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# Effect of electroacupuncture on the expression of agrin and acetylcholine receptor subtypes in rats with tibialis anterior muscular atrophy induced by sciatic nerve injection injury

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## ABSTRACT

**Objective** To investigate the effects of electroacupuncture (EA) on mRNA and protein expression of agrin, acetylcholine receptor (AChR)-ε and AChR-γ in a rat model of tibialis anterior muscle atrophy induced by sciatic nerve injection injury, and to examine the underlying mechanism of action.

**Methods** Fifty-four adult Sprague-Dawley rats were divided into four groups: healthy control group (CON, n=6); sciatic nerve injury group (SNI, n=24), comprising rats euthanased at 1, 2, 4 and 6 weeks, respectively, after penicillin injection-induced SNI (n=6 each); CON+EA group (n=12), comprising healthy rats euthanased at 4 and 6 weeks (after 2 and 4 weeks, respectively, of EA at GB30 and ST36); and SNI+EA group, comprising rats euthanased at 4 and 6 weeks (after 2 and 4 weeks, respectively, of EA). The sciatic nerve functional index (SFI), tibialis anterior muscle weight, muscle fibre cross-sectional area (CSA), and changes in agrin, AChR-ε, and AChR-γ expression levels were analysed.

**Results** Compared with the control group (CON), SNI rats showed decreased SFI. The weight of the tibialis anterior muscle and muscle fibre CSA decreased initially and recovered slightly over time. mRNA/protein expression of agrin and AChR-ε were downregulated and AChR-γ expression was detectable (vs zero expression in the CON/CON+EA groups). There were no significant differences in CON+EA versus CON groups. However, the SNI+EA group exhibited significant improvements compared with the untreated SNI group ($p<0.05$).

**Conclusions** EA may alleviate tibialis anterior muscle atrophy induced by sciatic nerve injection injury by upregulating agrin and AChR-ε and downregulating AChR-γ.

## INTRODUCTION

Nerve injury following injection is a type of iatrogenic peripheral nerve injury, with 80% of cases occurring during intramuscular injection into the buttock, most commonly in infants. Such injuries are avoidable through education about proper technique, but remain a concern particularly in developing countries.[1] Possible mechanisms of nerve injury include drug toxicity (major contributor), partial mass compression and direct injury due to the needle itself. Drug toxicity can cause nerve oedema and degeneration, which can lead to muscular atrophy and even disability.[2 3]

Transmission of signals between nerves and muscles occurs when acetylcholine (ACh) binds to ACh receptors (AChR) on the postsynaptic membrane. The dispersed gamma-acetylcholine receptors (AChR-γ) are expressed on the muscle cell surface during the embryo stage. Epsilon-acetylcholine receptors (AChR-ε) aggregate and replace AChR-γ after birth, and form the motor endplate; this process is regulated by agrin.[4] During denervation, agrin expression is downregulated and AChR-γ replaces AChR-ε expression, resulting in disordered neuromuscular transmission, muscle protein degradation and, finally, muscular atrophy.[5] Yamane *et al* showed that the maturity of animal muscle function is closely associated with completion of subunit conversion. Expression of the γ subunit corresponds to immature muscle function or dysfunction. Therefore, appearance of the γ subunit can be considered to be a sign of muscle dysfunction at any developmental stage or following muscle transplant. It may also indicate pathological conditions such as nerve damage, toxin-mediated blockade of neuromuscular synaptic transmission, or muscular atrophy.[6]

Currently, there is no established treatment for muscular atrophy. However, acupuncture has previously been shown to alleviate muscular atrophy induced by sciatic nerve transection or clamping injury.[7–9] We have previously demonstrated that acupuncture at GB30 and ST36 alleviated muscular atrophy by reducing nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) activity and collagen fibre proliferation in a rabbit model of sciatic nerve injection injury-induced calf muscular atrophy.[10] Another study showed that electroacupuncture (EA) at GB30 and ST36 upregulated the activity of AChR and acetylcholine transporters in rats.[11]

Although EA can alleviate sciatic nerve injury (SNI)-induced muscular atrophy, it is unclear whether the underlying mechanism involves changes in expression of agrin and AChR subtypes. Therefore, the aim of this study was to investigate the effect of EA on muscular atrophy as well as the levels of expression of AChR and agrin in rats with muscular atrophy caused by SNI following penicillin injection.

