Modulation of jaw reflexes induced by experimental muscle pain in anesthetized rats

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Abstract

Previous studies using the experimental muscle pain model have shown that jaw reflexes and activity patterns of the jaw muscles during movements are altered in the presence of jaw-muscle pain. However, it is still unclear which jaw reflex is more subject to jaw-muscle pain. To clarify this, effects of the application of mustard oil (MO), an inflammatory irritant, into the temporal (jaw-closing) muscle on 1) the jaw-opening reflex evoked by tooth pulp stimulation (TP-evoked JOR) as a nociceptive reflex, 2) jaw-opening reflex evoked by inferior alveolar nerve stimulation as a non-nociceptive reflex, and 3) jaw-closing reflex evoked by trigeminal mesencephalic nucleus stimulation as a proprioceptive reflex were investigated in anesthetized rats. The MO application induced suppression of all reflexes, and the effect on the TP-evoked JOR was more prominent than on the other reflexes. To elucidate the neural mechanisms for these effects, a systemic administration of naloxone following the MO application was conducted. Naloxone not only reversed the MO-induced suppressive effect on the TP-evoked JOR but also facilitated the reflex. The results suggest that nociceptive inputs from orofacial deep tissues alter the gain for jaw reflexes particularly for the nociceptive jaw reflex, and such alteration includes both facilitatory and inhibitory aspects. The results also suggest that pain modulatory mechanisms such as the descending modulatory system play a crucial role in the suppression of nociceptive transmissions related to nociceptive jaw reflex, and under such pathological states, defense reflexes may not be properly evoked.

Keywords: jaw-opening reflex, jaw-closing reflex, inflammatory pain, jaw muscle, rat

1. Introduction

Pain arising from orofacial deep tissues, such as the masticatory muscles and the temporomandibular joints (TMJ), are major symptoms of temporomandibular disorders (TMDs). Considerable evidence has been obtained by observing TMD patients affected by orofacial deep pain that impacts on orofacial motor functions, especially that related to jaw movements (e.g., voluntary jaw movement, unassisted jaw opening, electromyographic activities in the jaw muscles during chewing, biting force)(see Dubner et al., 1978; Lund and Sessle, 1994; Stohler, 1999, for reviews).

Recent studies using experimental deep-pain animal models have provided insights into the neural mechanisms underlying orofacial deep pain. For example, application of algesic chemicals such as mustard oil (MO) into the masseter muscle induced central and/or peripheral sensitization that resulted in the expansion of the cutaneous receptive field or hypersensitivity in the nociceptive neurons to the peripheral stimuli in anesthetized animals (Hu et al., 1992). In addition, noxious stimuli to deep tissues affect orofacial motor systems. For example, a sustained increase in EMG activity in jaw muscles was evoked by MO application into the TMJ region, but the activity was suppressed by the central opioid depressive mechanisms (Cairns et al., 1998; Yu et al., 1994; Yu et al., 1995). Such pain modulatory systems also affect the nociceptive jaw-opening reflex evoked by the stimulation of tooth pulp (Oliveras et al., 1974; Sessle and Hu, 1981; Tanaka and Toda, 1982; Toda et al., 1981; also see Sessle, 1987, for review). These studies have provided fundamental insights into the alteration of nociceptive transmission to both sensory and motor systems in the presence of deep pain. However, when considering the effect of deep pain on orofacial motor function such as jaw movements, the effects of deep pain on non-nociceptive jaw reflexes playing important roles in the control of jaw movements

(Dubner et al., 1978) should also be considered. Indeed, recent studies using jaw-closing muscle-pain models in humans have documented that experimental jaw-muscle pain facilitates the jaw-jerk reflex (Wang et al., 2002; Wang et al., 2001; Wang et al., 2000) but suppresses the masseteric inhibitory responses evoked by noxious electrical stimuli (Svensson et al., 1999; Wang et al., 1999) which may correspond to the nociceptive jaw-opening reflex. Yet the effect of jaw-muscle pain on the non-nociceptive jaw-opening reflex is unknown. It is also of interest which jaw reflex is more subject to jaw-muscle pain. Therefore, the present study was conducted to investigate the effects of the application of MO into the temporal (jaw-closing) muscle on 1) the jaw-opening reflex evoked by tooth pulp stimulation as a nociceptive reflex, 2) the jaw-opening reflex evoked by inferior alveolar nerve stimulation as a non-nociceptive reflex and 3) the jaw-closing reflex evoked by trigeminal mesencephalic nucleus stimulation as a proprioceptive reflex in anesthetized rats. Possible neuronal mechanisms and functional significance of the MO induced effect on each reflex are also discussed.

