Table 1. The plasma Nicotine Concentration, Heart Rate, Dominant Alpha Frequency, N2 and P2 Latencies and N2/P2 Peak-to-Peak Amplitude for Each Session

Session	PNC (ng/ml)	HR (/min)	AF (Hz)	N2l (ms)	P2l (ms)	NPa (μV)
Control						
Pre	2.5(1.2)	66(8.7)	10.0(0.3)	750(52)	875(76)	21.8 (4.3)
5 min	2.6(1.4)	63(7.4)	10.0(0.3)	760(70)	876(75)	19.7 (3.9)
20 min	2.5(1.1)	60(6.5)	10.1(0.3)	767(84)	883(85)	20.7 (4.6)
35 min	2.5(0.9)	62(7.7)	10.0(0.4)	752(53)	881(54)	16.0 (4.5)
60 min	2.6(1.2)	62(8.0)	10.2(0.5)	751(64)	886(52)	18.0 (4.1)
Smoking	3.3(1.9)	65(8.3)	10.2(0.4)	786(76)	890(80)	18.3 (3.8)
Pre	23.5 (17.7)	79(10.8)	11.1(0.8)	813(100)	947(105)	20.3 (3.3)
5 min	11.1 (8.3)	70(9.5)	10.5(0.5)	789(44)	881(51)	19.0 (5.8)
20 min	10.6 (7.1)	68(9.0)	10.2(0.3)	775(41)	877(56)	16.6 (4.1)
35 min	8.8(5.2)	64(7.3)	10.4(0.4)	806(46)	924(50)	18.6 (3.2)
60 min						

Data are given as mean (SD). PNC, plasma nicotine concentration; HR, heart rate; AF, alpha frequency; N2l, N2 latency; P2l, P2 latency; NPa, N2/P2 peak-to-peak amplitude.

0.32, $p = 0.04$, Fig. **3C**) were positively correlated with PNC in the Smoking session.

DISCUSSION

In the present study, a slight but significant facilitatory effect of smoking on C-fiber LEPs was found. The amplitude of LEPs was positively correlated with the plasma nicotine concentration (PNC). In the two-way ANOVA, a significant main effect of Run was found on the N2/P2 peak-to-peak amplitude, although the effect of Session was not significant. These results indicate that the C-fiber LEPs were enhanced by nicotine intake but not changed by smoking itself. We previously reported an inhibitory effect of smoking on late

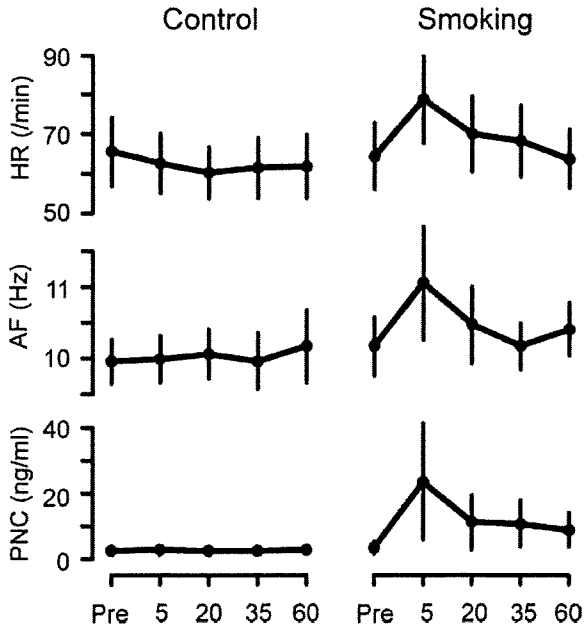

Fig. (1). Mean heart rate, dominant alpha frequency of the background EEG activity and plasma nicotine concentration for each run. The left and right columns correspond to the Control and Smoking sessions, respectively. Vertical bars show ± SD. HR, heart rate; AF, dominant frequency of the background EEG activity; PNC, plasma nicotine concentration.

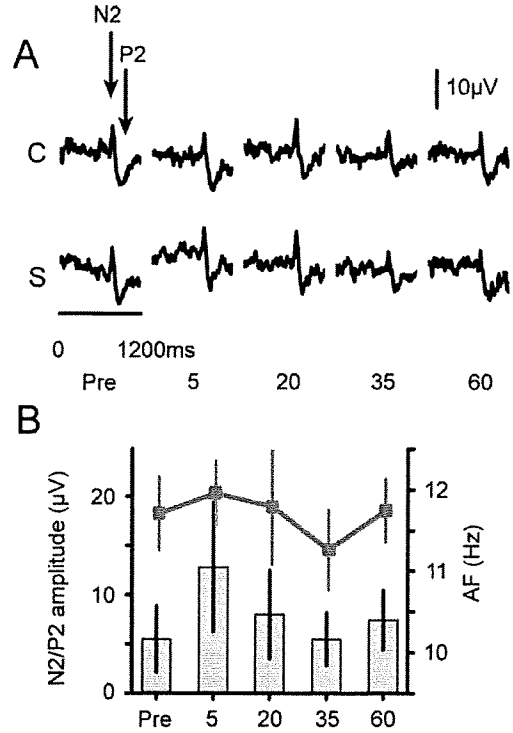

Fig. (2). Grand averaged waveforms of evoked potentials, and the N2/P2 amplitude and alpha frequency in the Smoking session. **(A)** Grand averaged waveforms for each run (Pre and 5, 20, 35 and 60 min) in the Control (C) and Smoking (S) sessions. **(B)** The N2/P2 amplitude (top) and dominant alpha frequency (bottom) for each run in the Smoking session. Vertical bars show ± SD.

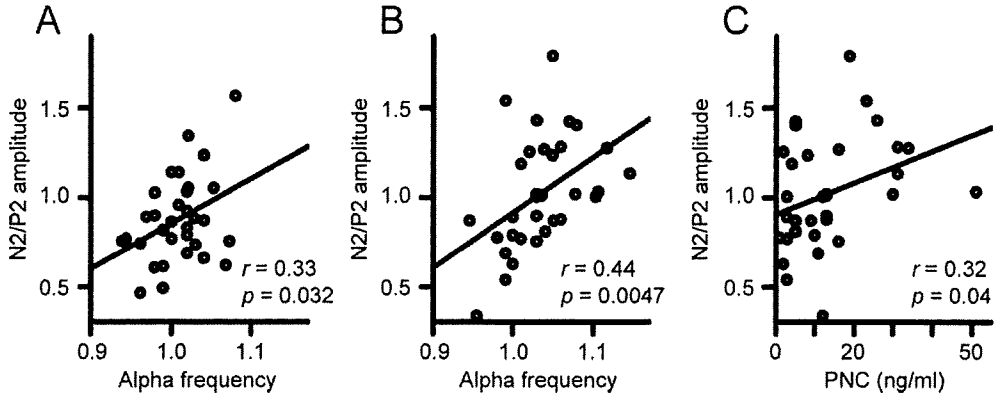

Fig. (3). Correlation of the N2/P2 amplitude with the dominant frequency of the background alpha activity and with the plasma nicotine concentration. A significant positive correlation was found between the N2/P2 amplitude and alpha frequency in the Control (**A**) and Smoking (**B**) sessions, and between the N2/P2 amplitude and plasma nicotine concentration in the Smoking session (**C**). Change from the baseline (Pre) was used for the N2/P2 amplitude and alpha frequency. AF, frequency of the background EEG activity; PNC, plasma nicotine concentration.

(Aδ fiber) laser evoked potentials (Miyazaki *et al.*, Submitted 2009). The P2 amplitude significantly decreased 5 minutes after smoking, and gradually increased. In addition, both the N2 and P2 amplitudes showed a significant negative correlation with PNC. In the present study, we examined whether tobacco smoking exerts a similar effect on ultralate (C-fiber) LEPs. Unexpectedly however, we found an effect of smoking on C-fiber LEPs in the opposite direction.

Most studies on effects of smoking and nicotine on EEG activities in humans support the hypothesis that smoking and nicotine have a facilitatory effect on the brain activity, by showing an increase in amplitude and/or a decrease in latency of evoked EEG components [1-4]. Many studies demonstrate that smoking and nicotine increase the dominant frequency of the alpha activity [14, 15]. The positive effect of nicotine on the C-fiber LEP amplitude found in the present study is consistent with these studies. The N2/P2 amplitude was positively correlated with the dominant alpha frequency not only in the Smoking session but also in the Control session, where PNC remained at a very low level, suggesting that the change in the N2/P2 amplitude was affected by the change in vigilance. This idea is also supported by the finding by Qiu *et al.* [16] that the amplitude of ultralate LEPs was slightly increased during attention and significantly decreased during distraction, relative to control. In the present study, both changes of the N2/P2 amplitude and dominant alpha frequency followed the change in PNC after smoking. Taken together, the positive effect of nicotine on the C-fiber LEP amplitude in this study seems to reflect the general facilitatory effect of smoking and nicotine on brain activities.

The different effects of nicotine on the LEPs between the first pain ascending through the Aδ fibers and second pain through C fibers suggest a difference in the processing of these two types of pain. Several studies recently reported on the differences in cerebral processing between the first and second pain using EEG, magnetoencephalography (MEG) and functional magnetic resonance imaging (fMRI). In summary, EEG and MEG results show a similar main activation in the secondary somatosensory cortex after Aδ and C fiber stimulation except for the mean latency [10]. Activation in the primary somatosensory cortex following Aδ and C fiber activation is still a matter of controversy [17-19]. By contrast, a clear difference was found using fMRI [20]. Brodmann's area 32/8/6 including the cingulate cortex and presupplementary motor area in the hemisphere ipsilateral to the stimulated side, and the bilateral anterior insula were significantly more activated following C fiber stimulation than Aδ fiber stimulation. Since these areas are thought to be involved in the emotional aspect of pain processing, second pain perception might be more related to the emotional aspect than first pain perception. Tamura *et al.* [21, 22] reported that repetitive transcranial magnetic stimulation over the primary motor cortex increased the LEP amplitude and subjective pain rating scores following Aδ fiber stimulation but decreased them following C fiber stimulation. This difference could be attributed to a difference in the activity of the insular and cingulate cortices between the two conditions. Based on these studies in humans, it seems considerable that there was a difference in the effect of nicotine on first and second pain perception.

