Effect of electroacupuncture at Zusanli (ST 36) on dorsal root nerve signals in normal rats

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Keywords: Electroacupuncture; Spinal nerve roots; Point ST36 (Zusanli); Intensity; Signal

Abstract

OBJECTIVE: To investigate how electroacupuncture (EA) at Zusanli (ST 36) with varying intensities of stimulation affects dorsal root nerve signals in normal rats.

METHODS: Adult female Wistar rats were examined after drug-induced anesthesia and isolation of the L4 dorsal root associated with the Zusanli (ST 36) acupoint, using bipolar platinum electrodes. We applied EA at Zusanli (ST 36) with a continuous wave waveform for 1 min, and recorded action potentials in the L4 spinal cord dorsal root nerve with a frequency of 5 Hz and various current intensity levels (approximately 1-4 mA). In addition, we tested EA with a frequency of 15 Hz and current intensity of 1-4 mA. The L4 spinal cord dorsal nerve bundle action potentials were measured for 1 min of EA. To analyze the discharge frequency, we used the maximum Lyapunov exponent and Lempel-Ziv (LZ) complexity.

RESULTS: At a fixed frequency, with increasing intensity of EA, dorsal root nerve filament discharge frequency revealed an initial increase, followed by a decreasing trend. A stimulation intensity of 3 mA induced a significantly greater discharge frequency, compared with stimulation intensities of 2 and 4 mA. EA stimulation evoked neuroelectric signals with chaotic characteristics. Increased intensity led to an initial increase in LZ complexity which then decreased, with a stimulation strength of 3 mA inducing the highest level of LZ complexity.

CONCLUSION: EA of different intensities can induce nerve action potential encoding with different features.

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INTRODUCTION

According to World Health Organization statistics, acupuncture is used in 183 countries. Although there is some evidence that acupuncture is effective in the treatment of certain diseases, current scientific knowledge regarding the underlying mechanisms of acupuncture is not comprehensive. One previous study reported that acupuncture causes information to be transmitted to the central nervous system through the peripheral
nervous system associated with the stimulated receptors, and information integration through the central nervous system regulates the neuro-endocrine-immune network, which in turn affects the target organ. In addition, previous studies have reported that acupuncture causes a large number of electrical signals and chemical signals to be generated in the body, which serve to achieve integration and regulation of the functional activities of the body. Thus, previous studies suggest that the generation and transmission of acupuncture nerve electrical signals can be elicited by applying a needle. Different acupuncture stimulation parameters can produce different effects. From the perspective of nervous system function, these effects are related to the characteristics of the type of afferent nerve fibers and the encoding of neuroelectrical information. Action potential transmission is an all-or-nothing process in which subthreshold stimulation cannot cause an action potential to occur. Once the threshold stimulus is reached, the resulting action potential generally does not affect the potential amplitude, only the frequency pattern of the discharge. Thus, the temporal pattern of stimulation is likely to have an impact on the effect of stimulation, and the nervous system expresses and transmits information in discrete action potential discharge sequences. Different types of information are transmitted by different combinations and sequences of action potentials, such as the National Natural Science Foundation of China (2006-2009, No. 50537030). Taking electrical signals as a starting point, and utilizing research from multiple disciplines, including electrical engineering, traditional Chinese techniques of acupuncture and moxibustion, systematic and modern detection, and signal processing techniques, a range of methods for peripheral afferent nerve and spinal dorsal horn neurons have been developed. Acupuncture-based manipulation of neuronal firing signals is associated with central nervous system function. Modern bio-signal analysis methods have been used to analyze the electrical signals associated with different acupuncture methods. Previous studies have reported that different acupuncture techniques result in differences in the characteristics of the elicited electrical signals, which may reflect the mechanisms by which different acupuncture methods produce different therapeutic effects.