2. Materials and Methods

2.1. Surgical procedures

The experiments were carried out in a total of 60 male rats (Wistar albino, 250-270 g) in accordance with the "Principles of Laboratory Animal Care" (NIH publication #86-23, revised 1996). The animal protocols were approved by the Intramural Animal Care and Veterinary Science Committee of the Niigata University. The animals were initially anesthetized with 2-3% halothane. Two percent lidocaine was injected into the skin to minimize surgical pain before the incisions were made. Cannulae were inserted into the trachea and the femoral vein for respiration and drug administration, respectively, and then

the anesthesia was maintained with the mixture of α-chloralose (50 mg/kg) and urethane (500 mg/kg) injected via the femoral vein. Depth of anesthesia was checked repeatedly throughout the experiment by pinching the paws; if a withdrawal reflex was elicited, a supplementary dose of chloralose-urethane mixture was administered. Rectal temperature was measured and maintained between 38°C and 39°C with a heating pad.

A midline incision was made along the ventral aspect of the mandible. Paired copper wire electrodes (0.12 mm in diameter, 3 mm interpolar distance) with an exposed tip (1 mm) were implanted bilaterally into the masseter (Mas) as a jaw-closing muscle and digastric (Dig) as a jaw-opening muscle to record electromyographic (EMG) activity. EMG electrode locations in each muscle were confirmed by post-mortem dissection.

In the present study, electrical stimulation of the tooth pulp (TP) of the rat's lower incisor was used as the noxious stimulation. For the purpose of limited stimulation of the pulpal fibers within the tooth crown, the electrodes were placed in the junction of the dentin and pulpal tissues of the apical part of the crown of the incisor, and the tips were never inserted into the pulp chamber. Two holes were made on the labial surface of the crown of the right lower incisor using a low-speed dental drill with a round tungsten carbide bur (#1/2) and water cooling: one hole was made 2 mm from the gingival margin (for the cathode), and the other hole was 4mm from the gingival margin (for the anode). The holes were rinsed with saline, and custom-made bipolar stimulating electrodes with two gold-coated metal pins (0.4 mm in diameter, 2 mm interpolar distance) were implanted in the holes to evoke the jaw-opening reflex (TP-evoked JOR). The positions of the electrode tips were adjusted to be at the border of the dentin and the pulp chamber, and the electrodes were fixed to the tooth with adhesive dental acrylic (SUPERBOND C&B, SUN MEDICAL, Shiga, Japan). To stimulate the inferior alveolar nerve (IAN) to evoke the jaw-opening

reflex (IAN-evoked JOR), a pair of Teflon-coated stainless-steel wire electrodes (0.1 mm in diameter, tip exposure 0.5 mm) was inserted into the right mental foramen 1 mm deep for the anode and 3 mm deep for the cathode, and fixed on the adjacent bone with dental acrylic adhesive.

The rat's head was then placed in a stereotaxic frame, the skin over the dorsal surface of the skull was reflected, and four screws were inserted into the frontal and parietal bone. These screws were attached to a vertical support bar with dental acrylic (UNIFAST II, GC, Tokyo, Japan), and the ear bars were removed. This facilitated access to the orofacial region without any interference. A part of the occipital bone overlying the cerebellum was removed to enable the microelectrode penetrations (glass coated tungsten microelectrode, 0.2-0.6 MΩ at 1 kHz) for the trigeminal mesencephalic nucleus (MesV) stimulation to evoke the jaw-closing reflex (MesV-evoked JCR). The microelectrode was introduced stereotaxically through the cerebellum into the MesV. Neuronal responses evoked by passive jaw opening or probing the belly of the masseter muscle were used to confirm that the electrode tip was located within the MesV.