The present results may indicate the general arousal effect of nicotine in tobacco smoke, consistent with previous ERP studies. This effect might also have taken place in the first pain perception, but a larger antinociceptive effect on the first pain might have concealed it. Although the exact reason for this difference between Aδ and C fibers is unknown, the pharmacological and physiological effects of nicotine on the first and second pain are clearly different.

CONCLUSION

This is the first study to investigate the effect of tobacco smoking on pain-related brain activity after selective laser beam C fiber stimulation. Smoking significantly increased the dominant alpha frequency. Both the N2/P2 amplitude of laser evoked potentials and dominant alpha frequency were positively correlated with the plasma nicotine concentration. Tobacco smoking/nicotine had the opposite effect on Aδ fiber-mediated first pain and C fiber-mediated second pain. These differences may be important in understanding the actions of nicotine on the first and second pain responses.

ACKNOWLEDGMENT

The nicotine assays were done by Dr. Linda von Weymarn and the Analytical Biochemistry Core at the Masonic Center, University of Minnesota Minneapolis, MN 55455, and supported by National Institute on Drug Abuse Grant DA 018974-01-03. This work was supported by a grant from the Smoking Research Foundation, Japan. The entire study has been conducted in a completely scientifically objective fashion. The authors caution that these research findings should not be interpreted as indicating tobacco smoking has any significant properties. Instead, the findings support basic scientific studies on the actions of nicotine in the brain.

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Received: July 28, 2009 Revised: September 16, 2009 Accepted: September 17 2009

Selective Stimulation of C Fibers by an Intra-Epidermal Needle Electrode in Humans

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Abstract: We recorded evoked potentials (EPs) induced by intra-epidermal electrical stimulation using a needle electrode with specific parameters. We identified the fibers activated by this specific stimulation by assessing the conduction velocity (CV) of the peripheral nerve. The EPs were recorded from the Cz electrode (vertex) of the International 10-20 system in ten healthy male subjects. The dorsum of the left hand and forearm were stimulated with an intensity of 0.01 mA above the sensory threshold. The mean P1 latency of EPs for the hand and forearm were 1007 ± 88 and 783 ± 80 ms, respectively, and the CV estimated from the latency of P1 was 1.5 ± 0.7 m/s. The CV indicated that the fibers activated by the stimulation were C fibers. Since the method of stimulation is convenient and non-invasive, it should be useful for investigating the functions of small fibers.

INTRODUCTION

In studies of sensory systems, a well-controlled stimulus is required to activate the system being examined. An experimental stimulus should be quantifiable, and reproducible (regularity of intensity and time distribution). Additionally, for clinical application, safety, low cost, and simplicity of use are required. Regarding the nociceptive system, no method of stimulation fulfilling these requirements is available, a technical drawback that has prevented progress within this field. The selective activation of C fibers with little or no concurrent activation of other sensory modalities is particularly difficult.

Various techniques (for review, see [1]) have been used to investigate C fiber-related cerebral processing [2-8] as well as conduction velocity (CV) [9], each with its own strengths and weaknesses.

The most common way of activating C fibers is to use a laser to stimulate a tiny area of skin [10]. Lasers can stimulate C fibers specifically with minimal effects on other fibers, but are expensive and hard to control. Electrical stimulation is also useful for investigating the nociceptive system, since the equipment is easier to use and the method itself is non-invasive. Despite its technical advantages, however, conventional electrical stimulation activates thicker fibers at a lower current intensity than C fibers.

We have developed a method of intra-epidermal electrical stimulation (IES) for the selective activation of Aδ fibers [11-16]. Since this method is easy to control, does not require special equipment, and provides a steep rise of stimulation, it would be good for studying the C fiber nociceptive system if it could activate C fibers selectively. We noticed in previous studies that IES actually activates C fibers when a stimulus intensity higher than the threshold for Aδ fibers is used. Similar results were reported by Nilsson *et al.* [17] and Nilsson and Schouenborg [18] who used a needle-like electrode. However, this indicates that the stimulus inevitably activates Aδ fibers at the intensity necessary to activate C fibers. Here we report that IES can activate cutaneous C fibers selectively when specific parameters are employed.

METHODS

Subjects

The experiments were performed on ten healthy male volunteers (25-43 years). The study was approved in advance by the Ethics Committee of the National Institute for Physiological Sciences, Okazaki, Japan, and written consent was obtained from all the subjects.

Stimulation

We used a method of intra-epidermal stimulation (IES) developed in our laboratory for the selective activation of Aδ fibers [12]. For IES, we used a concentric bipolar needle electrode (Nihon Kohden, Tokyo, Japan) which consisted of an outer ring 1.2 mm in diameter and an inner needle that protruded 0.1 mm from the outer ring. For the selective stimulation of C-fibers, the following parameters were used: 1) The anode was the inner needle and the cathode was the outer ring. 2) The electric pulse was a triangular wave with a rise and fall time of 1 ms. 3) The stimulation was a train of

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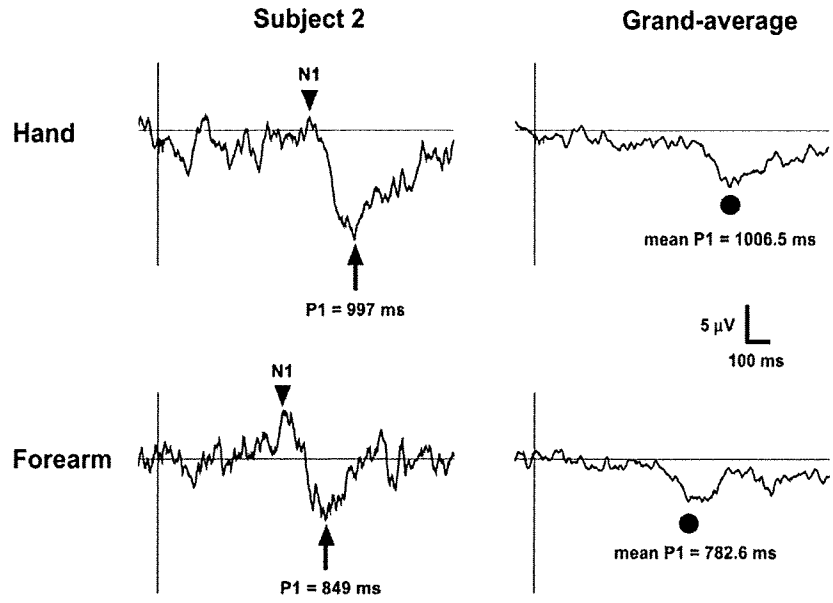

Fig. (1). Evoked potentials following intra-epidermal electrical stimulation recorded at Cz. Waveforms of evoked potentials in a representative subject (left) and grand-averaged waveforms (right). Arrowheads and arrows indicate the peak latency of the negativity and positivity, respectively. Circles in the right traces indicate the mean peak latency.

three pulses with an interstimulus interval (ISI) of 20 ms. 4) Three electrodes 10 mm apart were used for augmentation of the response. These parameters were determined based on results of preliminary experiments showing that 1) the standard cathodic stimulation always activated Aδ fibers at a lower intensity than that for C fibers, 2) a single pulse of anodal current rarely elicited C-fiber-related brain potentials or sensations while a train of 3-5 pulses was very effective at augmenting the response of C fibers, 3) thicker fibers seemed to prefer a steeper rise in the pulse, 4) a train of pulses with an ISI shorter than 20 ms more effectively augmented the activation of thicker fibers than C fibers, and 5) multiple electrodes were sometimes useful to elicit clear sensations due to the activation of C fibers. These results are generally consistent with the findings that 1) an anodal current is theoretically effective at stimulating cutaneous fibers running vertical to the skin's surface [19] such as branches of C-fibers innervating the epidermis [20, 21], 2) a pulse of longer duration is necessary to stimulate thinner fibers [22] and 3) a substantial spatial and temporal summation of C-fiber impulses is required to produce painful sensations [23, 24].

The electrical stimulus was applied to the dorsum of the left hand and forearm. The stimulation was started at with an intensity of 0.01 mA and increased in steps of 0.01 mA until the subject felt a sensation (threshold). Subjects were instructed to press a button quickly when they felt any sensation and the reaction time (RT) was measured. The mean sensory threshold was 0.04 ± 0.01 and 0.04 ± 0.01 mA at the hand and forearm, respectively. After confirming that the RT was in the range of C fiber transmission (700-1500ms), we recorded evoked potentials (EPs) following IES at an intensity 0.01 mA above the sensory threshold. At this intensity, single or double pulses did not elicit any sensations or EPs.

Recording of Evoked Potentials

EEG signals were recorded from the Cz electrode referenced to the linked earlobes (A1-A2) of the International 10-20 system. A pair of electrodes placed on the supra- and infra-orbit of the right eye was used for recording electro-oculograms. The impedance of the electrodes was kept below 5 kΩ. The EEG signals were recorded with a bandpass filter of 0.1-100 Hz at a sampling rate of 1000 Hz. The window of analysis was from 100 ms before to 1500 ms after the stimulus onset. The 100-ms period before the stimulus was used as the DC baseline. Since the sensation elicited by the stimulation was relatively weak, the subject was asked to attend the stimulus. At least ten artifact-free responses were collected and averaged for each stimulation site.

The peripheral CV was calculated by dividing the difference in peak latency between the EP responses following the hand and forearm stimulation, by the distance between the two sites. Data were expressed as the mean ± SD.

RESULTS

The stimulation did not elicit a C fiber-related sensation or EPs in two of ten subjects. Therefore, EP data obtained from eight subjects were used for the analysis. The sensations produced by IES were a weak painful sensation described as "flicking", "burning", or "long-lasting weak pricking" (n = 4), and a light touch sensation described as a "faint touch" or "light pressure" (n = 4).