Liu et al. studied the discharge sequence of spinal cord dorsal root nerve electrical signals at four frequencies (50, 100, 150, 200 beats/min). It was found that the frequency of spinal dorsal root nerves increased with the increase of acupuncture frequency. At the same time, the Interspike Interval (ISI) variance coefficient of different frequency interpolation signals is analyzed. The joint distribution map of the discharge frequency per unit time window shows that the response signal of spinal dorsal root nerve is at the discharge frequency under the stimulation of different frequencies. There is a clear distinction between trends and variability in ISI distribution.

Studies have shown differences in the discharge signals of the spinal dorsal root nerve caused by acupuncture at Zusanli (ST 36). Furthermore, the ISI of the four manual electrical signals was analyzed. One previous study found that the entanglement method exhibited clear distribution characteristics of cluster discharge, whereas the interpolation method did not. The entanglement method and the interpolation method by analyzing the ISI variance coefficient, revealing that the electrical signals of the four methods have unique characteristics.

Moxibustion is closely related to the effects of acupuncture, and has been found to have important effects in the nervous system. Acupuncture-induced information is transmitted via the peripheral nervous system, producing integration and regulation through the central nervous system. Thus, nerve signals are thought to be an important signal in the effects of acupuncture. Acupuncture can be divided into manual acupuncture and electroacupuncture (EA). EA has been widely used in research due to the precise control of the stimulation parameters and the flexibility in the amount of stimulation delivered. The parameters of EA include the frequency, intensity, time, and waveform. Several previous studies reported that different effects are produced by different stimulation parameters. Zhou et al. reported that EA with 3.5 mA stimulation produced a stronger analgesic effect for adjuvant arthritis in rats, compared with 5.5 mA EA. Liu et al. confirmed that 1 mA of EA for chronic pain in rats induced a stronger analgesic effect than 5 mA EA. Wang et al. tested EA with different intensities in normal rats, and found that the number of cells that were positive for p-Akt expression in the rat brain was higher for 2-4 mA stimulation compared with 5-7 mA stimulation. However, the neural mechanisms underlying the different effects associated with differences in EA intensity are currently unclear. Therefore, in the current study, we used nerve action potential encoding as a starting point to investigate the effects of EA on the strength of neural coding mechanisms.

The current study examined electrical signals in the spinal cord dorsal root nerve following different intensities of EA stimulation at Zusanli (ST 36). Our experimental results were analyzed using linear and nonlinear analysis methods, and the characteristics of the nerve signals coded by different intensities of EA were examined.

METHODS

Grouping
In this experiment, we tested EA stimulation frequencies of 5 and 15 Hz. Each level was divided into four groups with different current intensities, as follows: 1, 2, 3, and 4 mA (Table 1).
Experimental grouping: in this experiment, there were 10 healthy Wistar female rats weighing 220-240 g, 5 in the 5 Hz electroacupuncture group, and 5 in the 15 Hz electroacupuncture group. Stimulation method: rats were stimulated with electroacupuncture frequency of 5 Hz and intensity of 1 mA for 1 min, then rested for 10 min. As the elution period, after the rest, the rats were stimulated with the intensity of 2 mA for 1 min, then rested for 10 min until the intensity was 4 mA. The stimulation was over; the stimulation method of the 15 Hz electroacupuncture group was the same as that of the 5 Hz electroacupuncture group (Figure 1).