2.2. Stimulations and recordings

To evoke jaw reflexes, the TP, IAN and MesV were alternately stimulated at an interval of 5 s (test stimuli), i.e., each kind of test stimulus was applied at an interval of 15 s. The parameters for the test stimuli were as follows: a single pulse (0.2 ms duration) for TP stimulation, a single pulse (0.2 ms duration) for IAN stimulation and 3 trains of cathodal pulses (0.1 ms duration at 500 Hz) for MesV stimulation. The threshold was determined as the minimum stimulus current that consistently evoked EMG-detectable reflex response. During data recording, the stimulus current was set at 1.2 T for each reflex.

The EMG activities were amplified with custom-built AC amplifiers (band pass:

0.1-3 kHz), and the signals were fed into a computer equipped with a CED Power 1401 board and analysis software (Spike 2; Cambridge Electronic Design Ltd., Cambridge, UK). The sampling rate for the EMGs was 5000/s. Recorded EMG activity was stored electronically and analyzed offline. The stimulus pulses for the test stimuli were also fed into a computer with a CED Power 1401 board as event signals.

In the present experiment, the 60 animals were divided into the following four groups. The first group (Test stimuli only group, n=9) included the animals to which the repetitive test stimuli only was conducted for 120 min to test the effect of such repetitive stimuli on the reflex properties. The second group (MO group, n=25) included the animals in which a small volume of mustard oil (MO, 20% in mineral oil, 20 μl; Wako Pure Chemical Industries Ltd, Osaka, Japan) was injected into the temporal muscle (jaw-closing muscle) in additional to the repetitive test stimuli to test the effect of MO on the reflex properties evoked by the test stimuli. In the MO group, a 30-gauge needle connected by polyethylene tubing to a Hamilton syringe (50 μl) penetrated the midregion of the temporal muscle after the surgery, and the animal was observed for at least 30 min. The repetitive test stimuli were then started. Baseline EMG activity and each reflex were recorded for 10 min (control period), and then MO was injected into the temporal muscle over 5-10 s. After the MO application, the recordings were continuously made for 120 min. The third group (Naloxone only group, n=9) included the animals to which a systemic administration of opiate antagonist naloxone hydrochloride (i.v., 1.3 mg/kg in 0.5 ml isotonic saline, Sigma chemical Co, St Louis, USA) was conducted in additional to the repetitive test stimuli to test the effect of naloxone on the reflex properties evoked by the test stimuli. Baseline EMG activity and each reflex were recorded for 10 min (control period), and then naloxone was systemically administered over 50-60 s. After the naloxone administration, the

recordings were made continuously for 120 min. The fourth group (MO and naloxone group, n=17) included the animals to which a systemic administration of naloxone (same dose as used in the third group) was conducted 30 min after the MO application to test the effect of naloxone on the MO-induced effect on the reflexes evoked by the test stimuli. The procedure for MO application was identical to that in the second group. In the present experiment, all the test stimuli were not necessarily conducted successfully throughout the recording. If the reflex threshold was above 3 mA for TP stimulation, 300μA for IAN stimulation and 300μA for MesV stimulation when determining the threshold for each reflex, the reflex data were not analyzed since we noticed from the preliminary experiments that the electrode had a problem in such cases. In addition, MesV-evoked JCR data were excluded from the analysis if the position of the electrode was not histologically identified within the MesV (see below). Table 1 details how the data were obtained from the animals.

2.3. Histology

At the completion of the experiments, direct anodal current (20μA for 20 s) was passed through the MesV-stimulating microelectrode. The animals were deeply anesthetized with sodium pentobarbital and perfused through the heart with phosphate-buffered saline containing heparin followed by 10% buffered formalin. The block of brain was stored in 30% sucrose in 10% buffered formalin for 7-10 days and serial frozen sections (100μm thick) were prepared and stained to confirm the position of the tip of MesV-stimulating microelectrode.

