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Original Article

Effect of rocuronium on the level and mode of pre-synaptic acetylcholine release by facial and somatic nerves, and changes following facial nerve injury in rabbits

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Abstract: Muscles innervated by the facial nerve show differential sensitivities to muscle relaxants than muscles innervated by somatic nerves. The evoked electromyography (EEMG) response is also proportionally reduced after facial nerve injury. This forms the theoretical basis for proper utilization of muscle relaxants to balance EEMG monitoring and immobility under general anesthesia. (1) To observe the relationships between the level and mode of acetylcholine (ACh) release and the duration of facial nerve injury, and the influence of rocuronium in an *in vitro* rabbit model. (2) To explore the pre-synaptic mechanisms of discrepant responses to a muscle relaxant. Quantal and non-quantal ACh release were measured by using intracellular microelectrode recording in the orbicularis oris 1 to 42 days after graded facial nerve injury and in the gastrocnemius with/without rocuronium. Quantal ACh release was significantly decreased by rocuronium in the orbicularis oris and gastrocnemius, but significantly more so in gastrocnemius. Quantal release was reduced after facial nerve injury, which was significantly correlated with the severity of nerve injury in the absence but not in the presence of rocuronium. Non-quantal ACh release was reduced after facial nerve injury, with many relationships observed depending on the extent of the injury. The extent of inhibition of non-quantal release by rocuronium correlated with the grade of facial nerve injury. These findings may explain why EEMG amplitude might be diminished after acute facial nerve injury but relatively preserved after chronic injury and differential responses in sensitivity to rocuronium.

Keywords: Rocuronium, pre-synaptic, acetylcholine, quantal release, non-quantal release, facial nerve

Introduction

Evoked electromyography (EEMG) is widely used during surgery that is undertaken in proximity to the facial nerve to reduce the risk of iatrogenic facial nerve injury [1, 2]. The EEMG response depends on relatively intact electrophysiological signal transmission at the facial neuromuscular junction, which is blocked by muscle relaxants acting at the nicotinic acetylcholine receptor (nAChR). We have previously shown that when using a small dose of muscle relaxant to achieve partial neuromuscular blockade (PNMB) in skeletal muscles, we could achieve sufficient neuromuscular block to facilitate surgery while preserving sufficient facial nerve neuromuscular transmission to permit the use of EEMG [3]. These findings are consistent with those of other studies [4, 5], and suggest that differences might exist in sensitivity to neuromuscular blocking drugs between facial and skeletal muscles. This hypothesis has been confirmed both *in vitro* in rats and *in vivo* in rabbits [6, 7].

The amplitude of the EEMG response is reduced in patients whose facial nerve function is impaired before surgery [8], while monitoring has become increasingly important in identifying emerging irritation or developing injury to the nerve. For example, in the setting of tumor related facial nerve injury like acoustic neuroma with the intention of facilitating intervention to reverse neurophysiologic manifestations.

Our previous *in vitro* and *in vivo* studies found that the amplitude of contraction and EEMG response of the orbicularis oris were reduced if

the facial nerve had been injured [6, 7], and the extent of EEMG depression correlated with the severity and duration of facial nerve injury [7]. Therefore, evoked electromyography should therefore be interpreted with particular caution in patients with pre-existing impairment of facial nerve function.

Non-depolarizing muscle relaxants bind the nAChR in the post-synaptic membrane to block neuromuscular transmission, and inhibit the level of pre-synaptic acetylcholine (ACh) release [9]. We have found that differences in sensitivity to muscle relaxants between facial and skeletal muscles were explained by nAChR density at the endplates, the affinity of the nAChR to muscle relaxants [6], and the inhibitory effect of muscle relaxants on pre-synaptic ACh release [10].

Acetylcholine is subject to quantal and non-quantal release from the pre-synaptic membrane, representing vesicular and non-vesicular release, respectively. The former mainly mediates neuromuscular transmission, while the latter is an important trophic factor during endplate development and in the mature neuromuscular junction [11, 12]. Thus, we hypothesized that the extent of facial nerve injury might influence ACh quantal or nonquantal release, which meditates, at least in part, differences in sensitivities to muscle relaxants in the injured facial nerve-innervated muscles.

Therefore, we compared the effect of rocuronium on quantal and non-quantal ACh release at the neuromuscular junction of the facial and tibial nerves. We also examined the influence of mild, moderate and severe facial nerve injury on both types of ACh release over six weeks. Finally, we examined whether mild, moderate and severe facial nerve injury might contribute to differences in sensitivity to rocuronium at the neuromuscular junction with the orbicularis oris. We used a facial nerve injury rabbit model that allowed determination of the extent of facial nerve injury.

Materials and methods

The local ethics committee of Shanghai Jiaotong University (Shanghai, China) approved the conduct of the study. One hundred and fourteen male New Zealand rabbits (Experimental Animal Centre of the School of Medicine, Shanghai Jiaotong University, Shanghai, China), weighing 2.0-2.5 kg, were used in the study.

Facial nerve injury model

The rabbits were fasted but had free access to water for 12 to 18 hrs prior to surgery. Facial nerve injury was induced using the crush axotomy model as previously described. [13] Briefly, rabbits were anesthetized with intramuscular injections of ketamine at 40 mg/kg and diazepam at 5 mg/kg. The facial nerve was exposed on both sides through buccal incisions. A crush was made at the middle point of the buccal branch of the facial nerve between the auricle and the angulus oris. A standard crush using the same batch of vessel clamps was performed for a defined period resulting in a graded injury according to the Sunderland criteria [14], in which crushing for 30 sec results in a grade I injury, 60 sec for a grade II injury and 120 sec for a grade III injury [7]. Finally, the wound was closed using 4-0 skin sutures (Ethicon, Johnson & Johnson Medical Ltd, Shanghai, China). After surgery, all rabbits were allowed free access to food and water for 1-42 days before undergoing the second procedure. During the breeding period, the animal would be euthanized using an overdose anesthetic if the animal presented with eating difficulties or obvious weight loss.

