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Disinhibitory involvement of the anterior cingulate cortex in the descending antinociceptive effect induced by electroacupuncture stimulation in rats.

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Running title: Electroacupuncture-induced inhibition of ACCX neurons.

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ABSTRACT

The present study was conducted to clarify the role of the anterior cingulate cortex (ACCX) in acupuncture analgesia. Experiments were performed on 35 female Wistar albino rats weighing about 300 g. Single unit recordings were made from ACCX neurons with a tungsten microelectrode. Descending ACCX neurons were identified by antidromic activation from electrical shocks applied to the ventral part of the ipsilateral PAG through a concentric needle electrode. Cathodal electroacupuncture stimulation of Ho-Ku (0.1 ms in duration, 45 Hz) for 15 min was done by inserting stainless steel needles bilaterally. An anodal silver-plate electrode (30 mm x 30 mm) was placed on the center of the abdomen. Naloxone (1.0 mg/kg, i.v.) was used to test whether changes of ACCX activities were induced by the endogenous opioid system or not. Data were collected from a total of 73 ACCX neurons. Forty-seven neurons had descending projection to the PAG, and 26 had no projections to the PAG. A majority of descending ACCX neurons were inhibited by electroacupuncture stimulation. By contrast, non-projection ACCX neurons were mainly unaffected by electroacupuncture. Naloxone did not reverse acupuncture effects on the changes of ACCX neuronal activities. Acupuncture stimulation had predominantly inhibitory effects on the activities of descending ACCX neurons. Since the functional connection between ACCX and

PAG is inhibitory, electroacupuncture caused disinhibition of PAG neurons, whose activity is closely related to descending antinociception to the spinal cord. This disinhibitory effect elicited by acupuncture stimulation is thought to play a significant role in acupuncture analgesia.

Keywords

Acupuncture, Anterior cingulate cortex, Disinhibition, Periaqueductal gray matter, Opioid peptides, Pain, Rat.

INTRODUCTION

It is well known that descending inhibitory mechanisms are strongly involved in acupuncture analgesia (Lee*, et al.*, 2006; Toda, 1992). This descending inhibition is the most powerful analgesic mechanism in the central nervous system. Generally, descending pathways project to the spinal cord or spinal trigeminal sensory nucleus from various pain suppression centers as revealed by neuroanatomical studies (McMullan and Lumb, 2006). These centers include the periaqueductal gray matter (PAG), the nucleus raphe magnus (NRM), the arcuate nucleus of the hypothalmus, and other areas in the ventromedial medulla (Mason, 2005).

The anterior cingulate cortex (ACCX) is involved in modulating pain sensation and pain-related reactions in humans (Davis*, et al.*, 2000) and various species of animals (Kirzinger and Jurgens, 1982; Rainville, 2002; Vogt and Peters, 1981). In human studies, it was reported that lesion of the ACCX can alleviate emotional reaction to painful stimuli, while the ability to localize such stimuli remains unaffected (Davis*, et al.*, 1994). Behavioral studies in monkeys showed that stimulation of the ACCX could produce shrill vocalization, which was presumed to be associated with escape responses evoked by noxious stimulation (Johansen*, et al.*, 2001; Koyama*, et al.*, 1998). On the other hand,

anatomical studies indicate that there are dense descending projections from the ACCX to the PAG (An, *et al.*, 1998; Calejesan, *et al.*, 2000). Since the PAG is a key link in the descending pain control system projecting to the spinal cord or the trigeminal sensory complex through the NRM (Jiang and Behbehani, 2001; Willis, *et al.*, 1984), it can be assumed that the ACCX is concerned with the control of the descending analgesic system activated by acupuncture stimulation. However, there are no available data concerning the response properties of the ACCX neurons following acupuncture stimulation.

Therefore, this study was undertaken to reveal the effect of acupuncture stimulation on the responses of single ACCX neurons, and specifically to compare the responses of PAG-projection and PAG-non-projection ACCX neurons.

Methods

Experiments were performed on 35 female Wistar albino rats weighing about 300 g. The animals were lightly anesthetized with thiamylal sodium, initially given 80 mg/kg intraperitoneally and additionally given 10 mg/kg intravenously as needed throughout the experiment, via a cannula inserted into the femoral vein. The respiratory conditions,

the blood pressure, and the rectal temperature were continuously monitored and maintained within normal physiological levels throughout the recording session.

The skull over the medial part of the cerebral cortex was removed bilaterally, and the exposed brain surface was covered with liquid paraffin. The stereotaxic coordinates for the ACCX (A: 0.0-3.0 mm rostral to bregma, L: 0.0-1.2 mm from the midline: 1.0-1.4 mm ventral to cortical surface), and for the PAG (P: 6.3 -8.1 mm caudal to bregma, L: 1.0-1.4 mm from the midline, D: 5.3-6.8 mm ventral to cerebral surface) were taken from the brain atlas of Swanson (Swanson, 1992).

