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Acupuncture Effects on Bladder Activity and State of Vigilance Through GABAergic Neuronal Systems

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1. Introduction

It has been reported that acupuncture to several acupoints increases bladder capacity and that it is used effectively for therapy to overactive bladder or for improving the symptoms of nocturnal enuresis [1-3]. Acupuncture to other points can change the state of vigilance or improve insomnia [4-6]. Acupuncture effects are, thus, confirmed in various clinical treatments, however the neural mechanisms mediating these effects remain unknown.

We have shown that acupuncture to the sacral vertebra suppresses bladder activity [7], and the same point has an effect to induce the state close to deep sleep in urethane anesthetized rats [8, 9]. During this series of experiments, it became clear that bladder activity had a close relation with brain activity; that is, urinary bladder was more active when the rats were in a light anesthetized condition than in a deep anesthetized condition [10]. It can be expected that the same mechanisms mediating acupuncture effects on bladder activity and on the state of vigilance reside in the central nervous system.

In this article, based upon our neurophysiological data on the regulation of sleep and wakefulness and of micturition, we would introduce our hypothesis on the neural mechanisms mediating the effects of acupuncture to the sacral vertebra.

2. Methods

2.1. Animals

Male rats (Sprague-Dawley) were used for the experiments. Rats were anesthetized by intraperitoneal injection of urethane (1.0 g/kg) and additional doses (2 to 5 % of the initial dose)
of the same anesthetic were given to maintain an appropriate level of anesthetic. The animals were put in prone position with their heads fixed to a stereotaxic frame. All surgical wounds were infiltrated with 2% lidocaine. Rectal temperature was kept at 37 °C with a heating pad controlled through a feedback circuit. The present study was performed in Fukushima Medical University under the control of the Animal Research Committee in accordance with the Guidelines on Animal Experiments of Fukushima Medical University and the Animal Protection and Management Law of the Japanese Government.

2.2. Experimental procedures

Rats were fixed to a stereotaxic instrument in prone posture. To analyze the effects of acupuncture, activity of urinary bladder, electroencephalogram (EEG) and single neuronal activity from the brainstem were recorded.

For recording urinary bladder pressure, a polyethylene catheter (outer diameter, 0.96 mm) was inserted from the dome into the urinary bladder through a small incision in the lower abdominal wall, the catheter was fixed securely and the wound was closed. The catheter had a bifurcation, with one end connected to a pressure transducer (Nihon-Koden, TP400-T) and the other to a syringe filled with physiological saline. The latter was used to inject physiological saline through an infusion pump (Harvard, Pump 11) to the bladder at a rate of 0.2 ml/min until this induced spontaneous bladder contraction (0.4-1.0 ml). For recording electroencephalogram (EEG), stainless steel bolts 1.0 mm in diameter were screwed to the skull overlying the frontal and parietal cortices. Single neuronal activity was recorded through a glass pipette electrode filled with 0.5-M sodium acetate containing 2% Pontamine sky blue. The electrode was penetrated stereotaxically into the brainstem through a hole made in the skull overlying the cerebellum. To avoid penetration of the venous sinus, the electrode was angled posteriorly at 30° and lowered through the cerebellum. Neuronal activity was amplified through a high-impedance amplifier and then a conventional amplifier with a time constant of 0.01 s. Recording sites were marked by ejection of Pontamine sky blue from the recording electrode.

For acupuncture stimulation, an acupuncture needle (diameter 0.3 mm) was positioned almost vertically at the periosteum about 5 mm lateral to the midline of the lumbar and sacral vertebrae (L6 to S4) and rotated manually at a speed of about 1.5-2 turns/sec for one minute. The vertebra for stimulation was located by palpation and in some animals the stimulated vertebra was ground by a thick needle and accuracy of the target vertebra was checked after removing the muscle around the vertebrae. It was confirmed that an acupuncture needle inserted to the stimulation points and put there for one minute without rotation had no effect on bladder contractions or neuronal activity. Acupuncture stimulation was judged to affect bladder activity when the relaxation periods (time from the end of a bladder contraction to the beginning of the next contraction after the stimulation) just after the stimulus exceeded twice the average of five relaxation periods before stimulation.