Experimental protocol

The experiment consisted of two parts. The first was designed to compare the response of the orbicularis oris muscles innervated by uninjured facial nerve and gastrocnemius muscles innervated by the tibial nerve. Six rabbits were used. The effect of treatment with rocuronium was measured on muscles located on the left side of the animal, and the right sides were used as controls.

The second part compared the response of the orbicularis oris muscles that were innervated by a facial nerve that was subject to mild, moderate or severe injury according to the Sunderland scale; in this experiment 108 rabbits were used. The left orbicularis oris muscles were designated as the rocuronium treatment group, and the contralateral sides were designated the rocuronium non-treatment group. Muscles were incubated with an irreversible cholinesterase inhibitor in the second proce-

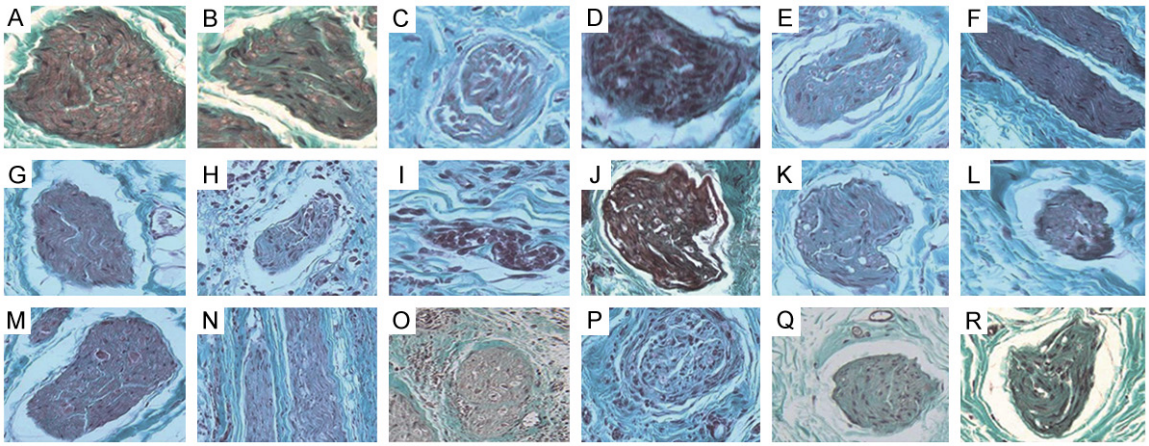

Figure 1. Histopathological specimens of injured facial nerve tissue after modified trichrome staining (× 400 magnifications). A-F: Sunderland I injured facial nerve after 1, 3, 7, 14, 28 and 42 days appear almost normal at each time point with the integrated perineurium and axonal continuity; G-L: Sunderland II injured facial nerves after 1, 3, 7, 14, 28 and 42 days show evidence of demyelination; M-R: Sunderland III injured facial nerves after 1, 3, 7, 14, 28 and 42 days show impairment of late axonal regeneration by the presence of intrafunicular fibrosis, which obstructs or diverts regenerating nerves from their proper paths.

dure to measure non-quantal release. Therefore, facial nerve injuries were required on both sides. Data that was collected from the normal facial nerve from the first part of the protocol were used as controls.

According to the random number table method, all rabbits were randomly divided into three groups containing 36 rabbits each, and according to the designated severity of injury from mild to severe: groups DI (Sunderland grade I injury) to DIII (grade III). Subsequently, each group was divided into six subgroups containing six rabbits each, in which intracellular microelectrode measurements could be undertaken 1, 3, 7, 14, 28 and 42 days after the facial nerve injury.

Preparation of in vitro specimens

Rabbits were anesthetized by an intravenous injection of sodium pentobarbital 25 at mg/kg. The buccal branches of the facial nerves were then exposed. The facial nerves with the accompanying orbicularis oris muscles were dissected into rectangles of approximately 1.5-2.0 cm in the direction of the muscle fibers and 2 cm perpendicular to the muscle fibers. Specimens were then placed in a tissue bath, and then perfused continuously with the Ringer-Krebs solution comprising (all in mmol/l): NaCl 120; KCl 5.0; $CaCl_2$ 2.0; $MgCl_2$ 1.0; $NaHCO_3$ 23; NaH_2PO_4 1.0 and glucose 11. The solution was maintained at a pH of 7.2-7.4, at 20°C and bubbled with 95% O_2 and 5% CO_2. Drugs of interest were added to the Ringer-Krebs solution as required [15].

Intracellular microelectrode recording

The isolated nerves connected to the muscle strips were mounted on the hook of the stimulating electrode. The distance between the two electrodes was 1 mm. The resistance of the recording electrode was 15-25 MΩ and the electrode was filled with 3 mmol/L potassium acetate. Microelectrodes were then inserted into nerve fibers within the muscle using an anatomical microscope. A bio-signal processing system (Model SMUP-E, provided by the Department of Physiology, School of Medicine, Fudan University, China) was used to record small changes in membrane potential from the resting potential, known as spontaneous miniature end-plate potentials (MEPP) that were caused by spontaneous release of individual vesicles. Then, motor nerves were subject to supra-maximal stimulation (square waves with a frequency of 1 Hz, a width of 0.1 ms and a voltage of 8 V) and the end-plate potentials (EPP) that were caused by the release of multiple vesicles were recorded. The ratio between EPP and MEPP (expressed as EPP/MEPP) reflected the quantal release of ACh from the pre-synaptic membrane [11, 16]. The specimens were treated with the irreversible cholin-