Single unit recordings were made from left or right ACCX neurons with a tungsten microelectrode (FDH 25-10, tip impedance 10-12 MΩ, Brunswick, USA). Electrical signals were amplified by a biological amplifier (DAM 80, WPI instruments, Sarasota, USA) and continuously recorded on the chart recorder (SEN-3, Sanei, Tokyo, Japan).

Descending ACCX neurons were identified by antidromic activation from electrical shocks applied to the ventral part of the PAG through a concentric needle electrode (outer diameter: 1.0 mm, inner diameter:0.1 mm) (Fig. 1A). Collision test was

performed to examine whether the unit being recording projected to the PAG or not (Fig. 1B).

Cathodal electroacupuncture stimulation was delivered to bilateral Ho-Ku (Large Intestine 4) by inserting stainless steel needles (0.2 mm in diameter) bilaterally to a depth of 2.0 mm. An anodal silver-plate electrode (30 mm x 30 mm) was placed on the center of the abdomen. Rectangular constant current pulses of 0.1 ms duration were delivered at 45 Hz for 15 min. The intensity of electroacupuncture stimulation was the one with which moderate muscle contraction was evoked very near to the needling point. These intensities (ranging from 40 to 70 μA) were about 5 times the threshold for evoking a minimum twitch. In this study, one to three times application of electroacupuncture stimulation was applied in one rat, because we have previously showed that the effects of electroacupuncture are reproducible through repeated trials (Toda*, et al.*, 1980). Naloxone (1.0 mg/kg) was injected into the femoral vein 60 s after the cessation of electroacupuncture stimulation to test whether changes of ACCX activities were induced by the endogenous opioid system or not.

At the end of experiment, the rat was killed by an overdose of thiamylal sodium. The

sites of recording in the ACCX were histologically identified by making an electrical lesion using a 2-mA DC current passing for 2 min.

The paired t-test was used to determine the statistical significance of the data before and after electroacupuncture stimulation. On-going activities of mean six bins (one bin=10 s) just before electroacupuncture stimulation for 60 sec and those of just after electroacupuncture for 60 s were compared in each unit. 1SD variation was checked to distinguish TYPE E (acupuncture-induced excitation), TYPE I (acupuncture-induced inhibition) and TYPE O (no change) ACCX units. Statistical significance was set at the 5% level ($P< 0.05$). The software Statview version 5.0 (SAS Institute, Cary, NC) aided in statistical analysis.

RESULTS

Data were collected from a total of 73 ACCX neurons. Forty-seven neurons had descending projection to the PAG, and 26 had no projections to the PAG. (Table 1).

Responses of projection neurons

Spontaneous activity in about 60% of the descending ACCX neurons (28/47) was inhibited after electroacupuncture stimulation. Fig.2 shows a typical example of on-going activity of an ACCX neuron before and immediately after the cessation of 15 min-acupuncture stimulation of Hoku, and the effects of naloxone injection. As clearly seen, firing rate and instantaneous frequencies of spontaneous activities were well inhibited (TYPE I). When naloxone was injected 60 sec after the cessation of acupuncture stimulation, acupuncture-induced decrease of on-going activities of ACCX neurons was still observed, indicating that endogenous opioids were not concerned with this inhibitory effect induced in the ACCX.

Six neurons (13%) were excited (TYPE E, Fig. 3) after electroacupuncture stimulation. Excited responses were not affected by naloxone injection similarly as seen in TYPE I neurons. Thirteen neurons (28%) were not changed (TYPE O) after acupuncture stimulation.

Mean firing rates before and after electroacupuncture stimulation were 3.94 and 1.89 Hz in TYPE I, and 2.67 and 9.13 Hz in Type E, respectively. Naloxone did not affect the

acupuncture induced-changes of ACCX neuronal activities (Fig. 4).

The highest percentage of the descending ACCX neurons were found in layer V; however, there was no significant tendency for the distribution of these three types ACCX neurons (Fig. 5).

Responses of non-projection neurons

In non-projection neurons, there were found only 4 TYPE I neurons (15%) after electroacupuncture stimulation. On-going unit activities in 3 neurons (8%) were excited (TYPE E) after electroacupuncture stimulation. Mean firing rates before and after electroacupuncture stimulation were5.53 and 2.06 Hz in TYPE I, and 4.43 and 8.67 Hz in Type E, respectively (Fig. 4). Similarly to projection neurons, naloxone did not alter these responses. Seventy-seven percent of the non-projection ACCX neurons were unaffected (TYPE O) by electroacupuncture stimulation. Histological investigations revealed that non-projection neurons were mainly found in laminar II to V of the ACCX (Fig. 5).

DISCUSSION

The present study demonstrated that the majority of descending ACCX neurons having PAG-projections are inhibited by electroacupuncture stimulation. By contrast, non-projection ACCX neurons were predominantly unaffected by electroacupuncture stimulation. In addition, naloxone did not affect these acupuncture-induced effects.

