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Effects of neuromuscular presynaptic muscarinic M_1 receptor blockade on rocuronium-induced neuromuscular blockade in immobilized tibialis anterior muscles

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Summary

This in vivo study tested the hypothesis that the modulation of acetylcholine (ACh) release by the M_1 muscarinic receptor (mAChR) in the neuromuscular junction of disused muscles may affect the tensions of the muscles during the neuromuscular monitoring of a rocuronium-induced neuromuscular block and compared the results with those obtained from normal muscles. A total of 20 C57BL/6 (wild-type) and 10 α7 knock out (α7KO) mice were used in this experiment. As a pre-experimental procedure, knee and ankle joints of right hind limbs were fixed by needle pinning at the 90° flexed position. After 2 weeks, the main experiment was performed. Both tendons of the tibialis anterior (TA) muscles were obtained, and the muscle tensions were recorded while the dose-responses of rocuronium were measured three times in the same mouse by the serial administration of pirenzepine (0, 0.001 and 0.01 μg/g). Weight losses were observed after 2 weeks of immobilization in both groups, and a decrease in the mass of TA muscles at the immobilized side was observed compared to those of the contralateral nonimmobilized side. Tension depression of the TA muscles at immobilized side of the α7KO group was faster than those of the wild-type group, but these differences decreased after the administration of pirenzepine. The tension depressions were similar regardless of the pirenzepine doses at the same side in the group. Tension depression may become more rapid in the α7 AChR-expressed disused muscles by the decreased release of ACh release upon neuronal firing by the blockade of facilitatory M_1 mAChR

KEYWORDS

acetylcholine release, muscarinic acetylcholine receptors, muscle atrophy, neuromuscular junction, neuromuscular nondepolarizing agents, rocuronium

1 | INTRODUCTION

Several types of receptors can be found at the neuromuscular presynaptic junction.[1,2] Among these receptors, facilitatory M_1 and inhibitory M_2 muscarinic receptors (mAChRs) regulate the fine-tuning actions of release of acetylcholine (ACh) upon neuronal firing, and these receptors are in turn regulated by purinergic receptors.[3-7] Facilitatory M_1 mAChRs are predominant under the low-frequency neuronal stimulation (less than 5 Hz).[4] At the same time, purinergic A_1 receptors are activated by low concentrations

of adenosine, which is coreleased with ACh during neuronal stimulation at the synapse.[5,6] On the other hand, when the frequency of evoked stimulation is high (> 50 Hz), the amount of adenosine is increased to levels capable of activating the facilitatory adenosine A_{2A} receptors which counteract the M_1 receptors and potentiates M_2 inhibitory receptors.[5-7]

In normal innervated muscles, the mature form of nicotinic acetylcholine receptors (nAChRs) is present only in the neuromuscular postsynaptic area and is involved in neurotransmission. When the neuronal influence or activity is depressed, the γ subunit containing immature acetylcholine receptors (AChRs) are upregulated and expressed throughout the muscle membrane.[8-10] Neuronal AChRs containing five homometric α_7-subunits, which were described previously only in the central nervous system, have more recently been described in the skeletal muscle after denervation only.[11] In some pathologic states, despite the intact innervations, the upregulation of immature or atypical AChRs occurs. Several studies have shown that the disuse of a muscle leads to muscle atrophy and the de novo expression of immature AChRs throughout the muscle membrane, despite the presence of continued innervation.[12-14] The expression of the immature AChRs in the junctional area has been assumed to contribute to the resistance to non-depolarizing neuromuscular blocking agents during immobilization. Recently, there have been some reports of α7 nAChR expression after immobilization that revealed some important roles in the resistance to a neuromuscular blocking agent, such as rocuronium.[14,15] In the clinical setting, there would be some changes on responses of neuromuscular blockade when the patient is in the long-time immobilized state, such as the cast of the part of the body or respiration therapy by the artificial respirator. In that situation, the modulation of the ACh release at the neuromuscular presynaptic side and thus the change of the concentration of ACh on the same neuronal firing would eventually affect the response of muscle on the nerve stimulation for neuromuscular monitoring.[3] This might be due to the mature form of nAChR, and α7 nAChR shows a different response to ACh or rocuronium.[14,16,17]

As such, in this study, we tested the hypothesis that the reduction in ACh release by the modulation of M_1 mAChRs in the neuromuscular junction (especially those of the muscles after immobilization) could influence the tensions of the muscles during neuromuscular monitoring of rocuronium-induced neuromuscular block. To accomplish this, mechanomyographic techniques together with pirenzepine in wild-type mice and mice genetically processed not to express the α7 subunits of the AChR (α7KO mice) were used. In addition, the hypothesis was tested in the immobilized tibialis anterior (TA) muscles of wild-type and αs7KO mice and compared with those in the contralateral normal ones.