Table 1. Effect of rocuronium on the amount of quantal and non-quantal ACh release in the muscles innervated by facial and tibial nerves (n = 6, $\bar{x}$ ± s)

	Orbicularis oris			*Gastrocnemius*		
	Non-treatment	Treatment	Discrepancy	Non-treatment	Treatment	Discrepancy
MEPP (mV)	0.90 ± 0.06	0.90 ± 0.50		0.94 ± 0.08	0.87 ± 0.06	
EPP (mV)	26.05 ± 0.69	23.00 ± 0.36[c]		25.04 ± 0.48[a]	17.00 ± 0.75[d]	
Quantal release	29.07 ± 2.22	25.58 ± 1.13[c]	3.49 ± 1.25	26.80 ± 2.22[b]	19.65 ± 1.95[e]	7.15 ± 2.36[f]
$RMP_{paraoxon}$ (mV)	-31.97 ± 0.21	-32.00 ± 0.67		-33.02 ± 0.30	-33.46 ± 0.63	
$RMP_{paraoxon + tubocurarine}$ (mV)	-43.00 ± 0.74	-42.08 ± 0.44		-44.63 ± 0.32	-45.00 ± 0.52	
Non-quantal release (mV)	11.02 ± 0.56	10.08 ± 1.02	0.95 ± 1.34	11.603 ± 0.56	11.54 ± 1.06	0.07 ± 1.14

[a]*P < 0.05*, *gastrocnemius vs. orbicularis oris* in the non-treatment group; [b]*P < 0.05*, gastrocnemius *vs.* orbicularis oris in the non-treatment group; [c]*P < 0.05*, treatment group *vs.* non-treatment group at orbicularis oris; ([d]*P < 0.01*, and [e]*P < 0.01*) treatment group *vs.* non-treatment group at gastrocnemius; [f]*P < 0.05* orbicularis oris *vs. gastrocnemius.*

esterase inhibitor, paraoxon (Sigma, USA) at a dose of 10 μmol/L for 30 min and then rinsed several times with 0.9% NaCl solution. The resting membrane potential (RMP) was then measured at the endplate zone. Then, preparations were treated with 10 μmol/L of tubocurarine (Sigma, USA) for 5 min and the RMP was measured again. The differences between the RMPs under these two conditions (known as the H-effect) are generally considered to represent the non-quantal release of ACh (expressed as $RMP_{paraoxon}$-$RMP_{paraoxon + tubocurarine}$). Next, 10 μmol/L of choline chloride (Sigma, USA) was added to the Ringer-Krebs solution to delay the post-denervation decrease in non-quantal ACh release [17].

Effect of rocuronium on quantal and non-quantal ACh release

The specimens in the treatment groups were bathed with rocuronium (Merck Sharp & Dohme, China) 10 μg/ml for 5 minutes before any other drugs were added. Then EPP and MEPP were recorded, and the amount of quantal ACh release was calculated. Then, RMPs at the endplate zone were recorded with the presence of paraoxon and tubocurarine successively to measure the amount of non-quantal ACh release. The effect of rocuronium was established by calculating the difference between the treatment and non-treatment groups.

Histopathologic examination

At the end of all experiments, tissue sections were obtained from the same segments of the injured facial nerves of each rabbit to ascertain the integrity of the facial nerve using modified trichrome staining under light microscopy (oil immersion objective at × 400 magnification). The investigators who took the pathological measurements were blinded to the severity of nerve injury. If the results of this examination did not match the expected degree of injury, data collected from that animal were excluded from the analysis [7].

Statistical analysis

Sample size was determined using an alpha value of P = 0.05 and a power of 90%. Taking into account the mean values and standard deviations for the primary outcome ACh quantal and non-quantal release, based on our pilot study we determined that the sample size should be at least five animals for each group. All data are presented as the mean ± standard deviation and were analyzed using the SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). The paired Student's t test was used to compare treatment and non-treatment groups. The intra-group and the inter-group mean values were analyzed by randomized block analysis of the variance, whereas the differences between any two groups were identified by the S-N-K test and Dunnett's T3 test for heterogeneity of variance. Correlation analysis of the extent of quantal and non-quantal ACh release with the extent of facial nerve injury was undertaken using Spearman's test. A P value of less than 0.05 was considered statistically significant.

Results

Histopathologic examination of the injured facial nerves was consistent with the characteristics of grade I to III nerve injury as described by Sunderland (**Figure 1**) [14]. As a result, no animals were excluded from the analysis.