A number of studies suggest that the ACCX, which is the one of the highest centers for sensory integration (Iwata*, et al.*, 2005; Park*, et al.*, 2006), is involved in modulating pain sensation or noxious responses in humans and animals (Kupers*, et al.*, 2005; Rainville, 2002; Zhuo, 2006). Also, It has been shown that cingulotomy can diminish affective responses to painful stimuli in humans (Cohen*, et al.*, 2001; Yen*, et al.*, 2005). Behavioral studies showed that stimulation of the ACCX could produce shrill vocalization, which might be associated with escape responses (Jürgens, 1998; LaGraize*, et al.*, 2004). These studies strongly suggest that the ACCX has an important functional role in the processing of pain sensation of emotional dimension.

More recently, Zhang et al. (Zhang*, et al.*, 2005) also suggested that supraspinal centers biphasically modulate nociceptive information at the spinal cord, including descending

inhibition and facilitation. Especially, the ACCX is not only involved in the transmission of pain sensation but also plays a role in processing pain-related emotion. The reason why these complicated effects can be produced is suggested by anatomical studies, which show that ACCX widely connects with relevant regions of the descending modulation system including the PAG (McMullan and Lumb, 2006). Since the PAG is a key link in the descending pain control system, it is assumed that the ACCX is concerned with control of descending analgesic system through PAG.

In the present study, electroacupuncture stimulation suppressed on-going activities of a majority of descending ACCX neurons that projected to the PAG. Our previous study showed that the main effect of ACCX stimulation on the descending PAG neuronal activities is inhibitory (Toda, 1992). Conversely, Calejesan *et al.* (2000) reported that ACCX stimulation facilitates behavioral nociceptive responses through PAG. Hence, inhibition of ACCX may induce antinociception by disinhibition of PAG neurons. Electroacupuncture-induced suppression of ACCX neuronal activities is thought to induce disinhibition of the descending PAG neuronal activities, and thus produce enhancement of the descending inhibitory system through PAG-NRM to the spinal cord.

A smaller number of descending ACCX neurons were excited (13%) or unchanged (28%). The functional role of these neurons was not elucidated clearly; however, a possible explanation is that responses of ACCX neurons to somatosensory stimulation were of a bimodal fashion (Calejesan*, et al.*, 2000; Vaccarino and Chorney, 1994). Therefore, electroacupuncture stimulation may also produce bimodal effects on the neuronal activities of ACCX neurons.

Among non-projection ACCX neurons, few neurons had their ongoing activities inhibited by electroacupuncture stimulation. Typically, the response pattern of non-projection ACCX neurons was Type O (unchanged); therefore, it is thought that these neurons were not concerned with producing acupuncture effects through descending analgesic pathways.

Naloxone did not affect neuronal responses in the ACCX induced by electroacupuncture, suggesting that endogenous opioids did not participate in the acupuncture-induced changes of the ACCX neuronal activities. In electroacupuncture analgesia, endogenous opioid may act at the brainstem level, not on at higher centers, including the ACCX (Han and Terenius, 1982; Staud and Price, 2006).

In conclusion, acupuncture stimulation can predominantly provoke inhibitory effects on the activities of descending ACCX neurons. Since the functional connection between ACCX and PAG is inhibitory, and disinhibition of the PAG is closely related to descending antinociception to the spinal cord, the preset results suggest that inhibition of ACCX neurons may be one of the mechanisms behind acupuncture analgesia.

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FIGURE LEGENDS

Figure 1.

Schematic drawing of the experimental design. (A) A tungsten microelectode was inserted into the ACCX for single-unit recordings of neuronal activity. Projection neurons were identified by antidromic activation of the PAG. (B) Collision test for projection neurons. Open circles indicate spontaneous spikes; asterisks indicate stimulus artifacts; and closed circle indicate evoked spike response. Note that collision occurred in b.

Figure 2.

Typical example of neuronal activity in a Type I ACCX neuron (A) before and (B) after EA stimulation and Naloxone injection (projection neuron). The ongoing activity in these neurons was inhibited by EA stimulation, and this inhibition was unaffected by Naloxone injection. (a) Spike events; (b) Firing rates; and (c) Instantaneous frequencies of neuronal activity.

Figure 3.

Typical example of neuronal activity in a Type E ACCX neuron (A) before and (B) after

EA stimulation and Naloxone injection (projection neuron). In these neurons the effect of EA stimulation was excitatory, but as in the Type I neurons, this effect was unaffected by Naloxone injection. (a) Spike events; (b) Firing rates; and (c) Instantaneous frequencies of neuronal activity.

Figure 4.

Effects of EA stimulation on the mean firing rate of ACCX neurons. Projection and non-projection ACCX neurons were divided according to their responses into Type I (inhibition) Type E (excitation) and Type O (unchanged). Asterisks indicate statistical significance ($P<0.05$) between control and after EA stimulation in the paired t-test. Significant differences were not found between after EA stimulation and after Naloxone (NAL) injection. The effect of Naloxone was not tested in the case of Type O neurons.