2 | RESULTS

Overall, decreases in bodyweight and TA muscle mass were observed during the immobilization period in all genotypes (Table 1). No significant differences in the bodyweight were observed between the wild-type and α7KO group. Weight losses were observed after 2 weeks of immobilization in both groups, and a decrease in the mass of TA muscles at the immobilized side was observed compared to those of contralateral non-immobilized side. After a 2-week period of immobilization, there was an average weight loss of 1.07 g and 1.81 g in the α7KO mice and wild-type mice, respectively. After completing the experiment, the TA muscles of both the immobilized and contralateral sides were harvested and their weights were compared. In all genotype mice, there were significant differences between the immobilized and the contralateral sides ($P < 0.05$). The mean differences of weights of TA muscles were 12.2 mg and 9.29 mg in the α7KO mice and the wild-type mice, respectively (12.2 (4.57) vs 9.29 (5.65), $P > 0.05$).

Initially, the rocuronium dose-responses to the different pirenzepine doses at the same side of the same genotype were compared. In the normal (contralateral) side of the wild-type mice, there were no significant changes in the rocuronium dose-responses by injecting and increasing pirenzepine. This was also observed in the immobilized side, but there was a decrease at low pirenzepine dose (0.01μg/g, PZP1) and an increase at high dose (0.1μg/g, PZP2). These findings were observed consistently in the α7KO mice (Figure 1).

The rocuronium dose-responses were compared at immobilized and contralateral side of the wild-type mice, and the same comparison was performed in the α7KO mice. In the wild-type mice, there were significant differences between the immobilized and the contralateral sides at the initial stage, but these differences disappeared at PZP1 and PZP2 (Table 2, Figure 2b,d,f). In α7KO mice, however, there were significant differences between both sides at the initial stage and these differences were maintained despite the injection of pirenzepine (Table 2, Figure 2a,c,e).

TABLE 1 Whole bodyweight at the initial and 2 weeks after immobilization, tibialis anterior muscle weight of immobilized side and contralateral side

	BW initial (g)	BW immo (g)	TA control (mg)	TA immo (mg)
Wild (n = 10)	27.32 (1.87)	25.51* (1.63)	46.09 (3.82)	35.77** (4.19)
α7KO(n = 10)	27.21 (1.55)	26.14* (1.49)	46.99 (3.11)	34.79** (5.80)

Data are expressed as the mean (SD). Wild, wild-type mice; α7KO, α7 knockout mice; BW initial, bodyweight at initial; BW immo, bodyweight at 2 weeks after immobilization; TA control, tibialis anterior muscle weight of the contralateral side; TA immo, tibialis anterior muscle weight of immobilized side.

*P = 0.001 and 0.000 in wild and α7KO, respectively.

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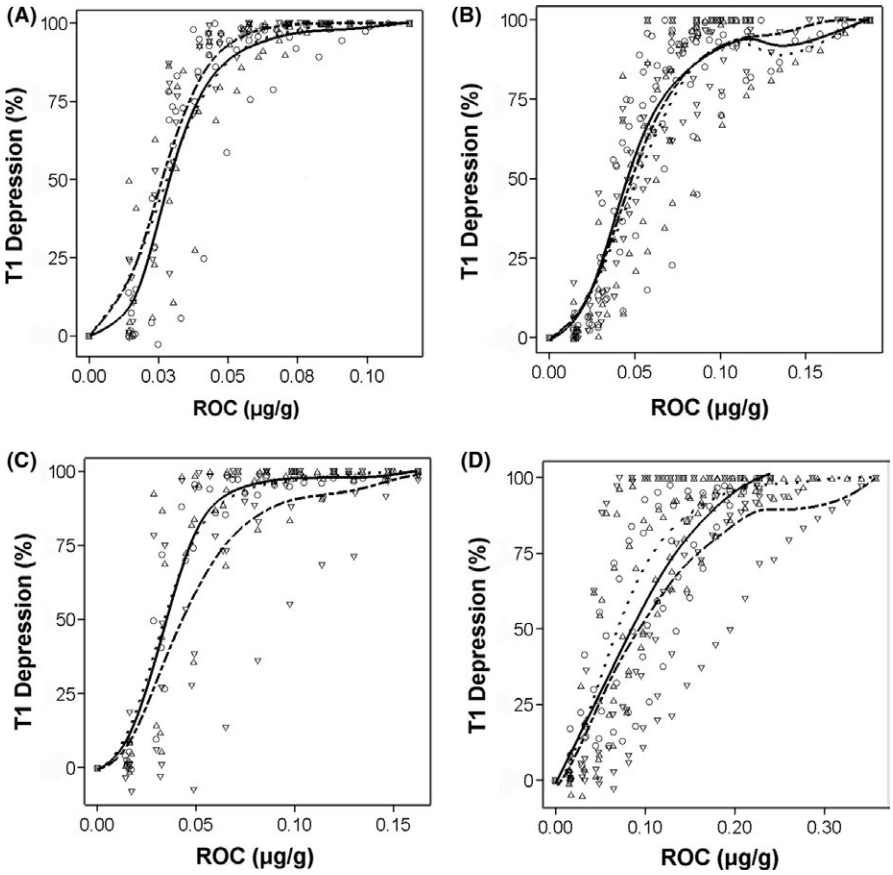