2.3. Data analysis

EMG signals were full-wave rectified and smoothed (time constant 20 ms) with Spike2 analysis software. The parameters analyzed to elucidate the features of each reflex included the mean latency, amplitude (area, A/D units) and duration. To define the onset

and offset of each reflex, the baseline EMG activity in each muscle was calculated for 2 min during the control period, and the onset was defined as the point in time when the EMG activity exceeded 2SD from baseline EMG activity. Likewise, the offset was defined as the point in time when the EMG activity fell below 2SD from the baseline EMG activity. The latency of the MesV-evoked JCR was regarded as the time from the first stimulus pulse to the onset of the reflex response. To elucidate the sequential effect of MO and/or naloxone on the reflexes, mean values of 20 reflexes during the control period were calculated for each parameter and ware considered control values. Then the mean values of four consecutive reflexes after the each time point of 0, 1, 2, 3, 4, 5, 6, 9, 10, 11, 14, 15, 16, 19, 20, 21, 24, 25, 26, 29, 30, 35, 40, 45, 50, 55, 60, 75, 90, 120 min after the start of the MO application were calculated. The mean values for each time point were normalized to the control value and compared with the control.

In addition to the reflex analyses, the effect of MO and/or naloxone on the baseline EMG activity was also analyzed. Increases in EMG activity after the application of MO or naloxone were regarded as significant if one or more EMG activity (area) exceeded 2SD from the baseline EMG activity. When a significant increase in EMG activity was noted, its latency and duration were calculated. The time from the beginning of the application of the chemical to the increase in EMG activity was regarded as the latency of the response, and that from the increase in EMG activity to its recovery to the baseline EMG activity was regarded as the duration of the response.

Effects of the application of MO or naloxone on each reflexes were statistically evaluated with a repeated measures one-way ANOVA and post-hoc comparisons (Tukey test) for parametric data or a repeated measures one-way ANOVA on Ranks and post-hoc comparisons (Dunn's method) for non-parametric data. A Mann-Whitney Rank Sum Test

was used to compare the reflex parameters between the MO group and the MO and naloxone group for each time point. For comparison of the parameters between the muscles, a one-way ANOVA and post-hoc comparisons (Tukey test) was used for parametric data and a one-way ANOVA on Ranks and post-hoc comparisons (Dunn's method) was used for non-parametric data. A paired t test was used for comparison of the jaw-opening reflex parameters between the right and left Digs. The values were expressed as mean +/- SD, and P values less than 0.05 were regarded as significant.

3. Results

3.1. Properties of the reflexes before the MO application

3.1.1 TP-evoked JOR

The threshold of the TP-evoked JOR was 1.6 +/- 0.8 mA (n=33). Different from other reflexes tested, the intensity of the stimulus current prominently affected the latency of the reflex. Figure 1 is an example showing the relationship between the stimulus intensity and the reflex response. At just above the threshold current (1.7 mA), response to the stimulation was evoked only in the Dig ipsilateral, and the latency was 22.0 ms (Figure 1A). When the stimulus current was increased to 1.2T, the response was consistently evoked in the bilateral Digs, and the reflex amplitude was increased by 437% in the ipsilateral Dig (Figure 1B). No remarkable change was noted in the latency at this current strength. However, when the current was increased to 1.4T, the response with short latency (i.e., short latency response: 6.4ms latency in the ipsilateral Dig and 6.7 ms latency in the contralateral Dig) appeared besides the response already noted at the lower current (i.e., long latency response: 21.4 ms latency in the ipsilateral Dig and 21.3 ms latency in the contralateral Dig). It should be noted that the amplitude of long latency responses became

smaller at this current strength than that evoked at 1.2T: it was decreased by 57% in the ipsilateral Dig and by 37% in the contralateral Dig (Figure 1C). When the stimulus current was further increased to 1.6T, the amplitude of short latency response was increased (526% of the response evoked at 1.4T in the ipsilateral Dig and 348% in the contralateral Dig), but the long latency response disappeared (Figure 1D). The finding that the reflex response initially evoked by the activation of the pulpal fibers was the long-latency responses, and its latency was much longer (> 5 times) than the JOR evoked by the low-threshold IAN stimuli (described later) suggests that the afferent fibers mediating the long-latency response are small-diameter fibers having a prominent role for nociceptive transmission. As noted in the methods section, the current strength was set at 1.2T during the recording period in the present experiment; and only the long latency responses were evoked in bilateral Digs in all the animals tested for TP stimulation (n=33).

