Effect of electroacupuncture on thermal pain threshold and expression of calcitonin-gene related peptide, substance P and γ-aminobutyric acid in the cervical dorsal root ganglion of rats with incisional neck pain

Li-na Qiao,1 Jun-ling Liu,2 Lian-hong Tan,1 Hai-long Yang,1,3 Xu Zhai,1 Yong-sheng Yang1

ABSTRACT

Objective Acupuncture therapy effectively reduces post-surgical pain, but its mechanism of action remains unclear. The aim of this study was to investigate whether expression of γ-aminobutyric acid (GABA) and the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) in the primary sensory neurons of cervical dorsal root ganglia (DRG) are involved in electroacupuncture (EA)-induced analgesia in a rat model of incisional neck pain.

Methods The pain model was established by making a longitudinal midline neck incision in 60 rats. Another 15 rats underwent sham surgery (normal group). Post-incision, 15 rats remained untreated (model group) and 45 rats underwent EA (frequency 2/100 Hz, intensity 1 mA) at bilateral LI18, LI4-PC6 or ST36-GB34 (n=15 each) for 30 min at 4 hours, 24 hours, and 48 hours post-surgery, followed by thermal pain threshold (PT) measurement. 30 min later, the rats were euthanased and cervical (C3-6) DRGs removed for measurement of immunoreactivity and mRNA expression of SP/CGRP and the GABAergic neuronal marker glutamic acid decarboxylase 67 (GAD67).

Results Thermal PT was significantly lower in the model group versus the normal group and increased in the LI18 and LI4-PC6 groups but not the ST36-GB34 group compared with the model group. Additionally, EA at LI18 and LI4-PC6 markedly suppressed neck incision-induced upregulation of mRNA/protein expression of SP/CGRP, and upregulated mRNA/protein expression of GAD67 in the DRGs of C3-6 segments.

Conclusions EA at LI18/LI4-PC6 increases PT in rats with incisional neck pain, which is likely related to downregulation of pronociceptive mediators SP/CGRP and upregulation of the inhibitory transmitter GABA in the primary sensory neurons of cervical DRGs.

INTRODUCTION

Postoperative pain control is an important concern for both patients and surgeons. After thyroidectomy, patients often require analgesics such as ketorolac, acetaminophen and morphine, which may aggrivate anaesthetic-induced side effects.1 2 Acupuncture therapy has been lauded as a promising alternative approach for postoperative pain relief.3 A systematic review and several recent clinical trials4–8 suggest that perioperative acupuncture may be a useful adjunct to standard postoperative pain management.6–8

It has also been demonstrated that electroacupuncture (EA) stimulation at LI18 (Futu), LI4 (Hegu) and PC6 (Neiguan)—traditional acupuncture points that have been classically used for analgesia in thyroid surgery patients9—is effective, and that EA treatment may reduce the required dosages of remifentanil and acetaminophen, with a superior effect compared with acetaminophen administration alone in the reduction of postoperative pain.10

Regarding the mechanisms of acupuncture for postoperative pain relief, it appears that central endorphin, supraspinal serotoninergic and noradrenergic descending projection systems, spinal γ-aminobutyric acid (GABA), and opioid neurotransmitters are involved.11 12 In recent years, we have investigated the spinal mechanisms underlying the effects of an EA intervention.
in relieving incisional neck pain in rats. Our results showed that the analgesic effect of EA at LI18, LI4 and PC6 is probably associated with the upregulation of mRNA and protein expression of 5-HT 2A receptor, substance P (SP) and calcitonin gene-related peptide (CGRP) in the C2–C5 spinal cord. However, to our knowledge, it is unknown whether the primary sensory neurons of the dorsal root ganglion (DRG), including the nociceptive neuropeptides and inhibitory neurons, are involved in postoperative acupuncture analgesia.

Primary sensory afferents of the DRG innervate the skin and detect a wide range of stimuli. Traditionally, the DRG neuron is considered to be a structure that merely ‘supports’ physiological communication between the peripheral and central nervous systems to convey peripherally encoded information to the spinal cord or trigeminal nuclei of the brain stem. However, new clinical data have shown that a pulsed radiofrequency current applied to the DRG may reduce pain intensity in patients with complex regional pain syndrome or lumbosacral radicular syndrome. Thus, the DRG is an active participant in the processing of pain information and therefore a robust target for neuromodulation therapies.