Representative EP waveforms of a single subject and the group-averaged waveforms are shown in Fig. (1). Similar to previous studies using laser stimulation [25], the stimulation evoked a negativity followed by a positivity (P1). However in some subjects, the negativity was unclear and its peak

Table 1. Peak Latency of EPs and CV

Subject	Peak latency of P1 (ms)		Distance (cm)	CV (m/s)
	Hand	Forearm		
1	992	717	24.3	0.9
2	997	849	27.4	1.9
3	991	650	28.8	0.8
4	955	792	26.2	1.6
5	1092	881	25.2	1.2
6	887	781	24.9	2.4
7	966	862	25.4	2.4
8	1172	729	25.6	0.6
mean	1006.5	782.6	25.9	1.5
SD	87.7	80.4	1.5	0.7

latency was difficult to identify. Thus, we used P1 for the analysis, which was large in amplitude and detected in all eight subjects. The mean P1 latency following hand and forearm stimulation was 1007 ms and 783 ms, respectively (Table **1**). The peripheral CV calculated from the latency difference of P1 was 1.5 ± 0.7 m/s.

DISCUSSION

The stimulation did not elicit a C fiber-related sensation or EPs in two of ten subjects. This might be due to individual difference in thickness of the corneum. The electrode we used was consisted of an inner needle that protruded 0.1 mm from the outer ring and we used a very weak current for selective stimulation of C-fibers. Therefore, in subjects with the thicker corneum, it is possible that the weak current could not reach to the epidermal area in which free nerve endings are located. Our previous study using a laser beam which is the most common way for activating C fibers also failed to elicit C fiber-related responses in four out of 17 subjects [26].

In the present study, P1 peaked at around 800 ms for the forearm and 1000 ms for the hand stimulation. In previous studies using a laser beam to a tiny area of skin [4, 9, 10, 27], which is known to selectively activate C fibers, the mean P1 latency for stimulation of the hand was 930-1144 ms, which is very similar to the present results. The estimated CV of 1.5 m/s in this study was also consistent with previous studies showing a value of 0.8 – 2.8 m/s by averaged EEG [4, 27, 28] and 0.4 – 1.8 m/s [29] by microneurography. In terms of the precise measurement of CV, microneurography is superior to EEG. However, since C-fibers have a very slow CV, the latency difference between the two stimulation sites was enough long (224ms) to correctly detect by EEG. Another weakness of the present method is that we could not know receptive properties of C fibers unlike microneurography (for review, see [30]).

In addition, we also considered that our method mainly stimulated C fibers, since C fiber-related EPs do not appear on the concomitant activation of other fibers. For example, laser stimulation that activates both Aδ and C fibers elicits only the Aδ components without responses at latencies compatible with ultra-late components related to C fibers [31-33]. Likewise, C fiber-related cortical responses are only recorded when the concomitant activation of Aδ fibers is suppressed, *i.e.* a pressure nerve block [34] or heating the skin below the Aδ threshold [4]. In the present study, since there was no consistent response at a latency earlier than 400 ms, the results suggested a selective activation of C fibers by our method.

Laser beams applied to a tiny area [4, 7, 9, 28, 31, 35] have been used to selectively activate C fibers, which is considered difficult to do with electrical stimulation, since C fibers generally have a high threshold to electrical stimulation. However, the present results indicate that this threshold is not so high when appropriate parameters are chosen. In support of this notion, unmyelinated afferents respond to skin deformation in mammals [36-38], and have a low mechanical threshold in human skin [39]. In addition, recent studies suggested that low-threshold C mechano-receptive afferents provide information about pleasant touch [40-42], although their functional role remains unclear. In the present study, we used a very weak current (about 0.05 mA) and half of the subjects felt the evoked sensation as a light touch. Therefore, it is possible that our method preferentially activates the low-threshold C fibers.

In conclusion, intra-epidermal electrical stimulation successfully activated cutaneous C fibers selectively. Because the method is easy to control and non-invasive, it should be useful for investigating the functions of small fibers both for basic research and for clinical examinations, although the parameters remain to be refined for a more consistent and stronger stimulation of C fibers, for example, by changing the waveform of the pulse or duration of the stimulus [43-45].

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Received: April 23, 2009 Revised: June 12, 2009 Accepted: June 17, 2009

BRAIN RESEARCH 1313 (2010) 185–191

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Research Report

The effect of smoking on pain-related evoked potentials

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ARTICLE INFO

Article history:
Accepted 8 December 2009
Available online 16 December 2009

Keywords:
Electroencephalography
Pain
Smoking
Event-related potential
Nicotine

ABSTRACT

The effects of human tobacco smoking and nicotine on pain-related brain activities were investigated. EEG responses evoked by a painful laser beam (laser evoked potentials; LEPs), and the plasma nicotine concentration (PNC) were measured. There were two sessions, one after smoking (Smoking session), and the other in no smoking (Control session). Subjective ratings of pain perception were also measured using the visual analog scale (VAS). Two major components, N2 and P2 of LEPs, were recorded. The amplitude of P2 was significantly smaller in the Smoking session than in the Control session. A significant negative correlation was found between PNC and the amplitude of N2 as well as P2. The results were consistent with the hypothesis that smoking and/or nicotine have an antinociceptive effect, which supports most non-human studies and some human studies. Smoking of a single tobacco cigarette did not show a subjectively perceivable extent of reduction in the intensity of evoked pain.

1. Introduction

Nicotine has been demonstrated to have various psychophysiological effects in humans. The effects of smoking or nicotine on event related potentials (ERPs) have been explored by numerous groups (Friedman et al., 1974; Houlihan et al., 2001; Knott et al., 1999; Woodson et al., 1982; for reviews, Knott et al., 1995 and Pritchard et al., 2004). Most of these studies reported increased amplitudes and/or decreased latencies supporting the notion that nicotine enhances brain processing.

On the other hand, non-human studies demonstrated an antinociceptive effect of nicotine. Mattila et al. (1968) reported that nicotine injected subcutaneously increased the threshold of pain in mice and rabbits. The antinociceptive effect of nicotine was shown to involve nervous systems with various neurotransmitters including μ-opioid (Berrendero et al., 2002; Biala and Weglinska, 2006), serotonin, and epinephrine (Cucchiaro et al., 2005; Cucchiaro et al., 2006; Iwamoto, 1991).

The effect of nicotine on pain perception in humans has also been explored by some groups, but the results of these studies have not been consistent. Pomerleau et al. (1984) reported that smoking increased the pain threshold time for arm immersion into ice water, whereas Mueser et al. (1984) found no significant effect of smoking on the perception of painful electric stimulation. Scott et al. (2007) explored effect

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doi:10.1016/j.brainres.2009.12.022

of smoking on μ-opioid receptor-mediated neurotransmission using positron emission tomography (PET). They reported that tobacco smoking enhanced μ-opioid receptor-mediated neurotransmission in the right anterior cingulate cortex and suppressed in the left amygdala, left ventral basal ganglia, and right thalamus after tobacco smoking, suggesting a possible role of smoking and nicotine on pain perception in humans through these brain areas, which are important in pain processing (Kakigi et al., 2005).

Pain-related evoked potentials are commonly used in studies that objectively evaluate pain-related brain activities in humans. N2 and P2 are the two major components constantly recorded in pain-related evoked potentials. They are thought to reflect the activities of operculoinsular and cingular cortices, respectively (Tarkka and Treede, 1993; Bromm and Chen, 1995; Valeriani et al., 1996; Iannetti et al., 2003; Kakigi et al., 2005). Their amplitudes are shown to be correlated to the magnitude of subjectively perceived pain (Kakigi et al., 1989; Bromm and Treede, 1991; García-Larrea et al., 1997). Knott (1990) and Knott and De Lugt (1991) recorded pain-related evoked potentials after electrical stimulation, but the results did not consistently support the notion that smoking attenuates their amplitude.

In the present study, we measured evoked potentials after painful laser stimulation (laser evoked potentials; LEPs) in smoking and non-smoking conditions. Painful laser stimulation activates Aδ fibers but not Aβ fibers; electrical stimulation activates both. Laser stimulation enables one to evaluate the brain activities related to Aδ fiber mediated pain information without confounded by Aβ fiber mediated tactile information. To evaluate the effect of smoking on LEPs over time, LEPs were measured in five runs in each of the two sessions on separate days (Smoking and Control sessions), which followed a 12-hour abstinence from smoking. Subjects smoked one cigarette after the first run in the Smoking session. Plasma nicotine and cotinine concentration (PNC and PCC) were also measured just before each run. We tested the correlation between amplitudes of LEPs and PNC, which rapidly decrease after smoking. Cotinine is the main metabolite of nicotine, which remains in the plasma with a $t_{1/2}$ of about 18 hours. It is used to assess current smoking status of subjects. To our knowledge, there have been no reports on the effect of smoking or the plasma nicotine concentration on laser-evoked potentials (LEPs).

2. Results

2.1. *Plasma nicotine and cotinine concentrations*

For all the 10 subjects, the PNC in the Control session and Pre in the Smoking session was less than 7 ng/ml, indicating that all the subjects had been abstinent from smoking before each session as instructed. Mean values and standard errors are shown in the results. The mean PNC for each run averaged over subjects was highest at 5 min after smoking in the Smoking session (29.8±6.9 ng/ml) and decreased as time passed in the following runs.

The mean PCC at Pre in the Control and Smoking sessions averaged over all subjects was 61.9±24.3 ng/ml and 74.0±23.1 ng/ml, respectively. The PCC in 4 of the 10 subjects was below the limit of detection in the Control session. For three of these four subjects, the PCC at Pre was also below the limit of detection in the Smoking session.

2.2. *Amplitudes and latencies of N2 and P2*

Fig. 1 shows superimposed waveforms of a representative subject in each run in each session. The N2 and P2 components of LEPs were consistently observed in each subject with a peak latency at around 200 and 300 ms, respectively. Fig. 2 shows the grand-averaged LEP waveforms for each run in the Smoking and Control sessions. The mean amplitudes of N2 and P2 averaged over subjects for each run are shown along with the PNC in Figs. 3a and b. The mean N2 amplitude, N2 latency, P2 amplitude, and P2 latency averaged over all subjects and all runs were 10.6±0.6 μV, 216±2 ms, 16.6±0.7 μV, and 330±2 ms, respectively. The amplitude of N2 is shown as a positive value.