At 1.2T, the reflex latency was 20.5 +/-1.7 ms (n=33) for the Dig ipsilateral to the stimulation and 21.2 +/- 1.5 ms (n=33) for the Dig contralateral to the stimulation, and the amplitude of the reflex was 9.3 +/- 6.9 A/D unit (n=33) for the ipsilateral side and 6.9 +/- 6.7 A/D unit (n=33) for the contralateral side. The latency was significantly shorter, and the amplitude was significantly larger for the ipsilateral side than for the contralateral side ($p<0.05$, paired t-test). The duration of the reflex was 8.1 +/- 1.9 ms (n=33) for the ipsilateral side and 7.6 +/- 2.7 ms (n=33) for the contralateral side. No significant difference was noted for the duration between the sides.

3.1.2 IAN-evoked JOR

The threshold of the IAN-evoked JOR was 68.7 +/- 29.3 μA (n=33). The reflex latency was 5.3 +/- 0.3 ms (n=33) for the Dig ipsilateral to the stimulation and 5.2 +/- 0.3 ms (n=33) for the Dig contralateral to the stimulation. The amplitude of the reflex was 19.3

+/- 9.8 A/D unit (n=33) for the ipsilateral side and 15.5 +/- 13.2 A/D unit (n=33) for the contralateral side when the stimulus current was 1.2T. The latency was significantly shorter, and the amplitude was significantly larger for the ipsilateral side than for the contralateral side ($p<0.05$, paired t-test). The duration of the reflex was 5.2 +/- 1.0 ms (n=33) for the ipsilateral side and 55.4 +/- 1.2 ms (n=33) for the contralateral side. No significant difference was noted for the duration between the sides.

3.1.3 MesV-evoked JCR

The threshold of the MesV-evoked JCR was 174.35 +/- 64.54 μA (n=31). The reflex response was evoked only in the Mas ipsilateral to the stimulation when the stimulus current was 1.2T. The latency, amplitude and duration of the reflex at 1.2T were 2.9 +/- 0.3 ms, 9.7 +/- 4.9 A/D unit and 4.0 +/- 1.0 ms, respectively (n=31). It was notable that the reflex was quite often facilitated when the MesV stimuli were applied during a spontaneous increase in the Mas EMG activity, even if it was small.

3.2. Effects of MO application

The baseline EMG activity in each muscle as well as the latency, amplitude and duration of each reflex did not change significantly throughout the recording period (120 min) in the Test stimuli only group (n=9).

3.2.1 Effect on baseline EMG activity

The MO application reflexly evoked a sustained increase in the EMG activity both in the Dig (jaw-opening) and Mas (jaw-closing) muscles (Figure 2A). Such EMG activity was evoked in 88.1% (37 out of 42 animals tested) of the Dig in the MO injection side, 80.1% (34 out of 42) of the Dig in the MO non-injection side, 57.1% (24 out of 42) of the Mas in the MO injection side and 16.7% (7 out of 42) of the Mas in the MO non-injection

side. The latency for the EMG activity was 9 +/- 6 s (n=37) for the Dig in the MO injection side, 11 +/- 9 s (n=34) for the Dig in the MO non-injection side, 12 +/- 11 s (n=24) for the Mas in the MO injection side and 12 +/- 7 s (n=7) for the Mas in the MO non-injection side. No significant difference was noted for the latency between the sides in either the Dig or the Mas. The duration of the sustained EMG activity was 128 +/- 84 s (n=37) for the Dig in the MO injection side, 104 +/- 76 s (n=34) for the Dig in the MO non-injection side, 65 +/- 50 s (n=24) for the Mas in the MO injection side and 24 +/- 19 s (n=7) for the Mas in the MO non-injection side. The duration was significantly longer for the Dig than for the Mas in both sides ($p<0.05$, Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's Method). No significant difference was noted between the sides in either the Dig or the Mas. The area under the EMG response curve was 988.9 +/- 849.6 A/D unit (n=37) for the Dig in the MO injection side, 535.5 +/- 504.4 A/D unit (n=34) for the Dig in the MO non-injection side, 347.3 +/- 317.6 A/D unit (n=24) for the Mas in the MO injection side and 128.4 +/- 106.8 A/D unit (n=7) for the Mas in the MO non-injection side. The amplitude was significantly larger in the Dig than in the Mas in the injection side ($p<0.05$, Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's Method). No significant difference was noted between the sides in either the Dig or the Mas.