**Surgical preparation**

The experiments were conducted on adult female Wistar rats (Certificate of Conformity 2009-003), weighing 200-300 g, obtained locally from Beijing Vital River Laboratory Animal Technology Co., Ltd. Rats were housed in pairs on a 12:12 h light/dark cycle. Food and water were provided ad libitum. All procedures were carried out in accordance with the USA Public Health Services Guide for Care and Use of Laboratory Animals, and the experimental protocols were approved by the Animal Use and Care Committee of Tianjin University of Traditional Chinese Medicine. All efforts were made to minimize the number of animals used and the number of procedures per animal. The experimental animals were anesthetized with ketamine and xylazine (150 mg/kg and 15 mg/kg, respectively) via intra-peritoneal injection and then placed in a heating blanket to maintain core temperature. The rats were placed in a water bath (HH-1, Shanghai Huyue Ming Scientific Instrument Co., Ltd.). At a temperature of approximately 37 °C, liquid paraffin was used to cover the spinal cord to avoid drying. Under the dissecting microscope (SZX-B1301F80929, Japan OLYMPUS), the dura mater was cut with hairspring tweezers, and the L4 intervertebral foramen was found on both sides. The L4 dorsal root was separated by a glass minute hand at the position of the corresponding intervertebral foramen, or slightly under the lumbar enlargement. The L6, L5, and L4 dorsal root nerves were sequentially distributed along the midline, and the L4 dorsal root was separated by a glass minute hand and slid down to determine whether it was from the L4 intervertebral foramen. The L4 dorsal root was isolated and cut using ophthalmology scissors near the lumbar enlargement.

**Separate dorsal root nerve filaments**

Nerve filaments were segregated under a dissecting microscope (SZX-B1301F80929, Japan OLYMPUS) with hairspring tweezers from the L4 dorsal root afferent. The nerve filaments were placed in a bipolar electrode of a platinum wire recording device. According to the amplitude and waveform of discharge, we made a preliminary judgment about whether we were observing a single nerve fiber discharge, and continued to subdivide small nerve bundles until a sensitive point in the

<table>
<thead>
<tr>
<th>Group</th>
<th>1 mA</th>
<th>2 mA</th>
<th>3 mA</th>
<th>4 mA</th>
</tr>
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<tbody>
<tr>
<td>5 Hz</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>15 Hz</td>
<td>5</td>
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Figure 1 Experimental stimulation method
Zusanli (ST 36) area of single nerve fibers was identified. Spinal cord dorsal root nerve fiber discharge was recorded by BIOPAC-MP150 (BIOPAC Corporation, USA). After completing the experimental procedures, the animals were sacrificed with an overdose of urethane.

Zusanli (ST 36) location
Zusanli (ST 36) was located according to the positioning method of comparative anatomy. The position of Zusanli (ST 36) was as follows: on the rat hind limb knee joint, approximately 5 mm below the fibular head.

Electrophysiological recording
Corresponding Zusanli (ST 36) nerve filament signals were obtained using EA with different parameters. The recorded stimulus frequencies were 5 and 15 Hz, with intensities of 1, 2, 3 and 4 mA. The stimulation time lasted 1 min, with a 10 min rest between each stimulus intensity interval. Different frequencies were utilized to eliminate differences between frequency and impact. Figure 2 shows the sequences of action potentials collected in the spinal cord dorsal root nerve fibers with EA with a stimulation frequency of 5 Hz and intensity of 2 and 3 mA, as well as EA with a stimulation frequency of 15 Hz and intensity of 2 and 3 mA.

Data analysis
A total of 30 sets of effective action potential data were recorded in this experiment. The data sampling frequency was 40 kHz in each condition, and data were analyzed for 60 seconds for each parameter. Using a signal selection method (sorting) for separation of single nerve fiber discharge, we recorded the unit discharge frequency of time windows, the largest Lyapunov exponent, and the Lempel-Ziv (LZ) complexity as measures of signal characteristics. Statistical comparisons were carried out using SPSS to perform one-way analysis of variance and post hoc tests. For all comparisons, $P < 0.05$ was accepted as the criterion indicating significant differences.

Unit time window discharge frequency
The frequency coding theory is a widely accepted theory of neural information coding. The underlying concept of this theory is that the only important property of a nerve bundle in a nerve discharge series is the discharge frequency, which carries the relevant information. In the nervous system, thin neural beam discharge of high and low frequency reflects the strength of stimulation. In most conventional electrophysiological observation indexes, neural discharge pulse counting is used to determine the activity level. Therefore, the current study used the concept of unit time window discharge frequency, examining the number of neural electrical signals within a specified timeframe. Unit time discharge frequency was measured by setting a time window, calculating the neural electrical signals in each time window, translating the time window, and analyzing the effects of different stimulation parameters on spinal cord dorsal root nerve discharge.