## METHODS

### Animal care, grouping and ethics statement

Fifty-four normal adult Sprague-Dawley rats, aged 7–9 weeks and weighing 150–250 g, were purchased from the Laboratory Animal Center of the Third Military Medical University (SCXK 2012-0005, Chongqing, China). The rats were housed in individual cages (470×300×150 mm) at a constant room temperature of 22±2°C and relative humidity of 65 ±5%, fed standard rat chow and given free access to water. Experiments began after a 1-week adaptation period. The animals were randomly divided into four groups: control (CON, n=6), SNI (n=24), CON+EA (n=12), and SNI+EA (n=12). In the SNI group, the rats were euthanased at 1, 2, 4 and 6 weeks (n=6 each) after penicillin injection-induced SNI. The CON +EA and SNI+EA comprised normal and SNI rats, respectively, that were euthanased at 4 and 6 weeks (n=6 each), respectively, following penicillin injection of the sciatic nerve, 2 and 4 weeks post-EA treatment at GB30 and ST36 for the SNI+EA group only. Thus, in total, there were nine subgroups (n=6 each). The protocol for this study was approved by the ethics review board of Zunyi Medical College.[12] All procedures were performed according to the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals' (National Academies Press, Washington DC, USA).

### SNI induced by penicillin injection

We used the third trochanter of the rat femur as a bony landmark to locate the sciatic nerve, which lies within 0.5 cm of its vicinity. The 36 Sprague-Dawley rats allocated to the SNI and SNI+EA groups underwent routine disinfection of the skin, and were subjected to intraperitoneal anaesthesia with 10% chloral hydrate at 0.3 mL/100 g. A 1.0 cm longitudinal incision was made in the right femoral area beneath the third trochanter. The sciatic nerve was then exposed using blunt dissection, after which 200 000 U (0.5 mL) penicillin sodium (0.48 g/0.8 million units, ref. A051134107, Harbin Pharmaceutical Group Pharmaceutical Factory) was injected using a no. 4 needle on the outer side of the neural stem.[10 13] The wound was then sutured in layers and disinfected after surgery. The 24 rats in the SNI group were serially euthanased at 1, 2, 4 and 6 weeks after penicillin injection into the sciatic nerve (n=6 per stage).

### EA treatment

Acupuncture points GB30 and ST36 (both distant from the site of nerve injury) were selected based on the principles and practice of Traditional Chinese Medicine for treating muscle atrophy caused by SNI. Stainless steel filiform needles (0.25×13 mm, Suzhou Medical Supplies Co, Ltd, China) were inserted unilaterally at GB30 and ST36 (on the injured side) and connected to a G6805-II type EA device (Qingdao Xinsheng Industrial Co, Ltd, China). The positive pole was connected to the needle inserted at GB30 and the negative pole was connected to ST36. Electrical stimulation was provided at 5 Hz frequency, 2 mA intensity and pulse width 0.5 ms for 30 min. A course of treatment consisted of EA on alternate days, three times a week for 2 weeks.[7] Treatment began 2 weeks after SNI modelling. In each of the CON+EA and SNI+EA groups (n=12 each), six rats received one 2-week course of treatment (before euthanasia at 4 weeks) and the other six received two 2-week courses of treatment (before euthanasia at 6 weeks).

### Sciatic nerve functional index testing

After observing their gait, the rats had coloured ink applied to the soles of their hind feet and were allowed to voluntarily walk from one end of a self-made footprint box to the other. Four to five clear footprints of each hind foot were recorded on each side (figure 1A). The print length (PL), toe spread (TS) and intermediary toe spread (IT) of normal (N) and experimental (E) hind feet, respectively, were measured and entered into the following (Bain) equation to calculate the sciatic nerve functional index (SFI), where 0 indicates normality and −100 indicates