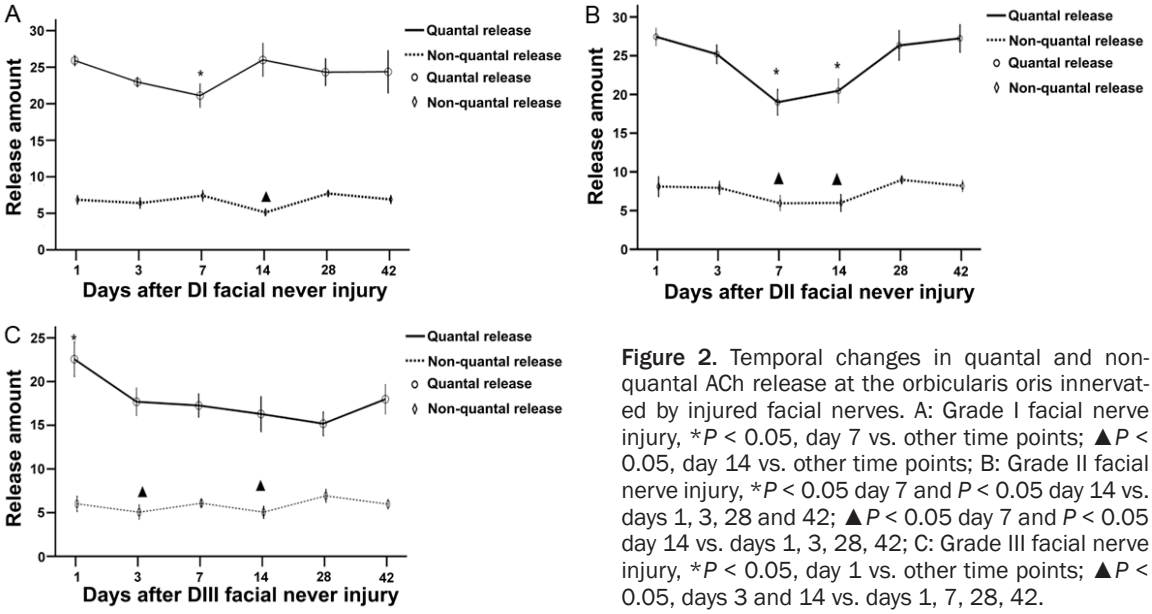

Figure 2. Temporal changes in quantal and non-quantal ACh release at the orbicularis oris innervated by injured facial nerves. A: Grade I facial nerve injury, **P* < 0.05, day 7 vs. other time points; ▲*P* < 0.05, day 14 vs. other time points; B: Grade II facial nerve injury, **P* < 0.05 day 7 and *P* < 0.05 day 14 vs. days 1, 3, 28 and 42; ▲*P* < 0.05 day 7 and *P* < 0.05 day 14 vs. days 1, 3, 28, 42; C: Grade III facial nerve injury, **P* < 0.05, day 1 vs. other time points; ▲*P* < 0.05, days 3 and 14 vs. days 1, 7, 28, 42.

Changes in the level of quantal and non-quantal ACh release: comparisons of facial and tibial neuromuscular junctions

There were no significant differences in the MEPP or amount of non-quantal ACh release at the facial or tibial neuromuscular junctions (*P* = 0.278 for MEPP, *P* = 0.118 for ACh non-quantal release). However, baseline EPP and quantal ACh release were greater in the orbicularis oris than in the gastrocnemius (*P* = 0.033 for EPP, *P* = 0.041 for ACh quantal release). As expected, the amplitude of the EPP and the extent of quantal ACh release decreased significantly after treatment with rocuronium (*P* = 0.006 for EPP, *P* = 0.023 for ACh quantal release), and this was significantly more apparent in the gastrocnemius than in the orbicularis oris (*P* = 0.004 for EPP, *P* = 0.001 for ACh quantal release). There were no significant differences seen in the frequency and amplitude of MEPP, $RMP_{paraoxon}$, $RMP_{paraoxon + tubocurarine}$, and the level of non-quantal ACh release that was found in either the rocuronium treatment or non-treatment groups or in the orbicularis oris and the gastrocnemius (**Table 1**).

Influence of nerve injury severity on the innervation of the orbicularis oris

We found that quantal release of ACh in facial nerve-orbicularis oris preparations was significantly reduced at a variety of time points after more substantial nerve injuries. In the least severely injured facial nerves (DI), quantal ACh release was significantly reduced at day 7 after injury as compared with other time points (*P* = 0.031). In the DII group it was significantly reduced at days 7 (*P* = 0.041) and 14 (*P* = 0.038), and in the most severely injured (DIII) preparations release was significantly reduced at all time points as compared with day 1 (*P* = 0.026) (**Figure 2A-C**). In contrast, non-quantal release of ACh was only significantly reduced after a couple of time points. Non-quantal ACh release after mild (DI) nerve injury was significantly reduced at day 14 as compared with other time points (*P* = 0.034). In the DII group, non-quantal release was significantly reduced at days 7 (*P* = 0.032) and 14 (*P* = 0.036), and in the DIII group it was significantly reduced on day 3 (*P* = 0.027) and 14 (*P* = 0.033, **Figure 2A-C**).

Treatment with rocuronium after mild, moderate and severe facial nerve injuries significantly reduced quantal ACh release at all times (*P* < 0.05, **Table 2**). Non-quantal release was also significantly reduced by rocuronium at all times after moderate and severe injuries, but only at day 7 after mild injury (*P* < 0.05, **Table 2**). We calculated the reduction value in quantal and non-quantal ACh release caused by treatment with rocuronium by subtracting the values in

Table 2. Effect of rocuronium on the level of quantal and non-quantal ACh release in *the orbicularis oris* innervated by injured facial nerves of differing severity and over time (n = 6, $\bar{x} \pm s$)