Maximum Lyapunov index
The Lyapunov exponent is the speed of divergence or
convergence of a given orbit in different directions, thus depicting the local instability of the orbit, and the quantitative discrimination of the initial sensitive dependence of chaos.

For an m-dimensional dynamical system

\[ X = f(X) \]

The initial conditional error \( \delta X_0 \) with time evolution would be expected to correspond to the following equation.

\[ \delta X = \frac{\partial f}{\partial X} \delta X_0 \]

After \( \tau \) moment, \( \delta X \) can be expressed as

\[ |\delta X| = |\delta X_0| e^{\lambda \tau} \]

Then, the Lyapunov index is

\[ \lambda = \frac{1}{\tau} \ln \frac{|\delta X|}{|\delta X_0|} = \frac{1}{\tau} \ln \left| \frac{\partial f}{\partial X} \right| \]

If \( \lambda < 0 \) the system along the direction I convergence to a stable point or a periodic orbit would appear to be dissipative, indicating asymptotic stability. If \( \lambda = 0 \), the system along the direction of the I exhibits neutral stability, indicating that it is conservative and in a stable state. If \( \lambda > 0 \), the system along the direction of the I exhibits chaos and instability.

For a chaotic system, there is at least one positive Lyapunov exponent and \( \sum \lambda < 0 \) (maintaining the global stability of the system). The greater the Lyapunov exponent, the more chaos in the system, and the more difficult the values are to predict. Because of the limited number of action potentials evoked by the peripheral and lower central nerve pathways in the 60 s EA period, the corresponding ISI sequence was relatively short. Therefore, the maximum Lyapunov index of the ISI sequence corresponding to the action potential sequence with different intensities of EA was estimated using a small data method.

**Lempel-Ziv complexity**

The LZ complexity of the algorithm was as follows:

A typical action potential duration is approximately 1 ms, and if the time resolution is 1 ms, the neuronal action potential sequence can be converted to a sequence of 0s and 1s. The action potential of the length of the time series is set to \( t \), and the window width \( TW \) is set to 1 ms with a sliding window on the action potential train if the sliding windows do not overlap. If there is discharge in the sliding window, the value is set to 1, and if there is no discharge, the value is set to 0. As a result, the length of \( N = T/TW \) symbol sequence.

\[ S = (S_1, S_2, \cdots, S_n) \]

So,

\[ S_i = \begin{cases} 1 & \text{if } i \in [(j-1)T_e, iT_e] \\ 0 & \text{Rest} \end{cases} \]

(2) Take character from sequence \( S' = (S'_1, S'_2, \cdots, S'_n) \), connect \( Q = (S'_1, S'_2, \cdots, S'_n) \), gain character \( SQ = (S', Q) \), change \( S' \), \( Q \) into \( S' \), \( Q' \). Estimate whether \( Q \) is from \( S', Q \). If not, set \( S'_i, Q \rightarrow S' \) and \( (S'_1, Q) \rightarrow Q \) to get \( \epsilon(n) + 1 \rightarrow \epsilon(n) \). If so, fix \( S'_i \) to get \( (S'_1, S'_2, \cdots, S'_n, Q) \rightarrow Q \).

(3) Repeat operation (2) to the symbol sequence, until the last character \( S' \) or \( Q \) is the end of the entire original string.

(4) Perform the final execution of \( \epsilon(n) + 1 \rightarrow \epsilon(n) \), and the LZ complexity equals \( C(n) \).

The complexity of LZ is related to the length of the string. To exclude the effect of the sequence length, normalize processing of \( C(n) \). For a sufficiently long (0, 1) sequence,

\[ \frac{\ln C(n)}{\ln(n)} \]

Thus, there is normalized LZ complexity

\[ C(n) = \frac{\epsilon(n)}{\ln(n)} \]

This function expresses the complexity of the time series, and some patterns exist in the sequence, the probability of the occurrence of 0 and 1 in the sequence changes, resulting in the complexity of the reduction. For a completely random sequence, \( C(n) > 1 \), while for a periodic sequence, \( C(n) > 0 \), namely \( C(n) \) more, indicating that a random dynamic system is enhanced as complexity increases, while power systems become more apparent.