In a two-way repeated-measures ANOVA (Session and Run) on the amplitude of P2, we found a significant interaction ($F(4, 36)=3.1$, $p=0.049$) and a significant main effect of Session ($F(1, 9)=11.0$, $p=0.01$). In a post hoc paired t-test for each run, the amplitude of P2 was found to be smaller in the Smoking session than in the Control session for the run 5 min after smoking ($t(9)=3.5$, $p=0.03$). The difference in the P2 amplitude between Runs in the Smoking session was not significant in a one-way repeated measures ANOVA ($F(4, 36)=2.53$, $p=0.10$). We did not find a significant interaction of Session and Run, or a significant main effect of Session or Run on the amplitude of N2 ($F(4, 36)=1.7$, $p=0.19$, $F(1, 9)=0.96$, $p=0.35$, and $F(4, 36)=2.7$, $p=0.07$, respectively), or on the latency of N2 or P2 in two-way repeated measures ANOVAs (Session and Run). PNC was negatively correlated to N2 amplitude ($R=-0.29$, $p<0.01$, Fig. 4a) and P2

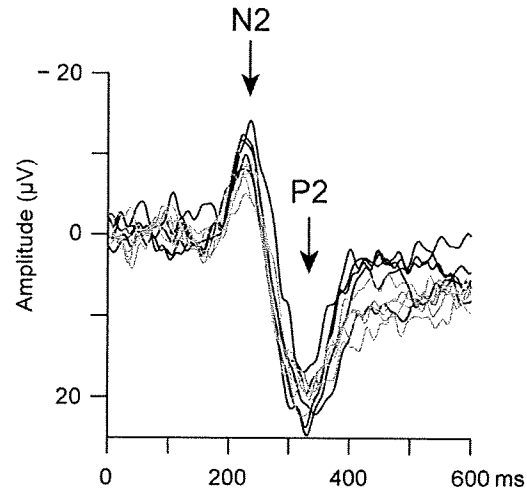

Fig. 1 – Superimposed waveforms of a representative subject in each run in the Control (black) and Smoking (gray) sessions. The waveforms of 600 ms from stimulus onset are shown. Each sweep represents the averaged waveform of 10 artifact-free epochs in a run. Each waveform is adjusted using the 100-ms prestimulus period as a baseline. N2 and P2 deflections (labeled with arrows) were constantly recorded throughout the experiment.

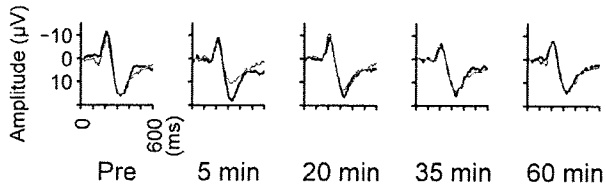

Fig. 2 – Grand averaged waveforms for each run in each session. Recordings in the Control (black) and Smoking (gray) sessions are shown. Each graph corresponds to each run. The horizontal axes indicate the time after stimulus onset.

amplitude ($R=-0.22$, $p=0.03$, Fig. 4b), but not to N2 latency ($R=0.05$, $p=0.62$) or P2 latency ($R=-0.08$, $p=0.41$, respectively).

2.3. VAS scores

The mean VAS score over all the measurements was 5.18±0.13. We found no significant interaction ($F(4, 36)=0.70$, $p=0.53$) or main effect of Session ($F(1, 9)=0.13$, $p=0.73$) or Run ($F(4, 36)=1.9$, $p=0.17$) on the VAS score after a two-way ANOVA (Session and Run). The correlation between PNC and the VAS score was not statistically significant ($R=-0.09$, $p=0.35$, Fig. 4c).

2.4. Heart rate (HR)

The mean HR over all the runs and subjects was 63.7±1.0/min. The mean HR over the Control session and over the Smoking session was 59.8±1.3/min and 67.6±1.4/min, respectively. In a two-way repeated-measures ANOVA (Session and Run), we found an interaction of Session×Run ($F(4, 36)=23.5$, $p<0.01$) and the main effects of Session ($F(1, 9)=11.5$, $p<0.01$) and Run ($F(4, 36)=11.5$, $p<0.01$). In a post hoc paired t-test for each run, the HR at 5 min after smoking was significantly larger in the Smoking session than in the Control session ($t(9)=4.9$, $p<0.01$). HR showed a significant positive correlation with PNC ($R=0.39$, $p<0.01$, Fig. 4d).

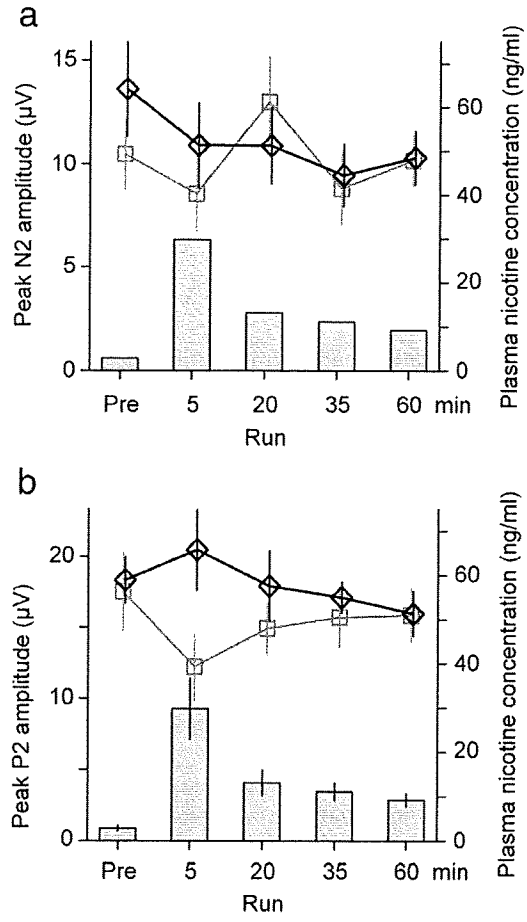

Fig. 3 – Mean±SE of peak N2 and P2 amplitudes, and the plasma nicotine concentration (PNC) for each run. Peak N2 (a) and P2 (b) amplitudes are plotted for the Control (black lines and open diamonds) and Smoking (gray lines and open squares) sessions using the left axes. PNCs are shown with gray bars using the right axes and duplicated in the two graphs. Error bars of PNC are only shown in (b).

3. Discussion

We found an effect of smoking to modulate pain related brain activities. The amplitudes of the N2 and P2 components of pain-related potentials were negatively correlated with plasma nicotine concentration. The amplitude of P2 was significantly smaller in the Smoking session. The amplitudes of N2 and P2 components are thought to reflect the intensity of perceived pain (Kakigi et al., 1989; Bromm and Treede, 1991; García-Larrea, 1997). The results of the present study are consistent with the idea that smoking and nicotine have an antinociceptive property.

The results of the present study need a careful interpretation. No placebo was used in the Control session and the psychological effect of smoking could have modulated the results. This point remains to be clarified until a double-blind study is made using denicotinized cigarettes that cannot easily be distinguished from normal cigarettes. The Control session was always conducted after the Smoking session. The order of sessions might have had some impact on the LEPs. However, the difference in the P2 amplitude was significant between sessions in the 5 min run but not in the other runs. The amplitude of N2 was not significantly different between the sessions, but was significantly negatively correlated to the plasma nicotine concentration. These results cannot be fully explained by the order of the sessions or psychological effect of smoking, but suggest that the amplitude of LEP components reflects the plasma nicotine concentration.

Although the P2 amplitude tended to change after smoking as can be seen in Fig. 3b, the difference in the P2 amplitude between the runs within the Smoking session was not statistically significant ($p=0.10$). Change in the P2 amplitude at each time point might have been too small to be detected without taking account of the actual plasma nicotine concentration at each time point.

The tobacco cigarette used in the present study might have been considerably different in the nicotine content or "taste" from cigarettes usually smoked by each subject. Although the subjects reported that the experimental cigarette was

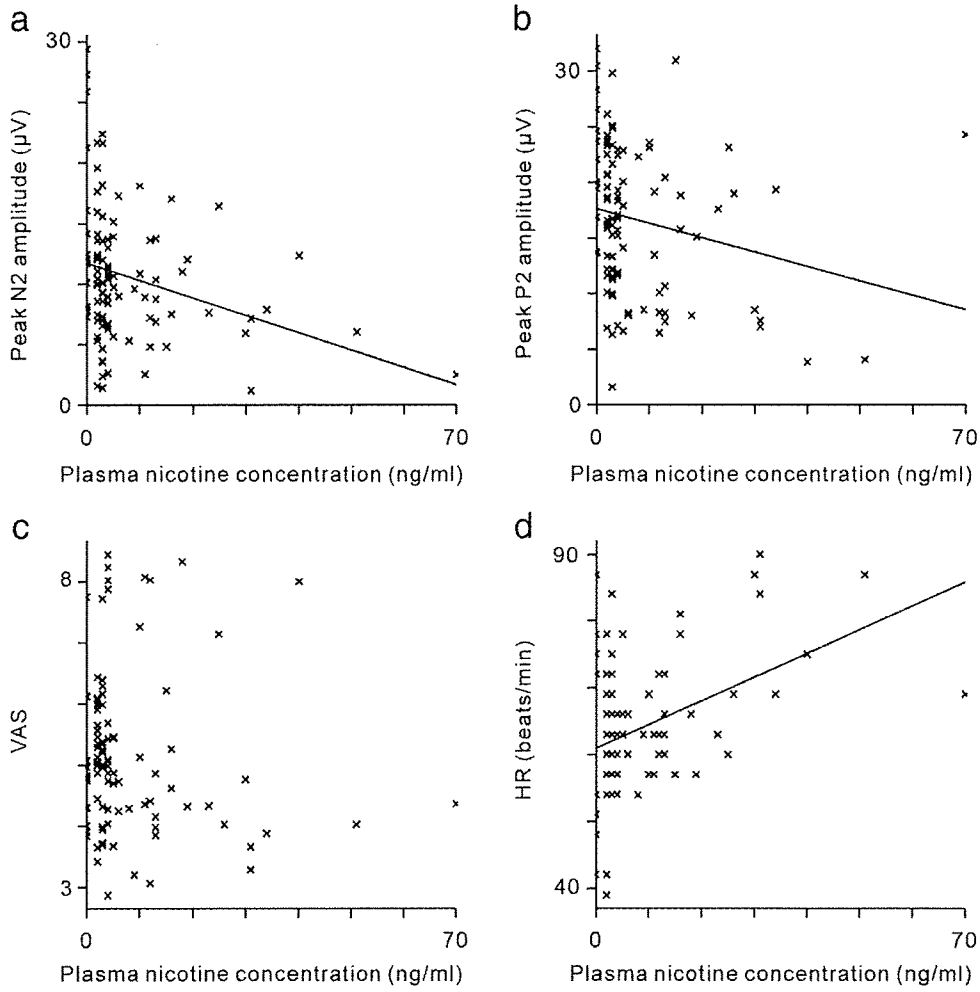

Fig. 4 – Scatter plots of peak N2 (a) and P2 (b) amplitudes, VAS scores (c) and HR (d) versus the plasma nicotine concentration (PNC). Data from both the Smoking and Control sessions are plotted. A fitted linear model is shown where a significant correlation was found (a, b, and d).

acceptable, the difference might have influenced subjects' smoking behavior or emotion and have modulated the effect of smoking and nicotine on LEPs.