Grade of Injuries	Recovery time (days)	ACh quantal release			ACh non-quantal release (mv)		
		Non-treatment	Treatment	Discrepancy	Non-treatment	Treatment	Discrepancy
Normal		29.1 ± 2.2	25.6 ± 1.1	3.5 ± 1.3	11.0 ± 0.6	10.1 ± 1.0	1.0 ± 1.3
DI	1	25.9 ± 1.0	22.0 ± 0.9*	3.9 ± 1.4	6.9 ± 0.9▲	7.2 ± 0.5	-0.3 ± 0.9△
	3	23.0 ±0.9▲	19.2 ± 2.9*	3.8 ± 2.9	6.4 ± 1.1▲	6.7 ± 0.9	-0.3 ±1.4△
	7	21.1 ± 2.4▲	15.2 ± 1.1*	5.9 ± 2.4	7.4 ± 1.1▲	3.8 ± 0.7*	3.6 ± 1.3▲,▽
	14	26.0 ± 3.4	18.7 ± 0.7*	7.3 ± 4.0	5.1 ± 0.7▲	5.2 ± 1.1	-0.1 ± 1.3△
	28	24.3 ± 2.7	20.1 ± 1.0*	4.2 ± 2.8	7.7 ± 0.6▲	8.5 ± 1.6	-0.8 ±1.9△
	42	24.4 ± 4.3	19.2 ± 2.5*	5.1 ± 3.7	6.9 ± 0.8▲	7.0 ± 0.9	-0.5 ± 1.6△
DII	1	27.4 ± 1.6	22.9 ± 1.7*	4.6 ± 3.2	8.1 ± 1.89▲	5.0 ± 0.8*	3.1 ± 1.4▲
	3	25.2 ± 1.7▲	21.6 ± 4.0*	3.6 ± 3.1	7.9 ± 1.3▲	5.9 ± 1.3*	2.1 ± 1.4
	7	19.0 ± 2.5▲	12.6 ± 3.9*	6.4 ± 4.7	5.9 ± 1.5▲	3.0 ± 1.2*	2.9 ± 2.2
	14	20.5 ± 2.3▲	14.2 ± 2.6*	6.3 ± 2.9	6.0 ± 1.6▲	2.9 ± 0.8*	3.1 ± 2.1
	28	26.3 ± 2.8	21.1 ± 4.1*	5.2 ± 2.6	9.0 ± 0.6▲	5.9 ± 0.6*	3.1 ± 1.0
	42	27.2 ± 2.6	23.1 ± 2.8*	4.1 ± 2.2	8.2 ± 1.0▲	5.9 ± 1.2*	2.3 ± 1.6
DIII	1	22.6 ± 2.9▲	17.5 ± 1.4*	4.8 ± 2.2	6. ± 1.3▲	3.7 ± 0.9*	2.3 ± 2.0◆
	3	17.7 ± 2.3▲	12.1 ± 1.2*	5.6 ± 2.5	5.1 ± 1.2▲	3.2 ± 0.9*	1.9 ± 0.9◆
	7	17.3 ± 2.0▲	7.2 ± 1.0*	10.1 ± 2.5▲	6.1 ± 0.8▲	1.8 ± 0.7*	4.3 ± 0.9▲
	14	16.3 ± 3.0▲	10.1 ± 3.4*	6.2 ± 3.9	5.1 ± 1.0▲	2.0 ± 1.1*	3.0 ± 1.1
	28	15.2 ± 2.0▲	10.1 ± 1.9*	5.1 ± 3.0	6.9 ± 1.1▲	4.1 ± 0.5*	2.8 ± 1.0
	42	18 ± 2.5▲	12.5 ± 3.0*	5.4 ± 4.2	6.0 ± 0.8▲	4.9 ± 0.9*	1.1 ± 1.0◆

*P < 0.05, treatment group *vs.* non-treatment group; ▲P < 0.05, injured facial nerve group *vs.* normal facial nerve group; △P < 0.05, DI *vs.* DII and DIII; ▽P < 0.05, day 7 *vs.* days 1, 3, 14, 28 and 42 in the DI group; ◆P < 0.05, days 1, 3 and 42 *vs.* days 7, 14 and 28 in the DIII group.

the treatment groups from the corresponding values in the non-treatment groups. The discrepancy in ACh quantal release caused by rocuronium was independent of the severity of facial nerve injury at all time points determined (P > 0.05, **Table 2**), but the discrepancy in the level of non-quantal ACh release was significantly different between the DI, DII and DIII nerve injury groups on days 1, 3, 14, 28 and 42. In addition, the discrepancy seen after DI injury was significantly less than that recorded after DII and DIII injuries (P < 0.05, **Table 2**). The discrepancy in the level of non-quantal release was significantly greater on day 7 in the DI group (P < 0.05, **Table 2**), not significantly different at any time in the DII group (P > 0.05, **Table 2**), and significantly greater on days 7, 14 and 28 in the DIII group (P < 0.05, **Table 2**).

The extent of quantal release of ACh was negatively correlated with the severity of facial nerve injury (**Figure 3**), but not the non-quantal release. On days 1, 3, 14 and 28 after injury, the discrepancy in non-quantal release of ACh caused by rocuronium was positively correlated with the severity of facial nerve injury. However, quantal release was only positively correlated with the severity of facial nerve injury on day 7 (**Figure 4**). Moreover, neither the discrepancy in quantal or non-quantal ACh release was correlated with the recovery time.

Discussion

This study is an evolution of our previous research, which indicated that the extent of pre-existing facial nerve injury influenced the magnitude of the EEMG response. However, that study did not demonstrate the sensitivity to muscle relaxants when using an automatically controlled and designated unilateral facial nerve injury model in rabbits *in vivo* [7]. The current study is aimed to test the hypothesis that the reduction of the EEMG response might result from changes in pre-synaptic ACh quantal or nonquantal release by facial nerve injury, and the related sensitivity to muscle relaxants. We used a bilateral facial nerve injury model in

