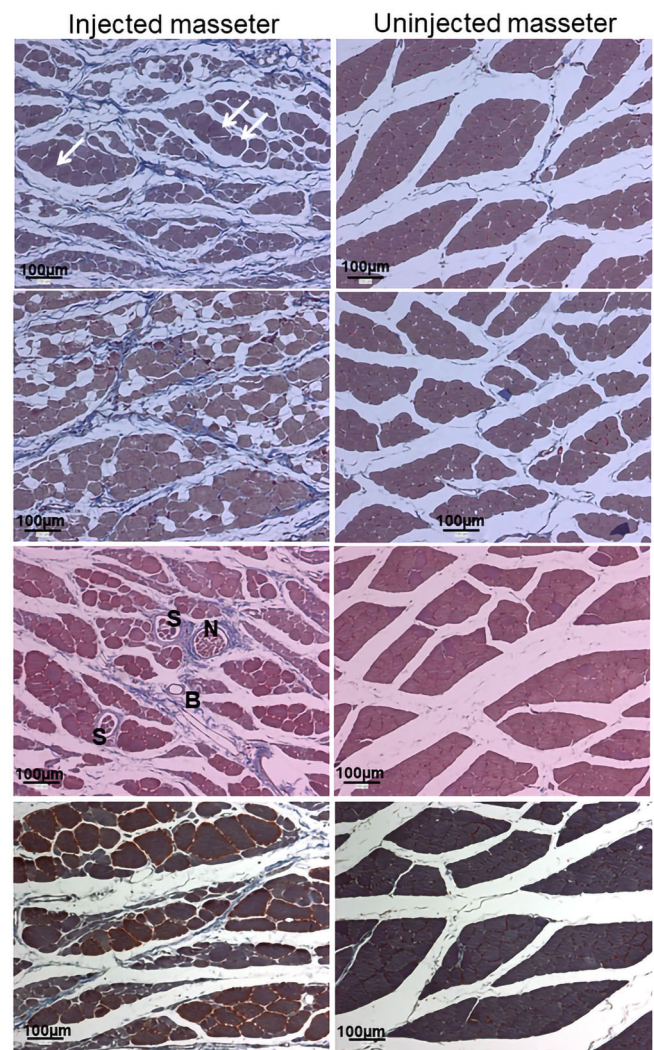


FIGURE 5 Exemplar masseter pairs from 12-week BoNT animals. The injected sides still evince many small fibers, but other fibers appear to be larger than normal (white arrows in top row). A number of fiber profiles appear empty (second row). Muscle spindles, nerve bundles and blood vessels are often prominent (third row). At the superior level (bottom row), distant from the injection sites, there are fewer small fibers but more large fibers, often much larger than any fibers at the injection level. Excess collagen is present but less apparent than at the injection level. The uninjected masseter always appears fully normal. N, nerve bundle; S, muscle spindle; B, blood vessel

injected side averaged 8.0 ± 1.2 BrdU-positive cells, and the uninjected side 1.8 ± 0.6 ($p < .001$, $n = 5$). At 12 weeks, injected masseters averaged 3.4 ± 1.1 positive cells and uninjected masseters 0.15 ± 0.07 ($p = .006$, $n = 5$). The difference between 4- and 12-week counts is likely real ($p < .001$ and $p = .02$ for injected and uninjected sides, respectively). Another interesting feature of these results was the location of the replicating cells.