FIGURE 1 Comparison of the progression of the *T*1 depression according to time. (A) Contralateral normal side of the α7KO mice. (B) Immobilized side of the α7KO mice. (C) Contralateral normal side of the wild-type mice. (D) Immobilized side of the wild-type mice. In the normal (contralateral) side of the wild-type mice, there were no significant changes in the rocuronium dose-responses by injecting and increasing pirenzepine. This was also observed in the immobilized side, but there was a decrease at low pirenzepine dose (0.01μg/g, PZP1) and an increase at high dose (0.1μg/g, PZP2). These findings were observed consistently in the α7KO mice. (○—) Initial; (△·····) PZP1, period at the injection of pirenzepine 0.01 μg/g; (▽·····) PZP2, period at the injection of pirenzepine 0.1 μg/g; ROC, cumulative doses of rocuronium

We also compared data by the genotype at the initial, PZP1 and PZP2, respectively. At the initial and PZP1 stage, there were no significant differences in ED_{50} and ED_{95} between the wild-type and α7KO mice in the contralateral (normal) side. The ED_{50} and ED_{95} in each genotype were different at PZP2. But in the immobilized side, this difference pattern was quite different. At initial, there were significant differences in ED_{50} and ED_{95} between wild-type and α7KO mice. These differences disappeared when the pirenzepine was injected (Table 3).

Tension depression of the TA muscles at the immobilized side of the α7KO group was significantly faster than those of the wild-type mice, but these differences were decreased after the administration of pirenzepine. Regardless of the administration of pirenzepine, no statistically significant differences on train-of-four (TOF) fade on the same side in the wild-type and α7KO group were observed ($P > 0.05$, Table 4). However, when compared with the immobilized and contralateral normal side, differences of TOF fade disappeared at the high dose of pirenzepine (PZP2) in the wild-type group ($P = 0.014$ and 0.001 in initial and PZP1, respectively, $P = 0.103$ in PZP2, Table 4).

3 | DISCUSSION

In this in vivo experiment, the action modulation of M_1 mAChRs by the specific antagonist, pirenzepine, affected the dose-responses of rocuronium and the rocuronium-induced TOF fade when compared in the immobilized and normal muscles.

At low-frequency (<5 Hz)-evoked stimulation, the facilitatory M_1 mAChR function is known to have a predominant role in the evoked release of ACh.[4] Because the M_1 mAChRs have a facilitator effect on the release of ACh, its antagonism by pirenzepine may decrease the amount of acetylcholine released by evoked stimulation. This, in turn, can affect the twitch tension of the TA muscle that is generated by indirect supramaximal stimulation of the ipsilateral sciatic nerve. In our previous ex vivo experiments,[3] it was demonstrated that the blockade of presynaptic M_1 mAChR with pirenzepine led to a lower requirement of rocuronium for a > 95% decrease in the twitch tension of the haemidiaphragm. In the present result, rocuronium needed for a >95% depression of the T1 twitch tension at the immobilized side was decreased in the PZP1 and to increased in PZP2 at the immobilized side of wild-type mice compared to the initial value. Although there were no significant differences between the initial, PZP1 and PZP2, these findings were comparable to those found at the previous ex vivo experiment.[3] The inclination for the decrease in rocuronium used in PZP1 might be due to the decrease in ACh release which is the blockade of the M_1 mAChR by pirenzepine. One limitation is that it is unclear whether the dose and incubation time of pirenzepine used in this in vivo experiment were appropriate. As there was no evidence or references of adoptable pirenzepine doses used in a similar study, this pirenzepine dose was initially set based on previous results of the ex vivo experiment,[3] bioavailability[18] and protein-binding affinity of pirenzepine (http://druginfo.co.kr/cp/msd/ingredient/ingre_view_cp.aspx?cppid=60973&cpingPid=1339&cpingPid_List=3253, written

