Title: Early and continued manual stimulation is required for long-term recovery after facial nerve injury

Maria Grosheva MD, PhD^{1*}, Svenja Rink MD^{2*}, Ramona Jansen BSc^{2*}, Habib Bendella MD³ Stoyan P. Pavlov MD, Ph.D⁴, Levent Sarikcioglu PhD⁵, Doychin N. Angelov MD, PhD^{2#}, Sarah A. Dunlop PhD^{6#}

¹Department of Oto-Rhino-Laryngology, University of Cologne, 50924 Cologne, FRG

²Anatomical Institute I, University of Cologne, Germany

³Department of Neurosurgery, University of Witten/Herdecke, Cologne Merheim Medical Center (CMMC), Cologne, Germany

⁴Department of Anatomy, Medical University Varna, Bulgaria

⁵Department of Anatomy, Akdeniz University Faculty of Medicine, Antalya, Turkey

⁶Experimental & Regenerative Neuroscience, School of Biological Sciences, The University of Western Australia

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Correspondence to:

Prof. Dr. Doychin N. Angelov, Institut 1 für Anatomie der Universität zu Köln, Joseph-Stelzmann-Strasse 9, D-50924 Köln, Germany; Telephone: +49-221-478-5654; Fax: +49-221-478-87893; email: angelov.anatomie@uni-koeln.de

*Share first authorship

Share last authorship

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ABSTRACT

Introduction. We showed previously that manual stimulation of vibrissal muscles for 2 months after facial nerve injury (FFA) in rats improves whisking and reduces motor endplate poly-innervation. Here, we asked whether discontinuing or delaying MS after FFA would also lead to similar results.

Methods. Rats were subjected to FFA and received MS for: i) 4 months (“early & continued”), ii) the first but not the last 2 months (“discontinued”), or iii) the last 2 months (“delayed”). Intact animals and those not receiving MS (“no MS”) were also examined.

Results. Early & continued MS restored whisking amplitude to 43°, a value significantly higher compared to the discontinued, delayed, and no MS groups (32°, 24°, and 10°). Motor end-plate poly-innervation occurred in all experimental groups, but it was significantly higher in the delayed group.

Discussion. Early & continued MS results in better recovery than when it is either discontinued or delayed.

Keywords: facial nerve, surgical reconstruction, manual stimulation, timing, recovery

INTRODUCTION

Traumatic facial nerve injury in humans is relatively common due to lacerations, iatrogenic injuries, and combat.¹⁻⁴ Such injuries often lead to permanent synkinesis, i.e. unintentional movements accompanying volitional movement, ocular complications, impaired speech, feeding difficulties, and inability to convey emotion through facial expression.⁵⁻⁶

Although experimental studies using growth factors, cell transplantation, and biomaterials have shown functional benefits,⁷⁻¹³ clinical translation remains a challenge.¹⁴⁻¹⁵ In the absence of effective cellular and molecular therapies, facial nerve injuries are managed acutely using dynamic surgical procedures, including nerve grafting, anastomosis, crossover, and muscle transfer to neurotized flaps.^{2,17-18} Nevertheless, surgery alone seldom delivers functional recovery.¹⁹⁻²⁰ Although physical therapies, including neuromuscular re-education, stretching, mime therapy, biofeedback, and massage, are a major management approach to treat chronic synkinesis and achieve normal movement and expression,²¹⁻²⁴ there is limited evidence regarding the timing of intervention.^{1,25}

Clinically, soft tissue massage is a non-invasive therapy that can be used following nerve damage to improve blood flow, facial symmetry, and smiling.²⁶ Based on these findings in humans²⁴ we used our experimental rat model of facial nerve injury followed by surgical reconstruction with end-to-end facial nerve anastomosis (FFA) to develop a soft tissue massage technique and test whether it was similarly effective in restoring function. We used gentle, rhythmic manual stimulation²⁴ of the vibrissal muscle pads and showed that this simple intervention promoted recovery of whisking function and reduced the abnormal levels of poly-innervation of motor end-plates, structures that are normally only mono-innervated.²⁷⁻²⁹ Furthermore, we showed that similar non-invasive interventions also restored the blink reflex after FFA as well as

tongue position, and therefore presumably feeding, after hypoglossal nerve injury and reconstruction.^{30-32,21,33}

