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Neuroanatomical characteristics of deep and superficial needling using LI11 as an example

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ABSTRACT

Objectives To compare the neuroanatomical characteristics of the deep and superficial tissues at acupuncture point LI11 using a neural tracing technique, in order to examine the neural basis of potential differences between deep and superficial needling techniques.

Methods In order to mimic the situations of the deep and superficial needling, the retrograde neural tracer Alexa Fluor 488 conjugate of cholera toxin subunit B (AF488-CTB) was injected into the muscle or subcutaneous tissue, respectively, at acupuncture point LI11 in eight rats (n=4 each). Three days following injection, the distribution of motor and sensory neurons labelled with AF488-CTB was examined in the spinal cord and dorsal root ganglia (DRG) under a fluorescent microscope.

Results For both types of injection, labelled motor and sensory neurons were distributed on the side ipsilateral to the injection in the spinal cord and DRG between spinal levels C5 and T1. The number of labelled motor neurons following intramuscular injection was significantly higher than subcutaneous injection. By contrast, the number of labelled sensory neurons following subcutaneous injection was significantly higher in number and extended over a greater number of spinal segments compared to intramuscular injection.

Conclusions These data indicate that the motor and sensory innervation of muscle and subcutaneous tissue beneath LI11 differ, and suggest that acupuncture signals induced by deep and superficial needling stimulation may be transmitted through different neural pathways.

INTRODUCTION

Deep and superficial needling are two fundamental methods of acupuncture treatment.^{1–4} Although it has been proposed that appropriate depth of needling is an important issue that directly

influences the effect of acupuncture, the underlying mechanisms remain obscure.^{5–8} Innervation of the various tissue layers from skin to muscle have rarely been assessed in relation to acupuncture but it is likely to be important, especially given that we know the nervous system is intimately involved in the actions of acupuncture.^{9–12} Therefore, an understanding of the neural properties of the different tissue layers underlying points of needle insertion is necessary in order to explore the relative advantages of deep needling or superficial needling.¹³ In order to obtain a detailed view of the neuroanatomical characteristics of the tissue layers encountered by an acupuncture needle at variable depth, a representative acupuncture point, LI11 (*Quchi*), was selected and examined using a neural tracing technique with Alexa Fluor 488 conjugate of cholera toxin subunit B (AF488-CTB), a sensitive tracer that has successfully been used to investigate the neuroanatomical characteristics of acupuncture points.^{14 15} The aim of the present study was to inject AF488-CTB into muscle or subcutaneous tissue under LI11 in rats to mimic deep and superficial needling, respectively, in order to examine the motor and sensory innervation of the muscle and subcutaneous tissue at this anatomical location.

METHODS

Experimental animals

A total of eight young adult male Sprague Dawley rats, aged 10–12 weeks and weighing 220–250 g, were used in this study. Animals were provided by the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (licence number SCKX (JUN) 2007-004). During the experiment, the animals were



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housed in a 12 h light/dark cycle with controlled temperature and humidity and allowed free access to food and water. This study was approved by the ethics committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (reference number 20140014). The handling and care of the experimental animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington DC, USA, 1996).

Microinjection of AF488-CTB

In the human body, LI11 is located on the lateral aspect of the elbow, at the midpoint of a line connecting acupuncture point LU5 (lateral aspect of biceps tendon) and the lateral epicondyle of the humerus. When the elbow is fully flexed, LI11 is located in the depression at the end of the cubital crease. A corresponding site for LI11 in the rat was determined by relative anatomy. The rats were divided into two groups, one receiving intramuscular injection (n=4) and one receiving subcutaneous injection (n=4) to represent deep and superficial needling, respectively. Under general anaesthesia with isoflurane, 4 μ L 0.1% AF488-CTB (Invitrogen-Molecular Probes, Eugene, Oregon, USA) solution was injected at LI11 using a fine tipped Hamilton microsyringe. For intramuscular injection, AF488-CTB was injected perpendicularly into the brachioradialis and extensor carpi radialis longus muscles, which are partly overlapped under LI11, via an open skin incision in the LI11 region that was sutured after injection. For subcutaneous injection, AF488-CTB was injected at 15° angles into the subcutaneous tissue beneath LI11. When the rats awoke from anaesthesia they were placed back into their raising box.