TABLE 2 Comparisons of dose-response of rocuronium between the control and immobilized side at initial, PZP1 and PZP2 in wild-type and α7KO mice

			Control	immobilized	P-value
Wild (n = 10)	Initial	ED_{50}	1.234 (0.248)	2.395 (0.482)	0.01
		ED_{95}	1.790 (0.460)	3.669 (0.677)	0.01
	PZP1	ED_{50}	1.148 (0.422)	2.239 (0.833)	0.08
		ED_{95}	1.608 (0.600)	3.298 (1.397)	0.10
	PZP2	ED_{50}	1.497 (0.553)	2.925 (1.475)	0.10
		ED_{95}	2.065 (0.816)	4.053 (2.043)	0.13
α7KO(n = 10)	Initial	ED_{50}	0.972 (0.230)	1.521 (0.485)	0.01
		ED_{95}	1.318 (0.327)	2.154 (0.799)	0.01
	PZP1	ED_{50}	0.909 (0.187)	1.612 (0.608)	0.01
		ED_{95}	1.259 (0.258)	2.289 (0.922)	0.02
	PZP2	ED_{50}	0.831 (0.095)	1.504 (0.404)	0.02
		ED_{95}	1.153 (0.137)	2.158 (0.816)	0.03

Data are expressed as mean (SD).
Wild, wild-type mice; α7KO, α7 knockout mice. The statistical significance was defined as the $P < 0.05$. PZP1, data obtained after injection of pirenzepine 0.01 μg/g; PZP2, data obtained after injection of pirenzepine 0.1 μg/g. Statistically significant differences were observed only at the initial period in the wild-type mice, but statistically significant differences were maintained at all times in the α7KO mice.

in Korean language), although most of those data are obtained from the pirenzepine administration per os.

In a α7KO mice, the dose-responses of rocuronium was expected to be similar to those wild-type mice in spite of administration of pirenzepine (Figure 1a,b) because there were no changes in the number or size of synapses in disuse atrophy and this was an experiment without denervation. On the other hand, α7 nAChR might be some interaction in presynaptic M_1 mAChR function. Pirenzepine-induced reduction in ACh release at the neuromuscular presynaptic membrane and thus the reduction in ACh amount per neuronal stimulation could make the differences of evoked muscular responses more prominent in the muscles in which the α7 nAChR is expressed compared to those in the normal muscles.

Normally, α7 nAChR is involved in the neuroprotection of nicotine during stress-induced memory impairment and other beneficial effects.[19-21] On the other hand, in the neuromuscular physiology of anaesthesiology, the expression of α7 nAChR showed unusual, unexpected and undesirable effects, particularly on the induced neuromuscular blockade.[8,22-25] The α7 nAChR at the neuromuscular junction is expressed on the pathologic condition such as immobilization, inflammation or burn. The muscles at which the α7 nAChR is expressed showed resistance to the neuromuscular blocking agents such as rocuronium.[14] In our results, along with the dose-responses of rocuronium, TOF fade was also affected by blockade of M_1 mAChR. In the immobilized sides of the wild-type mice, TOF fades were attenuated compared to those in the contralateral normal side when the pirenzepine was injected. However, in the immobilized sides of the α7KO mice, injection of the pirenzepine did not show the attenuation of TOF which was seen in the wild-type mice. We speculated that this attenuation is due to the pirenzepine-induced reduction in ACh release and thus decreased the T1 responses, rather than the increased the forth responses (T4) of TOF stimulation. This can be supported by the results of the in vitro experiment performed by Pereira et al.[4] Although the T1 is attenuated by the decreased release of ACh in the situation of blockade of M_1 mAChRs by pirenzepine, repeated stimulation and thus the accumulation of ACh at the neuromuscular junction might preserve the responses of T4.

The present study had several drawbacks and limitations. Because the M_1 mAChR is a facilitatory autoreceptor, it is believed that repeated dose or increased doses of pirenzepine might further influence the decreased of rocuronium requirement for depressing T1 > 95%. On the other hand, by increasing the dose from 0.01 to 0.1 μg/g, pirenzepine showed quite opposite action. That is, the rocuronium dose appeared higher in PZP2 than in PZP1 in the wild-type mice. Although these changes were statistically insignificant, high dose of pirenzepine might have some reaction other than blocking M_1 mAChR. As is already known, specific antagonists for muscarinic receptors are not quite specific.[26] Therefore, it is important to find the adequate dose or reaction time is crucial. As described in Table 4, there were statistically significant differences between the wild-type mice and α7KO mice in the contralateral side at the high dose of pirenzepine (PZP2). In the immobilized side, however, there was a significant difference at initial stage only. If these phenomena are true, this means that the blockade of M_1 mAChR and thus the decrease in ACh molecules at the neuromuscular synapse have some effect on the contralateral side at high doses. In other words, the tensions of muscles in which the α7 nAChRs were expressed could be influenced more by the decrease in ACh at the synaptic junction. However, we did not examine the precise amount of ACh at each neuromuscular junction in this experiment; only the functional data are compared. Therefore, a more discrete immunochemical investigation will be needed. Another limitation and drawback in the present experiment is that we extracted the data of PZP1 and PZP2 from the same mice. We allowed a