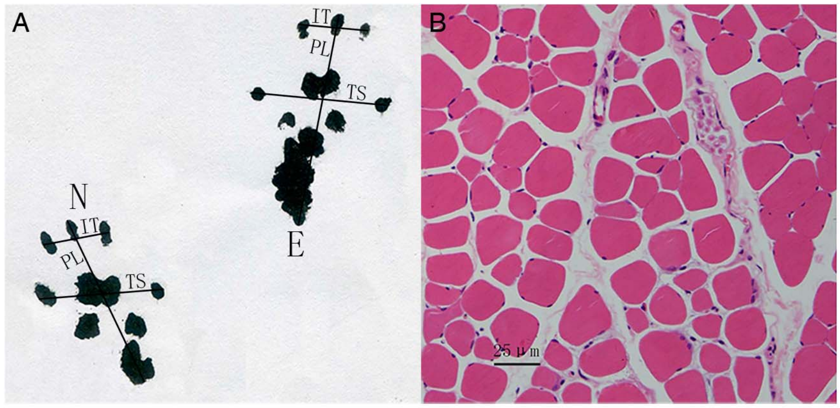


**Figure 1** (A) Example of footprints used for sciatic nerve functional index (SFI) measurements in a rat with sciatic nerve injury induced by penicillin injection: N, normal side; E, experimental side; PL, print length; TS, toe spread; IT, intermediary toe spread. (B) Representative H&E stained image of the tibialis anterior muscle of a healthy control rat (scale bar=25 μm).

complete damage: SFI=109.5 (ETS−NTS)/NTS−38.3 (EPL−NPL)/NPL+13.3 (EIT−NIT)/NIT−8.8.[14]

#### Morphological analysis

At the allocated time points (1–6 weeks) the rats were euthanased by carbon dioxide inhalation followed by decapitation. The tibialis anterior muscle was dissected out and weighed, and samples of the muscle belly were snap frozen and stored at −80°C for subsequent reverse transcriptase-PCR (RT-PCR) and Western blot analyses, or fixed in 10% neutral formalin for subsequent H&E staining. Paraffin-embedded muscle tissue was cross-sectioned into 8 μm thick slices. After H&E staining, the muscle fibre cross-sectional area (CSA) of the tibialis anterior muscle was measured using an Olympus DP26 with Cellsens standard 1.11 image analysis software (Olympus Corporation, Japan). A representative image is shown in figure 1B. Laboratory workers were kept blind to treatment allocation during the analysis.

#### RT-PCR

Total RNA was extracted from homogenised frozen samples of tibialis anterior muscle using TRIzol reagent (B511311, Sangon Biotech Co, Ltd, Shanghai, China) and 1 μL was reverse transcribed using FastQuant RT kits (KR106, Tiangen Biotech Co, Ltd, Beijing, China). Thereafter, the 2× Taq PCR Master Mix kit (KT201, Tiangen Biotech Co, Ltd) was used for PCR. The PCR electrophoresis bands were photographed using a gel imager and the optical density values of each band were measured using Quantity One software (Bio-Rad Co, Ltd, California, USA). The primer sequences for AChR-ε, AChR-γ, agrin and β-actin were designed and synthesised by Sangon Biotech Co, Ltd and are detailed in table 1.

#### Western blotting

Equivalent amounts of protein extracts were run on a 4–12% gradient polyacrylamide gel (Beyotime Institute of Biotechnology, China). Separated proteins were transferred to a PVDF (polyvinylidene difluoride) membrane, which was probed with specific antibodies (Santa Cruz Biotechnology Inc, USA), namely, goat anti-mouse AChR-ε (sc-1455), goat anti-human AChR-γ (sc-1453) and goat anti-rat agrin polyclonal (sc-6166), and developed using a chemiluminescent substrate.

#### Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS) V.17.0 (SPSS Inc, Chicago, Illinois, USA). Groups were compared using one-way analysis of variance and post hoc Tukey test. The level of formal statistical significance was set at $\alpha=0.05$.

## RESULTS

#### General observation

Compared with rats in the control (CON) group, SNI rats developed clubfeet and their hind legs became limp and had to be dragged. Muscle mass at 1 week diminished slightly and was followed by significant muscle atrophy with demonstrable thinning of the legs at 2, 4 and 6 weeks in the SNI groups. No such changes were observed in the CON+EA group and a

**Table 1** Primer sequences for agrin, AChR-ε, AChR-γ and β-actin

| Gene | Primer sequence |
|---|---|
| Agrin-F | 5′-CCATCAACAGCCCTCAAAGT-3′ |
| Agrin-R | 5′-AGAGCCAGAGCCAGGAAATC-3′ |
| AChR-ε-F | 5′-GCCTGTATCACCATCTCTTCG-3′ |
| AChR-ε-R | 5′-GCCTCCCTCATAGACCAGAAC-3′ |
| AChR-γ-F | 5′-GAACTCTGTGGTCGTGCTCA-3′ |
| AChR-γ-R | 5′-GCTCTGCCTCATTTCTGGAC-3′ |
| β-actin-F | 5′-GAGAGGGAAATCGTGCGTGAC-3′ |
| β-actin-R | 5′-CATCTGCTGGAAGGTGGACA-3′ |

AChR, acetylcholine receptor.