Perfusion

Three days following injection, the rats were euthanased with urethane and transcardially perfused with 100 mL of 0.9% saline immediately followed by 300 mL of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). The cervical (C) and thoracic (T) spinal cord and associated dorsal root ganglia (DRG), as well as the local tissues in the LI11 region, were dissected out and stored in 25% sucrose PBS at 4°C.

Sectioning

Frozen sections were used in this study. Serial tangential sections of local tissues in the LI11 region, transverse sections of spinal cord, and sagittal sections of DRG were cut at a thickness of 20 μ m on a cryostat (Thermo, Microm International FSE, Walldorf, Germany) and mounted on silane-coated glass slides. Before observation, the slides were coverslipped using 50% glycerine to improve visualisation of labelling.

Without the need for any staining, neurons labelled with AF488-CTB can be directly observed on the sections under a fluorescent microscope.

Observation

AF488-CTB labelling was viewed and recorded using a fluorescent microscope (BX53, Olympus, Tokyo, Japan) equipped with a digital camera (DP73, Olympus). Digital images were then processed with Adobe Photoshop CS5 and Adobe illustrator CS5 (Adobe Systems, San Jose, California, USA). The anatomical structure of the spinal tissue sections was determined cytoarchitecturally based on the atlas of Paxinos and Watson,¹⁶ and segments of DRG were identified according to location of the vertebra. The number of labelled motor neurons were counted in five representative sections from each spinal cord and the number of labelled sensory neurons were counted in three sections from each DRG in all rats.

Statistical analysis

Data are presented as mean \pm SD and were processed with statistical software SPSS V.16.0 (SPSS Inc, Chicago, Illinois, USA). Data were collected and analysed by Dongsheng Xu who was kept blind to the group allocation.

RESULTS

At 3 days post-injection, AF488-CTB was still clearly present at the sites of injection. Following intramuscular injection, AF488-CTB was mainly limited to muscle with a longitudinal arrangement parallel with the muscle fibres (figure 1A). Following subcutaneous injection, AF488-CTB was distributed extensively around the site of injection up to a diameter of 2 mm within the subcutaneous tissue (figure 1B). For both types of injection, labelled motor and sensory neurons formed a segmental pattern on the side ipsilateral to the injection.

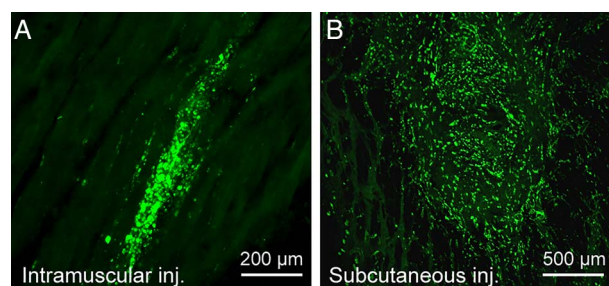


Figure 1 Distribution of the retrograde neural tracer Alexa Fluor 488 conjugate of cholera toxin subunit B (AF488-CTB) in local tissues from the region of acupuncture point LI11. (A) After intramuscular injection, AF488-CTB was limited to a relatively small area within the muscle tissue. (B) After subcutaneous injection, AF488-CTB spread widely in subcutaneous tissue.

Motor innervation

In both types of injection, labelled motor neurons were located at the mediolateral part of the ventral horn of the spinal cord (figure 2A). Following intramuscular injection, labelled motor neurons were distributed from C5 to T1 with highest concentrations at the C8 segment (presented in figure 2A). Meanwhile, following subcutaneous injection, labelled motor neurons were limited to the C7 and C8 segments (figure 2B). A total of 18 labelled motor neurons were counted among the four cases of subcutaneous injection (5 ± 1.3) and 80 counted among the four cases of intramuscular injection (20 ± 2.6), which was four times that of subcutaneous injection.

Sensory innervation

For both types of injection, labelled sensory neurons were distributed from the C5 to T1 DRG with highest concentrations in the C6 to C8 DRG (figures 3 and 4). A total of 340 labelled sensory neurons were counted among the four cases of intramuscular injection (85 ± 15.9), while 478 labelled sensory neurons were counted among the four cases of subcutaneous injection (120 ± 12.6), which was about 1.4 times that of intramuscular injection.