Figure 5.

Drawings of coronal brain sections showing the positions of the recording electrodes in the ACCX. We could not distinguish any particular pattern of spatial distribution for each neuronal type. Section levels are relative to Bregma.

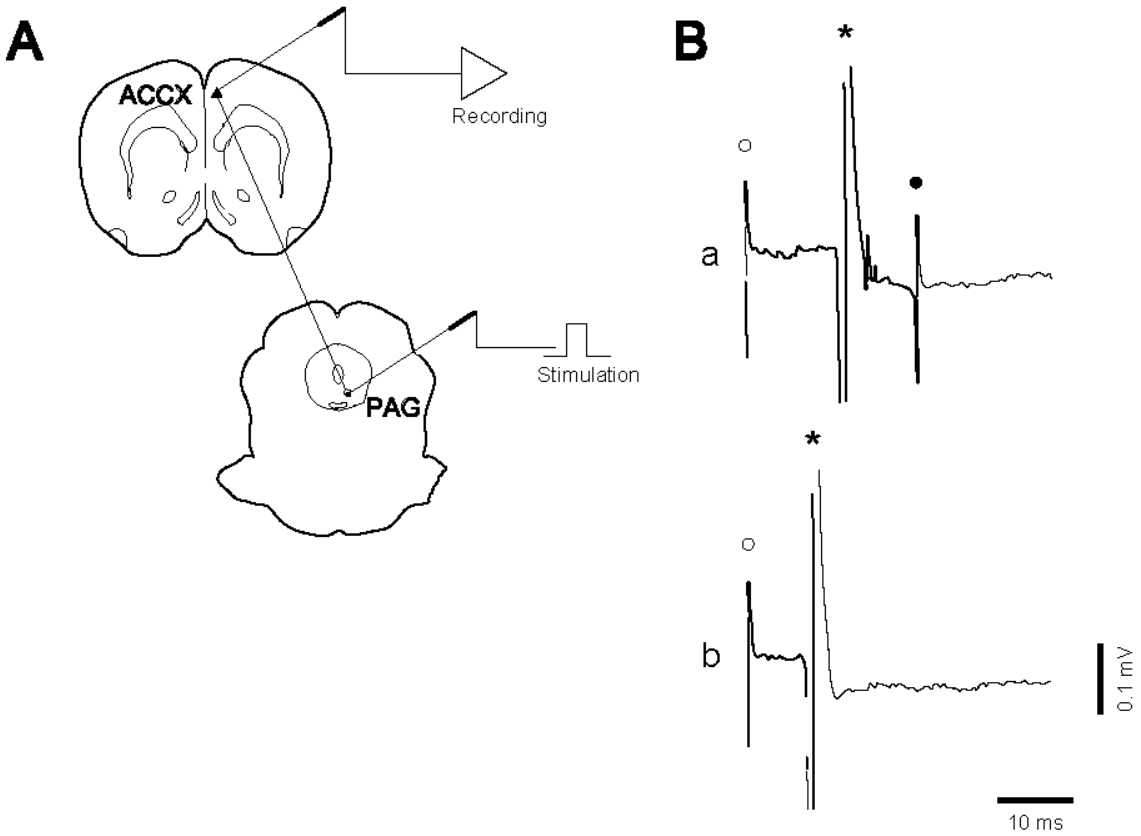


Figure 1

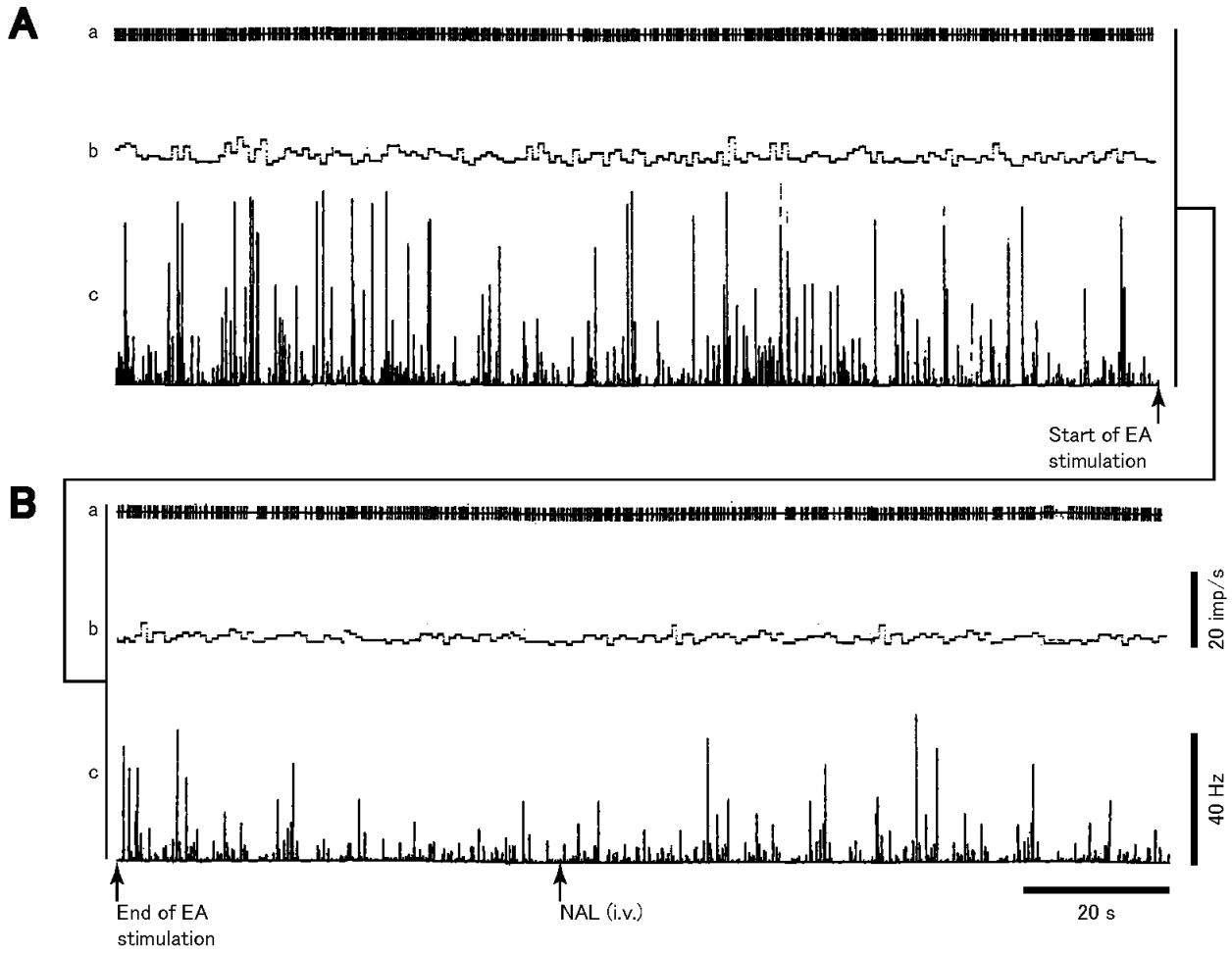
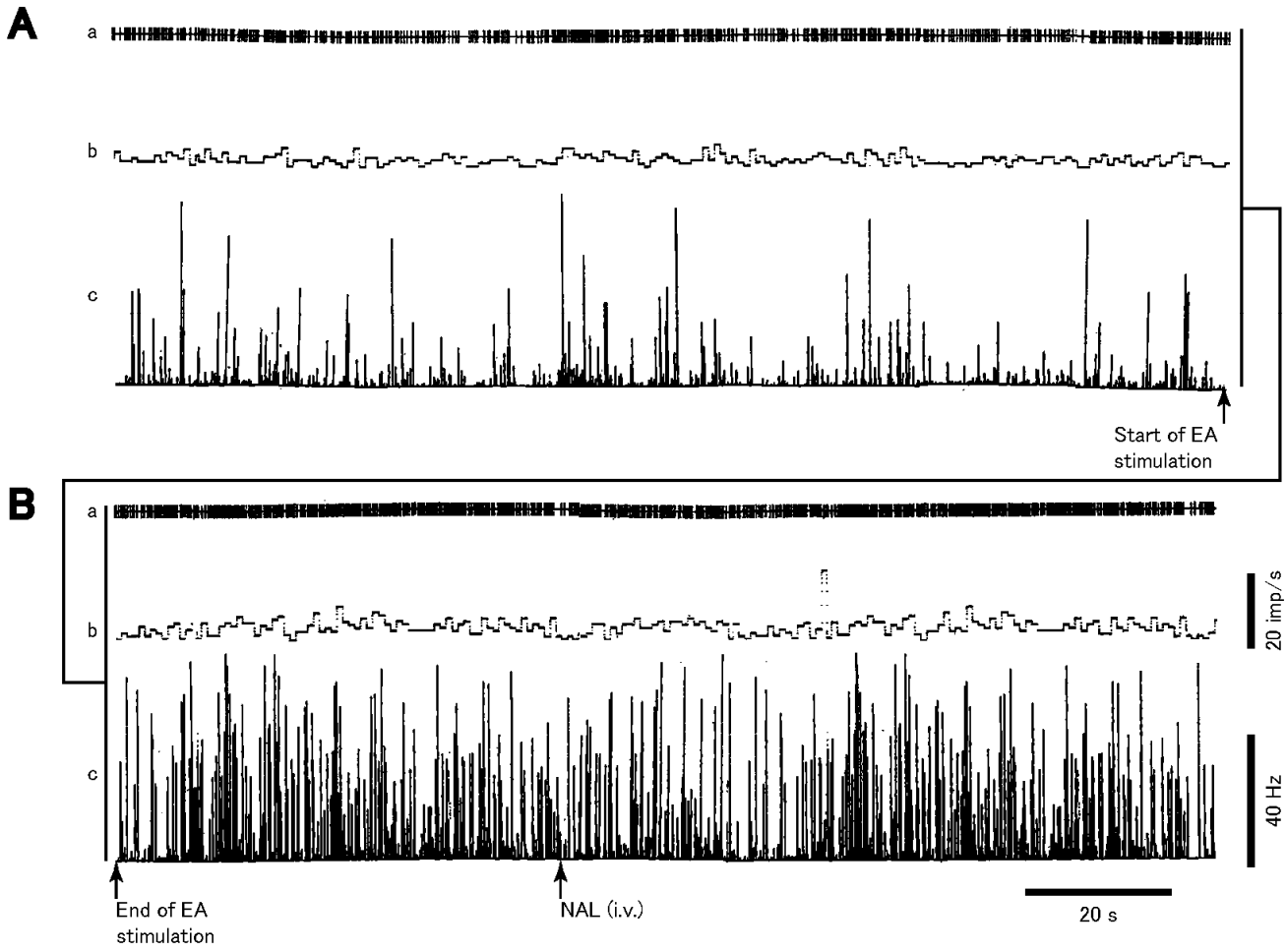