Manual stimulation therefore appears to be generalizable in its ability to restore function after injury to specific peripheral nerves. However, we had no evidence regarding the timing of treatment, i.e. whether it can be stopped or delayed.^{1,25} Our previous studies involved, “continuous” MS (i.e. 5 minutes per day, 5 days per week) for either 2 or 4 months.^{27,33} Here, we used the same rodent model to ask whether vibrissal function could still be restored to normal after FFA and the extent of motor end-plate poly-innervation reduced if MS was either: 1) given for 2 months and then stopped for 2 months, or 2) its start delayed for 2 months but then given for 2 months.

MATERIALS AND METHODS

Animals

Thirty-two female adult (175 - 200 g) Wistar rats (strain HsdCpb:WU, Harlan-Winkelmann, Borcheln, Germany) were used. Rats were fed standard laboratory food (Ssniff, Soest, Germany), provided tap water *ad libitum*, and kept in an artificial light-dark cycle of 12 hours on, 12 hours off. All experiments were conducted in accordance with the German Law on the Protection of Animals, and procedures were approved by the local Animal Care Committee (Az: 50.203.2-K 35,6/06; Az: 9.93.2.10.31.07.299).

Surgery

Experimental rats (n = 8 in each group) received unilateral transection and suture of the right facial nerve (facial-facial-anastomosis, FFA). The procedures were performed by Dr. M. Grosheva and Dr. Svenja Rink using surgical anesthesia (Ketamin/Xylazin (100 mg Ketanest[®], Parke-Davis/Pfizer, Karlsruhe, Germany, and 5 mg Rompun[®], Bayer, Leverkusen, Germany, per kg body weight; i.p.) as described earlier²⁷. In brief, the

facial nerve trunk was exposed and transected close to its emergence from the stylomastoid foramen (figure 1). The proximal stump was microsurgically reconnected to the distal stump with 2 11-0 atraumatic sutures (Ethicon, Norderstedt, Germany).

Manual mechanical stimulation of vibrissal muscles

Rats in Group 1 served as intact controls. Rats in Group 2 received no post-operative treatment (no MS). Rats in Groups 3-5 received MS of the right whisker pad muscles for 4 months (Group 3, “early & continued”), the first 2, but not the last 2 months (Group 4, “discontinued”), or only the last 2 months (Group 5, “delayed”).

Manual mechanical stimulation was started in Groups 3 and 4 the day after surgery and in Group 5 on the first day of the third postoperative month. Stimulation involved gentle rhythmic forward stroking of the right vibrissae and whisker pad muscles by hand for 5 minutes per day, 5 days per week and mimicked natural active vibrissal movements during whisking, i.e. active protraction and passive retraction.²⁷ Animals became accustomed to this procedure within 2-3 days and did not show any signs of stress such as freezing, trying to bite, weight loss, or lack of grooming.

Vibrissal motor performance and its analysis

In intact animals, whisker movements are coordinated and comprise either large-amplitude exploratory vibrissal sweeps through protraction/retraction cycles (frequency of 5-11 Hz) and low-amplitude “foveal” or “palpating” whisker movements (15-25 Hz).³⁴ Facial nerve transection abolishes exploratory sweeps, and whiskers become caudally oriented and either motionless or showing denervation-induced tremors.³⁵ With time, and as we show here, varying levels of motor performance are regained. The return of exploratory sweeps can be readily assessed using non-invasive video-based motion analysis.^{27,29}