To examine the involvement of the GABAergic system in acupuncture regulation, a dose of 2-4 mg/kg of bicuculline (GABA receptor antagonist, Sigma) was intraperitoneally injected and the effects of acupuncture stimulation were compared with those before the injection. Effects of acupuncture stimulation on neural activity were judged by comparing the mean
firing rate measured for five contraction-relaxation cycles before stimulation with that measured after stimulation. When the mean firing rate after stimulation increased to more than 150% of that before stimulation, it was judged to be excitatory, while when the value was less than 50% of that before the stimulation, it was judged to be inhibitory.

2.3. Histology

After the experiment, the animal was deeply anesthetized with pentobarbital, and perfused transcardially with 300 ml of 4% paraformaldehyde in 0.1-M phosphate 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, sections were processed for NADPH-diaphorase, a specific marker of brainstem cholinergic neurons [11]. The sections were then counterstained by Neutral Red.

3. Results

3.1. Relation of bladder activity and state of vigilance

Under urethane anesthesia, rats alternatively exhibited two patterns of electroencephalogram (EEG): larger amplitude slow wave, indicating a state of deep anesthesia and smaller and slightly faster wave, sign of a light anesthesia. During a light anesthesia state, bladder contractions occurred regularly and contentiously, while during a deep anesthesia state, bladder contractions were completely suppressed (Fig. 1). This suggests that there is a close relationship between the micturition regulating system and vigilance state or sleep-waking regulating system. This article will try to elucidate the neural mechanisms of acupuncture effects on micturition system and sleep-waking system, so the neural substrates for the micturition system and sleep-waking system will be reviewed briefly in the following sections.

![Figure 1](http://dx.doi.org/10.5772/55405) 79

**Figure 1.** Changes in bladder activity and electroencephalogram (EEG) under urethane anesthesia. Under urethane anesthesia, EEG exhibits alternative patterns, large amplitude slow wave and small amplitude faster wave. Urinary bladder contracts when EEG shows smaller amplitude, while it becomes flaccid when EEG shows large amplitude. EEG, electroencephalogram; UBP, urinary bladder pressure.
3.2. Micturition center neurons in the brainstem regulating bladder contraction

The brain area regulating micturition was first investigated by Barrington [12], who revealed that a restricted area from the caudal mesencephalon to rostral pons functions as a micturition center and the locus was named, after him, the Barrington’s nucleus. His work was confirmed by electrical or chemical brain stimulation studies [13-15] or neuroanatomical studies showing efferent and afferent connections between the micturition center and the lumbosacral spinal cord [16-19]. We have emphasized that the most effective stimulus site for inducing bladder contraction is restricted to, as well as the Barrington’s nucleus, the area ventral to the anatomically defined Barrington’s nucleus [14].

We have recently revealed, through single neuronal recording studies, that there are three types of neurons in and around the Barrington’s nucleus, exhibiting discharges modulated in relation to spontaneous contraction of the urinary bladder (bladder activity-related neurons).
The three types were named Type E1, Type E2 and Type I neurons (Fig. 2). In this study, it was found that Type E1 neurons started to discharge prior to the onset of bladder contraction but the discharge declined before or soon after the onset of contraction, then the neurons became almost inactive at the latter half of the contraction or the following relaxation period (Fig. 2A). Type E2 neurons showed tonic firing during bladder contraction. The rate of firing changed in parallel with bladder pressure (Fig. 2B). In a majority of type E2 neurons, firing started to increase prior to the start of contraction, but the preceding time (the period between onsets of firing and contraction) was clearly shorter than that of type E1 neurons. Type I neurons showed a firing property which was mirror image of Type E2 neurons, that is, they discharged during relaxation; their discharge was suppressed at the rising phase of bladder pressure and was strongly suppressed during the contraction period. The strong suppression of firing occurred soon after the bladder pressure started to rise steeply, but the onset of the decrease of firing seemed to be prior to the start of bladder contraction (Fig. 2C).