A
a
b
c
Start of EA
stimulation
B
a
b
c
20 imp/s
40 Hz
End of EA
stimulation
NAL (i.v.)
20 s

Figure 2


A
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Start of EA
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40 Hz
End of EA
stimulation
NAL (i.v.)
20 s

Figure 3

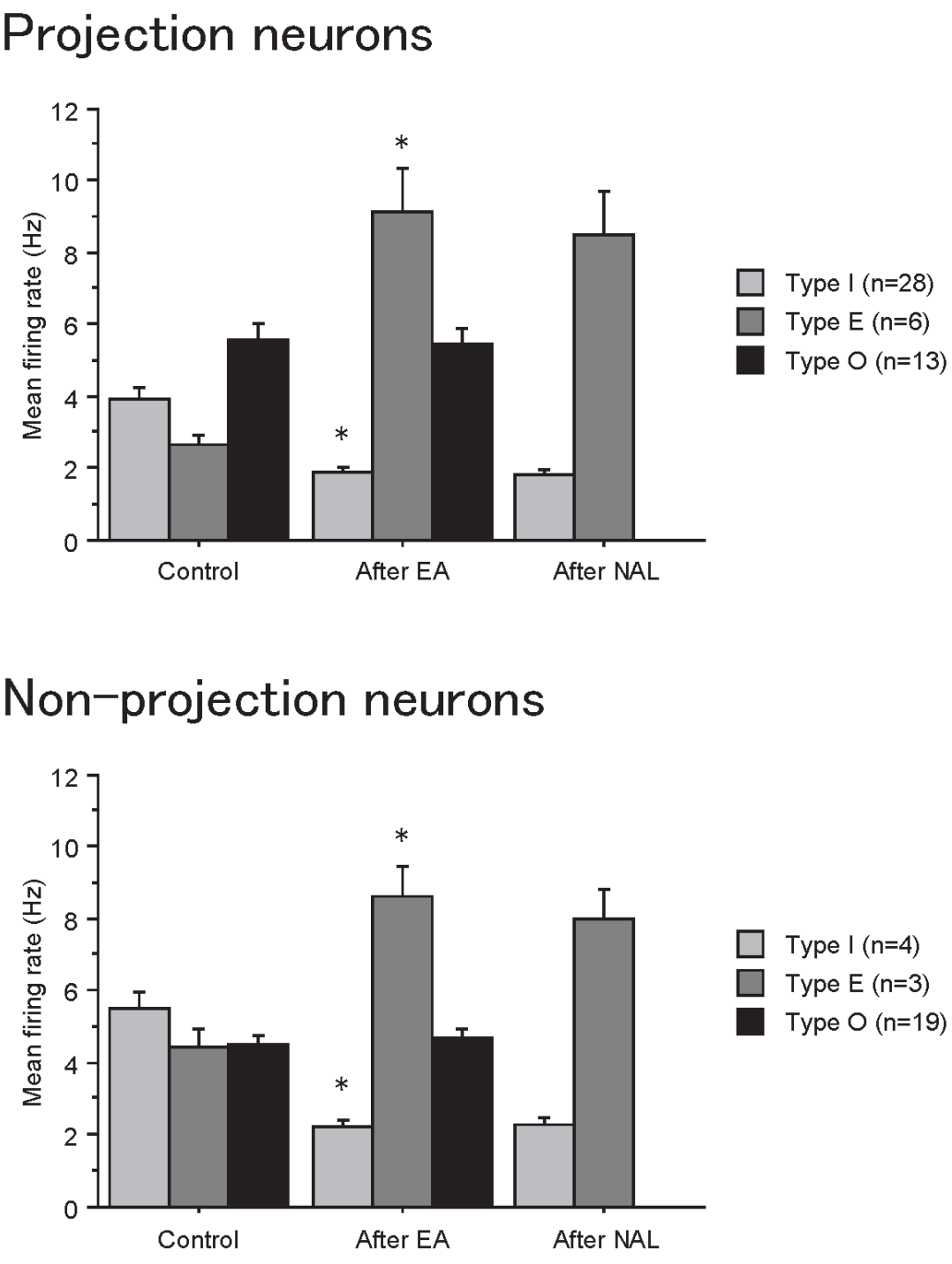
Projection neurons
Mean firing rate (Hz)
12
10
8
6
4
2
0
*
*
Control
After EA
After NAL
Type I (n=28)
Type E (n=6)
Type O (n=13)
Non-projection neurons
Mean firing rate (Hz)
12
10
8
6
4
2
0
*
*
Control
After EA
After NAL
Type I (n=4)
Type E (n=3)
Type O (n=19)