In brief, with the exception of 2 large vibrissae in row C on each side of the face, all vibrissae were clipped under light anesthesia, and animals were videotaped for 3-5 minutes during active exploration (Digital Camcorder: Panasonic NV DX-110 EG; 50 Hz; 50 fields per second; shutter open for 4 ms per cycle). Sequences in which the animal's head was stable for 1.5 s were selected for analysis of the following parameters: i) amplitude (difference between maximal retraction and maximal protraction in degrees; ii) whisking frequency (cycles of protraction and retraction, i.e. backward movement, per second); iii) protraction (forward vibrissal movement, measured by the angle (in degrees) between the rostral midsagittal plane and the whisker shaft (Figure 1 B & C; lower angles represent maximal protractions, i.e. rostrally directed, while higher angles represent reduced protractions, i.e. more caudally directed); and iv) angular velocity during protraction (degrees per second). Measurements were performed by 3 observers (S.K. Angelova, D. Bösel, D. Felder) blinded to treatment.

We are aware that facial paralysis deviates slightly the peak of rat's nose from the midline toward the non-injured side of the face and that this deviation has an impact on the measurements. Nevertheless, since this deviation remains more or less the same for all experimental groups, we feel confident that our comparisons are objective and plausible.

Video-based motion analysis on intact rats has been undertaken and published previously (Group 1, Intact #1, n = 16, Table 1).²⁷ To check that these original data were robust, we also undertook video-based motion analysis on a separate group of intact animals (Group 1, Intact #2, n = 12, Table 1). As expected, there was no significant difference between the Group 1, Intact #1 and Group 2, Intact #2 for any parameter ($P > 0.05$) thus confirming the invariant nature of whisking behavior in normal animals. Data from Group 1, Intact #1 were used for comparison with the 4 experimental groups using 1-way ANOVA with *post-hoc* Tukey tests and significance

level of 0.05 (Statistica 6.0 software; StatSoft, Tulsa, OK, USA).

Analysis of target muscle reinnervation

As described previously³⁶, the levator labii superioris (LLS) muscle was dissected, cryoprotected in sucrose, and sectioned (longitudinal; 30 μ m). Sections were immunostained (rabbit polyclonal antibody against neuronal class III β -tubulin; Covance, Richmond, CA, USA, No. PRB-435P, 1:1000; Cy3-conjugated anti-rabbit IgG; 1:400; Sigma). Motor end-plate boundaries were visualized using acetylcholine receptor staining with Alexa Fluor 488-conjugated alpha-bungarotoxin (Molecular Probes, 1:500).

The LLS is an extrinsic vibrissal muscle responsible fundamentally for whisker retraction. Still, a great amount of this movement is due to the viscoelastic properties of upper lip tissue. Thus, it is at least of equal importance to evaluate intrinsic muscle innervations, since they are responsible for whisker protraction and, therefore, for most whisking mechanics. Since, the intrinsic vibrissal (sling) muscles are very thin (<1 mm) and distributed along the convex surface of the whisker pad (animal's snout), a thorough histological evaluation using a plausible section selection strategy would be very difficult.

Numbers of axonal branches that either entered or left individual end-plates were counted directly under the fluorescence microscope (objective x40) by observers blinded to treatment group. Endplates with 1 axon were identified as "mono-innervated", and those with 2 or more axonal branches were identified as "poly-innervated". We also determined whether there were any denervated motor endplates i.e. with no visible axon. Poly-innervated endplates are morphologically similar to those seen after other types of nerve damage in which collateral (arising from nodes of Ranvier), and/or terminal (arising near the end-plates), branching occurs.³⁷⁻³⁸ Individual motor endplates may therefore be innervated by >1 motoneuron, (poly-neuronally

innervated). We use the term poly-innervated, rather than poly-neuronally to reflect numbers of axons innervating motor endplates rather than their perikaryal origin.

We are aware of the fact that, although an axonal branch may cross a given end-plate, this is not sufficient proof of poly-innervation; it only demonstrates exuberant sprouting of the axon terminal but not necessarily abnormal establishment of multiple functional synaptic contacts on a single end-plate. In any event, this methodological restriction was valid for all experimental groups.

