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[Original Article]

SUPPRESSIVE EFFECT OF ACUPUNCTURE STIMULATION TO THE SACRAL SEGMENT ON THE STATE OF VIGILANCE AND THE BRAINSTEM CHOLINERGIC NEURONS

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Abstract: The effects of acupuncture stimulation to the sacral segment on the electroencephalogram (EEG) and activity of the cholinergic neurons in the laterodorsal tegmental nucleus (LDT) were examined in urethane-anesthetized rats. When EEG was small amplitude and higher frequency, the stimulation to the sacral segment induced large amplitude and slow EEG with latencies ranged from 45 sec to 12 min, and durations from 48 sec to 56 min. The stimulus induced EEG is composed of significant increase in delta power and significant decrease in theta and beta powers. Firing rate of the cholinergic LDT neurons significantly decreased from 2.9 ± 1.5 Hz to 1.1 ± 0.8 Hz after the stimulus ($n=12$, $p<0.05$). The decrease of neuronal activity always preceded to the start of large and slow EEG, while the increase of the activity always preceded to the change of EEG from large slow wave to small faster wave. These results suggest that the acupuncture stimulation to the sacral segment changes the state of the animals from light anesthesia to deep anesthesia, and that the change is mediated by the suppression of the cholinergic neurons in the LDT.

Key words: acupuncture, electroencephalogram, sacral segment, laterodorsal tegmental nucleus, cholinergic neuron

INTRODUCTION

Acupuncture has been used to treat various diseases in China for more than 2,000 years. Such a therapeutic technique has recently been clinically applied to the patients with sleep disturbances particularly insomnia[1-4] instead of using sleeping

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pills which cause side effects[5]. However, the mechanism of acupuncture to improve the sleep diseases remains to be known. In animals, several experiments have performed using acupuncture to investigate the effect on the state of vigilance[6-8]. Both in clinically and animal studies, it has been proved that acupuncture stimulation to the sacral segment has suppressive effect on bladder activity[9,10] and we also found that the bladder activity is closely related with the change of electroencephalogram (EEG) or the state of vigilance[11]. So, it is required to clarify whether the acupuncture stimulation to the sacral segment affects the state of vigilance.

It is well known that the state of vigilance is regulated by the monoaminergic and cholinergic neurons in the brainstem[12,13]. Among them, the cholinergic neurons in the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT) have a crucial role in the regulation of REM sleep and waking[14,15]. A population of cholinergic neurons in the LDT/PPT increase their activity specifically during REM sleep, while another population shows the increased firing both during REM sleep and waking[16-18]. So, when the acupuncture stimulation to the sacral segment affect the state of vigilance, it is possible that such change is mediated by the brainstem cholinergic neurons. In the present study, using anesthetized rats, we examined the effect of acupuncture stimulation to the sacral segment on the electroencephalogram (EEG) and the activity of the brainstem cholinergic neurons.

MATERIALS AND METHODS

Experimental animals

The present study was carried out under the control of the Animal Research Committee in accordance with the Guideline on Animal Experiments in Fukushima Medical University and Animal Protection and Management Law of Japanese Government.

Sprague-Dawley male rats weighing 300-500 g (Japan SLC Inc) were used. They were anesthetized with urethane (1.0 g/kg, intraperitoneally) and additional doses of the same anesthetic were given to maintain an appropriate level of anesthesia. The head was fixed in a stereotaxic instrument and a small piece of skull overlying the cerebellum was trephined. Stainless steel bolts (diameter, 1.0 mm) were screwed into the skull over the frontal and parietal cortices to record the electroencephalogram (EEG).

Experimental procedure

For acupuncture stimulation, a fine acupuncture needle with diameter of 0.35 mm was positioned at the periosteum of sacral segment (S2 or S3) by palpation and rotated manually for 1 min. Under urethane amesthesia, the EEG showed a periodical alternation between two states: slow wave with large amplitude, which is indicative of deep anesthesia, and faster wave with small amplitude, which is

indicative of light anesthesia. Acupuncture stimulation was applied when EEG showed fast wave with small amplitude.