For 4 of the 10 subjects, the plasma cotinine concentration was below the limit of detection in the Control session, suggesting that these subjects smoked less regularly than the other subjects. To assess the difference in the effect of smoking and nicotine on LEPs between regular and non-regular smokers, studies on larger number of subjects are needed.

The present study does not directly link smoking or nicotine with brain activities that underlies the processing of intensity or other aspects of pain. However, the most important finding is that the change in the amplitudes of laser-evoked potentials was negatively correlated to the plasma nicotine concentration in contrast to other types of brain activities (Friedman et al., 1974; Woodson et al., 1982; Knott et al., 1999; Houlihan et al., 2001). The mechanism underlying this effect needs to be studied using fMRI or PET, antagonists of nicotine, opioid, or other neurotransmitters. On the other hand, the present study was not designed to distinguish the effect of smoking and nicotine from the effect of recovery from the abstinence. The effect on LEPs could be explained as recovery from a temporary sensitization to pain stimuli as a part of withdrawal symptoms induced by abstinence from smoking, instead of a direct effect of nicotine. To answer this question, studies are needed on non-smokers with administration of nicotine without an exposure to tobacco smoke, for example, using nasal spray or intravenous injection.

We found a difference in the results in the ANOVAs on the amplitudes of N2 and P2. Both the interaction of Session and Run and the main effect of Session were significant on the amplitude of P2 but not on that of N2. In Fig. 3, the difference in the amplitude between the sessions was obvious in the P2 component but not in the N2 component. The N2 and P2 components are thought to reflect the activities of operculoinsular and cingular cortices, respectively (Tarkka and Treede, 1993; Bromm and Chen, 1995; Valeriani et al., 1996; Iannetti et al., 2003; Kakigi et al., 2005). The difference in the results on the N2 and P2 amplitudes might indicate that the modulative effect of smoking is stronger or more lasting on the cingular cortex than on the operculoinsular cortex. Scott et

al. (2007) showed that smoking increased the activity of μ-opioid receptor-mediated neurotransmission in the right anterior cingulate cortex but decreased it in the left amygdala, left ventral basal ganglia, and right thalamus, using positron emission tomography. If the effect of smoking on the LEP amplitudes is mediated by a differential modulation on μ-opioid receptor mediated neurotransmission in these brain areas, the difference in the effect of smoking on the amplitudes of the two LEP components might be consistent.

In contrast with the results in LEPs, we did not find a significant effect of smoking or nicotine to reduce the intensity of subjectively perceived pain. The dose of nicotine by smoking of one cigarette might have not been enough to cause an obvious change in the intensity of perceived pain. Subjective ratings by visual analog scale can considerably fluctuate throughout an experiment and might not be sensitive enough to detect a slight change in perceived pain over time. It might also be possible that smoking does not modulate much the sensory-discriminative aspect of pain, if smoking mainly attenuates the activities in the cingulate cortex but not much in the operculoinsular cortex or other brain areas. Most of human studies that found an antinociceptive effect of smoking and/or nicotine used other techniques to measure the change in subjectively perceived pain. Pomerleau et al. (1984) reported that the smoking of normal cigarettes resulted in a longer pain awareness threshold in a cold pressor test than did the smoking of zero-nicotine cigarettes. Perkins et al. (1994) reported a significant negative effect of the plasma nicotine concentration on the latency of pain detection to a radiant heat stimulus after an administration of nicotine via a nasal spray.

Some previous studies reported that no significant antinociceptive effect was found after smoking or a nicotine dosage in humans. Mueser et al. (1984) did not find any significant effect of smoking on the pain or tolerance threshold, or pain discrimination capacity in experiments with painful electric stimulation. Knott (1990) and Knott and De Lugt (1991) reported that there was no consistent effect of smoking on pain-evoked EEG responses or subjective ratings after painful electrical stimulation in a condition either with or without a warning before the stimulus. In the present study, we used painful laser stimulation and found the effect of smoking to reduce the amplitudes of pain-related evoked potentials. It is difficult to find the cause of the discrepancies in the results of these studies. But one possible cause is that the difference in the method of painful stimulation. Electric stimulation at the surface of the skin activates both Aδ and Aβ fibers, whereas laser stimulation can activate Aδ fibers without activating Aβ fibers. Since the tactile information through Aβ fibers could reach the brain faster than pain information through Aδ fibers, brain processing after an electric stimulus might not be changed by smoking or nicotine. In addition, the dose of nicotine that is not acutely harmful for a human might be too small to elicit a significant antinociceptive effect. The dose of nicotine through tobacco smoking in a usual way might not significantly affect pain-related cognition or behaviors. To obtain more conclusive results, we might need studies using a less toxic nicotine receptor agonist at a higher dose or a more sensitive method to measure subjectively perceived pain.

4. Experimental procedures

4.1. *Subjects*

Ten healthy male volunteers who varied in their smoking habit, aged 23 to 43 (mean 33±6 SD), participated in the study. The mean number of years that the subjects have smoked was 12.4±7.0 years. The mean number of cigarettes they were smoking per day was 13.9±8.0. The subjects were free of any history of psychiatric or neurological disorders, or substance abuse. The study was approved in advance by the Ethics Committee of the National Institute for Physiological Sciences, Okazaki, Japan. Written consent was obtained from all the subjects.

4.2. *Stimulation and recordings*

A Tm:YAG laser (BLM1000S, Carl Baasel Lasertechnik, Starnberg, Germany) was used for noxious stimulation. The laser pulses were 2000 nm in wavelength, 1 ms in duration, and 3 mm in diameter. The laser beam was applied to the dorsum of the right hand between the first and second metacarpal bones. The subjects were instructed to rate the perceived pain sensation after each laser beam on a 10-cm horizontal bar, where the left margin indicated no painful sensation at all, and the right margin the most intense imaginable pain (visual analogue scale, VAS). Before starting each session, laser simulation was tested on each subject to determine the energy of laser to be used in the session and to make the subject accustomed to the stimulation. The laser energy was adjusted to a level at which stimulation produced a VAS score of 5–6. As a result, the same intensity was chosen for both sessions for each subject (mean 157.5±8.4 mJ). The stimulation intensity was kept constant through runs. To avoid tissue damage, the irradiated points were moved slightly for each stimulus.

LEPs were recorded using a scalp electrode placed at Cz referred to the linked earlobes (A1+A2) according to the International 10/20 system. The EEG signals were recorded with a band-pass filter of 0.1–100 Hz at a sampling rate of 1000 Hz, and then digitally filtered with a 50 Hz low-pass filter. The window of analysis was from 100 ms before to 600 ms after the stimulus onset, and the prestimulus period was used as the DC baseline. The impedance of the electrodes was kept below 5 kΩ. A pair of electrodes placed on the supra- and infra-orbit of the right eye was used for the rejection of trials containing artifacts due to blinks. An electrocardiogram was recorded to calculate heart rate, using a pair of disk electrodes placed on each forearm.

4.3. *Procedures*

The experiments were conducted in two sessions, Smoking and Control, with different conditions on separate days. Each session started at 9 AM or 1 PM. The starting time was counterbalanced between subjects and between sessions. The Control session was conducted 5 to 20 (mean 11.2) days after the Smoking session for each subject. The procedures of the experiment were the same for the two sessions, except that subjects smoked a cigarette in the Smoking session but not in

the Control session. For each session, subjects were required to be abstinent from smoking, alcohol, drugs, and caffeine for at least 12 hours before the experiment. Subjects were seated in an armchair in a quiet electrically shielded room, the temperature of which was controlled at 24 to 26 °C. Before the experiment, an indwelling catheter for collecting venous blood samples was placed in the left cephalic vein. There were five runs of recordings at different times: before smoking (Pre), and 5, 20, 35 and 60 minutes after smoking (Fig. 5). Just before the beginning of each run, venous blood was collected for measurements of plasma nicotine and cotinine concentrations, and an electrocardiogram was recorded for 20 s for heart rate calculation. In each run, 12–15 stimuli were applied and LEPs were recorded. The interstimulus interval was varied at random between 14 and 19 s. In the Smoking session, subjects smoked a tobacco cigarette for 5 minutes just after all data were obtained for Pre, and then the remaining four runs followed. The tobacco cigarette of a common brand in Japan with 1.0 mg of nicotine (Japan Tobacco Inc., Tokyo) was used and was reported to be acceptable by all the subjects. The plasma samples were frozen with dry ice and kept at −80 °C until assay. The plasma nicotine and cotinine concentrations were measured using gas chromatography–mass spectrometry. The method was similar to that used previously to analyze urinary cotinine concentrations (Hecht et al., 1999) with the addition of a solid phase extraction step carried out on an MCX column (Water Corporation, Milford, MA). The MCX column was prepared and the sample eluted as described previously (Murphy et al., 2007).