Every third section was analyzed. All individual motor endplates were identified (using α -bungarotoxin) and numbers of axons (using β III-tubulin) crossing the boundaries of the end-plate determined by focusing through the depth of the section. We included all individual endplates except those on the upper or lower surface that appeared to be cut. Measurements were performed by 3 observers (R. Jansen, D. Bösel, D. Felder) blinded to treatment. Data from intact rats (Group 1, Intact #1, n = 8, Table 2) showing 100% mono-innervation have been published previously²⁷ and, as expected, did not differ from the 100% mono-innervation observed in the separate group of intact animals (Group 1, Intact #2, n = 6, Table 2). Data from Group 1, Intact #1 were used for comparison with the 4 experimental groups. Frequencies of mono-, poly-, and non-innervated end-plates were expressed in percentage of the total population and group mean values compared with the 2-sided *t*-test for independent groups.

RESULTS

Early & continued manual stimulation resulted in the best long-term recovery of vibrissal whisking

Amplitude of whisking in intact animals was $57.0 \pm 13.0^\circ$ (Table 1; Figure 2). Following FFA, the best outcome was seen in animals that had MS starting within 1 day of facial

nerve reconstruction and that continued daily for 5 days per week for 4 months (Group 3, early & continued: $43.0 \pm 9.0^\circ$). This value was significantly higher than in animals that received MS for only the first, but not the last, 2 months (Group 4, discontinued: $32.1 \pm 17.8^\circ$, $P < 0.05$) or only the last 2 months, (Group 5, delayed: $24.4 \pm 10.8^\circ$; $P < 0.05$). However, values in the group receiving early & continued MS were nevertheless significantly lower than those in the intact group (25% less; $P < 0.05$; Table 1, Figure 2). As expected, the worst outcome was observed in animals that did not receive any MS for the 4-month period (Group 2, $10.5 \pm 5.4^\circ$).

With respect to the other parameters, there was no difference in frequency, angle of maximal protraction and angular velocity during retraction between Group 1 (intact) and any of the experimental groups (Table 1).

The degree of poly-innervation of motor endplates was highest in animals with delayed MS.

In intact animals, all motor end-plates were innervated by 1 axon i.e. they were mono-innervated (Figure 3). We did not observe any non-innervated motor endplates (Table 2). Compared to intact animals, the proportion of poly-innervated motor endplates, i.e. those innervated by ≥ 2 axons, was significantly increased in all experimental groups ($P < 0.05$) (Figure 3). Comparison of the 4 experimental groups (Table 2) showed that there was a significantly ($P < 0.05$) higher proportion of poly-innervated motor endplates in Group 5 (delayed: $15.8 \pm 5.6\%$) compared to the other experimental groups (Group 2, no MS: $7.1 \pm 1.1\%$; Group 3, early & continued: $8.4 \pm 0.9\%$; Group 4, $8.4 \pm 1.9\%$).

Functional outcome is not reflected by the degree of motor endplate poly-innervation

When considering data for the amplitude of whisking and that for poly-innervation of motor endplates, there appears to be no correlation between the degree of functional

recovery and the quality of target muscle re-innervation. Thus, although the amplitude of whisking was significantly higher in Group 3 (early & continued) compared to Group 2 (no MS) and Group 4 (discontinued), the degree of motor endplate poly-innervation did not differ (Tables 1 and 2). Furthermore, although the amplitude of whisking in Group 5 (delayed) did not differ compared to Group 2 (no MS) and Group 4 (discontinued), the degree of poly-innervation was significantly ($P<0.05$) higher.

DISCUSSION

We extend our experimental evidence for the contribution of manual stimulation to recovery of vibrissal function in rodents following facial nerve injury.^{27,33} We show that MS started within 1 day of FFA and continued for 4 months resulted in the best whisking amplitude, although performance was significantly less than in intact animals. Discontinuing MS after 2 months or delaying its start for 2 months did not result in any better recovery than in animals without MS. In addition, whereas we have previously reported an association between improved whisking amplitude and reduced motor endplate poly-innervation over a 2-month period, here we did not find such an association over a 4-month period.