The direct EEG signal was filtered with a band pass width of 0.5 to 300 Hz through a bioelectric amplifier and passed to the signal acquisition system (CED1401, Spike 2, Cambridge Electronic Design, Cambridge, UK). To record single neuronal activity, a glass pipette electrode filled with 0.5 M sodium acetate containing 2% Pontamine sky blue was used. A small hole was made in the skull overlying the cerebellum, and the electrode was introduced stereotaxically through the hole to the brainstem. To avoid penetration of the venous sinus, the electrode was angled posteriorly at 30 degrees. The neuronal activity was amplified through a high impedance amplifier and then a conventional amplifier with a time constant of 0.01 second. All signals were digitized and stored on a personal computer hard disk for off-line analysis using Spike 2 software.

The cholinergic neurons in the laterodorsal tegmental nucleus (LDT) were discriminated from the non-cholinergic neurons by the width of action potential or with a shoulder at the descending phase[19]. The neurons were judged to be cholinergic when a positive component of the spike was broader than 0.8 ms or with a shoulder at the descending phase (Fig. 2C). Change of firing rate was determined when the firing rate per 1 bin (1sec) exceeded±SD (standard deviation) of the mean (obtained from measurement of preceding 30 seconds) for more than 5 consequence bins. Recording sites were marked by ejection of Pontamine sky blue from the recording electrode.

Histology

After the experiment, the animal was deeply anesthetized with pentobarbital, and perfused transcardially with 300 ml of 4% paraformaldehyde in 0.1 M phosphare buffer (pH 7.4). The brain was removed from the skull and post-fixed in the same solution overnight, immersed in 30% sucrose for several hours, and cut on a freezing microtome at 50 μm in the frontal plane. To identify cholinergic neurons, the sections were processed for NADPH-diaphorase, which is a specific marker of the brainstem cholinergic neurons[20].

RESULTS

Changes in EEG pattern

Urethane anesthetized rats showed two EEG patterns which changed alternatively ; slow wave with large amplitude and faster wave with small amplitude. The former indicates the state of deep anesthesia, while the latter is that of light anesthesia. When EEG was small and faster, the acupuncture stimulation to the sacral segment increased the amplitude and decreased the frequency of EEG. When the sacral segment S3 (bar in Fig. 1A) was stimulated by acupuncture needle, the amplitude of EEG increased and the frequency decreased with a latency of 152

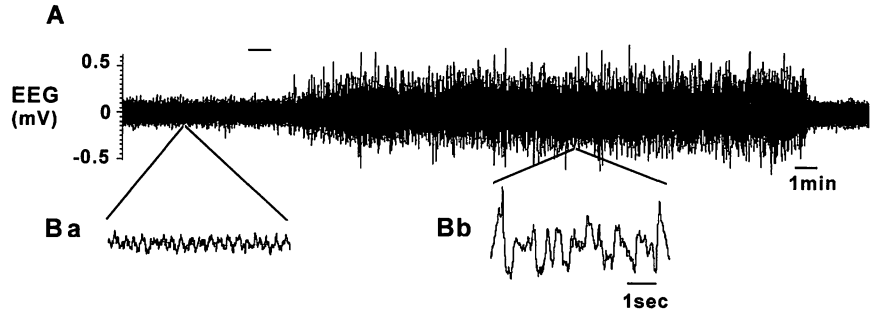

Fig. 1. Effect of acupuncture stimulation to the sacral segment on EEG change. A, EEG change after the acupuncture stimulation to the sacral segment S3. A bar above the EEG record indicates the period of acupuncture stimulation. Ba, Bb, Time expanded EEG trace before (Ba) and after (Bb) the acupuncture stimulation. EEG, electroencephalogram.

seconds and the large EEG continued for 23 minutes. Time expanded trace of EEG indicates that the main EEG frequency before the stimulus (3.0 Hz) (Fig. 1Ba) decreased to 1.5 Hz after the stimulus (Fig. 1Bb).