**RESULTS**

The effects of different intensities of EA stimulation on dorsal root nerve discharge frequency are shown in Figure 3. As shown in Figure 3A, EA strength of 2 mA caused significant nerve fiber discharge, with increasing intensity causing an initial increase in discharge frequency followed by a decreasing climate. A stimulation strength of 3 mA induced a significantly higher discharge frequency compared with 2 and 4 mA stimulation \((P < 0.00001)\).

Figure 3B shows that a current strength of 2 mA induced clear nerve fiber discharge. With increasing current intensity, the discharge frequency exhibited a climax of initial increase followed by a decrease. A current strength of 3 mA induced a significantly higher discharge frequency compared with 2 and 4 mA currents \((P = 0.0285 < 0.05, P < 0.000001, \text{respectively})\). The frequencies of EA were 5 and 15 Hz. When the intensity changed, the largest Lyapunov exponents of the mean dorsal root nerve discharge frequency are shown in Figure 4. As shown in Figure 4A, when the EA frequency was 5 Hz, the degree of ISI sequence LZ complexity revealed a trend of an initial increase followed by a decrease with increasing intensity. In addition, LZ complexity was greatest with a stimulation strength of 3 mA, compared with strengths of 2 and 4 mA \((P < 0.00001)\). As shown in Figure 4B, we tested EA fre-
spinal dorsal root discharge signals. The maximum Lyapunov exponent was positive, indicating that the nervous system was sensitive to the initial value. The local structurally unstable nature of this chaotic movement causes nerve filaments in particular environments to change quickly from one mode to another, while producing a large amount of high discharge mode, and different effects of acupuncture points are generated by different intensities of electrical stimulation.

Because there was no current before EA stimulation to induce neural excitation, there was no discharge. While a current intensity of 1 mA was too weak to cause neural excitation, only sporadic discharge was found during the experiment. With increasing intensity, the firing rate initially increased, then showed a decrease, reaching the highest value with a strength of 3 mA. Similarly, LZ complexity initially increased with greater intensity, then decreased. A strength of 3 mA caused the greatest complexity. Thus, EA of 3 mA intensity had the greatest effect on the nervous system. Animals exhibited slight twitching with a stimulation strength of 4 mA, and the discharge rate began to decline, suggesting that these EA stimulation parameters may have caused pain responses.

The results obtained in the current study are consistent with previous findings reported by Han et al showing that electrical stimulation with a current strength of 1-3 mA was most appropriate, and that excessively strong stimuli produced pain. Thus, a strength of 3 mA appears to be a critical level, and the current results provide experimental support for the quantification of EA strength.

The nervous system converts external stimuli into information via space-time coding. Acupuncture acts on the body surface receptors of the human body, and is closely related to the nervous system. Various neural signals can be induced through different combinations of stimulation parameters.

Different intensity EA causes the nervous system to produce or alter neural electrical signals, indicating that incoming neuronal signals are encoded into combined spatiotemporal sequences. Several previous studies have reported that nerves can exert a regulatory effect on the body by coding external stimuli. However, there has been little research into the relationship between EA strength and induced discharge. Thus, the mechanisms of the effects of different intensity EA on the nervous system and the mechanisms of encoding and expressing neural electrical signals remain unclear.

In conclusion, the current findings suggest that comprehensive discharge frequency and LZ complexity may be valuable measures for determining the mechanisms underlying acupuncture stimulation. The time series of neuron discharge frequency and LZ complexity reflect the cluster characteristics of different intensity EA. Since acupuncture involves physiological sensory stimulation, a range of factors (such as acupuncture intensity, frequency, needle retention time, acupuncture...
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REFERENCES