The rodent vibrissae as a model for human facial nerve injury

Facial nerve innervation of rodent whiskers is equivalent to human facial muscle innervation and distinct from facial hairs, which are innervated by noradrenergic sympathetic axons, not peripheral motor axons.^{48,49} After facial nerve injury in humans, rehabilitation aims to restore the subtle facial movements that underpin communication and quality of life.²⁵ The rodent whisker system is equally crucial, since in these largely nocturnal animals, vibrissae convey information about the surrounding world to the brain.⁵⁰

Timing of intervention in relation to surgery and facial nerve regeneration

In our model, we have consistently started MS the day after surgery to allow recovery as well as to minimize any possibility that MS, albeit a gentle rhythmic massaging motion, would disrupt the surgical repair.^{27,30,28,39,33}

The time course of facial nerve regeneration in the rat has been examined in several models. In THY1-gfp transgenic rats, after nerve crush, regenerating axons cross the injury by 7 days, reach recipient stumps by 14 days, and have increasing axonal labeling between 4 and 8 weeks as axons reach target muscles.⁴⁰ Similarly, semi-quantitative analysis of vibrissal function after nerve crush shows initial movements at 17 days, indicating some target muscle reinnervation, and there is improvement in whisking function between 3 and 4 weeks.⁴¹ These findings after crush injury accord with our earlier observations of some, albeit abnormal, whisking function between 2 and 4 weeks after facial nerve section and end-to-end anastomosis.⁴² In our paradigm, surgical repair is early and reflects a general clinical consensus for the benefits of early surgical intervention.⁴⁶⁻⁴⁷ Also, MS is started before, and coincides both with axon regeneration and the first signs of weak whisker movement.⁴³⁻⁴⁵ Early MS in our model thus also reflects clinical consensus that some acute retention of function leads to better prospects for rehabilitation.⁴⁶⁻⁴⁷

Promoting recovery and the timing of intervention after facial nerve injury

We have previously shown that MS started 1 day after surgery and continued 5 days per week for 2 months resulted in whisking amplitude that did not differ from intact animals.^{27,33} By contrast, we report here that MS started 1 day after surgery and continued 5 days per week for 4 months post-operatively resulted in whisking function that was significantly lower than in intact animals, and these values agree with a

previous study that also delivered MS for 4 months.³³ The data suggest that, MS for 2 months after FFA restores normal whisking amplitude. However, continued MS for twice the time (4 months) results in whisking function that, although only somewhat lower than normal, is nevertheless significantly so. Studies that extend the time course to beyond 4 months after FFA will be required to determine whether this level of whisking function is maintained by MS or whether it continues to decline further.

Our findings also show that either delaying MS for 2 months or discontinuing it after 2 months resulted in a worse outcome compared to a regimen of continued MS for 4 months, although this latter regimen was sub-optimal. Our relatively simple model suggests that early and continued MS results in a better outcome (whisking amplitude) compared to delayed, discontinued, or no MS.

Compared to our rodent model, not unsurprisingly, the clinical situation is more complex. Although it has been previously reported that patients referred for rehabilitation months to years after nerve repair surgery are unlikely to benefit from neuromuscular retraining²⁵, evidence is accumulating that rehabilitation is beneficial even if it is delayed. A randomized controlled trial for patients with long-term facial paralysis (>9 months) showed significant improvements after mime therapy.²⁶ A larger analysis examining acute (days) to chronic (months – years) facial paralysis showed reasonable evidence for the effectiveness of rehabilitation and mime therapy.¹ Our study showing that continued MS is required to maintain function in the rat is supported by a large study (N = 303) in which patients had long lasting improvements with continued treatment that included education, neuromuscular training, and massage over 5 years.²¹

Our findings showed that MS that was either delayed for 2 months or discontinued after 2 months gave the worst outcome. This contrasts with a single case study which showed that 13 years after facial nerve injury, 7 weeks of intensive facial

exercise followed by 16 weeks of moderate training resulted in significant improvements in facial muscle strength which was maintained even after 24 weeks rest.²³ However, this single case study involved a 10 year-old at the time of injury, which suggests that juveniles respond better than adults. We used adult rats (12 weeks old), thus experiments in juvenile rats will be required to examine this issue further.