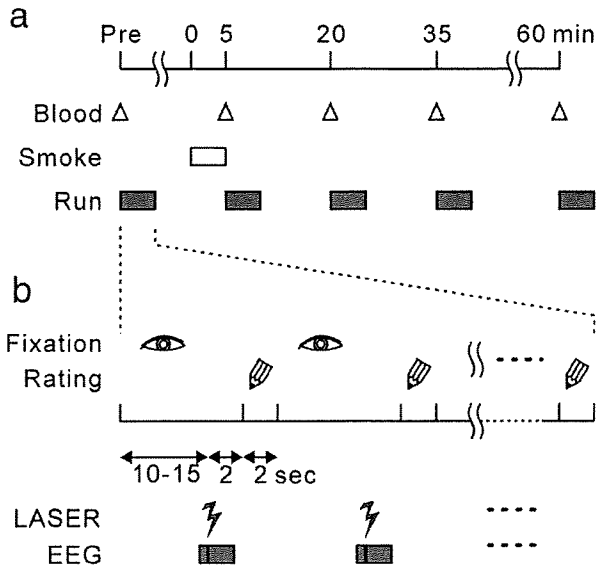

Fig. 5 – Experimental design. (a) Scheme representing the Smoking session. A blood sample was collected just before each run. The procedure for the Control session was the same except for the absence of smoking. (b) At least 12 trials of LEP recording were included in each run. In each trial, the subject fixated on a "fixation" sign on the screen. About 10–15 s after the fixation sign appeared, a laser stimulus was given to the dorsum of the right hand of the subject. EEG was recorded from 100 ms before to 600 ms after the stimulus. After 2 s, a "rating" sign appeared on the screen, and the subject rated the perceived pain on a form. The fixation sign for the next trial appeared in 2 s.

4.4. *Data analysis*

For each run of LEP recordings, 10 artifact-free epochs were averaged after all epochs with eye movements and/or blinks were visually inspected and rejected. When more than 10 artifact-free epochs were found in a run, only the first 10 epochs were averaged and used in further analyses. The mean amplitude of the 100-ms period just before the stimulus was used to adjust the averaged waveform for each run in each session and subject. The peak amplitude and latency of N2 and P2 were calculated using time frames with a latency period of 180–250 ms and 250–360 ms, respectively. The VAS scores were averaged for each run. The effect of smoking was assessed in ANOVAs and post hoc tests using the Control session as a baseline. A two-way (Session and Run) repeated-measures ANOVA was done on each of the amplitude and latency of N2 and P2, averaged VAS scores, and heart rate (HR). The Greenhouse–Geisser epsilon was used in the correction of degrees of freedom when appropriate. Post hoc tests have been operated using paired *t*-test with Bonferroni's correction between the sessions for each run when an interaction was found in the ANOVAs. Effect of Run was also assessed with a one-way repeated-measures ANOVA within the smoking session. Pearson's correlation coefficient was calculated between the plasma nicotine concentration (PNC) and each of the amplitudes and latencies of N2 and P2, VAS score, and HR, and was tested using *t*-test with Fisher transformation. Statistical analyses were carried out with SPSS 15.0J.

Acknowledgments

The nicotine/cotinine assays were done by Dr. Linda von Weymarn and the Analytical Biochemistry Core at the Masonic Center, University of Minnesota Minneapolis, MN 55455, and supported by National Institute on Drug Abuse Grant DA 018974-01A2. The authors would like to thank Prof. Kenneth L Casey for helpful suggestions. This work was supported by a grant from the Smoking Research Foundation, Japan. The entire study has been conducted in a completely scientifically objective fashion. The authors caution that these research findings should not be interpreted as indicating tobacco smoking produces clinically significant analgesia. Instead, the findings support basic scientific studies on the actions of nicotine in the brain.

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The Journal of Pain, Vol ■, No ■ (■), 2009: pp 1-7
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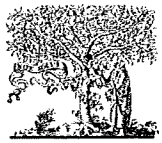

ELSEVIER

Assessing Aδ Fiber Function With Lidocaine Using Intraepidermal Electrical Stimulation

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Abstract: **The functions of small fibers can be impaired in peripheral neuropathies, and screening tests for clinical use are required. To verify whether intraepidermal stimulation (IES) is useful for assessing the functions of Aδ fibers in the superficial layer, we investigated sensory thresholds and evoked cortical responses in healthy volunteers before and after a transdermal administration of lidocaine. Pain and tactile thresholds were studied using IES and transcutaneous electrical stimulation (TS), respectively, in 10 healthy volunteers before, and 1 hour, 3 hours, and 5 hours after a local anesthesia with lidocaine. Cortical potentials evoked with IES and TS were also studied in 12 healthy volunteers before and 5 hours after the anesthesia. Although the local anesthesia had no effect on the evoked potentials or the tactile threshold for TS, it markedly increased the pain threshold and almost abolished the evoked potentials for IES. These results suggest that IES is a sensitive tool for detecting functional changes of cutaneous Aδ fibers.**

Perspective: ***Compared with other methods of stimulation used to investigate Aδ fiber function, our method is easy to apply and less invasive and can stimulate any site of the body. Therefore, it should be useful as a screening test for patients with neuropathy.***

Key words: *Aδ fibers, evoked potentials, intraepidermal electrical stimulation, neuropathy, pain.*

The impairment of small fibers can occur in various peripheral neuropathies.[13] Diabetes mellitus is the most common cause, with small fiber involvement beginning in distal parts of the limbs.[23] For an early diagnosis and treatment, screening tests suitable for clinical use are required. Researchers studying small fiber function in patients with peripheral neuropathies have applied various techniques including laser stimulation,[11] a cutaneous silent period,[17] a cooling detection threshold,[29] and warm and heat pain thresholds.[21,25]

Recently, intraepidermal nerve fiber density (IENFD) has been considered a reliable tool for diagnosing conditions affecting small fibers.[19-21,25-27] Shun et al[25] showed that IENFD was much lower in diabetic patients than matched control subjects, with IENFD per millimeter being about 1.8 and 9.4, respectively. Detecting these morphological changes with an electrophysiological test would be of great help for clinical diagnosis.

We have developed a method of electrically stimulating the epidermal area for the selective activation of Aδ fibers.[5] The method is easy to use and can be applied to various cutaneous sites. In the present study, we investigated whether this method is suitable for evaluating the functions of Aδ fibers located in the superficial layer of the skin by measuring pain threshold and evoked potentials before and after the transdermal application of lidocaine in healthy volunteers. We considered the effects of epidermal stimulation after the cutaneous application of lidocaine to mimic the early stages of diabetic neuropathy with small fiber involvement beginning in distal parts of the limbs. Because our method targets Aδ nociceptors or their fibers in the superficial layer of the skin, the results of the epidermal stimulation would be sensitive to the transdermal application of lidocaine, whose effects are stronger for the superficial layers than for the deeper layers.[3]

Received May 1, 2009; Revised July 9, 2009; Accepted October 2, 2009.
Supported by grants from the Ministry of Education, Sports, Sciences, and Technology and from the Ministry of Health, Labour, and Welfare, Japan.
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1526-5900/$36.00

doi:10.1016/j.jpain.2009.10.001

Methods

Subjects

The experiments were performed on 12 healthy volunteers (3 women and 9 men; age, 25 to 43 years). Ten volunteers participated in Experiment 1, all 12 in Experiment 2, and 5 in Experiment 3. The study was approved in advance by the Ethics Committee of the National Institute for Physiological Sciences, Okazaki, Japan, and written consent was obtained from all the subjects.

Stimulation

For nociceptive stimulation, we used a method of intraepidermal electrical stimulation (IES) developed in our laboratory[5] for the selective activation of cutaneous Aδ fibers without the activation of thicker fibers (ie, Aβ fibers). In this study, we used a stainless steel concentric bipolar needle electrode (Nihon Kohden, Tokyo, Japan) for IES.[8] The anode was an outer ring 1.2 mm in diameter and the cathode was an inner needle that protruded 0.1 mm from the outer ring. By pressing the electrode against the skin gently, the needle tip was inserted in the epidermis where nociceptors are located, while the outer ring was attached to the skin surface. The electrical stimulus was a constant current of double pulses (interstimulus interval, 10 ms) lasting 1 ms each. We used double pulses to augment the response. For tactile stimulation, similar cutaneous sites were stimulated using a bipolar felt tip electrode (NM-420S; Nihon Koden), 0.9 mm in diameter with a distance of 23 mm between the anode and cathode (transcutaneous electrical stimulation, TS). The stimulus parameters were the same for IES.

Sensory Threshold and Pain Rating

IES and TS were applied to the dorsum of the hand and foot and the sensory threshold was measured. For IES, we started stimulation with an intensity of 0.01 mA and increased the current in steps of 0.01 mA until the subject felt a pricking sensation, and then decreased it in steps of 0.01 mA to the point where the sensation disappeared. Usually, the pricking sensation disappeared with a decrease of 0.01 mA, but some subjects could feel a similar but weaker sensation at this intensity. Under the pain threshold, no sensations occurred in any subject. Because the threshold was slightly different at each penetration, the measurement was performed at 5 locations, and the mean value was used for the subsequent analysis. The upper limit of the intensity of IES was set at 1.0 mA. The threshold of tactile sensations for TS was measured similarly.

In the threshold measurement experiment for IES, subjects were also asked to score the intensity of perceived sensations on a visual analog scale (VAS), with zero indicating "no pain" and 100 meaning "worst possible pain" at the 5 different locations before the local anesthesia.

Recording of Evoked Potentials

EEG signals were recorded at Cz referenced to linked earlobes (A1-A2) of the International 10-20 system as described previously.[5] We focused on evoked responses recorded at Cz, since in a preliminary study, the maximum response was recorded from the Cz derivation, similar to our previous report.[10] The impedance of the electrodes was kept below 5 kΩ. The EEG signals were recorded with a bandpass filter of 0.1 to 100 Hz at a sampling rate of 2000 Hz. The window of analysis was from 100 ms before to 600 ms after the stimulus onset. The 100-ms period before the stimulus was used as the DC baseline. For each stimulus (TS and IES), at least 10 artifact-free responses were collected and averaged.

Local Anesthesia

To mimic the impairment of cutaneous Aδ fibers in the superficial layer, a lidocaine tape (Penles; NittouDenkou, Tokyo, Japan) was used. The tape was 30.5 mm × 50.0 mm and contained 18 mg of lidocaine. It was chosen because it could be used less invasively than injections.