The segmental and regional distribution of the labelled motor and sensory neurons following

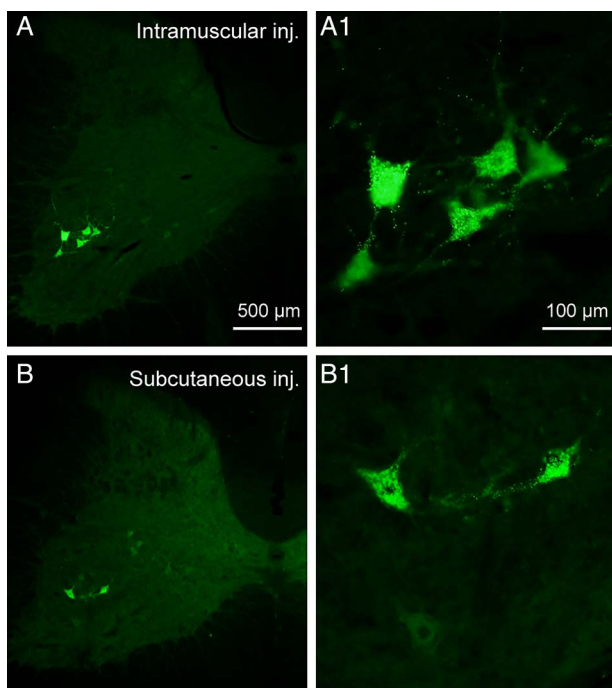


Figure 2 Labeled motor neurons in representative transverse sections of the ventral horn of C8 spinal cord (at two different levels of magnification) from eight experimental rats 3 days following intramuscular (A, A1) or subcutaneous (B, B1) injections of the retrograde neural tracer Alexa Fluor 488 conjugate of cholera toxin subunit B (AF488-CTB) at acupuncture point LI11 ($n=4$ each). The same scale bars apply to A and B and A1 and B1, respectively.

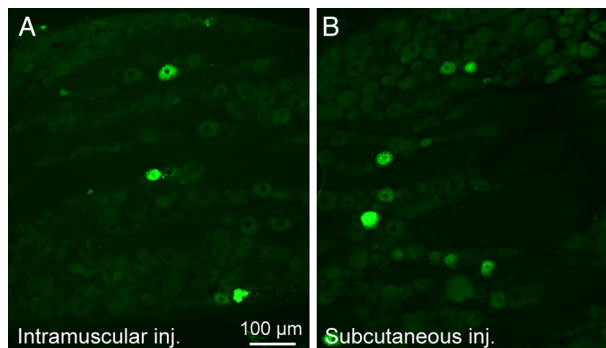


Figure 3 Labeled sensory neurons in representative sagittal sections of the C8 dorsal root ganglia (DRG) from eight experimental rats 3 days following intramuscular (A) or subcutaneous (B) injections of the retrograde neural tracer Alexa Fluor 488 conjugate of cholera toxin subunit B (AF488-CTB) at acupuncture point of LI11 ($n=4$ each). The same scale bar applies to A and B.

intramuscular and subcutaneous injection is summarised in figure 5, which reflects the neuroanatomical characteristics of the two different tissue layers in relation to deep and superficial needling at LI11.

DISCUSSION

Based on conventional human anatomy textbooks, the dermatomes and myotomes of the upper limb arise from spinal nerves C5–8 and T1. The innervation of the skin overlying LI11 corresponds to the C5/C6 dermatome, while the brachioradialis and extensor carpi radialis longus muscles correspond to the C5/C6 and C6/C7 myotomes, respectively. However, considerable overlap exists between adjacent dermatomes and myotomes innervated by the nerves derived from consecutive spinal cord segments. The present results in a small animal are generally consistent with the dermatomes and myotomes of the upper limb in the human, although it covers more spinal segments. The

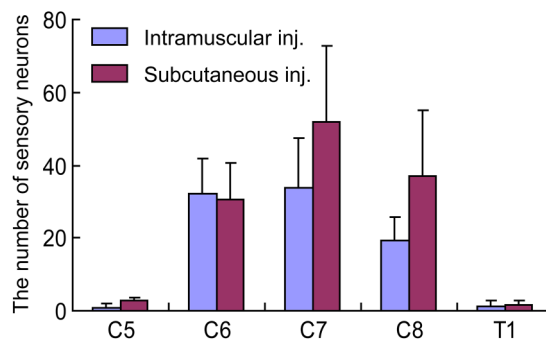


Figure 4 Average number of labeled sensory neurons at each spinal level from C5 to T1 in eight experimental rats 3 days following intramuscular (blue) or subcutaneous (purple) injection of the retrograde neural tracer Alexa Fluor 488 conjugate of cholera toxin subunit B (AF488-CTB) at acupuncture point of LI11 ($n=4$ each). Data are expressed as mean \pm SD.