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gradual improvement in this pattern was subjectively observed in the SNI+EA group, by comparison.

### Sciatic nerve functional indices

Figure 2A shows the SFI results of the rats in each experimental group. In the SNI group, SFI was significantly lower than in the CON group at all time points (all $p<0.001$). It was lowermost 1 week after SNI due to penicillin injection and gradually recovered thereafter, being significantly greater at 4 and 6 weeks compared to 2 weeks ($p=0.008$ and $p=0.003$, respectively). There were no statistically significant differences between the CON+EA and CON groups at any point. Compared with the SNI group at 2 weeks, SFI values in the SNI+EA group were 11.2% and 18.2% greater at 4 and 6 weeks, respectively. This increase was notably larger than the equivalent increase seen at 4 and 6 weeks within the SNI group (4.61% and 6.72%, respectively, compared to 2 weeks). Moreover, SFI values were significantly increased at 4 and 6 weeks in the SNI+EA group compared with the (untreated) SNI group at the equivalent time points ($p=0.005$ and $p<0.001$, respectively).

### Muscle weight

The weight of the tibialis anterior muscle in each group is shown in figure 2B. Compared to the muscle weights in the CON group (0.51±0.01 g), those in the SNI group were significantly decreased. The greatest reduction was observed at 4 weeks, and appeared to be followed by a slight (9.8%) recovery at 6 weeks ($p=0.011$, vs 4 weeks). Muscle weights in the CON +EA group did not differ significantly from the CON group ($p=0.934$ and $p=0.867$ at 4 and 6 weeks, respectively). Compared to the SNI group at 2 weeks, muscle weights in the SNI+EA group at 4 and 6 weeks were increased by 27.5% and 35.3%, respectively (both $p<0.001$), which represented a significant increase in weight at both 4 and 6 weeks compared to the (untreated) SNI group at 4 weeks ($p<0.001$) and 6 weeks ($p<0.001$).

### Muscle fibre CSA

The CSA of the tibialis anterior muscle fibre of CON rats was 510.0±2.58 $\mu m^2$ (figure 2C). Muscle fibre CSA in the SNI group was greatly reduced compared to that of the CON group, with the greatest degree of atrophy observed at 4 weeks. As noted for muscle weight, values recovered by 8.8% at 6 weeks ($p=0.01$, vs 4 weeks). No statistically significant differences were found between the CON+EA and CON groups. In the SNI+EA group, muscle fibre CSA was 22.1% and 35.0% greater at 4 and 6 weeks, respectively, compared to the SNI group at 2 weeks. When directly compared with the SNI group at 4 and 6 weeks, CSA was significantly increased in the SNI +EA group at both 4 and 6 weeks ($p<0.001$).