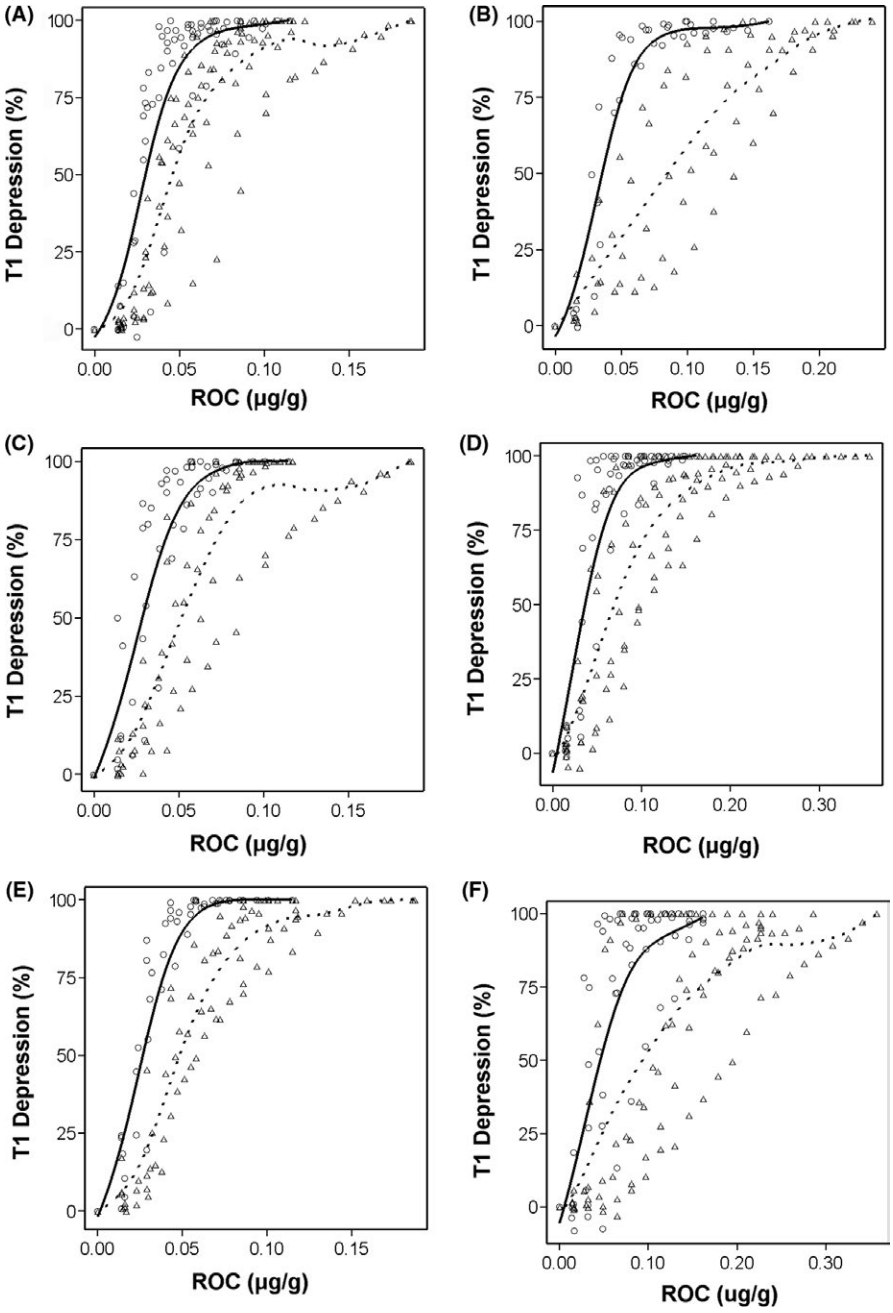

FIGURE 2 Comparison of the progression of the *T*1 depression according to the side. In the α7KO mice, *T*1 depression of immobilized side and contralateral normal side at: (A) the initial stage; (C) PZP1; and (E) PZP2 was displayed; (○—) α7KO_control; (△·····) α7KO_immo. In the wild-type mice, *T*1 depression of immobilized side and contralateral normal side at: (B) the initial stage; (D) PZP1; and (F) PZP2 was displayed; (○—) wild_control; (△·····) wild_immo. The regression equation was set, which has a R^2 more than 0.8 and the constants representing their slopes were compared. Statistically significant differences in the slopes in the α7KO groups were observed throughout the entire period, but those of the wild-type group showed statistical significance only at the initial period. Initial, reference *T*1 depression; α7KO_immo, immobilized side of α7KO; α7KO_control, contralateral normal side of α7KO; Wild_immo, immobilized side of wild-type mice; Wild_control; contralateral normal side of wild-type mice; PZP1, period at the injection of pirenzepine 0.01 μg/g; PZP2, period at the injection of pirenzepine 0.1 μg/g; ROC, cumulative doses of rocuronium

40-minute recovery time before the next session of the experiment was initiated, however, there is a lack of agreement that 40 minutes is a sufficient recovery time. This is the result obtained from our previous and pilot experiment. We considered full recovery to be when there was >95% recovery of *T*1 twitch tension and no tetanic fades by 50 Hz 5-s tetanic stimulation. These results were usually obtained within 30 minutes after the *T*1 twitch tensions were reappeared during the pilot study. We also confirmed that there were no differences in recovery indices (the time interval from 25% to 75% recovery of *T*1 twitch tension) by performing repeated dose-response study of rocuronium after allowing 40-minute recovery time in the pilot study. We did not perform these procedures in the main experiment, however, fearing that such intense stimulations (50 Hz tetanic stimulation) might influence the next dose-response results. Instead, we allowed 10 minutes more for the recovery time in the main experiment. That is why we set the 40-minute recovery time in this experiment.