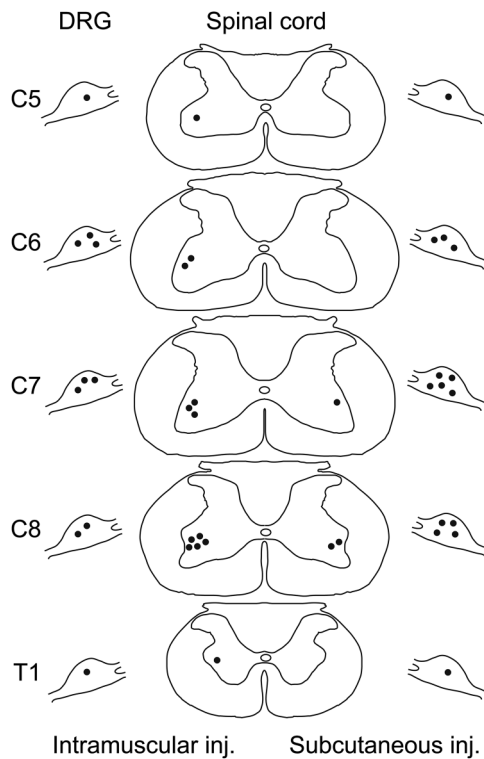


Figure 5 A series of line drawings through spinal levels C5 to T1 illustrating the dorsal root ganglia (DRG) and transverse spinal cord showing the general distribution of sensory and motor neurons labelled with the retrograde neural tracer Alexa Fluor 488 conjugate of cholera toxin subunit B (AF488-CTB) 3 days following intramuscular injection (left side) or subcutaneous injection (right side) at acupuncture point LI11 in the rat ($n=4$ each). The black dots in the DRG and spinal cord represent the labelled sensory and motor neurons respectively, and the numbers of dots reflect the relative quantities of labelled neurons between rats receiving intramuscular and subcutaneous injection at each spinal level.

different neuroanatomical characteristics of the deep and superficial tissue layers at LI11 suggest that the depth of needling is an important consideration in acupuncture treatment at this point. It should be noted that the present study mainly focused on the *depth* of needling rather than location, hence the lack of a control point. However, comparisons can be made with our previous studies of the neuroanatomical characteristics of other acupuncture points including BL57, BL40 and GB30.^{14 15}

Neuroanatomical considerations

Injection of AF488-CTB into the muscle and subcutaneous tissue at LI11 in rats resulted in effective retrograde labelling of motor and sensory neurons in the spinal ventral horn and DRG. It indicates that motor and sensory innervations of the muscle and subcutaneous tissues are different, suggesting that the neural mechanisms underlying the effects of deep and superficial needling may be functionally distinct.

Previous studies from our laboratory have provided experimental evidence that motor and sensory neurons in the vicinity of classical acupuncture points follow a segmental pattern in the nervous system.^{14 15 17} In the present study, we have confirmed that this is also true of acupuncture point LI11, while further demonstrating that the motor neurons of the deep tissue layer spread over a greater number of spinal segments and outnumber those of the superficial tissue layer. Although the sensory neurons associated with both deep and superficial tissue layers distribute similarly across the same spinal segments, the number of sensory neurons in the superficial tissue layer is greater than that of the deep tissue layer. In considering the distribution and number of motor and sensory neurons associated with the deep and superficial tissue layers, it is clear that there are quantitative differences between the motor and sensory innervation of these two layers, which are potentially involved in deep versus superficial needling, respectively.