3.2.2 Effect on TP-evoked JOR

The latency of the TP-evoked JOR was not significantly affected by the MO application. However, significant alteration occurred for other parameters (i.e., amplitude and duration) after the MO application. Figure 3A shows the time course of the amplitude of the TP-evoked JOR before and after the MO application. The reflex was significantly decreased immediately after the MO application in bilateral Digs (60.4 +/- 33.3% of control

value for the Dig in the MO injection side, 54.3 +/- 35.0% for the Dig in the MO non-injection side, n=13, see arrow a in Figure 3A, also see Figure 2C for an example). This immediate suppression was observed even during the sustained increase in the Dig EMG activity (11 out of 13 in bilateral Digs, Figures 2A, C). The reflex was further suppressed with time, and the peak suppression was noted 19 min after the MO application in bilateral Digs (12.6 +/- 14.8% of control value for the Dig in the MO injection side, 16.3 +/- 15.3% for the Dig in the MO non-injection side, n=13, see arrow c in Figure 3A, also see Figure 2D for an example). The suppression then gradually declined with time, with activity returning to 41.2 +/- 28.0% (Dig in the MO injection side, n=13) or 44.79 +/- 27.90 % (Dig in the MO non-injection side, n=13) of the control level by 60 min after the MO application (see arrow d in Figure 3A, also see Figure 2E for an example). Although a further decline of the suppressive effect was noted in few animals, the reflex amplitude did not return to the control level at the end of the recording period in the majority of animals tested (11 out of 13 for the Dig in the MO injection side, 10 out of 13 for the Dig in the MO non-injection side). The mean reflex amplitude at the end of the recording period was 58.5 +/- 38.2% of the control (Dig in the MO injection side, n=13) or 59.0 +/- 38.5 % of the control (Dig in the MO non-injection side, n=13). A significant decrease in the reflex amplitude was noted from immediately after the MO application to 90 min after the MO application (Dig in the MO injection side) or from 3 min after the MO application to 60 min after the MO application (Dig in the MO non-injection side)($p<0.05$, one-way repeated measures ANOVA followed by Tukey post-hoc test)(Figure 3A). The time course of the MO induced suppressive effect on the TP-evoked JOR was generally the same between the sides, and no significant difference in the suppressive effect was noted between them for each time point throughout the recording period. The duration of the TP-evoked JOR was

also decreased. A significant decrease in the reflex duration was noted from 11 min after the MO application to 35 min after the MO application (Dig in the MO injection side) or from 14 min after the MO application to 29 min after the MO application (Dig in the MO non-injection side)($p<0.05$, one-way repeated measures ANOVA followed by Tukey test). No significant difference in the suppressive effect on the reflex duration was noted between the sides for each time point throughout the recording session.

3.2.3 Effect on IAN-evoked JOR

The latency and duration of the IAN-evoked JOR were not significantly affected by the MO application. However, significant alteration in the amplitude occurred after the MO application. Figure 3B shows the time course of the amplitude of the IAN-evoked JOR before and after the MO application. Different from the TP-evoked JOR, the strong and immediately appearing suppression did not occur in the IAN-evoked JOR after the MO application. No significant change was observed in the reflex amplitude immediately after the MO application in bilateral Digs (90.5 +/- 20.2% of control value for the Dig in the MO injection side, 99.3 +/- 18.1% for the Dig in the MO non-injection side, n=13, see arrow a in Figure 3B, also see Figure 2C for an example). It should be noted that no facilitation was observed even during the sustained increase in the Dig EMG activity evoked by the MO application (11 out of 13 in Dig in the MO injection side and 10 out of 13 in Dig in the MO non-injection side, see also Figures 2A, C). The reflex was then gradually suppressed until the suppressive effect reached its peak 19 min after the MO injection in the bilateral Digs (60.9 +/- 12.4% of control for the Dig in the MO injection side, 58.5 +/- 17.8% for the Dig in the MO non-injection side, n=13, see arrow b in Figure 3B, also see Figure 2D for an example). After the suppression reached its peak, it gradually declined with time, with activity returning to 86.6 +/- 23.3% (Dig in the MO injection side, n=13) or 84.0 +/- 16.1%