When the urinary bladder was extended by infusion of saline, activity of about half of Type E neurons were modulated. Almost all (93%) of Type E neurons showed excitatory response, while only 3% showed inhibitory response. In the about 66% of Type I neurons whose firing was modulated by bladder extension, half were excited and the remaining half were inhibited [20].

Figure 3. Locations of bladder activity-related neurons plotted on diagrams of serial coronal sections at intervals of 300μm. The small dots represent the exact distribution of NADPH-diaphorase-positive (i.e., cholinergic) neurons in the laterodorsal tegmental nucleus (LDT) in one animal. Circles, type E1 neurons. Squares, type E2 neurons. Triangles, type I neurons. Bar, Barrington’s nucleus. LC, locus coeruleus.
As shown in Fig. 3, bladder activity-related neurons were located not only in the Barrington’s nucleus but also outside of the Barrington’s nucleus. Of these, Type E2 neurons were encountered more frequently in the Barrington’s nucleus than outside of it. Type E1 neurons were frequently located ventral or ventromedial to the Barrington’s nucleus. Type I neurons were distributed in the reticular formation more widely and more distant from the Barrington’s nucleus than Type E neurons.

Based on these firing profiles of bladder activity–related neurons, we could draw hypothetical brainstem-spinal circuits regulating bladder activity (see Fig. 10). Anatomical studies have demonstrated the neurons in and around the Barrington’s nucleus directly project to the spinal (sacral) cord [17, 18]. Pseudorabies virus injected into the urinary bladder was retrogradely and transsynaptically transferred to the Barrington’s nucleus [19]. Since location of Type E2 neurons looks to correspond to the area where the pseudorabies virus was retrogradely transferred, Type E2 neurons would, directly projecting to the sacral spinal cord, command the bladder contraction (② in Fig. 10). Considering the time course of Type E1 and Type E2 neurons, Type E1 neurons would send excitatory drive to Type E2 neuron to initiate contraction. Different responses (excitation and inhibition) of Type I neurons to bladder distension suggest two populations of Type I neurons. One type would be tonically active during resting period and suppresses Type E neurons. Another type would receive excitatory input from the urinary or spinal level, and would suppress Type E neurons during contraction-relaxation cycles.

### 3.3. Cholinergic and aminergic neurons in the brainstem regulating sleep and wakefulness

Based up the pioneering work by Moruzzi and Magoun, it became clear that there were neural populations in the reticular formation of the brainstem that activate the entire cerebral cortex, which they proposed as ascending reticular activating system [21] (Fig. 4A). Recent neuroanatomical and neurophysiological studies have revealed the transmitter phenotype of the neurons which compose the ascending reticular activating system. These neurons, locating in the area extending from caudal mesencephalon to the rostral pontine tegmentum, include the cholinergic neurons in the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT), noradrenergic neurons in the locus coeruleus (LC) and serotonergic neurons in the dorsal raphe (DR). They send their axons widely to the cerebral cortex, thalamus or basal forebrain, and exert influences on the cerebral cortex directly or through the thalamus or basal forebrain (Fig. 4B). Single neuronal recording studies in animals under natural sleep/waking cycles have revealed that some of the cholinergic neurons in the LDT/PPT are highly active during waking and REM sleep, while the noradrenergic neurons in the LC and serotonergic neurons in the DR are specifically active during waking (Fig. 4C). These neurons are, therefore, considered to be involved in inducing or maintaining wakefulness and are called waking promoting neurons.

The cholinergic and monoaminergic neurons can be discriminated from other phenotype of neurons by the shape of action potentials (spikes) recorded extracellularly or juxtacellularly [22, 23]. The neurons recorded from the LDT that generate spikes of a longer duration with a shoulder at the falling phase of the spike are cholinergic (Fig. 5C). Noradrenergic neurons in the LC and serotonergic neurons in the DR also generate spikes of similar shape. Based on this
finding, the neurons displaying spikes of a shorter duration (brief spikes) are considered to be non-cholinergic and non-monoaminergic.
Under urethane anesthesia, these waking promoting neurons change their activity in relation with state of anesthesia. They are more active during light anesthesia, and less active or completely silent during deep anesthesia.