In conclusion, in the wild-type mice, resistance to neuromuscular blocking agents in the immobilized side was reduced when the M_1 mAChR was blocked by a specific antagonist, pirenzepine. In the α7KO mice, resistance to the neuromuscular blocking agents at the immobilized side was less than wild-type mice in the initial stage. However, these differences between the immobilized and contralateral sides were maintained when the muscarinic M_1 receptor was blocked by specific antagonist, pirenzepine. The expression of α7 nAChR due to the disuse atrophy might have a different response to

TABLE 3 Comparisons of the dose-responses of rocuronium according to the genotype

		Control side			Immobilized side		
		Wild (n = 10)	α7KO(n = 10)	*P*-value	Wild (n = 10)	α7KO(n = 10)	*P*-value
Initial	ED_{25}	1.03 (0.17)	0.84 (0.20)	>0.05	1.92 (0.42)	1.29 (0.37)	0.03
	ED_{50}	1.23 (0.25)	0.97 (0.23)	>0.05	2.40 (0.48)	1.52 (0.49)	0.02
	ED_{75}	1.44 (0.33)	1.10 (0.27)	>0.05	2.87 (0.55)	1.76 (0.60)	0.02
	ED_{95}	1.79 (0.46)	1.32 (0.33)	>0.05	3.67 (0.68)	2.16 (0.80)	0.02
PZP1	ED_{25}	1.03 (0.17)	0.78 (0.16)	>0.05	1.84 (0.64)	1.36 (0.49)	>0.05
	ED_{50}	1.22 (0.25)	0.91 (0.19)	>0.05	2.24 (0.83)	1.62 (0.61)	>0.05
	ED_{75}	1.43 (0.33)	1.04 (0.21)	>0.05	2.63 (1.04)	1.87 (0.72)	>0.05
	ED_{95}	1.79 (0.46)	1.26 (0.26)	>0.05	3.30 (1.40)	2.29 (0.92)	>0.05
PZP2	ED_{25}	1.29 (0.47)	0.71 (0.08)	0.02	2.505 (1.273)	1.26 (0.32)	>0.05
	ED_{50}	1.50 (0.55)	0.83 (0.10)	0.04	2.925 (1.475)	1.50 (0.40)	>0.05
	ED_{75}	1.71 (0.65)	0.95 (0.11)	0.04	3.346 (1.684)	1.75 (0.49)	>0.05
	ED_{95}	2.07 (0.82)	1.15 (0.14)	0.04	4.053 (2.043)	2.16 (0.65)	>0.05

Data are expressed as mean (SD).
Statistical significance was defined as the $P < 0.05$. α7KO, α7 knockout mice; PZP1, data obtained after injection of pirenzepine 0.01 μg/g; PZP2, data obtained after injection of pirenzepine 0.1 μg/g; Wild, wild-type mice.