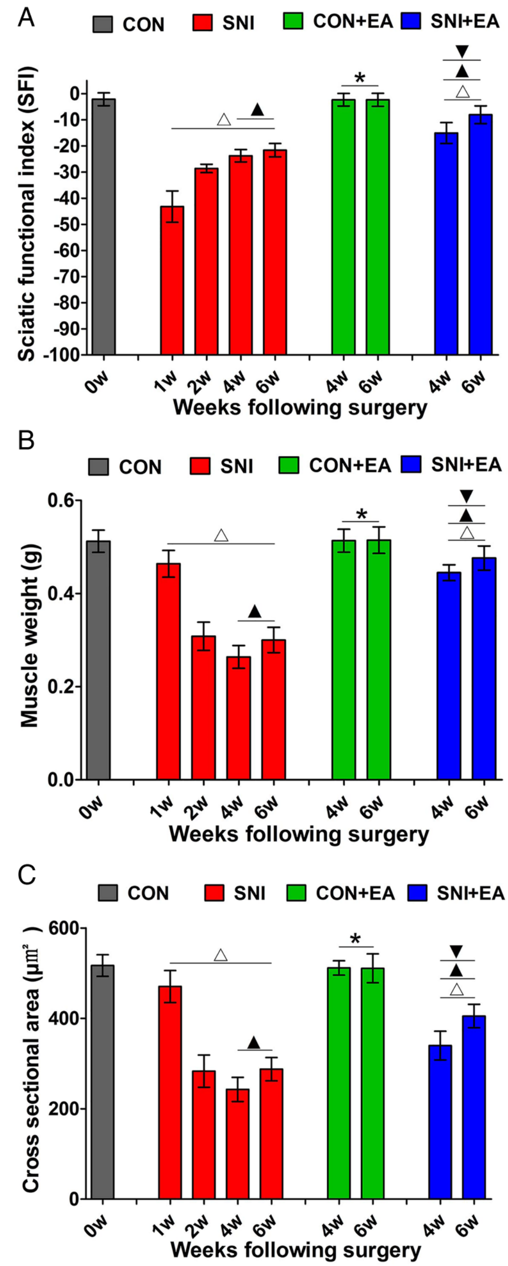


**Figure 2** Sciatic nerve functional index (SFI) measurements (A), tibialis anterior muscle weight (B) and cross-sectional area (C) in 18 healthy control rats left untreated (CON group, n=6) or treated with electroacupuncture (CON+EA group) for 2 or 4 weeks (n=6 each) before euthanasia at 4 or 6 weeks, respectively, and 36 rats with sciatic nerve injury (SNI) induced by penicillin injection left untreated (SNI group) and euthanased at 1, 2, 4 or 6 weeks (n=6 each) or treated with electroacupuncture (SNI+EA group) for 2 or 4 weeks (n=6 each) before euthanasia at 4 or 6 weeks, respectively. △$p<0.05$ vs CON; *$p>0.05$ vs CON; ▲$p<0.05$ vs SNI at 2 weeks; ▼$p<0.05$ vs SNI at 4 or 6 weeks.

### mRNA expression of agrin, AChR-ε and AChR-γ

The mRNA expression of agrin, AChR-ε and AChR-γ in each experimental group is shown in figure 3. The optical density value of AChR-ε in the CON group was 0.87±0.01, while AChR-γ was not detectable. The AChR-ε expression levels in the SNI group were