During deep needling at LI11, the needle is inserted into the muscles. It is clear that skeletal muscles are supplied by both motor (efferent) and sensory (afferent) nerves. Axons derived from α and γ motor neurons in the spinal ventral horn innervate skeletal muscle fibres and spindles, respectively, while sensory fibres are mainly associated with specialised sensory receptors within muscle spindles.¹⁸ During superficial needling, the needle mainly makes contact with the skin (including the epidermis and dermis) and subcutaneous tissue. These tissue layers contain a wide variety of sensory receptors that detect mechanical, thermal, or nociceptive stimuli applied to the body surface.¹⁹ These receptors include bare nerve endings, Pacinian corpuscles, Merkel's discs, Meissner's corpuscles, Ruffini endings, and follicle receptors.^{12 18} Given the location of these pieces of sensory apparatus, in contrast to deep needling, neural signals originating from superficial needling may be transported through different sensory receptors to the central nervous system. The reason why such a relatively small number of motor neurons were labelled with AF488-CTB after subcutaneous injection is unclear. A possible explanation is that only a small number of motor innervations exist in skin, although the blood vessels, arrector pili muscle, and sweat glands are well supplied with motor nerve endings.

Based on our current knowledge, acupuncture is believed to exert its effects through sensory afferent stimulation. Although we are unable to assess, based on the present results, whether acupuncture works via stimulation of motor nerves, as the study was neuroanatomical rather than neurofunctional in nature, it is clear that tracer can be transported in a retrograde manner to the cell body of motor neurons. This phenomenon implies that motor neurons could be directly stimulated by acupuncture. Irrespective, our

results provide neuroanatomical evidence that suggests there may be differences between the neural mechanisms underlying deep and superficial needling at LI11.

Clinical considerations

From a clinical point of view, it is important to consider the advantages and disadvantages of deep versus superficial needling.² Deep needling is theoretically associated with a higher risk of damage to nerves, blood vessels and other structures, and a greater incidence of post-treatment soreness. In contrast to deep needling, superficial needling only involves penetration of the dermal and subcutaneous layers, and is easily carried out with a lower risk of damage to the surrounding structures.² Although acupuncture signals may be more strongly elicited by deep needling compared to superficial needling, we should carefully consider the relative advantages and disadvantages to select the appropriate depth of needling and degree of stimulation according to the clinical scenario.

Research prospects

Even though the functional significance of deep versus superficial needling remains far from clear,^{2, 7} the findings of the present study provide us with a clue as to the neuroanatomical basis of the pathways involved, which originate in the deep and/or superficial tissue layers at the needling site, which should help us better understand the neural mechanisms underlying the action of deep versus superficial acupuncture stimulation. Recent studies have shown that sensory neurons with different chemical phenotypes, such as somatostatin-, vanilloid receptor-, substance P-, and calcitonin gene related peptide-positive neurons, are characterised by axons that extend to muscle and cutaneous tissue;^{20–22} these subtypes of sensory neuron may play a critical role in deep and superficial needling stimulation and/or production of the *de qi* sensation. The roles of different types of neurons will be examined by future research.

Limitations of the study

It is a technical limitation that AF488-CTB cannot be used to label nerve fibres and axonal terminals like conventional CTB;¹⁷ however, it remains an appropriate method to label sensory and motor neurons associated with acupuncture points in a retrograde manner.^{14, 15} Although, in the present study, the tracer was separately injected into the muscle or subcutaneous tissue in the experimental animals (through the use of a skin incision), in clinical practice the acupuncture needle will always pierce the dermal layer before reaching the muscle layer. Therefore, when evaluating the effect of deep needling, the neuroanatomical characteristics associated with both the muscle and the subcutaneous tissue layers should be considered together.

CONCLUSION

The present study provides neuroanatomical evidence demonstrating that the motor and sensory neurons in the deep and superficial tissue layers beneath LI11 are different and may therefore participate in deep and superficial needling in distinct ways. Although it cannot be concluded which method of acupuncture treatment is better, this study provides new insights into the neural pathways involved in deep and superficial acupuncture stimulation.

Contributors The work presented here was carried out in collaboration between all authors. MW and JC contributed equally to this work. WB, JC and XJ designed the study. MW, DX and KZ carried out the laboratory experiments and analysed the data. JC and WB wrote the paper. All authors read and approved the final manuscript.

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Competing interests None declared.

Ethics approval This study was approved by the ethics committee at Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, Beijing, China.

Provenance and peer review Not commissioned; externally peer reviewed.

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