Experimental Procedures

Experiment 1 (Threshold Measurements)

Effects of lidocaine on the sensory threshold of IES-evoked pain sensations and TS-evoked tactile sensations were examined by varying the period of application. If the IES method was sensitive enough to detect change of function of Aδ fibers, the sensory threshold of IES-evoked pain sensations would increase with an increase in the period of lidocaine application. IES and TS were applied to the dorsum of both hands and both feet. After the threshold was measured (control), the lidocaine tape was applied to the left hand and left foot. Changes in the sensory threshold were examined 1 hour, 3 hours, and 5 hours after the local anesthesia. Therefore, there were 4 runs. At each run, the tape was removed to stimulate and then replaced after the recordings for the next run. The electrode was placed at around the center of the tape (approximately 20 × 40 mm area). Threshold measurements of IES were performed at 5 different points, and the mean value was used for subsequent analyses.

Experiment 2 (EP Measurements)

To obtain objective evidence that the IES method is sensitive enough to detect changes of function of Aδ fibers, we recorded evoked potentials (EPs) after IES and TS before and 5 hour after the lidocaine tape was used. IES and TS were applied to the dorsum of both hands. After the control response was recorded, the lidocaine tape was applied to the left hand for 5 hours, and EPs were recorded again. The intensity of TS was 1.5 times the tactile threshold at each time point. Clear tactile sensations were elicited without any painful sensations using these parameters. The intensity for IES in the control session was 1.5 times the pain threshold. Because none of the subjects felt sensations elicited by IES after the local application of lidocaine in the intensity range (0 to

Table 1. Evoked Potential Values and Thresholds With Each Stimulus Condition (TS and IES) Before and After a 5-Hour Application of Local Anesthesia

		N2 Latency (ms)	*P2 Latency (ms)*	*N2-P2 Amplitude (μV)*	*Threshold (mA)*
Left hand (IES)	Before	204.7 ± 17.2	299.7 ± 28.7	23.9 ± 9.8	0.11 ± 0.04
	5-h application	—	—	—	*
Right hand (IES)	Before	200.4 ± 14.1	310.6 ± 37.7	22.5 ± 7.3	0.13 ± 0.03
	5-h application	202.5 ± 22.1	306.2 ± 31.1	20.8 ± 7.1	0.12 ± 0.03
Left hand (TS)	Before	150.0 ± 10.3	225.9 ± 23.9	17.3 ± 4.6	0.75 ± 0.24
	5-h application	147.9 ± 14.2	218.0 ± 25.7	15.7 ± 5.8	0.81 ± 0.22†
Right hand (TS)	Before	147.3 ± 8.2	224.7 ± 26.7	15.3 ± 3.5	0.75 ± 0.2
	5-h application	150.7 ± 7.4	225.8 ± 24.9	14.8 ± 5.9	0.81 ± 0.25†

Abbreviations: IES, intraepidermal stimulation; TS, transcutaneous electrical stimulation.
*Only 1 subject perceived a tactile sensation at 0.65 mA.
†When changes in TS threshold between the before and 5-hour runs were calculated in each subject and compared between the left and right hands, there was no significant difference ($P = .47$, paired t test).

1.0 mA), we used the stimulus intensity of the control session in the 5-hour session.

Experiment 3 (IES-Evoked Potential Measurements with Time)

We examined the time course of the effects of lidocaine on IES-induced EPs to know whether there is a gradual change that would reflect the number of fibers activated under the pharmacological effect of lidocaine or more importantly, the gradual loss of fibers in peripheral neuropathy. IES was applied to the dorsum of both hands. After the control response was recorded, the lidocaine tape was applied to the left hand (affected side). Changes in the EPs evoked with IES were examined after a 1-hour, 3-hour, and 5-hour application of the local anesthesia. We used the same stimulus intensity (1.5 times the pain threshold for the control session) in all sessions.

Analysis

In Experiment 1, effects of the local anesthesia on the tactile threshold of TS were evaluated statistically using a 1-way repeated-measures analysis of variance (ANOVA). In Experiment 2, IES and TS elicited a similar negative-positive sequence (N2-P2) at different latencies. The peak latency of N2 and P2 was determined during a latency period of 120 to 180 ms and 180 to 280 ms for TS and 170 to 250 ms and 250 to 350 ms for IES, respectively. The local anesthetic effects on the latency and amplitude of each component were assessed with a 2-way (local anesthesia × run) repeated-measures ANOVA. In Experiment 3, the local anesthetic effects on the latency and amplitude of each component were assessed with a paired t test at each time point.

Results

Experiment 1

IES produced a pin-prick sensation, and TS, a clear tactile sensation (tapping or throbbing) before the local anesthesia. The mean pain rating for the control condition (before) at the left hand and foot was 37.5 ± 4.9 and 31.8 ± 7.2, respectively.

The mean tactile threshold for TS in each run (before, 1 hour, 3 hours, and 5 hours) was 0.75, 0.7, 0.76 ,and 0.81 mA, respectively, for the hand and 0.8, 0.9, 0.92, and 0.92 mA for the foot. The tactile threshold did not differ significantly among the 4 runs at each site (ANOVA, $P = .31$ for the hand, $P = .46$ for the foot).

The 1-hour application of lidocaine did not affect the pain threshold for IES for the hand (before, 0.11 ± 0.03 mA; after, 0.11 ± 0.02 mA). Among the 30 measurements (5 measurements × 6 subjects) in the third run (3 hours), however, no sensation could be elicited within the intensity range up to 1.0 mA for 12 measurements. Using the remaining 18 measurements, the mean threshold was 0.41 ± 0.35 mA. In the fourth run (5 hours), 5 of the 6 subjects did not feel a sensation in any measurement. Results of the foot stimulation were similar. The mean pain threshold was 0.14 ± 0.02 mA for the control run, 0.14 ± 0.03 mA for the 1-hour run, and 0.20 ± 0.09 mA for the 3-hour run. In the 5-hour run, IES did not elicit any sensation up to 1.0 mA in 8 of 30 measurements. Using the remaining 22 measurements, the mean threshold was 0.56 ± 0.18 mA. Taken together, the local application of lidocaine clearly increased the pain threshold but did not affect significantly the tactile threshold as expected.

Experiment 2

The peak latency and peak amplitude of evoked potentials and the threshold for each measurement are shown in Table 1. The N2-P2 complex after TS that ranged from about 150 to 230 ms in latency was clearly identified in each subject in the control and 5-hour runs (Fig 1). Results of the 2-way ANOVA (lidocaine × run) showed no significant effect of these 2 factors on the latency and amplitude of the N2-P2 complex. The mean TS-evoked sensation threshold was 0.75 ± 0.24 in the control run and 0.81 ± 0.22 after the 5-hour application of lidocaine for the left hand and 0.75 ± 0.2 and 0.81 ± 0.25, respectively, for the right hand. The mean threshold did not differ significantly between the 2 runs for either hand (paired t test, $P > .05$), and therefore the persistence of the response to TS after the application of lidocaine was not due to a higher intensity of stimulation.

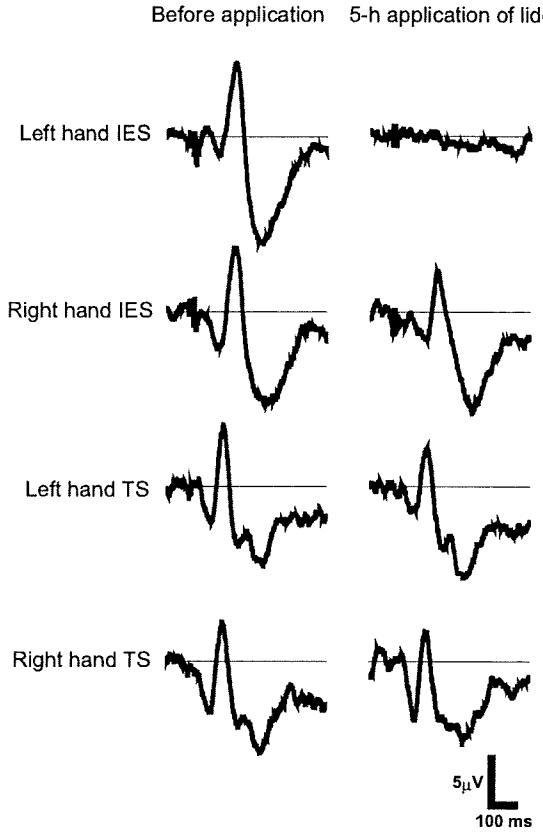

Figure 1. Grand-averaged waveforms of potentials evoked by intraepidermal stimulation (IES) and transcutaneous electrical stimulation (TS) recorded at Cz. Left traces, before lidocaine. Right traces, after a 5-hour application of lidocaine taped to the left hand.

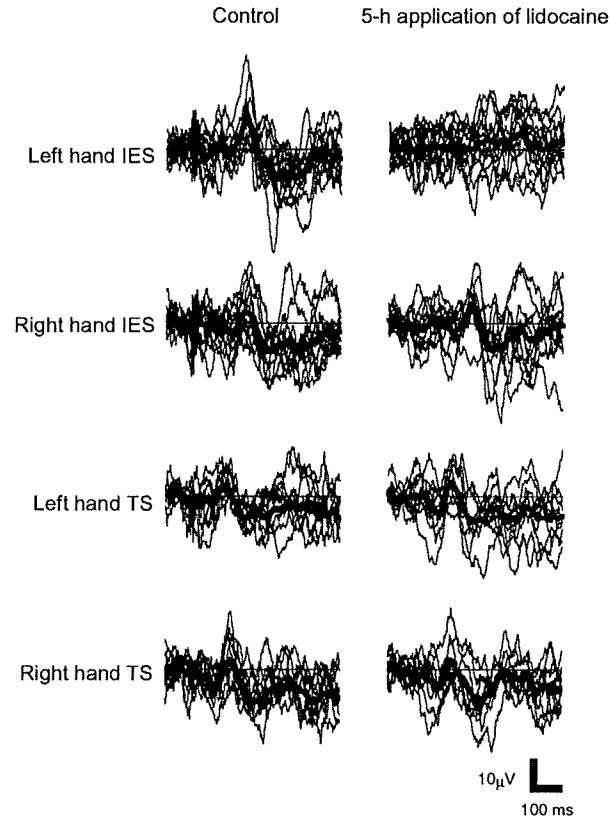

Figure 2. Superimposed waveforms of potentials evoked by intraepidermal stimulation (IES) and transcutaneous electrical stimulation (TS) recorded at Cz in a representative subject. Left traces, before lidocaine. Right traces, after a 5-hour application of lidocaine taped to the left hand.