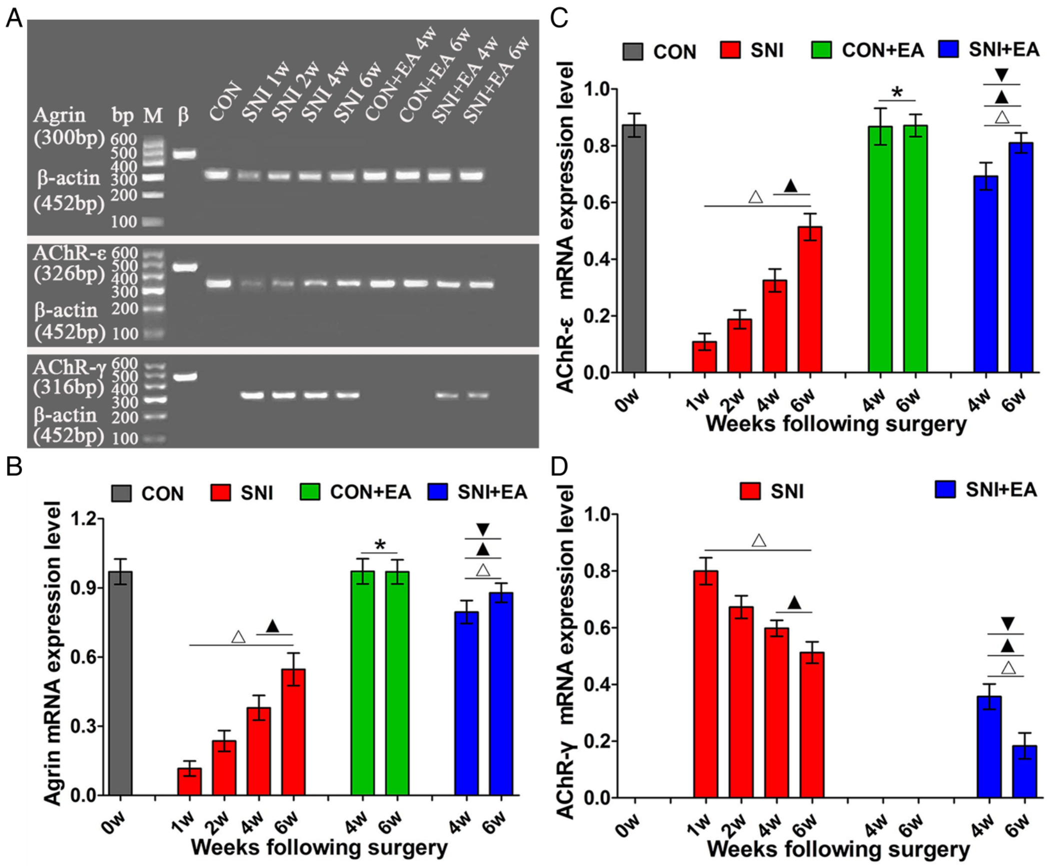


**Figure 3** Representative reverse transcriptase-PCR products (A) and mRNA expression of agrin (B), acetylcholine receptor (AChR)-ε (C) and AChR-γ (D), expressed relative to β-actin, in tibialis anterior muscle from 18 healthy control rats left untreated (CON group, n=6) or treated with electroacupuncture (CON+EA group) for 2 or 4 weeks (n=6 each) before euthanasia at 4 or 6 weeks, respectively, and 36 rats with sciatic nerve injury (SNI) induced by penicillin injection left untreated (SNI group) and euthanased at 1, 2, 4 or 6 weeks (n=6 each) or treated with electroacupuncture (SNI+EA group) for 2 or 4 weeks (n=6 each) before euthanasia at 4 or 6 weeks, respectively. △p<0.05 vs CON; *p>0.05 vs CON; ▲p<0.05 vs SNI at 2 weeks; ▼p<0.05 vs SNI at 4 or 6 weeks.

reduced relative to the CON group, and AChR-γ expression became measurable. The mRNA results for agrin mRNA were essentially the same as AChR-ε with minimal differences. No statistically significant differences were found between the CON+EA and CON groups. In the SNI group, AChR-ε expression at 4 and 6 weeks increased by 15.77% and 37.39%, respectively, compared with the same group at 2 weeks, while AChR-γ expression was reduced by 11.94% and 23.88%, respectively, ($p \leq 0.001$). In the SNI+EA group, levels of AChR-ε expression at 4 and 6 weeks were 46.34% and 71.63% greater, respectively, and AChR-γ expression levels were 46.26% and 73.13% lower, respectively, than the equivalent levels in the SNI group at 2 weeks. mRNA expression levels of all three factors differed significantly in the SNI +EA versus SNI groups at both 4 and 6 weeks (all $p<0.001$).

### Protein expression of agrin, AChR-ε and AChR-γ

Protein expression levels of the same three factors in the tibialis anterior muscle are shown in figure 4. Protein expression of agrin and AChR-ε was downregulated in SNI versus CON groups, and AChR-γ expression, which was absent in the normal rats in keeping with the mRNA data, reappeared in the SNI group and appeared to reduce gradually over time (between 1 and 6 weeks). The CON+EA group did not differ significantly from the CON group at either 4 or 6 weeks. The SNI+EA group demonstrated attenuated AChR-γ expression and higher agrin and AChR-ε expression at 4 and 6 weeks relative to the SNI group at 4 and 6 weeks (all $p<0.001$).