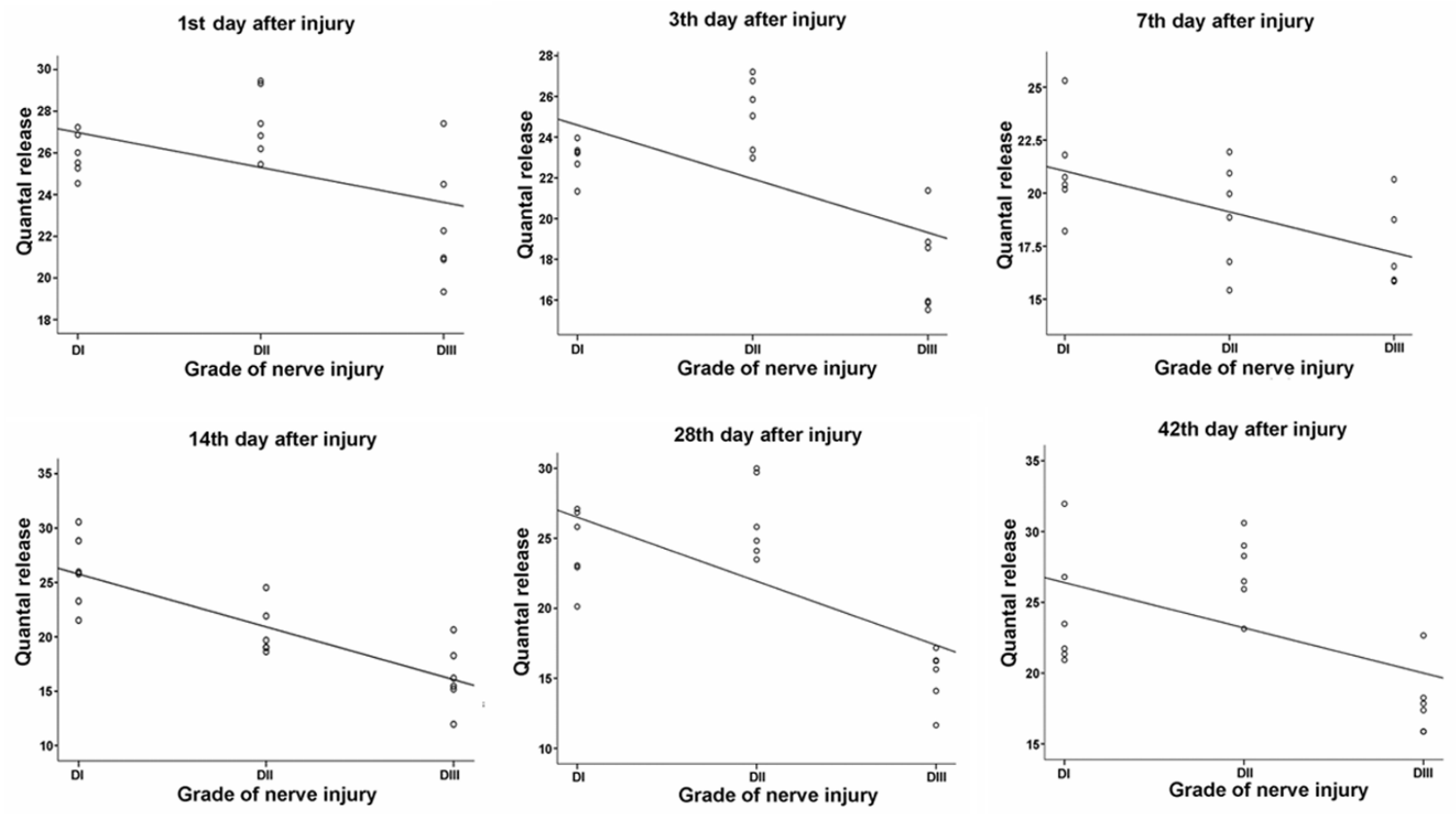

Figure 3. Correlation analysis of quantal ACh release by DI-III injured facial nerves at each post-injury time point. Day 1: r = -0.472, *P* = 0.027; Day 3: r = -0.610, *P* = 0.015; Day 7: r = -0.599, *P* = 0.037; Day 14: r = -0.829, *P* = 0.023; Day 28: r = -0.639, *P* = 0.035; Day 42: r = -0.693, *P* = 0.034.