Timing of intervention and poly-innervation

Poor recovery following facial nerve injury is related to collateral axonal branching⁵², failure of axons to enter their original nerve fascicles, and misrouting of axons to target muscles⁵³. Intramuscular axonal sprouting also leads to re-innervation of incorrect muscle fibers and poly-innervation of normally mono-innervated motor-endplates.^{54,43,37} Misrouting and poly-innervation are maladaptive, since they can result in a single muscle fiber being controlled by 2 or more asynchronous, and often functionally different, motoneurons.^{54,37}

Here, we also report increases in motor endplate poly-innervation in target muscles. Compared to intact animals, about half of all motor end-plates are poly-innervated at 2 months after FFA without MS; MS halves the amount of poly-innervation.²⁷ In this study, we were initially surprised that whisking amplitude in the early and continued group at 4 months was reduced compared to 2 months of MS.²⁷ Furthermore, we are also surprised that the reduced whisking amplitude was accompanied by lower, rather than higher, poly-innervation.²⁷ An explanation is therefore required for the lower percentages of poly-innervation seen in this study with a 4-month end-point compared to the study by Angelov et al.²⁷ with a 2-month end-point. It is reasonable to assume that, at the beginning of the intervention in the delayed group, the degree of poly-innervation would have been the same as animals receiving no MS for 2 months (i.e. 53%).²⁷ If so, then the degree of poly-innervation at 4

months in the delayed group (15%) is similar to that originally reported after just 2 months MS (22%).²⁷ In other words, it appears that MS can reduce poly-innervation even if the intervention is delayed. It would be interesting to determine whether MS continued beyond 4 months in a delayed group would result in a further drop in poly-innervation.

The low degree of poly-innervation in this study is supported by a companion study with a 4-month end-point, in which MS following FFA resulted in low values (~6%).³³ Taken together, the data suggest that, regardless of the above interventions post-FFA, there is a natural attrition of poly-innervated motor endplates over the extended 4-month time-course. Furthermore, it appears that, whereas we have previously hypothesized that poor vibrissal function is associated with a high degree of poly-innervation of motor endplates in target muscle,^{27,29} our data showing the same degree of poly-innervation in animals with good (Group 3: early & continued) as well as poor (Group 2: no intervention; Group 4: discontinued) whisking amplitude does not support this hypothesis. Other factors centrally will need to be considered to determine efficacy of manual stimulation in restoring functional recovery after facial nerve injury including peripheral^{55 57 39,56} and cortical changes.^{58, 59,,60}

ABBREVIATIONS

FFA	facial-facial anastomosis
GABA	gamma-amino-butyric acid
MS	manual stimulation
Thy-1 gfp	thymocyte antigen-1, green fluorescent protein

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FIGURE LEGENDS

Figure 1: Surgical procedure. (a) Schematic drawings of the infratemporal portion of the rat facial nerve and 3 of its sub-branches adapted from Dörfl⁴⁸. Transection and immediate end-to-end suture of the facial nerve trunk, i.e. facial-facial anastomosis (FFA), indicated by an arrow. Diagrams show derivation of angles of protraction (b) and retraction (c) for video-based motion analysis. Op-side is operated side, Fr – Occ denotes the mid-sagittal line. Adapted from Guntinas-Lichius et al.³⁶

Figure 2: Amplitudes of vibrissal whisking. The best recovery (significantly greater than Groups 2, 4, and 5, $P < 0.05$) was observed in Group 3 which received early and continued MS for 4 months; however, values were nevertheless significantly lower in intact animals.

Figure 3: Motor end-plates in levator labii superioris muscles of intact and surgically treated rats visualized by staining of the motor end-plates with Alexa Fluor 488 α -bungarotoxin (green fluorescence) and immunostaining of the intramuscular axons for neuronal class III β -tubulin (Cy3 red fluorescence). (A) Example of a monoinnervated end-plate (arrow) with several preterminal rami. (B) Example of a polyinnervated end-plate by 3 axonal branches (arrows). (C) Percentage of polyinnervated end-plates. All experimental groups had significantly increased proportions of polyinnervated end-plates compared to intact animals ($P < 0.05$). However, animals which received MS for the last 2 months (delayed) had the highest proportion of polyinnervated end-plates ($P < 0.05$).