## DISCUSSION

The aim of this study was to better understand the mechanisms underlying the improvement observed after EA treatment of muscular atrophy in rats with iatrogenic SNI. To this end, we analysed the effect of EA on the expression levels of AChR-ε and AChR-γ, which are considered to be indicators of neuromuscular function recovery,[6] as well as the putative adjustment effect of agrin on AChR-ε aggregation.[15] Hereby we have provided a new perspective on the treatment of muscular atrophy.[16] Intramuscular injection is usually performed in the outer upper quadrant



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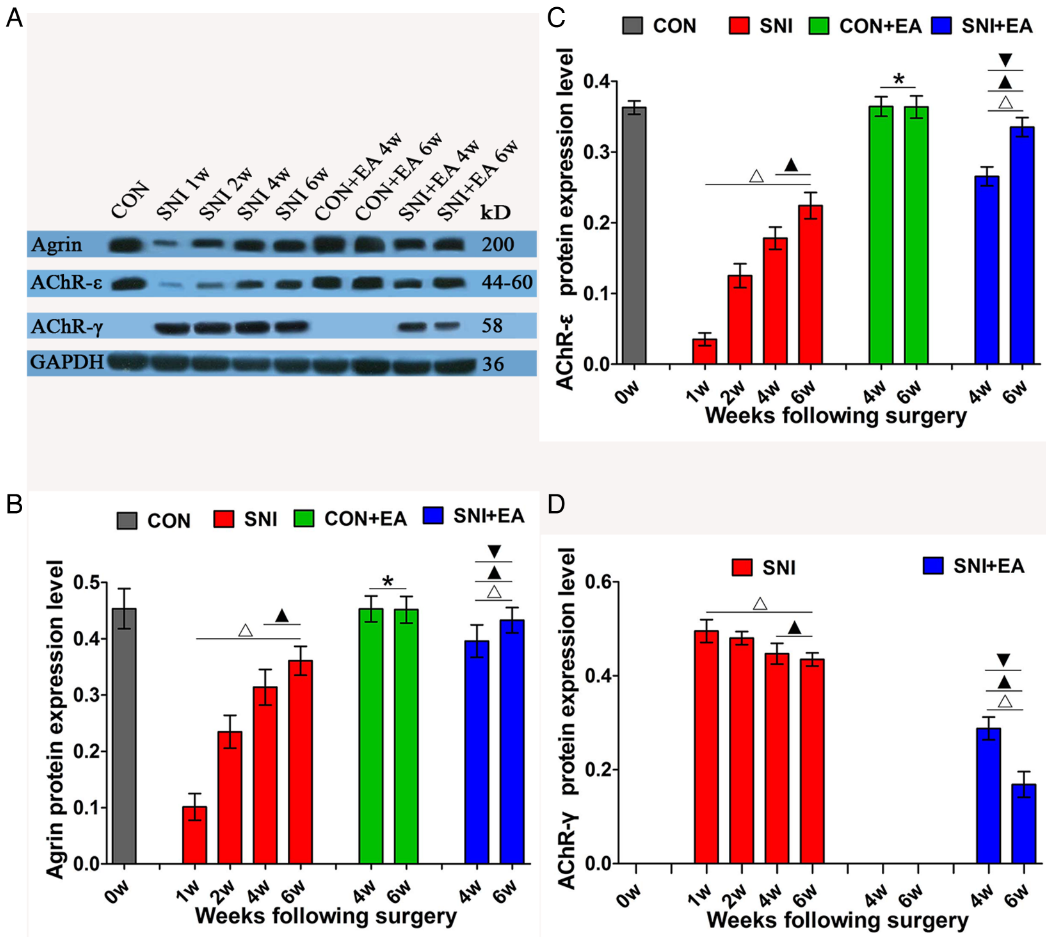


**Figure 4** Representative Western blots (A) and protein expression of agrin (B), acetylcholine receptor (AChR)-ε (C) and AChR-γ (D) in tibialis anterior muscle from 18 healthy control rats left untreated (CON group, n=6) or treated with electroacupuncture (CON+EA group) for 2 or 4 weeks (n=6 each) before euthanasia at 4 or 6 weeks, respectively, and 36 rats with sciatic nerve injury (SNI) induced by penicillin injection left untreated (SNI group) and euthanased at 1, 2, 4 or 6 weeks (n=6 each) or treated with electroacupuncture (SNI+EA group) for 2 or 4 weeks (n=6 each) before euthanasia at 4 or 6 weeks, respectively. △p<0.05 vs CON; *p>0.05 vs CON; ▲p<0.05 vs SNI at 2 weeks; ▼p<0.05 vs SNI at 4 or 6 weeks.

of the buttocks, and thereby most commonly affects the peroneal nerve.[1] However, in this study, we intentionally injected penicillin, which is extremely neurotoxic, into the outer side of the sciatic nerve stem in order to create a rat model of SNI.