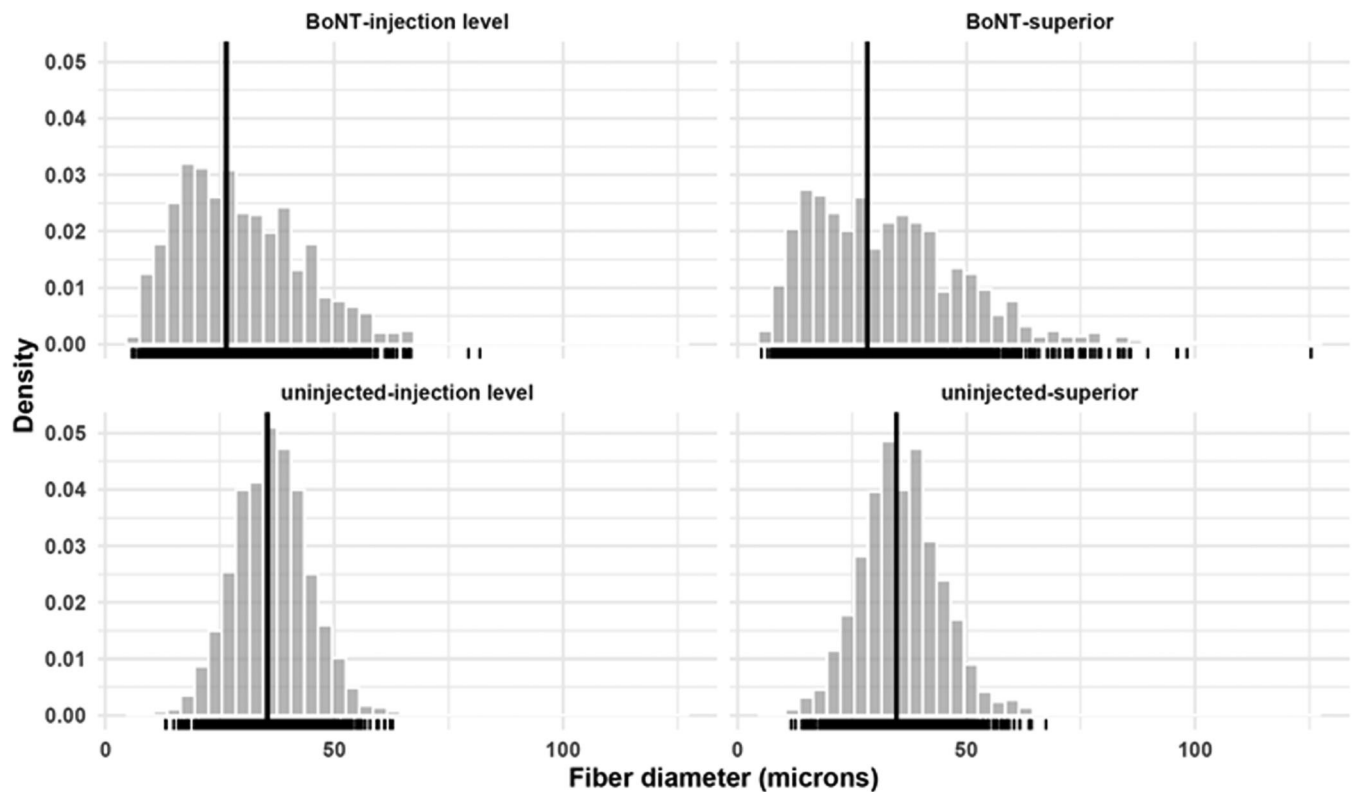


FIGURE 6 Density plots of muscle fiber least diameters by treatment and level 12 weeks after unilateral BoNT injection into the masseter. Each plot encompasses 960 diameter measurements, and the bin size is 3 μm . Rugplots on the x-axis depict individual fibers. Vertical lines indicate geometric mean values: BoNT-injection level, 26.4 μm (95% CI 25.6, 27.2); uninjected-injection level, 35.4 μm (34.9, 35.9); BoNT-superior level, 28.3 μm (27.4, 29.3); uninjected-superior level, 34.7 μm (34.1, 35.2). At both levels, two-sample Kolmogorov–Smirnov tests comparing empirical distribution functions between BoNT and uninjected were significant, and positive skewness and kurtosis were both greater in the BoNT compared to noninjected distributions. For BoNT but not uninjected muscles, values were greater at the superior level than at the injection level (see text)

TABLE 4 Injection level masseter sections: Total area and grid intersections, percent muscle, collagen, and interstitial space (mean \pm SD)