In the DRG, nociceptive cells are composed of small diameter non-peptide- and peptide-expressing CGRP and SP cells, and small- and medium-sized neurons with finely myelinated (Aδ fibre) or unmyelinated (C fibre) axons. Both CGRP and SP are important molecules in nociceptive processing. The GABAB1 subunit has been shown to be co-localised to >97% of DRG neurons immunoreactive (IR) for CGRP. Loss of GABAB receptors on primary afferent neurons may lead to mechanical allodynia, while application of GABA agonists to the DRG attenuates pain behaviour in rats with neuropathic pain.

Accordingly, the present study was designed to examine the analgesic effects of EA in relation to the balance between CGRP/SP and GABA expression in cervical DRGs, in order to explore the peripheral mechanisms involved in the relief of thyroid surgery-induced pain by EA.

METHODS

Animals and grouping
Seventy-five adult male Sprague-Dawley rats (weighing 200–250g) were purchased from Beijing Union Medical College and housed under standard laboratory conditions (12 hours alternate light-dark cycle) and given free access to standard chow pellet diet and water. The experimental protocols were approved by the ethics committee of the Institute of Acu-moxibustion, China Academy of Chinese Medical Sciences (reference no. 201400014) and conformed to the Guidelines for Laboratory Animal Care and Use of Chinese Ministry of Science and Technology (2006) and the National Research Council’s ‘Guide for Care and Use of Laboratory Animals’ (National Academies Press, Washington, DC, USA). The rats were randomly assigned to one of five groups (n=15 each): control, model, LI18, LI4-PC6, and ST36-GB34. Rats in the control group received only isoflurane anaesthesia, while those in the model group underwent neck incision under isoflurane anaesthesia, and those in the LI18, LI4-PC6 and ST36-GB34 groups received EA (at the respective points or combination of points) plus neck incision under isoflurane anaesthesia.

Establishment of incisional neck pain model
The incisional neck pain rat model was established by making a 1.5 cm longitudinal incision along the midline of the neck under isoflurane (1–2% in oxygen) delivered via an anaesthesia unit (Matrix Company, Midmark Animal Health, Versailles, OH, USA), followed by repeated blunt dissection stimulation of the bilateral sternohyoideus muscles in the region of the thyroid gland for about 10min using a pair of forceps. The incision was then sutured in layers with 4.0 surgical silk braided suture at intervals of about 0.5 cm.

Measurement of thermal pain threshold
In a subgroup of animals (n=8 per group), the thermal pain threshold (PT) of the neck incision region was measured at baseline and 48 hours later (after surgical incision ± EA) using a tail-flick unit (37360, UGO Basile, Italy). The heat intensity was set to 50 units with a cut-off time of 30 s to avoid tissue damage. The withdrawal latency of the neck was detected three times for each rat, at intervals of about 5 min, and the average value was used. The researcher recording thermal PT measurements was blind to group assignment and not involved in the acupuncture procedure.
Electroacupuncture intervention

Under anaesthesia with 1.5% isoflurane, rats allocated to the three EA groups underwent EA stimulation following insertion of filiform needles (32 gauge, 0.5 inch length, Suzhou, China) bilaterally at LI18, LI4 and PC6, or ST36 and GB34 (to a depth of about 2–3 mm), respectively, according to individual group allocation. In humans, LI18 is located at the posterior border of the sternocleidomastoid, 3 cun (about 6 cm) lateral to the laryngeal prominence. In rats the equivalent location is about 1 cm lateral to the thyroid cartilage. The remaining points were located according to an atlas of experimental animal acupuncture points, as follows: LI4 was located between the first and second metacarpal bones; PC6 was located about 3 mm to the transverse stripe of the wrist at the axopetal end; ST36 was located about 5 mm inferior to the capitulum fibulae and posterolateral to the hindlimb knee joint; and GB34 was located about 5 mm superolateral to ST36.21 After insertion, the needle handles were connected to a HANS EA apparatus (Hans-100A, China) and stimulated for 30 min at an alternating frequency of 2 Hz/100 Hz, pulse width of 0.2–0.6 ms and intensity of 1 mA at 4, 24 and 48 hours after modelling. Animals of the model group underwent the same anaesthesia procedures but without EA stimulation.

Necropsy and tissue preparation

Following thermal PT measurement after completion of EA treatment (n=40 rats only), all rats were deeply anaesthetised with 20% urethane and transcardially perfused through the ascending aorta with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4) and the C3-C6 DRGs were carefully removed. DRGs from the 40 rats that had undergone PT behavioural testing were kept in 0.1 M PBS containing 30% sucrose at 4°C for subsequent immunofluorescence testing. DRGs from the remaining 35 rats (n=7 per group) were kept on an ice plate before being snap frozen in liquid nitrogen and stored at −80°C for RNA extraction later.