The large amplitude slow EEG was induced by acupuncture stimulation in 18 of 26 trials (69%) with latencies from 45 to 730 seconds and durations from 48 seconds to 56 minutes. Under urethane anesthesia of the present dose (1.0 g/kg), the average interval of spontaneously occurring large EEG was about 800 sec ($n=9$, observation time 36 hours). So, in the present experiment, the response with latency less than 800 sec was considered to be stimulus evoked. While those with longer latencies were spontaneously occurring EEG change.

Changes in neuronal activity

Figure 2 shows the activity of the cholinergic neurons in the LDT when the sacral segment stimulation induced large amplitude slow EEG. The neuron, which discharged rather tonically about 4.7 Hz before the stimulation, decreased and completely stopped firing when the large EEG was induced. The firing gradually increased at the latter period of large EEG and completely recovered when the small amplitude faster EEG appeared (Fig. 2A). Time expanded trace around the initiation of large EEG indicates that the significant decrease of neuronal firing occurred about 43 seconds before the EEG change (Fig. 2Ba), while when the large EEG changed to be small, the increase of firing occurred about 1.6 seconds before the change of EEG (Fig. 2Bb). Of 12 trials, when the stimulation induced large EEG and the neuronal activity was successfully recorded (Fig. 3), 9 cholinergic neurons in the LDT significantly decreased the firing rate. The firing rate of these nine neurons changed from 3.1-1.7 Hz (before the stimulus) to 1.0-0.5 Hz (after the stimulus). Of 9 neurons, five completely stopped firing from 42 to 445 seconds during the large EEG state (Fig. 2A and Ba spike and rate). Although the firing change was not significant in the remaining three, the firing rate decreased to 64-77

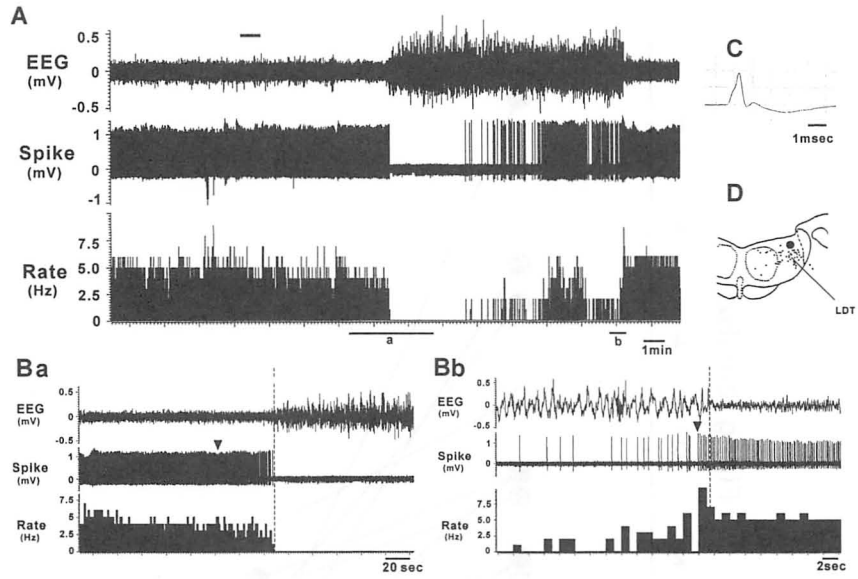

Fig. 2. A, Change of the activity of the cholinergic neuron in the laterodorsal tegmental nucleus (LDT) when EEG enlargement was induced by the acupuncture stimulation to S3 (bar). Ba and Bb, Time expanded scales of bar a and bar b in Fig. 2A, respectively showing the changes of neuronal activity in relation with EEG change. Arrowheads: the start of the changes of the neuronal activity. Broken lines: the start of the EEG change. C, averaged shape of the action potential recorded from site in D. The neuron was considered to be cholinergic because of the positive component with duration of 1.1 msec. D, Recording site (●). Spike, raw trace of action potential; Rate, firing rate per second of the neuron.