TABLE 4 Comparison of λ of the train-of-four ratios

Genotype	Time	Side		*P*-value (by side)
		Control	Immobilized	
Wild (n = 10)	Initial	288.93 (131.7)	38.84. (26.5)	0.014
	PZP1	587.14 (287.1)	213.29 (108.3)	0.001
	PZP2	576.78 (317.6)	310.22 (184.6)	0.103
	P-value (by time)	0.428	0.458	
α7KO(n = 10)	Initial	750.61 (645.2)	169.84 (89.78)	0.038
	PZP1	487.27 (102.7)	221.49 (65.3)	0.001
	PZP2	661.78 (222.8)	281.45 (108.7)	0.009
	P-value (by time)	0.651	0.855	

Data are expressed as mean (SD). Statistical significance was defined as the $P < 0.05$.
There were no statistical intergroup differences when comparing by time. α7KO, α7 knockout mice. PZP1, data obtained after injection of pirenzepine 0.01 μg/g; PZP2, data obtained after injection of pirenzepine 0.1 μg/g; Wild, wild-type mice.

the blockade of the presynaptic M_1 receptor which made diminished release of ACh upon neuronal stimulation.

4 | MATERIALS AND METHODS

This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) at the Asan Institute for Life Sciences (IACUC No. 2015-13-149). A total of 20 C57BL/6 (wild-type) and 10 α7 knock out (α7KO) mice were used in this experiment. The pinning-immobilization model was used for the current studies. The sample size was estimated by considering the previous ex vivo study[3] and the preliminary pilot test. We considered that a sample size of 10 was sufficient when the 20% allowable error of ED_{50}, 0.05 of α, 0.80 of power and 10% of drop rate was adopted. Among 20 wild-type mice, 10 were used for the pilot study and another 10 were used for main experiment. Each mouse was anaesthetized with tiletamine (Zoletil 50, 50–70 mg/kg intraperitoneal). The knee joints were immobilized by the pinning of a 23-gauge hypodermic needle through the proximal tibia into the distal femur to cause 90° flexion at the knee. The ankle joints were immobilized using a 26-gauge needle through the calcaneus into the distal tibia to fix the ankle joint at 90°. The contralateral hind limb served as the control. After recovery from anaesthesia, the mice were returned

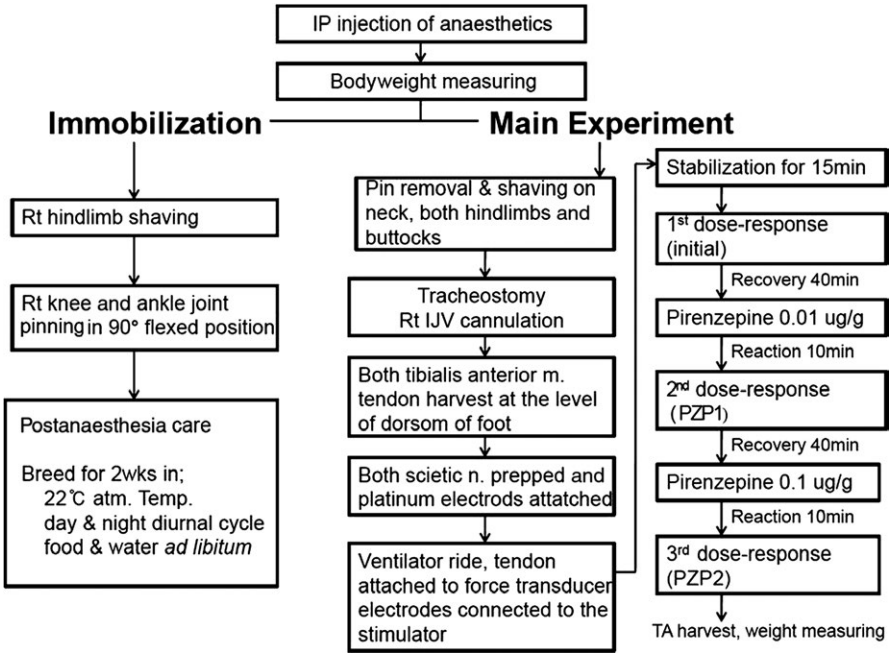

FIGURE 3 Study diagram. A total of 20 C57BL/6 (wild-type) and 10 α7 knock out (α7KO) mice were used in this experiment. The entire experiment process was divided into two phases; Phase 1 is the preliminary process for right hindlimb immobilization and phase 2 is the main experiment. In the main experiment, the recovery time allowed was 40 minutes, and full recovery was considered when there was no tetanic fade by 50 Hz stimulation for 5 s. IVJ, internal jugular vein; TA, tibialis anterior muscle

to their cages. Each mouse was housed for 2 weeks in a cage at 22°C in a 12-hour light and dark cycle with food and water supplied ad libitum.