Immunofluorescence assay

In the subset of 40 rats, DRG tissues were sectioned on a freezing microtome (Thermo, Germany). For immunofluorescence double-labelling, sections (30 µm) were blocked with 5% donkey serum for 30 min and then incubated with β-III tubulin (a marker for neurons; ab68193, Abcam, Cambridge, UK) plus either glutamic acid decarboxylase 67 (GAD67, a marker for GABA; ab104561, Abcam), SP (sc58591, Santa Cruz, Dallas, TX, USA) or CGRP (ab181887, Abcam) at 4°C overnight. Sections were washed three times with 0.1 M PBS then incubated in donkey anti-rabbit IgG conjugated Alexa Fluor594 (Invitrogen, Carlsbad, CA, USA) plus either donkey anti-goat IgG conjugated Alexa Fluor488 (Invitrogen) or donkey anti-mouse IgG conjugated Alexa Fluor 488 (Invitrogen) for 2 hours at room temperature. Control immunostaining was performed by substituting the primary antibody with normal serum.

SP, CGRP, GAD67 and βIII tubulin-IR positive cells in the bilateral DRGs (C3-C6) were detected in three randomly selected sections of each DRG by a technician who was blind to the grouping, and the percentages of SP, CGRP and GAD67 positive neurons were calculated...
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using an optical imaging analysis system (DXM1200c, Nikon, Japan). Representative photographs of sections were taken using a laser scanning confocal microscope (FV1000, Olympus Co, Tokyo, Japan) and the resultant digital images were processed with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA).

Figure 3 (A) Representative photomicrographs showing immunofluorescent staining of calcitonin gene-related peptide (CGRP; green) and βIII-tubulin (red); and (B) bar chart demonstrating the percentage of CGRP-positive neurons in the C3-6 dorsal root ganglia of eight healthy rats (Normal group) and 32 rats with incisional neck pain that remained untreated (Model group, n=8) or received electroacupuncture at LI18 (n=8), LI4-PC6 (n=8) or ST36-GB34 (n=8). Data are presented as mean±SD. Scale bar=200 µm. ▲p<0.05 versus Normal group. ★p<0.05 versus Model group. ▲p<0.05 versus LI18 group. #p<0.05 versus LI4-PC6 group.

Figure 4 (A) Representative photomicrographs showing immunofluorescent staining of glutamic acid decarboxylase 67 (GAD67; green) and βIII-tubulin (red); and (B) bar chart demonstrating the percentage of GAD67-positive neurons in the C3-6 dorsal root ganglia of eight healthy rats (Normal group) and 32 rats with incisional neck pain that remained untreated (Model group, n=8) or received electroacupuncture at LI18 (n=8), LI4-PC6 (n=8) or ST36-GB34 (n=8). Data are presented as mean±SD. Scale bar=200 µm. ▲p<0.05 versus Normal group. ★p<0.05 versus Model group. ▲p<0.05 versus LI18 group. #p<0.05 versus LI4-PC6 group.

Quantitative real-time PCR

In a subset of 35 rats, total RNA was extracted with Trizol (CW0581, China) and reverse transcribed into cDNA using a cDNA Synthesis Kit (CW0744, China). Gene expression levels were measured by qRT-PCR (LightCycler480, Switzerland) using the following
primer sequences for SP (forward 5’-TGTTTGCA-GAGGAATCGGTG-3’ and reverse 5’-GAACGTGCTAGCGCTTG-3’), CGRP (forward 5’-CCTTTCCGTGTTGTCAGCATC-3’ and reverse 5’-CAGTAGGCGAGCTTCTTCTTCA-3’), GAD67 (forward 5’-CCCTGTATCGTAGGAGACGTCAT-3’ and reverse 5’-GACTCATCGTACTCCCTGCTG-3’), and β-actin (forward 5’-GGAGATTACTGCCCTGGCTCCTA-3’ and reverse 5’-GACTCATCGTACTCCCTGCTG-3’).

Each reaction mixture consisted of 2 µL cDNA, 10 µL REAL SYBR Mixture (2×), 0.8 µL (10 µmol/µL) of both forward and reverse primers, and 7.2 µL PCR-grade water, yielding a final volume of 20 µL. PCR was performed under the following conditions: 30 s at 95°C, followed by 45 cycles of 5 s at 95°C, and 40 s at 60°C. Relative expression was calculated according to the ∆∆Ct method. Relative mRNA levels were expressed as 2^−∆∆Ct values. The researcher evaluating mRNA expression was blind to group assignment and not involved in the acupuncture procedure.