Muscle weight and muscle fibre size are indicators of skeletal muscle atrophy.[10 17] Our results showed that, by 1 week after SNI, the rats had developed clubfeet and their hind legs had become limp. The tibialis anterior muscle became smaller and was accompanied by a decrease in muscle weight, muscle fibre CSA and SFI. Moreover, muscle weights and muscle fibre CSA in the SNI group further reduced at 2 weeks. These results suggest that our model of muscular atrophy secondary to SNI induced by penicillin injection was successful. SFI showed the most significant decline at 1 week in the SNI rats. A possible reason for this observation might be the acute nerve injury caused by partial compression resulting from entry of penicillin into the nerve stem. The gradual restoration of SFI over 6 weeks in the SNI group may have occurred as the penicillin was gradually absorbed, which would have lessened the toxicity and compression, and may have led to nerve regeneration.

During embryonic development, AChR-γ is dispersed throughout the muscle cell surface in tiny clusters before the nerve endings reach the muscle cells. In the early postnatal stage, after the muscle cells have become innervated by nerve cells, AChR-ε gradually replaces AChR-γ.[15] Agrin synthesised by motor neuron cell bodies is transported to the nerve endings via the axon and released at the endplate, inducing AChR-ε aggregation and promoting postsynaptic membrane differentiation.[18] After nerve injury, agrin expression is significantly downregulated, and endplate area and receptor density decrease over time. As a result, AChR-ε stops aggregating and gradually subsides, and is replaced by upregulation of AChR-γ expression,[5 19–21] which interferes with neuromuscular transmission and subsequently causes muscular atrophy. The results of the present study showed that SNI-induced tibialis anterior muscular atrophy resulted in the downregulation of agrin and AChR-ε expression and the reappearance of AChR-γ expression. While acupuncture at GB30 and ST36 had no effect on the levels of expression of these three factors in normal (control) rats, it did significantly increase agrin and AChR-ε expression levels in the tibialis anterior muscle and reduce AChR-γ expression when administered following SNI. Finally, the mild recoveries observed in the (untreated) SNI group at 4 and 6 weeks might have reflected the ability of the rats' own nerves to regenerate.

In this study, expression levels of agrin, AChR-ε and AChR-γ changed in line with the SFI changes. Interestingly, there was a slight delay in the recovery of both muscle weight and muscle fibre CSA, which we suspect might be because restoration at a molecular level precedes that at a morphological level. Alternatively, the effect of EA on these factors may have been greater than other key factors involved in recovery from muscle atrophy. However, this is only a hypothesis and still needs to be verified.

Our study has some limitations. Although we successfully established an animal model of SNI by surgically exposing the sciatic nerve and directly injecting penicillin into the nerve trunk,[13] resulting in muscle atrophy, this model does not fully replicate the injury induced by percutaneous intramuscular injection. Therefore, future studies should consider developing methods more closely resembling intramuscular injection to create a more robust animal model of SNI-induced muscular atrophy. Furthermore, although AChR aggregation by agrin is recognised, the upstream regulation of agrin remains poorly understood. Therefore, further studies must investigate whether the observed upregulatory effect of EA on agrin is central or local. In addition, it is possible that there may have been expression of AChR-γ in the control groups that fell just below our lower limit of detection for the assays utilised.

In conclusion, we have shown that, in tibialis anterior muscular atrophy induced by SNI, EA stimulation at ST36 and GB30 alleviates muscle atrophy, upregulates agrin and AChR-ε expression, and downregulates AChR-γ expression. These effects would be expected to facilitate the recovery of neuromuscular signal transmission. In addition to acupuncture, pharmacological agents capable of targeting agrin, AChR-ε and/or AChR-γ levels may have a role in the treatment of muscular atrophy, or indeed other types of iatrogenic injuries, such as trauma, or those related to neuropathy.

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**Contributors** SY conceived and designed the experiments. JY and MW performed the experiments. JY, MW and SY analysed the data. SY, JY, MW, XZ and JL contributed reagents/materials/analysis tools. SY and JY drafted the manuscript and all authors approved the final version for publication.

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**Competing interests** None declared.

**Provenance and peer review** Not commissioned; externally peer reviewed.



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