Two weeks after immobilization, the main experiments were performed. Each mouse was anaesthetized with tiletamine (Zoletil 50, 50–70 mg/kg intraperitoneal), and a tracheostomy was performed for mechanical ventilation with ambient air at 140–150 breaths/minute with a tidal volume of 6–8 mL/kg (MiniVent Type 845; Hugo Saches Electronik-Harvard Apparatus Gmbh, March-Hugstetten, Germany). An adequate depth of anaesthesia was confirmed by the absence of a withdrawal response to intermittent toe clamping. The jugular vein was cannulated for fluid and drug administration. Anaesthesia was maintained with supplemental intermittent doses of tiletamine 10–20 mg/kg intraperitoneally. Supplemental doses were administered every 15–20 minutes empirically. The body temperature was monitored by using a rectal thermistor and maintained at 35.5–37°C with a heat lamp.

Neuromuscular transmission was monitored by the mechanomyography with a force transducer (FT03, Grass Technologies, West Warwick, RI, USA) along with the evoked indirect nerve stimulation using a peripheral nerve stimulator (S88; Grass Technologies). With the mice in dorsal recumbency, the tendons of both TA muscles were exposed surgically at both dorsi of the feet. The insertion points of the tendons of both TA muscles were separated and attached individually to separate FT03 force displacement transducers. Both sciatic nerves were exposed at their exit from the lumbosacral plexus at the thigh and tied with ligatures for indirect nerve stimulation of the muscles. Distal to the ligatures, platinum electrodes were attached for nerve-mediated indirect stimulation of the tibialis muscle. Both knees were fixed rigidly with clamps to prevent limb movement during nerve stimulation. Resting tensions of 50 mN, which yielded optimal evoked tensions, were applied to the immobilized and contralateral TA muscles. The tensions of the respective TA muscles, which were generated by evoked stimulation of the respective sciatic nerves, were calibrated in grams of force, recorded via a Grass P122 amplifier and displayed using LabChart 7 Software (AD Instruments, Sydney, Australia). The sciatic nerves were stimulated with the supramaximal electrical stimuli at 2 Hz for 2 s (TOF pattern) every 20 s using a Grass S88 stimulator and SIU5 stimulus isolation units (Grass Technologies).

The sciatic nerve/TA muscle preparations were stabilized for at least 15 minutes. In the initial set of experiments, the cumulative dose-response data of rocuronium were obtained with loading dose of 0.4 μg and 0.2 μg of booster doses injected repeatedly until >95% depression of the TA muscle tension was observed. The next injection of boost dose was considered when the muscle twitch tension depression was less than 3% or inclined to increase compared with the previous twitch tension. Spontaneous recovery of the neuromuscular blockade was provided after confirming that there were no tibialis muscle responses to the sciatic nerve stimulation. Full recovery from the initial rocuronium dose-response study was confirmed by a *T*1 twitch tension, and TOFR was recovered at 95% of the initial value and these commonly took 30–40 minutes after a 95% blockade of the *T*1 twitch tension. After confirming the full recovery, pirenzepine 0.01 μg/g was injected via a jugular catheter and allowed 10 minutes for reaction time. Subsequently, another cumulative dose-response of rocuronium was administered, which was considered as the PZP1. Finally, the data for PZP2 with 0.1 μg/g of pirenzepine were obtained with same sequence of PZP1. The present study protocol was summarized in Figure 3.

For statistical analysis, the values are expressed as the mean (SD). The differences in bodyweights before and after immobilization and weights of TA muscles of immobilized and control sides were analysed using a paired-sample *t* test, and the differences in the bodyweights of each genotype were analysed using independent *t* test. The changes in percentage

twitch depression (*T*1) were plotted and analysed by nonlinear regression using SPSS 13.0 Software (SPSS, Chicago, IL, USA). Comparisons according to time (control, PZP1 and PZP2) were analysed by ANOVA and Bonferroni as a post hoc test. The equation for TOFR was as follows; $y = 1-\lambda x^2$ where *y* represents the TOFR progression, *x* is the concentration of rocuronium, and λ represents the slope of the regression curve. The mean values of λ were compared between the groups using a Kruskal-Wallis test. The rocuronium EC_{50} and EC_{95} values for twitch tension data were calculated by fitting nonlinear regression curves to group data. *P*-values < 0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors have no potential conflict of interests to declare in association with this work.

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