Statistical analysis
Data were analysed using the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc, Chicago, IL, USA). Data were expressed as mean±SD and were analysed by one-way analysis of variance (ANOVA), followed by post hoc test of least significant difference for multiple comparisons between groups. A value of p<0.05 was considered to be statistically significant and a tendency towards a statistically significant difference was defined as p=0.05–0.1.

RESULTS
Effects of EA on thermal PT
As shown in figure 1, at 48 hours after neck incision, the thermal PT in the model group was significantly lower than in the normal group (p<0.001). Relative to the (untreated) model group, after EA, thermal PT values in the LI18 and LI4-PC6 groups were considerably higher (both p<0.001) while the ST36-GB34 group only showed a tendency towards a significant difference (p=0.053).

Effects of EA on SP and CGRP immunoreactivity
Immunofluorescence staining showed that SP- and CGRP-IR positive substances were granular in appearance and distributed throughout the cytoplasm. The SP- and CGRP-IR positive nerve fibres were also found between neuronal cell bodies. The diameters of SP- and CGRP-IR positive neurons ranged from 8–40 µm, being in the range of small- (diameter <30 µm) and medium- (31–40 µm in diameter) sized cells (figures 2A and 3A). The β III-tubulin-labelled DRG neurons were in the range of small- (<30 µm, 39.52±6.10%), medium- (31–40 µm, 35.07±5.99%) and large-sized cells (>40 µm, 25.41±3.53%). As shown in figures 2B and 3B, the proportions of SP- and CGRP-IR positive neurons were notably greater in the model group relative to the normal group at 48 hours post-surgery (p=0.01 and p<0.001, respectively). After EA, compared with the (untreated) model group, these proportions were markedly lower in the LI18 group (p=0.006 and p<0.001, respectively) and LI4-PC6 group (p=0.007 and p<0.001, respectively). By contrast, the proportion of CGRP-IR positive neurons tended to be higher in the ST36-GB34 group versus the model group (p=0.07), while the proportion of SP-IR positive neurons did not differ significantly (p=0.396).

Effects of EA on GAD67 immunoreactivity
As demonstrated by the photomicrographs presented in figure 4A, GAD67 expression was found in larger neurons (>40 µm in diameter) and was distributed throughout the cells. Compared with the model...
Effects of EA on mRNA expression of SP, CGRP and GAD67

The results of the qRT-PCR showed that, compared with the normal group, mRNA expression of SP and CGRP in the C3-6 DRGs was significantly increased in the model group versus the normal group (p<0.001 each; figure 5A,B). After the EA intervention, the increased mRNA expression of SP and CGRP was greatly downregulated in LI18 and LI4-PC6 groups (all p<0.001), but only slightly lower in the ST36-GB34 group, once again demonstrating a statistical tendency towards a significant difference relative to the model group (p=0.058 and p=0.054, respectively). After modelling, mRNA expression of GAD67 did not change (p=0.55; figure 5C); however, it was significantly increased in both the LI18 and LI4-PC6 groups (p<0.001 and p=0.019, respectively) relative to the model group. By contrast, no significant change in GAD67 mRNA expression was found between the ST36-GB34 group and the (untreated) model group (p=0.561).

DISCUSSION

To our knowledge, the present study report has, for the first time, characterised changes in mRNA and protein expression of SP, CGRP and GAD67 in the C3-C6 DRG in rats with incisional neck pain. Our results showed that 48 hours after neck incision, the regional thermal PT was significantly decreased, and both mRNA and protein expression of SP and CGRP were considerably increased. Peptidergic neurons in the C3-6 DRGs have been implicated in pain processing following neck incision, and our findings have mirrored the increase in SP and CGRP within the spinal dorsal horns of rats with incisional neck pain observed in our previous study.14 EA at LI18 and LI4-PC6 reversed the decrease in regional thermal PT and increase in mRNA and protein expression of SP and CGRP, and also upregulated the mRNA and protein expression of GAD67 in C3-C6 DRGs. This suggests that the increase in GABA and decrease in SP and CGRP expression may have contributed to EA analgesia in the LI18 and LI4-PC6 groups, probably (at least in part) by balancing the activity of these nociceptive and inhibitory neurons. However, no significant changes were found in thermal PT and levels of SP, CGRP and GAD67 expression after EA at ST36-GB34, which suggests a neuro-segmental analgesic action of EA. Our earlier study revealed that the afferent nerves from the regions of the thyroid gland and LI18 mainly converge on the C1-4 DRGs, and those from LI4 and PC6 regions chiefly distribute in the C3-7 DRGs.22 The afferent nerve fibres from the ST36 region mainly synapse with the L3-S1 DRG segment in rats,23 and both GB34 and ST36 are innervated by the deep and superficial peroneal nerves and therefore have a similar central projection. Thus, the spinal segments stimulated by acupuncture at LI18, LI4-PC6 and ST36-GB34 are likely to be equivalent, adjacent and distant, respectively, relative to the neck incision locus, which may explain the differential effects observed in the present study. Clearly, segmental needling in the neck had a much greater effect on thermal PT and expression of SP, CGRP and GABA in DRGs than distal needling; however, differences in thermal PT in the ST36-GB34 group came close to significance compared with the model group, suggesting an analgesic effect that may reflect central (supraspinal) mechanisms. These results provide experimental evidence for the clinical application of LI18 and LI4-PC6 EA analgesia to thyroidectomy. Until now, there have been few studies comparing local, segmental and distal effects of EA in this way.