	Muscle area (mm^2)	Total grid intersections	Muscle tissue (%)	Collagenous tissue (%)	Interstitial space (%)
Control ($n = 3$) plus 4-week sham ($n = 1$)	164.4 \pm 10.8	135 \pm 9	61.6 \pm 6.5	25.2 \pm 5.5	13.2 \pm 2.9
4-week injected side ($n = 7$)	135.1 \pm 15.0 ^{a,b***}	112 \pm 13 ^{b*}	58.5 \pm 11.3	32.9 \pm 9.8 ^{b*}	8.6 \pm 5.4 ^{b*}
4-week uninjected side ($n = 7$)	199.1 \pm 30.5	164 \pm 25 ^{a*}	62.9 \pm 9.4	17.4 \pm 3.5 ^{a*}	19.7 \pm 9.2
12-week sham ($n = 4$)	201.7 \pm 53.4	166 \pm 44	67.8 \pm 5.4	22.0 \pm 7.3	10.3 \pm 6.5
12-week injected side ($n = 8$)	184.5 \pm 17.2 ^{b*}	151 \pm 13 ^{b*}	64.4 \pm 5.5	26.7 \pm 4.5 ^{b*}	8.9 \pm 3.0 ^{b*}
12-week uninjected side ($n = 8$)	208.7 \pm 25.2	171 \pm 22	67.2 \pm 3.4	18.9 \pm 4.6	13.9 \pm 4.5

^aDifferent from control/sham (Mann–Whitney test, * $p < .05$, ** $.01$). For clarity, superscripts are shown only for the BoNT animals (both sides).

^bDifference between injected versus uninjected masseters of the same animal (Wilcoxon test, * $p < .05$, ** $.01$, *** $.001$). For clarity, superscripts are shown only for the injected masseters.

A majority of the labeled cells were found adjacent to connective tissue rather than muscle fibers and hence were probably fibroblasts and not satellite cells or myoblasts. However, while this discrepancy was almost complete in the uninjected masseters (one 12-week

muscle showed a single labeled cell associated with a muscle fiber), 4 of 5 injected masseters at each time-point showed muscle fiber-associated cells (1–5 per section) as well as connective tissue-associated positive cells.

4 | DISCUSSION

BoNT treatment of the masseter is a common treatment for bruxism, pain conditions, and cosmetic shaping of the lower face, but other than gross assessments of muscle size, little is known about the course of nerve and muscle recovery. In this study on rabbits, we found that even after a 3-month interval, muscle histology was far from normal, possibly because of incomplete reinnervation.

4.1 | Aim 1: Status of the muscle

The first goal of this study was to assess atrophy, fibrosis and cell replication in the masseter muscle after injection of BoNT. BoNT causes flaccid paralysis, which is rapidly followed by disuse atrophy. Unlike disuse from softened diets, BoNT administration abolishes even weak activation of affected fibers. Unlike disuse from denervation, some fibers probably escape poisoning, because the toxin is unlikely to reach all of the dispersed masseteric neuromuscular junctions even after multiple injection locations. Muscle mass and cross-sectional area are usually considered proxies for recovery of activity and force production.

Although muscle mass (Table 3) failed to show reduction in the 4-week BoNT-injected masseters relative to controls, the muscle cross-sectional areas did (Table 4), and both types of analysis indicated hypertrophy in the uninjected side masseters, a pattern that was reduced but still evident 12 weeks after the injection. The probable explanation for this is that at 4 weeks, the uninjected masseter was working to compensate for the loss of the injected masseter, whereas at 12 weeks the injected masseter would largely have recovered strength (Rafferty et al., 2012) and compensatory activity would no longer have been needed. However, both mass and cross-section include collagenous tissue and interstitial space. A better assessment for recovery is the fiber diameter, which should shrink with denervation, recover with reinnervation, and enlarge with increased demands.

The mean fiber diameter results at 4 weeks do echo the uninjected side hypertrophy and injected side atrophy shown for mass and cross-section. However, a much richer view is offered by the examination of minimum and maximum fiber diameter. On the injected side, BoNT caused both atrophy and hypertrophy of individual fibers, both of which were greater at 12 weeks than at 4 weeks. Simultaneous hypertrophy and atrophy is a standard feature of BoNT studies, including those in humans (Borodic & Ferrante, 1992; Schroeder et al., 2009), and also of neurodegenerative and dystrophic disorders generally (Botzenhart et al., 2020; Carpenter & Karpati, 2001). It is,

however, surprising that these changes were not reversed when functional recovery was nearly complete at 12 weeks postinjection (Rafferty et al., 2012) but rather were more extreme, with empty profiles suggesting muscle fiber death, confirming a previous observation (Korfage et al., 2012), and some fibers reaching giant proportions, as verified by the analysis of diameter distribution (Figure 6). These changes did not result from aging (Cicek, Tumer, & Unsal, 2020) because the uninjected side remained normal. Instead, as discussed below, incomplete reinnervation may account for the changes. A similar worsening of empty profiles and fatty infiltration with time was reported after BoNT injection in rabbit quadriceps (Fortuna et al., 2011), so this is a toxin effect rather than a masticatory muscle problem. Although Botzenhart et al. (2020) found no necrotic fibers in wild-type murine masseters after BoNT administration, necrosis has been reported from mouse hindlimb (Duchen & Strich, 1968) and human masseter (Kim et al., 2005).