TABLE LEGENDS

Table 1: Vibrissal motor performance after facial nerve reconstruction and manual stimulation for different time periods. ^{#1} indicates previously published data from intact rats.²⁷ ^{#2} indicates data from another group of intact rats. There were no significant differences between the 2 groups; data from Group 1, Intact ^{#1} were used for statistical analysis. FFA is facial – facial nerve end-to-end anastomosis. MS is manual stimulation given for varying times. Values are means $\pm$ standard deviation. Superscript numbers indicate group/s that are significantly different.

Table 2: Quality of target muscle reinnervation after facial nerve reconstruction and manual stimulation for different time periods. Groups are as in Table 1.. Values are % (means $\pm$ standard deviation) of mono-innervated, poly-innervated or non-innervated motor end-plates. Superscript numbers indicate group/s that are significantly different.

Table 1

Group	Amplitude (degrees)	Frequency (Hz)	Angle at maximal protraction (degrees)	Angular velocity during protraction (degrees/sec)
Group 1, Intact # ¹ (N = 16)	57.0±13 ^{2, 3, 4, 5}	7.0 ± 0.8	62.0 ± 13 ^{2, 3, 4, 5}	1238 ± 503 ^{2, 3, 4, 5}
<i>Group 1, Intact #²</i> (N = 12)	<i>55.5 ± 5.7</i>	<i>6.7 ± 0.7</i>	<i>61.8 ± 5.4</i>	<i>1149 ± 159</i>
Group 2: FFA only for 4 months (“no MS”)	10.5 ± 5.4 ^{1, 3}	6.5 ± 1.0	102.0 ± 18.1 ¹	680 ± 440 ¹
Group 3: FFA+MS for 4 months (“early and continued”)	43.0±9.0 ^{1,2,4,5}	5.8 ± 1.0	93.7 ± 16.0 ¹	505 ± 101 ¹
Group 4: FFA+MS for the first 2, but not the last 2 months (“discontinued”)	32.1 ± 17.8 ^{1, 3}	6.9 ± 0.8	78.1 ± 23.1 ¹	570 ± 242 ¹
Group 5: FFA+MS for the last 2 months only (“delayed”)	24.4 ± 10.8 ^{1, 3}	6.4 ± 0.9	77.3 ± 31.3 ¹	441 ± 186 ¹

Table 2

Group	Mono-innervated motor end-plates	Poly-innervated motor end-plates	Non-innervated motor end-plates	Total number of motor end-plates examined
Group 1, Intact ^{#1} (N = 8)	100 ± 0 ^{2, 3, 4, 5}	0 ± 0 ^{2, 3, 4, 5}	0	1,543 ± 132
Group 1, Intact ^{#2} (N = 6)	100 ± 0	0 ± 0	0	100 per animal
Group 2: FFA only for 4 months (“no MS”)	92.8 ± 1.2 ^{1, 5}	7.1 ± 1.1 ^{1, 5}	0.1 ± 0.1	1,514 ± 204
Group 3: FFA+MS for 4 months (“early and continued”)	91.2 ± 1.1 ^{1, 5}	8.4 ± 0.9 ^{1, 5}	0.4 ± 0.2	1,259 ± 283
Group 4: FFA+MS for the first 2, but not the last 2, months (“discontinued”)	91.3 ± 1.8 ^{1, 5}	8.4 ± 1.9 ^{1, 5}	0.3 ± 0.2	1,286 ± 155
Group 5: FFA+MS for the last 2 months only (“delayed”)	84.1 ± 5.4 ^{1,2,3,4}	15.8 ± 5.6 ^{1,2,3,4}	0.1 ± 0.1	1,365 ± 184