Figure 4

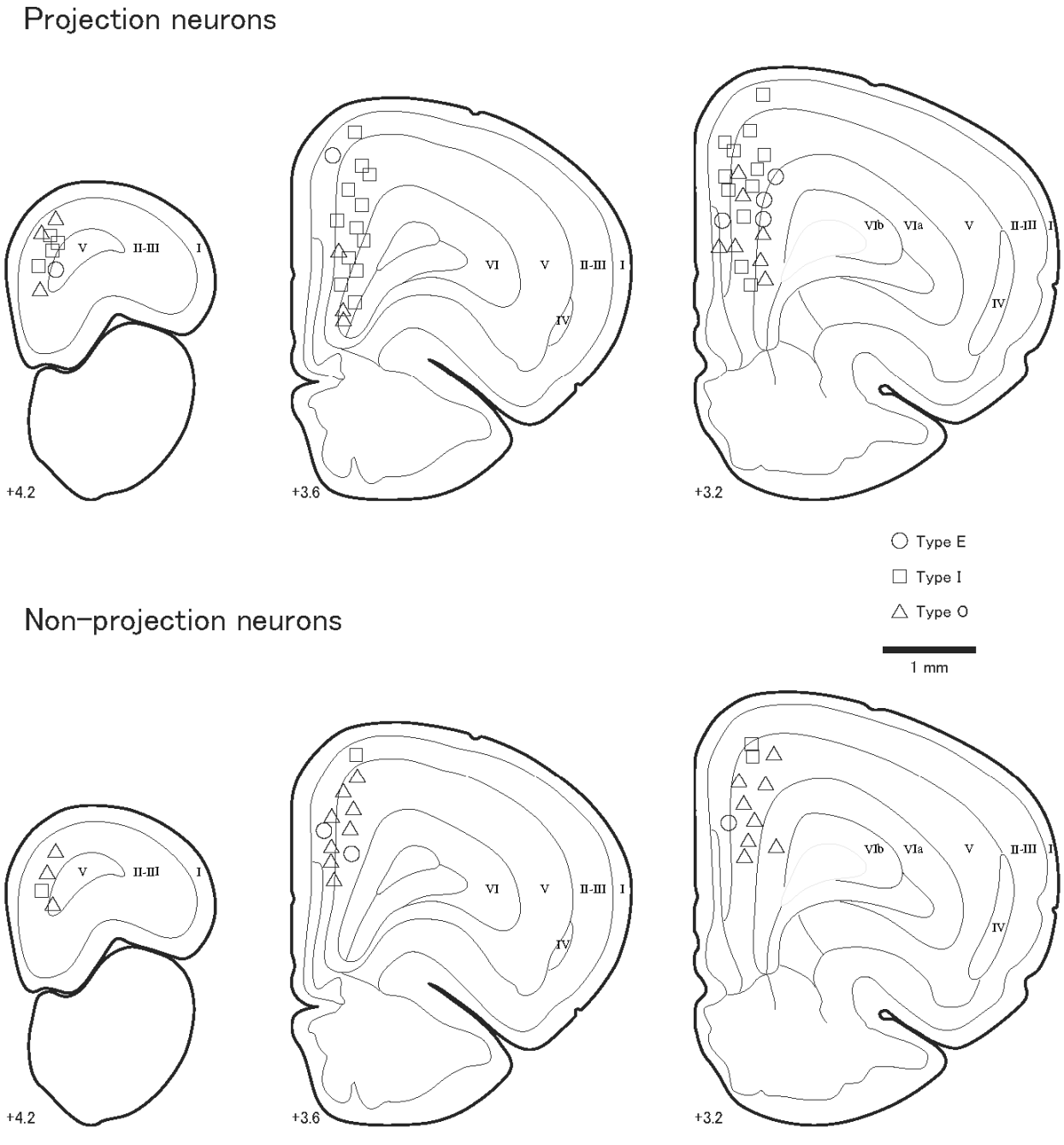
Projection neurons
V
II-III
I
+4.2
VI
V
II-III
I
IV
+3.6
VIb
VIa
V
II-III
I
IV
+3.2
Type E
Type I
Type O
1 mm
Non-projection neurons
V
II-III
I
+4.2
VI
V
II-III
I
IV
+3.6
VIb
VIa
V
II-III
I
IV
+3.2