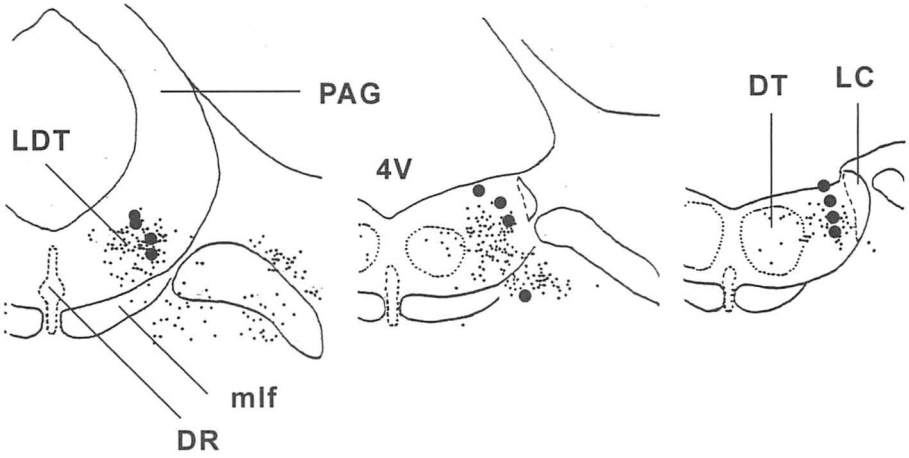

Fig. 3. Locations of the recorded neurons plotted on diagrams of serial coronal sections with interval of 0.3 mm. Small dots are the single cholinergic LDT neurons in one template animal identified by NADPH diaphorase histochemistry. LDT, laterodorsal tegmental nucleus. DR, dorsal raphe nucleus. DT, dorsal tegmental nucleus. LC, locus coeruleus. mlf, medial longitudinal fasciculus. PAG, periaqueductal gray. 4V, 4th ventricle.

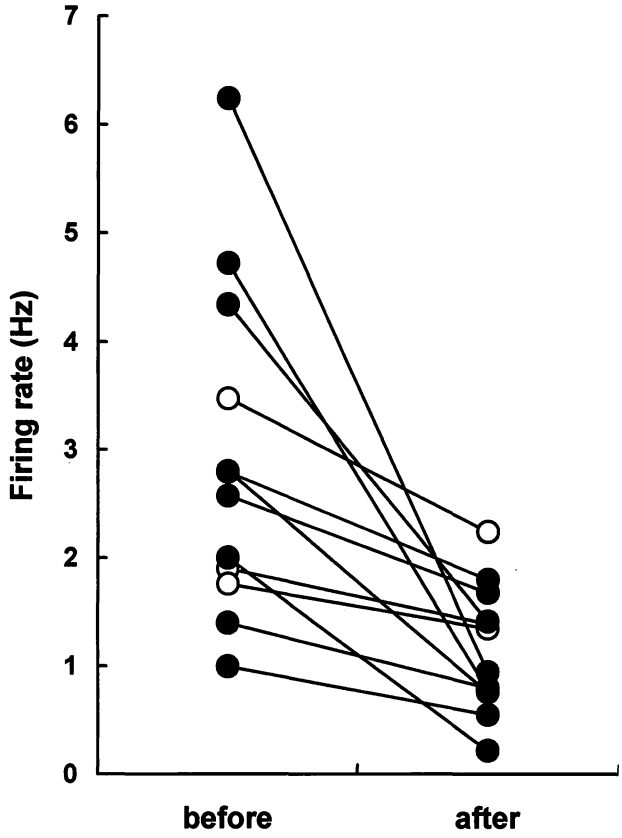

Fig. 4. Mean firing rate of the cholinergic LDT neurons ($n=12$) before and after acupuncture stimulation. Before, firing rate measured more than 90 sec before the acupuncture stimulation. After, firing rate measured during the large amplitude slow EEG period after the stimulation. Closed circles, firing rate change of the neurons that decreased significantly after the stimulus ($n=9$). Open circles, firing rate change of the neurons with the decrease of which was not significant ($n=3$). The firing rate of 12 neurons after the stimulus significantly decreased from that before the stimulus ($p<0.05$ by Student's t-test).