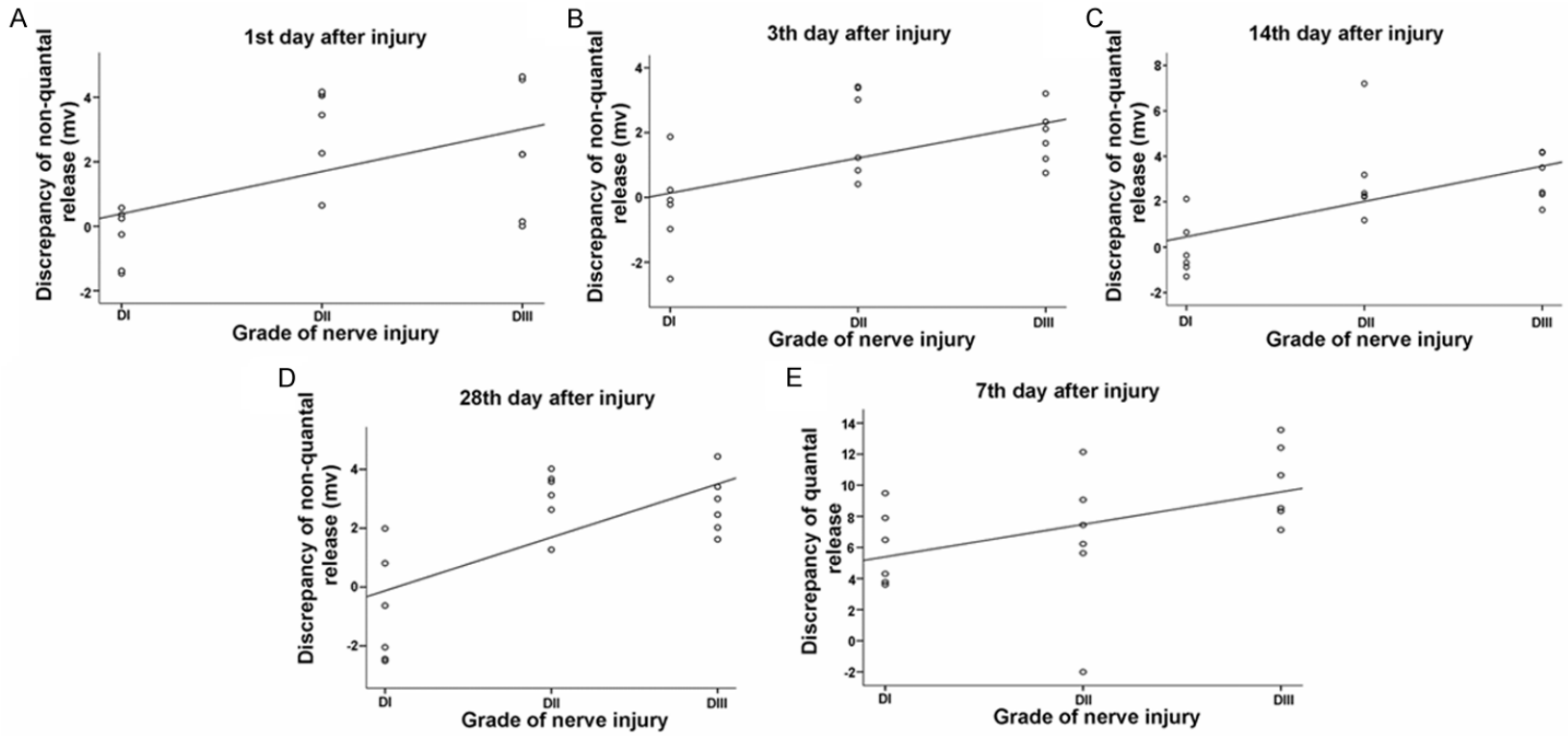

Figure 4. Correlation analysis *showing the effect of rocuronium on* ACh release by DI-III injured facial nerves at each post-injury time point. (A: r = 0.498, *P* = 0.026; B: r = 0.564, *P* = 0.043; C: r = 0.631, *P* = 0.038; D: r = 0.686, *P* = 0.040; E: r = 0.538, *P* = 0.045).

rabbits because of the use of an irreversible cholinesterase inhibitor, which is necessary for detecting nonquantal ACh release. The results demonstrated that rocuronium was more inhibitory on quantal ACh release at the gastrocnemius than at the orbicularis oris. ACh release is reduced after facial nerve injury, and the extent of quantal release is negatively correlated with the severity of facial nerve injury, but not the non-quantal release. Rocuronium significantly reduces ACh quantal release after facial nerve injuries, although this appears not to correlate with the severity of injury. Rocuronium does not affect non-quantal ACh release at the normal neuromuscular junction, but reduces non-quantal release in injured facial nerves. Our observations provide some new information to help explain the differences that are evoked by the electromyographic responses in surgeries referring to normal or pre-surgically injured facial nerves, as well as the strategy for the use of muscle relaxants in these situations, at least from the perspective of the presynaptic view.

The MEPP reflects minute changes in the resting potential of the pre-synaptic membrane at the neuromuscular junction caused by spontaneous or random release of a quantum (or a vesicle) of ACh. The EPP is produced by the simultaneous release of ACh from multiple quanta (e.g., 100-300 vesicles). The amplitude of the EPP changes continuously with the release of ACh. Therefore, the EPP/MEPP ratio reflects the amount of ACh quantal release [11]. Our previous study detected the effect of rocuronium on the EPP and MEPP responses in orbicularis oris muscle strips that were innervated by facial nerves and gastrocnemius muscle strips innervated by tibial nerves. We found that rocuronium had no effect on the frequency or amplitude of MEPP but it significantly decreased the amplitude of EPP. This suggested that rocuronium inhibits simultaneous release of multiple pre-synaptic vesicles, and hence reduces muscle contraction [10]. Our findings were consistent with other studies of the neuromuscular junction between the phrenic nerve and the diaphragm [18, 19]. We also compared differences in quantal ACh release at the facial and tibial neuromuscular junctions and confirmed that the differences we found might have contributed to the distinct sensitivities of the two to rocuronium [6, 10]. However, we did not detect an effect of rocuronium treatment on non-quantal ACh release and the correlated changes after nerve injury.

This study was based on our previous approach, which used the "H-effect" theory to measure the amount of non-quantal ACh release. Theoretically, if acetylcholinesterase (AChE) is inhibited at the neuromuscular junction, sufficient ACh should accumulate in the synaptic gap to cause significant post-synaptic membrane depolarization. In fact, only a small and slowly-evolving depolarization of the muscle fibers is evident, which does not evoke muscle fiber fasciculation that can be eliminated by post-synaptic nicotinic receptor blockers, such as (+)-tubocurarine or α-bungarotoxin (αBGT). The method of inducing hyperpolarization by applying (+)-tubocurarine or αBGT into the synaptic zone (or into the bath) through a micropipette has been referred to as the "H-effect" [12]. Therefore, the amount of non-quantal ACh release can be measured by subtracting the RMP measured in the presence of an irreversible AChE inhibitor (e.g., paraoxon) from the RMP that is measured after the addition of post-synaptic nAChR blockers (e.g., tubocurarine). Thus, non-quantal ACh release more closely reflects the amount of ACh released from the motor nerve terminal in its resting state, which may comprise 98% of the total ACh release, at least according to some reports [20]. We found that rocuronium did not change the RMP in the presence of paraoxon and tubocurarine, showing that rocuronium does not affect the amount of non-quantal ACh release, and only inhibits quantal ACh release evoked by an action potential. This result showed that rocuronium, which is a clinically and widely used muscle relaxant, inhibited presynaptic neuronal signal transmission that was evoked by a nerve impulse, in addition to an effect on the post-synapitc nAChRs.