3.4. Afferents from the urinary bladder have influences on the vigilance state through the waking promoting neurons

During deep anesthesia, while the animals were showing a large amplitude slow EEG, extension of the urinary bladder by infusion of saline caused activation of the cholinergic LDT neurons (Fig. 5A). Within a few seconds after the increase of firing, the EEG changed to that of light anesthesia [24]. Similar results were obtained from the noradrenergic neurons in the LC [25]. These results suggest that the state of urinary bladder affects the vigilance state by acting on the waking promoting neurons in the brainstem.

3.5. Acupuncture effects on state of vigilance and on wake promoting neurons

Acupuncture stimulation to the sacral vertebrae, when given during light anesthesia, induced changes in the state of vigilance; changes in the state from light anesthesia to deep anesthesia [8]. As shown in Fig. 6, when a smaller amplitude and faster EEG was observed, acupuncture stimulation to the sacral vertebra induced a change to a larger amplitude and slower EEG (Fig. 6A).

FFT analysis revealed that the peak frequency before the stimulus appeared in the delta band (1.46–2.44 Hz) and the theta band (3.92–4.9 Hz) (Fig. 6B), while after the stimulus, the peak shifted to the lower frequency delta band (0.98–1.95 Hz) (Fig. 6C). Changes in the EEG pattern occurred from 22 seconds to 31 minutes after the stimulus. The most frequent latency ranged from 100 seconds to 150 seconds. The latencies less than 450 seconds constituted one peak in the histogram of latency distribution and occupied 63% (71/112) of the response after the
stimulus. Under urethane anesthesia, the spontaneous transition of EEG from smaller to larger occurred at mean intervals of about 800 sec. So, in the present experiment, the responses with latency less than 450 seconds were considered to be stimulus evoked, while those with longer latencies to be spontaneous occurring EEG changes. Acupuncture’s effect on vigilance state was different across segments. Stimulation to the S2 vertebra induced a longer duration of large amplitude EEG than stimulation to other vertebrae (S1, S3, and S4). E. Effect of intraperitoneally injection of bicuculline (Bic) on stimulus-induced EEG changes. Large-amplitude slow EEG induced by stimulation (bar 1) was not observed after Bic injection (arrow), even when the same stimulus (bar 2) was applied. About 50 min after Bic injection, acupuncture stimulation (bar 3) again induced EEG changes.

Figure 6. Effects of acupuncture stimulation to sacral vertebra on EEG changes. A, EEG change after acupuncture stimulation to sacral vertebra S3 (bar). Time-expanded EEG traces before and after acupuncture stimulation are shown below the contentious recording of EEG. B, EEG power spectrum obtained before the stimulus (broken line on top left). C, EEG power spectrum obtained after the stimulus (broken line on top right). D, Regional differences in effect of acupuncture stimulation to different sacral vertebrae. Acupuncture stimulation to S2 (S2(1) and S2(2)) was more effective in inducing large-amplitude slow EEG than stimulation to other vertebrae (S1, S3, and S4).

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The acupuncture-induced EEG changes were suppressed by intraperitoneal injection of GABA_A receptor antagonist bicuculline (Bic). In Figure 6E, a large amplitude slow EEG induced by stimulation to sacral vertebra S3 (bar 1) was not observed when the same stimulation (bar 2) was applied 5 minutes after Bicuculline injection (arrow). These results suggest that the acupuncture effects on the state of vigilance are mediated through GABAergic neural system.

Changes of vigilance state or its associated physiological changes were induced by acupuncture to several other points; Shenmen, Yongquan, or Neiguan is used in therapy for insomnia [5, 26], while stimulation to Hegu or Zusanri induced analgesia [27,28]. However, stimulation to these acupoints induced smaller effects than stimulation to the sacral vertebrae on the change of the EEG pattern from light anesthesia to deep anesthesia [8].