% of that before the stimulus. In these 12 neurons the firing rate after the stimulus significantly decreased from that before the stimulus ($p<0.05$, by t-test, Fig. 4). Of all nine neurons in which the firing decreased significantly after the stimulus, the decrease of firing occurred before the EEG changed from small to large. When the large EEG returned to small, all 12 neurons increased the firing before the EEG change.

DISCUSSION

In the present study, we revealed that in urethane anesthetized rats acupuncture stimulation to the sacral segment induced EEG change from small amplitude faster

wave to large amplitude slow wave. It has been reported that the acupuncture stimulation to the sacral segment improves urinary incontinence in patients with an overactive bladder[21,9,22]. In urethane anesthetized rats, the acupuncture stimulation to the sacral segment suppressed the bladder activity[10]. We also found that the bladder activity was closely related with the state of anesthesia[23], that is, urinary bladder was active when the EEG displayed small and faster wave (light anesthesia) and became less active when the EEG became large and slower (deep anesthesia)[11]. So, it is possible that the EEG change after the sacral segment stimulation would be induced by the suppression of bladder activity. However the present stimulation was done without bladder activity, indicating that the acupuncture stimulation to the sacral segment induces the change of vigilance state without mediating the bladder activity.

Under urethane anesthesia, the EEG showed a periodical alternation between large amplitude slow wave and small amplitude faster wave, which always remains the possibility that the response after the stimulus reflects the spontaneous occurring change. However, the mean interval of spontaneous occurring large EEG was about 800 sec. So, in the present experiment, we considered that the response with the longest latency 730 seconds were stimulus evoked. The duration of the stimulus evoked large amplitude EEG ranged 48 seconds to 56 minutes. It is possible that the stimulus evoked response sometimes shifted to the spontaneous occurring large EEG which often continued for several tens minutes.

Anatomical studies have revealed the projection of the cholinergic LDT neurons in the LDT to the wide areas in the brain including the thalamus hypothalamus or cerebral cortex, areas that are closely involved in the regulation of wakefulness[24,25]. Electrophysiological studies have shown that some of the cholinergic neurons in the LDT increase their activity during waking as well as REM sleep, and that the increase of their activity preceded to the initiation of waking or REM sleep[16-18]. In addition, the LDT cholinergic neurons have excitatory influences on the thalamic neutrons[26]. These studies suggest that the cholinergic neurons in the LDT are involved in initiation or maintenance of wakefulness under natural sleep-waking cycles.

Even under urethane anesthesia, the cholinergic neurons in the LDT show remarkable changes in activity, lower activity during large EEG state (deep anesthesia) and higher activity during small EEG state (light anesthesia)[27]. After the sacral segment stimulation the firing of the cholinergic neurons in the LDT decreased in close relation with EEG change. The increase in firing rate always preceded to the change of EEG pattern from large EEG to small EEG, and the decrease in firing rate occur prior to the EEG change from small EEG to large EEG. The result suggests that the acupuncture stimulation affected the vigilance state at least in part through the cholinergic system in the LDT.

The mechanisms of suppression of the cholinergic neurons are unknown. Since the activity of the LDT cholinergic neurons are influenced by GABAergic sys-

tem[14,28], it is possible that the GABAergic inhibition on the cholinergic neurons is important to mediate the acupuncture effect to the LDT cholinergic neurons. Since some acupuncture effects are mediated by endogenous opiate system[29-32], it is also possible that the acupuncture to the sacral segment would be mediated by opiate system. In the present study the effect of acupuncture was obtained with a long latency that exceeded 12 minutes. It would be difficult to ascribe such long latency only to the neuronal circuit. Some humoral factors may be involved in the induction of acupuncture effect.

In traditional Chinese medicine acupuncture can obtain a variety of therapeutic effects, including analgesia[7], regulation of cardiovascular or autonomic nervous system[33-35] or modulation of neuronal activity in some brain areas[36], among them the acupuncture stimulation to Shenmeng, Anmian, Sishencong or Neikuan are used for the therapy of insomnia[1,2,4]. However, no report has been made on the effect of the sacral segment stimulation on the state of vigilance. The present results would greatly contribute to the therapy of sleep disturbances or insomnia.

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