It has been well documented that, during peripheral tissue injury or regional inflammation, the primary sensory neurons release specific mediators, such as ATP, CGRP and SP.24 The tachykinin SP, synthesised in the DRG, is released by the C-type primary afferent terminals of the small DRG neurons and interacts with neurokinin (NK)-1 receptors in the spinal dorsal horn to activate nociceptive signal transmission pathways, thereby inducing hyperalgesia. The small DRG neurons also express the NK-1 tachykinin receptor,25 which mediates SP-induced inhibition of GABA-activated current and membrane depolarisation.26 CGRP, a nociceptive neurotransmitter, is also expressed in the C5-C8 DRGs of the rat, in which it constitutes 41.5±5.4% of all neurons,27 and has been shown to be increased in the L5 DRG neurons in rats following L5-6 spinal nerve transection (SNT).28 Both GABAA and GABAB receptors are expressed in DRG sensory neurons. After application of selective GABAA (but not GABAB) receptor agonists, an analgesic effect is seen in rat models of inflammatory and neuropathic pain.29 30

In addition, it has been found that both CGRP and endomorphin-2 are co-localised with SP in the DRG neuron of rats,30 31 suggesting that interaction of these peptides may be involved in pain modulation. Acupuncture-induced analgesia is a complex process of physiological adjustment that involves both the central and peripheral nervous systems. An earlier study in rats showed that EA at LI4-PC6 for 10–30 min significantly decreased pain sensation in the cervical region and significantly increased the conduction velocity and amplitude of the electrical activities of the great auricular nerve.32 Furthermore, transcutaneous electrical nerve stimulation applied to the thigh reduced persistent postoperative mechanical hypersensitivity in rats following skin/muscle incision and retraction and reversed the upregulation of N-methyl-D-aspartate receptor 1 and SP levels observed in the DRG.33
Overall, it is possible that the positive effects of acupuncture in peripherally-generated pain conditions may be associated with decreased synthesis of pro-nociceptive mediators in the DRG and reduced activity of primary afferents. On the other hand, long-lasting downregulation of GABA tone or the sensitivity of DRG neurons has been confirmed in neuropathic rats and the activation of GABA-mediated inhibitory inputs from sensory neurons could be beneficial in the alleviation of pain, partly through selective inhibition of enhanced SP-like immunoreactivity. Thus, upregulation of GABA induced by the EA intervention may contribute to its analgesic effect by downregulating the expression of both SP and CGRP in the DRG of rats with incisional neck pain.

In conclusion, the findings of the present study suggests that small- and medium-sized SP- and CGRP-positive neurons in the cervical DRG may play an important role in mediating pain sensation after neck incision in rats. EA stimulation at LI18 and LI4-PC6 appears to effectively alleviate hyper-sensitivity by reducing levels of SP and CGRP and upregulating levels of GAD67 in the cervical DRG. These results may help to reveal a new target for EA therapy with the aim of reducing pain following thyroid surgery. Future research will focus on trying to delineate the exact mechanisms associated with EA analgesia that may underly the regulation of cross-talk between different types of neurons, or between nociceptive and anti-nociceptive molecules.

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Contributors  L-QN and J-LL performed the experiments and prepared the manuscript. J-LL designed the study and revised the manuscript. Y-SY supervised the experiments performed the experiments and analysed the data. J-LL and Y-SY provided assistance in applying for the grant. H-YL, L-HT and XZ assisted with the experimental work and data collection. All authors read and approved the final version of the manuscript accepted for publication.

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