When the acupuncture stimulation induced a large amplitude slow EEG, the activity of the cholinergic neurons in the LDT and the noradrenergic neurons in the locus coeruleus was remarkably suppressed [8,9]. The cholinergic neurons in the LDT (Fig. 7), which showed tonic discharge about 4.7 Hz before the stimulation, decreased and completely stopped firing during the large EEG period. At the later period of large EEG, the firing gradually increased and completely recovered when the small amplitude faster EEG reappeared. In most cases, the decrease in firing started prior to the induction of large EEG, while the increase in firing started prior to the EEG change from large to small amplitude. As shown in Figure 7Ba, the significant decrease of neuronal firing (indicated by triangles), occurred about 43 seconds before the EEG change, while in Figure 7Bb, when the large and slow EEG changed to a smaller and faster one, the increase of firing occurred about 1.6 seconds before the change of the EEG [8]. Similar results were obtained from the noradrenergic LC neurons [9].
Taken together, it can be concluded that acupuncture stimulation to the sacral vertebrae suppress the activity of waking promoting neurons in the brainstem through GABAergic system and, under urethane anesthesia, lead to the changes in the state from light anesthesia to deep anesthesia. Under unanesthetized condition, noradrenergic neurons in the LC and cholinergic neurons in the LDT/PPT have some roles, in addition to sleep-waking regulation, in maintaining arousal level or attention to novel stimuli, in the process of anxiety or pain, learning or reward [29,30]. It is highly probable that acupuncture to the sacral vertebrae has some influences on these higher brain functions.

Figure 8. Effect of intraperitoneal injection of bicuculline (Bic) on stimulus-induced suppression of bladder activity. A, Bladder activity suppression induced by acupuncture stimulation (bar 1) before Bic injection. B, Bladder activity suppression by acupuncture stimulation after Bic injection. Bars 2, 3, and 4 represent periods of stimulation about 27, 38, and 48 minutes after Bic injection, respectively. C, Time course of changes in "Ratio" values before and after Bic injection. Ratio (vertical scale) indicates the ratio of average relaxation period just after stimulation to that before stimulation, a degree of suppression induced by acupuncture stimulation.

3.6. Acupuncture effects on bladder activity and bladder activity–related neurons

When the urinary bladder was exhibiting rhythmical contraction, acupuncture stimulation to the sacral vertebra suppressed the contraction [31]. As shown in Figure 8, the suppression started just after the acupuncture to the S2 vertebra (bar 1) and continued for 13 minutes. The
same stimulus induced changes in EEG (Fig. 6), however in this case, no EEG change was observed, meaning that bladder activity is more strongly affected than state of vigilance by acupuncture stimulation to the sacral vertebra. The suppression started within a few minutes after the stimulus and continued from 30 seconds up to 40 minutes. Across several vertebrae (from L6 to S1), acupuncture stimulation to the S2 vertebra suppressed the bladder activity most effectively, while stimulation to the S4 was a least effective. The segmental effects on bladder activity was similar to the effect on state of vigilance. Other aspects of the autonomic nervous system, including respiration, blood pressure, and heart rate were unaffected by the sacral stimulation [31].

The stimulus-induced suppression of bladder activity was blocked by intraperitoneal application of bicuculline (Bic). About 27 minutes after Bic injection, the acupuncture stimulation failed to suppress bladder contraction (Fig. 8B, bar 2). The stimulation about 38 minutes after Bic injection also had no suppressive effect on bladder contraction (Fig. 8B, bar 3). Forty-eight minutes after Bic injection, the stimulus again suppressed bladder activity (Fig. 8B, bar 4). Figure 8C indicates the time course of stimulus-induced suppression after Bic injection, that is, the ratio of the mean relaxation time after the stimulus to that before the stimulus. When the contractions were suppressed by acupuncture stimulation, the values increased according to the duration of suppression. The value 1.0 indicates that the relaxation time is the same before and after the stimulus, meaning that the stimulus had no effect. Figure 8C indicates that the effects of Bic lasted for about 30 minutes. These results indicate that acupuncture suppression on bladder activity is mediated through GABA_{A} receptor systems.