Fibrosis is a common feature of denervation (Carpenter & Karpati, 2001). Cranial muscles may be prone to fibrosis due to characteristics of the neural crest-derived fibroblasts (Rosero Salazar, Carvajal Monroy, Wagener, & von den Hoff, 2020). Fibrosis exhibited in the BoNT-treated mouse masseter was speculated to relate to inflammation (Botzenhart et al., 2020). Fibrosis is linked to robust angiogenesis followed by vascular regression (Johnson & DiPietro, 2013), a situation which occurs in the BoNT-treated masseter of rabbits (Matic, Lee, Wells, & Gan, 2007). Denervation alone suffices to produce this transient increase and subsequent decrease of intramuscular capillarity (Kääriäinen & Kauhanen, 2012)). Moreover, fibrosis routinely accompanies muscle fiber atrophy, which is always prominent with denervation and BoNT treatment; whether this increase in connective tissue stiffens muscles is unclear, but if so BoNT may be contraindicated for treatment of spastic muscles (Pingel et al., 2016).

Cell replication associated with muscle fibers and central myonuclei suggests the recruitment of satellite cells and the regeneration of muscle fibers. Central nuclei increase modestly in mouse hindlimb muscles (Duchen & Strich, 1968) and masseter (Botzenhart et al., 2020) recovering from BoNT treatment, but cell replication has not been previously assessed. In the present study, we found increases of replication associated with muscle tissue at both timepoints and of central myonuclei at 12 weeks, mostly but not exclusively in the BoNT-injected sides. We could not determine whether these signs of recovery related to atrophied muscle fibers returning to normal size or already recovered fibers undergoing hypertrophy.

The morphological features of BoNT-injected masseter 12 weeks after treatment resemble those of permanently

denervated rabbit tibialis anterior (Ashley et al., 2007) and thus suggest that return of functional innervation was incomplete.

4.2 | Aim 2: Status of innervation

Our second goal was to test the functional integrity of neuromuscular junctions by stimulating the masseteric nerve and recording the evoked EMG potentials. The short stimulus duration (0.5 ms) and the electrical isolation of the nerve were intended to ensure that EMG resulted from nerve-generated action potentials and not direct stimulation of muscle fibers.

Both the amplitude and duration of evoked EMG were reduced 4 weeks after injection. The poor response at the 4-week timepoint can be understood as a manifestation of the recent poisoning of the nerve endings and the consequent paralysis. In a study on mouse sternomastoid muscle, de Paiva et al. (1999) were not able to produce a twitch through nerve stimulation until 4 weeks, and this response was ascribed to the “remedial” new junctions formed by nerve sprouts. Even if reinnervated, the still-atrophied muscle fibers would not be expected to produce large-amplitude action potentials. Moreover, new neuromuscular junctions were likely still immature and old ones were likely not yet functional. Notably, the superior row of electrodes, at a level distant from the injections and therefore less disrupted, tended to show higher amplitudes.

However, when reinnervation occurs and electrical activity resumes, the fibers should normalize, both in their size and the functionality of their neuromuscular junctions. This occurred by about 12 weeks in the mouse sternomastoid study (de Paiva et al., 1999). Thus, we expected recovery of the evoked EMG response 12 weeks after treatment. Our findings indicated that evoked potential duration was indeed normalized at 12 weeks, but amplitude was not improved. The return of normal potential duration suggests that some neuromuscular junctions were functioning normally, but the continuing reduction in amplitude suggests that motor units were smaller and/or fewer in number. This in turn implies that not all muscle fibers had been reinnervated by 12 weeks.