Generally speaking, factors affecting ACh release include Na^+-K^+-ATPase [21, 22], mitochondria [23], pre-synaptic nAChRs [24] and voltage-dependent calcium channels (VDCC) [25-27]. Current thinking is that quantal release of ACh relies on vesicle exocytosis that is triggered by the activation of pre-synaptic nAChRs and the opening of VDCCs; while non-quantal release of ACh is a consequence of sporadic random vesicle exocytosis and pre-synaptic ACh reuptake [12]. Some investigators have reported that rocuronium might inhibit ACh quantal release by blocking pre-synaptic nAChRs [9]. We have previously reported that there is a higher density of nAChRs with a lower

affinity to rocuronium at the neuromuscular junction of the facial nerve with the orbicularis oris as compared with that found between the tibial nerve and the gastrocnemius [6]. We thus speculated that differences in nAChR distribution and their affinity to rocuronium might explain differences seen in quantal release of ACh. The lack of an effect of rocuronium on non-quantal release might be due vesicle exocytosis playing only a relatively minor role.

The extent of facial nerve injury peaked between day 7 and 14 days. If we define day 1 as the hyper-acute period, days 2 to 3 as the acute period, days 7 to 28 as the intermediate period and days 28 to 42 as the chronic period, the most severe damage was evident under light microscopy in the intermediate period. Accordingly, quantal ACh release was diminished most after more severe nerve tissue injury (**Figure 3**). For the same severity of injury, quantal ACh release was reduced in the intermediate period and then recovered gradually, except in the most severely injury group (DIII). This might explain our previous finding of a relationship that was observed between the severity of facial nerve injury and the magnitude of the reduction in the EEMG response [7]. It also concurs with the work of Weber *et al* who showed that if the nerve had only been stretched and not seriously bruised or torn, then there might be a complete recovery of function within 6 weeks. However, under conditions of a more severe stretch and some bruising, then the nerve could take about 3 months to recover [28]. In contrast, the change in non-quantal ACh release showed different relationships with the extent of nerve injury at different times. However, it was reduced in all levels of injury as compared with the controls. In the hyper-acute and the intermediate phases it was not affected by the severity of nerve injury, whereas in the acute and chronic phases, it was reduced in the more extensively injured nerve tissues.

Nerve injury is associated with impaired mitochondrial function [29], and thus the function of Na^+-K^+-ATPase in turn reduces pre-synaptic ACh reuptake, which is the main source of ACh for non-quantal release [23]. Due to the large number of pathophysiologic factors influencing nerve injury, we cannot draw any firm conclusions about the nature of the changes during non-quantal ACh release over time after facial nerve injury. The current study provided a profile of the fluctuation of neurotransmitter during the development of facial nerve injury and gave information that could help guide management of evoked electromyography with the aim of directly targeting the pathologic course.

Rocuronium reduced the amount of quantal ACh release and the corresponding EPP values at each level of facial nerve injury and at all times afterwards when compared with controls. However, there was no significant difference seen in the extent of reduction, suggesting that there is no link between the pharmacological effect of rocuronium and the pathophysiology of nerve injury. We have previously reported that the density of nAChRs at the neuromuscular junction between the facial nerve and the orbicularis oris was significantly reduced after facial nerve injury. However, the affinity of the remaining nAChRs to rocuronium did not change, which led to reductions in the muscular tension amplitude *in vitro* [6] and the EEMG amplitude *in vivo*. By contrast, the non-affected EEMG response to rocuronium in the chronicity of the injuries [7]. Therefore, we reasoned that a reduced density of pre-synaptic nAChRs that retained normal affinity to rocuronium might explain the relationship between ACh quantal release and severity of nerve injury. However, we found that inhibition of non-quantal ACh release by rocuronium was influenced by the severity of facial nerve injury (**Figure 4**), making a pre-synaptic mode of action less likely, as vesicle exocytosis plays a relatively unimportant role in non-quantal ACh release.

The limitation of the study includes such factors as the MEPP amplitude being miniscule and easily subject to interference by baseline noise. The extent and duration of facial nerve injury is challenging to precisely quantify for individual variances. Rocuronium does not represent a mechanism of all kinds of muscle relaxant. Findings in animal models might also not be generalized to human subjects or to clinical practice. Finally, we have discussed the study without due consideration to the non-muscle-relaxant anesthesia; for example, in the use of remifentanil. Overall, in the situations in which remifentanil induced circulation depression, it should be avoided, or it should

not be present on the formulary of the institution. Finally, partial neuromuscular blockade should be an easily managed anesthetic technique.

In conclusion, rocuronium inhibits quantal ACh release less effectively at the facial nerve-orbicularis oris neuromuscular junction than at a somatic neuromuscular junction. Quantal ACh release is also reduced after facial nerve injury, the extent of reduction correlates with the severity of injury, which likely explains the reduction in EEMG response seen in clinical practice. The facial nerve injury also influences the pharmacologic effect of rocuronium on ACh quantal release, although this appears not to correlate with the severity of injury. Rocuronium does not affect non-quantal ACh release at the normal neuromuscular junction, but non-quantal release by the facial nerve is also influenced by the severity of nerve injury. Our findings suggest that one of the mechanisms for using partial neuromuscular blockade during surgical anesthesia that requires facial nerve monitoring may be due to the differential inhibitory effect of rocuronium on ACh quantal release. Thus it will not further impair pre-synaptic neurotransmitter release, which has already been influenced by nerve injury itself.

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Disclosure of conflict of interest

None.

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