When bladder activity was suppressed by acupuncture stimulation, almost all of Type E neurons (both Type E1 and Type E2 neurons) decreased firing. As shown in Fig. 9A, some Type E2 neurons stopped firing just after the acupuncture stimulation simultaneous with suppression of bladder contraction. The neuronal firing recovered when the bladder regained contraction. In other Type E2 neurons, the decrease in firing appeared before the suppression of bladder contraction (Fig. 9B), while in the third class of Type E2 neurons, the rhythmic firing continued after the suppression of bladder contraction (Fig. 9C). In contrast to Type E neurons, some of Type I neurons exhibited an increase in firing after acupuncture stimulation (Fig. 9D). The increase in firing started before the suppression of bladder activity. The firing further increased and, during the suppression period of bladder activity, exceeded the value during the relaxation period of spontaneous contraction before the stimulation. Of 14 type I neurons, 4 exhibited increased firing, 3 exhibited decreased firing and the remaining 7 exhibited no response to acupuncture stimulation.

In total, acupuncture stimulation suppresses Type E neurons and activates half of Type I neurons, leading to suppression of bladder activity. Another half of Type I neurons which were inhibited by acupuncture might be receiving neural inputs from urinary bladder and be working to maintain the basic level of bladder pressure which would be crucial to continue contraction. Time course of suppression in Type E neurons differ among neurons (Fig. 9 A-C). Acupuncture stimulation, therefore, would disturb the synchronous firing in Type E neurons rather than suppress all of the neurons in a similar magnitude, resulting in irregular and insufficient bladder contraction at the beginning of suppression.
4. Conclusion

Acupuncture stimulation to the sacral vertebrae in rats suppressed bladder activity and altered the firing profiles of bladder activity-related neurons in and around the micturition center. Acupuncture stimulation to the sacral vertebrae also affected the state of vigilance by inhibiting the activity of wake-promoting neurons. These effects were blocked by GABA$_A$ receptor antagonist bicuculline.

Since acupuncture stimulation to the sacral segment suppressed overactive bladder in spinal injured patients [32,33], it is highly possible that the acupuncture effect is mediated through the spinal level. However, the present study showed that when acupuncture was applied, a
population of bladder activity-related neurons changed firing before the suppression of bladder contraction, suggesting the possibility that the acupuncture effect is mediated through the central nervous system, especially through the brainstem micturition center. In a similar way, suppression of firing in waking promoting neurons occurring before the changes in the state of vigilance suggests that the acupuncture effect is mediated through the waking promoting neurons.

Figure 10. Schematic diagram representing neural circuit mediating acupuncture effects on micturition system and waking system. ACh, acetylcholine. ACP, acupuncture. NA, noradrenaline. SP, spinal cord. UB, urinary bladder. W wake-promoting neuron.

4.1. Hypothetical neural pathways mediating acupuncture effects on micturition and state of vigilance

Taking these findings in account, we could draw hypothetical schema explaining the neural mechanism mediating acupuncture effects on micturition and state of vigilance (Fig. 10). In summary, as is mentioned in Section 1 and Section 4, the waking center in the brainstem and micturition center in the Barrington’s nucleus are mutually excitatory each other (① in Fig. 10). Type E2 neurons in the micturition center exert excitatory drive on urinary bladder (②) and inputs from the urinary bladder have excitatory influences on Type E neurons and a half
of Type I neurons, while they have inhibitory influences on another half of Type I neurons (3). They also have excitatory influences on waking promoting neurons in the waking center (4). Acupuncture stimulation to the sacral vertebra causes excitation in GABAergic neurons of somewhere (5) which inhibit the pathways activating wakefulness (6) or facilitating micturition center (7). The location of GABAergic neurons and sites of action of GABAergic inhibition are still unknown (broken lines), and would be a subject of future study.

4.2. Clinical implications

The present findings provide a scientific validity for the therapy of overactive bladder in spinal injured patients [32,33] or nocturnal enuresis [1-3]. If the sacral acupuncture is effective for changing the state of vigilance in human, it would lead to a therapy for insomnia or would be useful for putting a patient in sedation.

Acupuncture has been used traditionally in China for thousands of years and is growing prominent in the Western countries. Acupuncture, thus, plays a crucial role in complementary and alternative medicine. To establish a better supported therapy of acupuncture more confirmedly, integration with the Western medicine is indispensable. Further studies to elucidate the neural mechanisms of acupuncture action would be urgently required.

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