As explained above, we found that atrophy was not reversed in many 12-week muscle fibers. While this in itself may relate to the continuing low amplitude of the response, the fact that these fibers were still atrophied implies continued disuse, which would result if neuromuscular junctions had not yet normalized. It is also plausible that some fibers did not become reinnervated at all. This would account for the observation of empty profiles, presumed to represent dead fibers, at 3 months. A

study on evoked stimulation of the rabbit quadriceps muscle also indicated a profound failure of reinnervation even 6 months after BoNT treatment (Fortuna et al., 2013). In short, amplitude of evoked EMG remained low most likely because there were few fibers receiving the stimulus.

The normalized duration of evoked EMG at 12 weeks indicates that some fully functional motor units were present. These must have included the many hypertrophied muscle fibers observed at this time point. Only at 12 weeks was fiber diameter of the BoNT-injected muscles correlated with evoked EMG, especially at the superior level where hypertrophy was extreme. Hypertrophy implies strong activity, in this case compensatory for the atrophy observed in other fibers. As suggested above, the hypertrophied fibers may have been unaffected by toxin, perhaps because little or no BoNT reached their motor endplates. Alternatively, their molecular make-up (e.g., fiber type) may have been relatively resistant to the effects of the A serotype of BoNT (Coffield, Bakry, Maksymowych, & Simpson, 1999; Liu, Rafferty, Ye, & Herring, 2015) or able to recover rapidly. Type I (slow-twitch) fibers in rabbit masseters exhibited less atrophy than type II (fast-twitch) fibers after BoNT treatment (Korfage et al., 2012) and were less affected by dietary disuse than fast-twitch fibers (Takasu et al., 2019), although this latter finding may be due to their continued use even with a liquid diet. In BoNT-injected mouse hindlimb muscles, the slow-twitch soleus atrophied faster than the fast-twitch gastrocnemius, but also recovered much faster and more completely owing to more active nerve regrowth; necrotic fibers were seen only in the gastrocnemius (Duchen, 1970). Thus at least some of the recovered or hypertrophied fibers may have been Type I, and those that died Type II.

Oddly, the evoked EMG amplitude, although not duration, of the uninjected masseters was reduced compared to controls, although less so than the injected masseters. As discussed below, the effects of BoNT can be transmitted to the opposite side. However, it would be surprising for such effects to persist for 3 months, and the uninjected side muscles were always morphologically normal. Moreover, our previous study did not indicate any decrease in masticatory EMG or muscle force on the uninjected side at either 4 weeks or 12 weeks after treatment (Rafferty et al., 2012). Thus it is likely that the low evoked EMG on the uninjected side was an experimental artifact. The control animals were younger than the BoNT animals because they were processed soon after arrival; they were also from a different cohort of rabbits, and the evoked EMG procedures were performed at a different time, perhaps with an unnoticed change in technique. It is conceivable that younger animals actually

have a greater evoked EMG response or that the cohorts differed in some other way.

4.3 | Aim 3: How extensive are the effects of BoNT?

Our final goal was to assess whether the effects of the toxin were limited to the level of injection in the multipennate, short-fibered masseter muscle of the rabbit. It is not commonly appreciated that BoNT diffuses widely in an injected muscle. Using fiber size variability as an assessment, a low dose (1 unit) in rabbit longissimus dorsi showed diminishing effects 1.5–3 cm from the injection site, whereas a higher dose of 10 units (which is the amount we used for the rabbit masseter) showed no diminishment even 4.5 cm distant from the injection (Borodic, Ferrante, Pearce, & Smith, 1994). It is possible, however, that this is an overestimate of toxin spread, because the distant location might have sampled the same muscle fibers at different locations.

We found clear evidence of physiological and morphological effects, including fiber atrophy and hypertrophy, at a distance of about 1 cm from injection level, but these effects were attenuated compared to injection level, and compensatory hypertrophy was more apparent. It is possible that this was an artifact of sampling the same fibers twice, but masseter fibers are relatively short, and not many of them were likely to have been sectioned at both levels. An alternative (but nonexclusive) explanation is that there was toxin diffusion within the masseter. The smaller amount of toxin received would account for the lessened atrophy and fibrosis distant from the injection site.