The N2-P2 complex after IES that ranged from about 200 to 300 ms in latency was clearly identified in each subject for the control condition, whereas stimulation at the same intensity elicited neither a pinprick sensation nor evoked potentials in any subjects after the 5-hour application of lidocaine (Fig 1). The peak latency and peak amplitude of the N2-P2 complex on the control-side (right hand) did not differ between before and 5 hours after the local anesthesia. Fig 2 shows the superimposed waveforms in a representative subject.

Experiment 3

The peak latency and peak amplitude of evoked potentials are listed in Table 2. The 1-hour application of lidocaine did not affect the peak latency or peak amplitude of evoked potentials, whereas after the 3-hour application, 4 of 5 subjects did not feel any sensation at a intensity of 1.5 times the control threshold. After the 5-hour application, none of the 5 subjects felt a sensation. Similarly, EPs were not detectable in 4 of 5 subjects in the 3-hour run and in any of the 5 subjects in the 5-hour run. The grand-averaged waveforms across all the subjects for each run are shown in Fig 3. The peak latency and peak amplitude of the N2-P2 complex on the control side (right hand) did not differ among the 4 (pre, 1-hour, 3-hour, and 5-hour) runs.

Discussion

In the present study, we tested whether IES can be used to evaluate the function of Aδ fibers in the superficial layer of the skin. Results indicated that IES could be a sensitive tool to find impaired Aδ fibers in the superficial layer of the skin and a potential screening test for diabetic neuropathy. The method has several advantages with respect to sensitivity, cost, invasiveness, and convenience. Given the extensive impairment of Aδ fibers in the early stages of neuropathy, affected individuals are expected to have an increased pain threshold with intact tactile sensations. Because this method can be applied to various parts of the body, one can also test whether the degree of impairment differs between distal and proximal areas. Recordings of evoked potentials can also provide objective evidence.

Theoretically, a concentric bipolar electrode can be regarded as a radial assembly of an infinite number of tripolar electrode arrays that can reduce undesired current spread.[16] In the case of our concentric bipolar needle electrode, the passing current is expected to be restricted to the needle tip, where free nerve endings exist without loop pathways extending to deeper layers. In previous studies, we reported that (1) IES elicits a clear pricking sensation without any tactile sensations, (2) the peripheral conduction velocity of signals evoked by IES is about

Table 2. Peak Latency and Amplitude of IES-Evoked Potentials Before and After 1-Hour, 3-Hour, and 5-Hour Applications of Lidocaine

		N2 Latency (ms)	*P2 Latency (ms)*	*N2-P2 Amplitude (μV)*
Left hand (lidocaine)	Before	192.8 ± 12.9	307.8 ± 23.0	21.6 ± 8.5
	1-h application	194.4 ± 19.4	312.4 ± 35.2	19.9 ± 6.2
	3-h application	—	—	—
	5-h application	—	—	—
Right hand (control)	Before	197.2 ± 7.3	318.4 ± 32.8	21.6 ± 6.3
	1-h application	200.2 ± 17.9	312.2 ± 42.0	20.3 ± 5.8
	3-h application	195.4 ± 11.2	307.2 ± 38.0	18.3 ± 8.7
	5-h application	204.0 ± 13.2	288.6 ± 44.2	19.0 ± 9.4

Abbreviation: IES, intraepidermal stimulation.

15 m/s, and (3) IES evokes pain-related potentials or magnetic fields similar to those evoked by laser beams.[5-7] These findings indicate that IES can selectively activate Aδ fibers. The present results that the local application of lidocaine affected IES-induced pain sensations but not TS-induced tactile sensations are consistent with this notion. The difference in latency of the N2/P2 component, about 60 ms, between IES and TS can be well explained by the difference in the peripheral and spinal conduction velocities between Aδ and Aβ fibers.[7] Because each conduction velocity is similar between the peripheral nerve and spinal cord,[9,28] a gross calculation reveals that it takes 20 ms to travel 120 cm (from the hand to brain) at a conduction velocity of 60 m/s (Aβ fibers), whereas it takes 80 ms at 15 m/s (Aδ fibers). Although it is well recognized that pain-related EPs are influenced by the subject's attention or other internal states, the present results showed that EPs and the sensory threshold in the control condition (right hand) did not differ between runs (before and after the 5-hour application). Therefore, the influence of such factors was minimal in the present study.

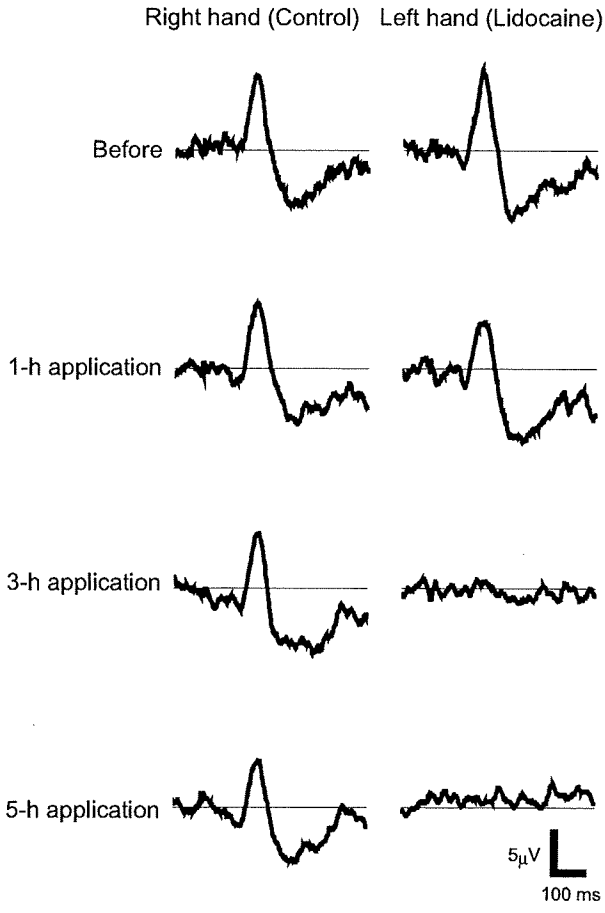

Figure 3. Gradual change of potentials evoked by intraepidermal stimulation (IES) with various application times (1-hour, 3-hour, and 5-hour application).

In a previous study using laser stimulation, small-diameter fibers were selectively blocked by a subcutaneous injection of lidocaine.[22] In the present study, we used the transdermal application of lidocaine for a local anesthesia. Lidocaine would penetrate the stratum corneum and then diffuse into the epidermis and dermis. The diffusion seemed slow, because there was no effect on the pain threshold and EPs after a 1-hour application of the lidocaine tape.

There are several possible explanations for the present results. First, the effect of lidocaine was selective regarding the Aδ fibers. That is, lidocaine was sensitive to the IES-evoked activation of Aδ fibers but not to the TS-evoked activation of Aβ fibers. Second, the effect of lidocaine was stronger for the superficial layer of the skin than deeper layers, and therefore the greater effects of lidocaine on the sensations of pain and EPs induced by IES than those induced by TS might be due to the difference in the depth of each receptor/fiber. Anatomic findings show that nociceptive fiber terminals are located in the epidermis and superficial layer of the dermis,[12,15] whereas the other fibers run more deeply in the dermis.[14] In support of the second view, warm sensations were unaffected by the application of EMLA (Eutectic Mixture of Local Anesthetics) cream despite marked effects on pain sensation, a finding interpreted to mean that C-fiber warm receptors could be located in slightly deeper layers of the skin.[3] Although the results of the present study did not favor one particular explanation over the other, the lidocaine-induced impairment of Aδ fibers with intact Aβ fiber function in either case mimicked the predominant impairment of small fibers in some kinds of neuropathies.

Another possible explanation for the different effects of lidocaine on Aβ and Aδ fibers is that the site of activation, that is, receptors or fibers, differs between TS and IES. Because lidocaine may preferentially act at the site

of receptors (impulse generation) more strongly than at the site of fibers (conduction block),[24] its effects would differ between TS and IES if they activate different sites more strongly. However, the fact that both laser beam–induced pain[3] and IES-induced pain are attenuated by lidocaine implies that lidocaine suppresses the activation of both receptors and fibers, that is, both nerve conduction and impulse generation. Laser beams activate cutaneous nociceptors, whereas IES would bypass the transduction process. Although it is not clear whether the effect of transdermal lidocaine was truly related to a selective effect on small-diameter fibers or related to the location of the receptors in the skin, the present results showed that the IES method could detect the impaired functioning of Aδ fibers in the superficial layer of the skin.

Recently, IENFD has emerged as a reliable tool for diagnosing conditions affecting small fibers,[19,21,25-27] and studies have indicated that changes in IENFD correlate with other measures, for example, warm,[25] cold,[26] and heat pain threshold.[21] If changes in IENFD partly reflect the impairment of Aδ fibers, results of IES pain threshold examinations in patients with peripheral neuropathies would also correlate with changes in IENFD, although the present method cannot identify changes in C-fibers. We recently developed a method using IES for the selective stimulation of cutaneous C-fibers.[18] A combination of the 2 (ie, IES for Aδ and C fibers) might be a good tool for diagnosing small fiber impairment.

In recent European guidelines on neuropathic pain assessment, laser stimulation was recommended as the best neurophysiological tool.[4] In some studies, lasers that can activate Aδ fibers were used to evaluate small-fiber function in diabetic neuropathy,[1,2,11] but their clinical usefulness is limited in that such devices are expensive and hard to control. In contrast, an electrical stimulator is more convenient and less invasive and therefore may be a better alternative for clinical examinations. Further clinical studies, for example, of the correlation of the IES-evoked pain threshold with the clinical manifestations of neuropathies or results of other clinical examinations, are necessary to evaluate the advantages of this method.

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