Figure 5

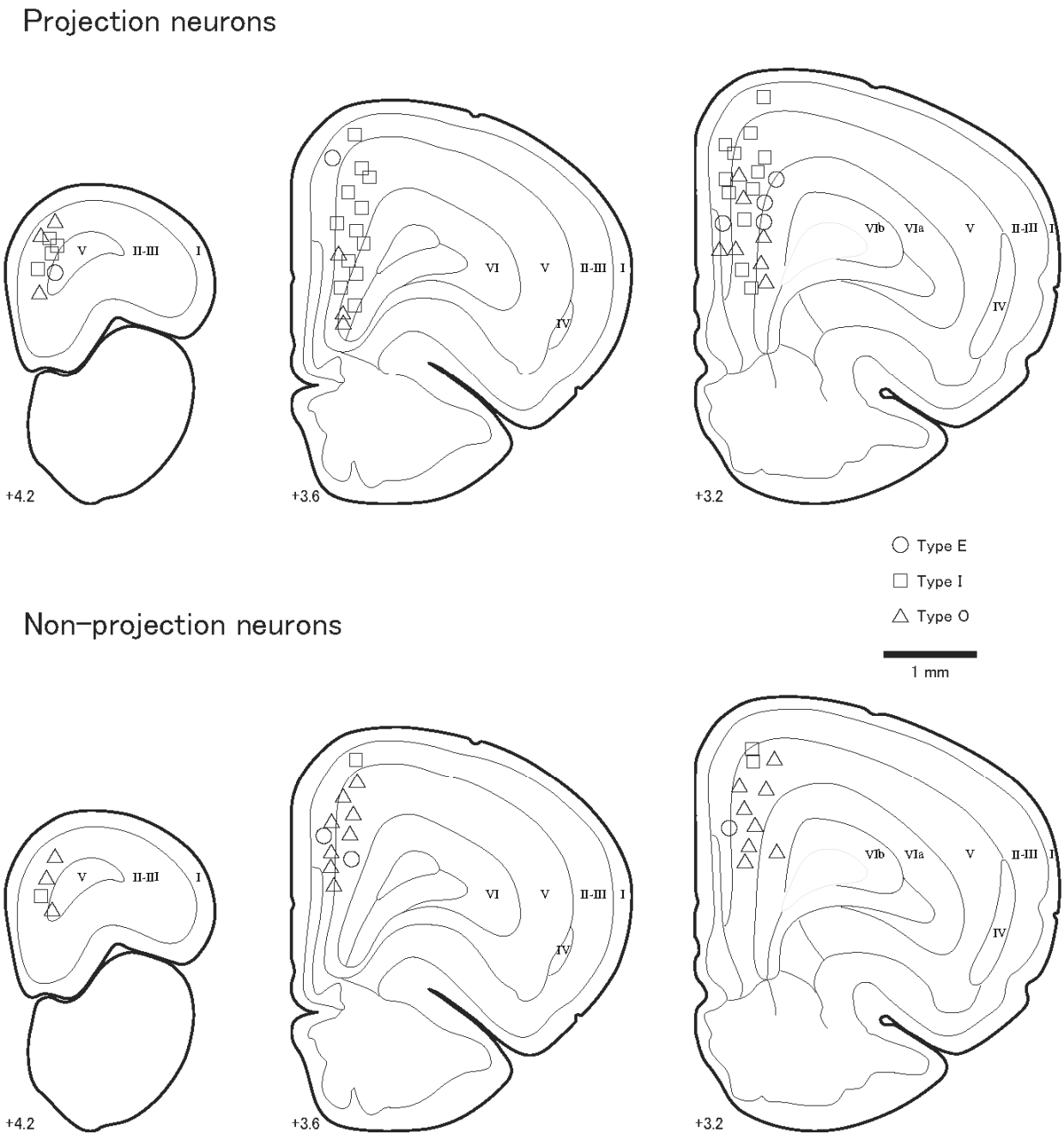
Projection neurons
V
II-III
I
+4.2
VI
V
II-III
I
IV
+3.6
VIb
VIa
V
II-III
I
IV
+3.2
Type E
Type I
Type O
1 mm
Non-projection neurons
V
II-III
I
+4.2
VI
V
II-III
I
IV
+3.6
VIb
VIa
V
II-III
I
IV
+3.2

Figure 6

Table 1. Characteristics of ACCX-neuron response to acupuncture stimulation.

	Projection (count)	Non-projection (count)
Type I	28	4
Type E	6	3
Type O	13	19
Total	47	26

Type I: acupuncture-inhibited; Type E: acupuncture-excited; Type O: not changed.