BoNT can also reach adjacent muscles by diffusion (Botzenhart et al., 2020; Duchon & Strich, 1968), although we did not assess this in the present study. More surprisingly, distant muscles, including contralateral muscles, are sometimes affected. Larger doses in the rabbit longissimus study were associated with increases in acetylcholinesterase staining in both injected and contralateral muscles (Borodic et al., 1994), and repetitively injected rabbit hindlimbs showed substantial weakness and atrophy on both sides as well (Fortuna et al., 2011). Atrophic changes are unlikely to be due to adaptive modifications in motor behavior and must result from the toxin itself. Both central nervous system reactions and toxin entry into the circulatory system have been suggested (Ramirez-Castaneda et al., 2013). Occasional reports of generalized botulism resulting from single muscle injections (e.g., Fan, Wang, Chu, & Leung, 2016) would tend to support circulatory delivery of the toxin to the whole body. However, as reviewed recently (Muñoz Lora et al., 2019), BoNT also undergoes retrograde

transport to the central nervous system and interneuron transport within it. Although the possibility of experimental error cannot be eliminated, a generalized trigeminal depression could explain why the uninjected masseters of BoNT animals showed less evoked EMG amplitude than did control masseters.

4.4 | Limitations

The use of 4-week and 12-week endpoints was intended to coincide with maximal degeneration and full recovery, respectively. In retrospect, however, maximal degeneration probably occurs about a week earlier (Rafferty et al., 2012). In contrast, the 12-week recovery endpoint, while typical of rabbit studies (Korfage et al., 2012; Rafferty et al., 2012), may have been too brief, considering the slow recovery of human masseters (Ma et al., 2018). It remains unknown whether the changes we observed would ultimately resolve or worsen.

Rather than untreated controls, it might have been preferable to use unilaterally saline-treated shams, and it was not ideal to combine the two groups. However, our previous study (Rafferty et al., 2012) indicated no effect of saline treatment on either the treated or the untreated side, so this was probably not an important source of error.

The evoked EMG technique was imprecise, as the closely spaced EMG electrodes likely picked up signals from other locations. Also, both the nerve stimulation and EMG response depend on direct contact between electrodes and tissue, which may not have been fully maintained, leading to high variability. These problems, as well as the small sample size, may explain our inability to demonstrate regional differences or correlations between EMG and fiber diameter.

Finally, the control animals were used at a different age (5 vs. 6 and 8 months) and after different durations of residence in our facility (1 week vs. 5 or 13 weeks) than the BoNT rabbits. These differences may relate to the smaller maximum fiber diameters in the controls (Table 3) as well as their greater evoked EMG amplitudes relative to the uninjected muscles of the BoNT animals.

5 | CONCLUSIONS

Neuromuscular blockage of rabbit masseter fibers caused by a single BoNT application is not only responsible for widespread atrophy 1 month after injection, but also for persistent damage seen after 3 months. Improved strength and functional activity of the muscle at 3 months may be related to a population of muscle fibers that recovered early and hypertrophied in response. Within the treated muscle,

effects were attenuated at a distant location where neuromuscular junctions would have received less toxin. Both these observations suggest that the muscle effects are neurogenic in origin and are primarily due to failed or delayed reinnervation. Although some muscle fibers appeared to have died at 3 months, others were likely still recovering, as evidenced by modestly increased numbers of dividing cells and fibers with central nuclei. It is unknown if the changes in neuromuscular function and masseter histology are ultimately reversible, nor whether the human masseter reacts similarly. Nevertheless, these potential negative effects of BoNT application serve as a caution that this treatment may lead to permanent damage to neuromuscular function, or at the very least, a longer recovery than currently suggested.

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AUTHOR CONTRIBUTIONS

Michael C. Baldwin: Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); software (equal); visualization (lead); writing – original draft (supporting); writing – review and editing (supporting). **Zi Jun Liu:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (supporting); software (supporting); supervision (supporting); validation (supporting); visualization (equal); writing – original draft (supporting); writing – review and editing (supporting). **Katherine L. Rafferty:** Conceptualization (supporting); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (supporting); supervision (supporting); validation (supporting); writing – review and editing (supporting). **Andrew Keith:** Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Basma Tamamas:** Formal analysis (supporting); investigation (supporting); methodology (supporting); validation (supporting); writing – review and editing (supporting). **Karl Kaiyala:** Conceptualization (supporting); formal analysis (supporting); methodology (supporting); software (equal); validation (equal); writing – original draft (supporting); writing – review and editing (supporting).

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