(Dig in the MO non-injection side, n=13) of the control level by 60 min after the MO application (see arrow c in Figure 3B, also see Figure 2E for an example). The mean reflex amplitude returned to the control level 75 min after the MO application in bilateral Digs, and no significant alteration occurred after that (Figure 3B). A significant decrease in the reflex amplitude was noted from 15 min after the MO application to 24 min after the MO application and 24 min after the MO application (Dig in the MO injection side) or 15min after the MO application and from 19min after the MO application to 29 min after the MO application (Dig in the MO non-injection side) ($p<0.05$, Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's Method). The time course of the MO induced suppressive effect on the IAN-evoked JOR was generally the same between the sides, although the effect was significantly stronger for the Dig in the MO injection side at few time points (i.e., 4 and 5 min after the MO application).

3.2.4 Comparison of the MO effect between TP-evoked JOR and IAN-evoked JOR

The MO induced suppressive effect was significantly larger for the TP-evoked JOR than that for the IAN-evoked JOR for each time point except for 75 min after the MO application in bilateral Digs ($p<0.05$, Mann-Whitney Rank Sum Test). It was notable that a part of the stimulus-response curve of the TP-evoked JOR after the MO application was similar to that of the IAN-evoked JOR. Namely, after the rapid and strong suppression had occurred, the TP-evoked JOR response was further suppressed by time, and the suppressive effect reached its peak at around 20 min. The suppression then gradually declined with time although the reflex activity did not recover to the control level, probably due to the strong, long lasting and naloxone sensitive suppression discussed above.

3.2.5 Effect on MesV-evoked JCR

The latency and duration of the MesV-evoked JCR were not significantly affected

by the MO application. However, considerable alterations in the amplitude occurred after the MO application. Figure 3C shows the time course of the amplitude of the MesV-evoked JCR before and after the MO application. Different from the JOR evoked by TP or IAN stimulation, the effect immediately after the MO application was somewhat variable between the animals. The sustained EMG increase in the Mas in the MO injection side (i.e., the muscle in which the reflex was evoked) was noted in 7 out of 13 animals tested. Different from the control period, no prominent alteration in the reflex amplitude was noted during the sustained EMG increase in all 7 animals in which the sustained EMG increase was evoked (see Figure 2C for an example). However, after cessation of the sustained EMG increase, the reflex was facilitated in 3 of these 7 animals. In contrast, the reflex was strongly suppressed immediately after the MO application in 2 of 6 animals in which the sustained EMG increase was not evoked. No prominent alteration was noted in the remaining 4 animals in which the sustained EMG increase was not evoked. As a result, no significant change was noted in the reflex amplitude immediately after the MO application due to the large variation caused by the response variability between the animals when the data from the animals were put together (111.8 +/- 48.8 % of control, n=13, see arrow a in Figure 3C). However, with time, the reflex was gradually suppressed in the animals in which facilitation or no remarkable change was noted. The amplitude of the reflex 19 min after the MO application was 77.7 +/- 22.0% of the control level (see Figure 2E for an example), and the peak suppression was noted 29 min after the MO injection (57.76 +/- 27.86% of the control level). The suppression then gradually declined with time, with activity returning to 74.3 +/- 22.6% of the control level by 60 min after the MO application (see arrow c in Figure 3C, also see Figure 2E for an example). The mean reflex amplitude returned to the control level 90 min after the MO application, and no significant alteration

occurred after that (Figure 3C). A significant decrease in the reflex amplitude was noted 29 min after the MO application ($p<0.05$, Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's Method).

3.3. Effect of naloxone administration

No significant alteration was noted in the baseline EMG activity in each muscle or in all of the parameters tested (i.e., latency, amplitude and duration) for each reflex in the Naloxone only group (n=9) throughout the recording period.

3.3.1 Effect on baseline EMG activity

There was no significant difference between the baseline EMG activity of each jaw muscle at the control period and that at 30 min after the MO application in all animals receiving MO into the temporal muscle (n=42). The systemic administration of naloxone 30 min after the MO application did not induce any significant effect in the baseline activity in the MO and naloxone group (n=17).

3.3.2 Effect on MO induced modulatory effect on TP-evoked JOR

Consistent with the animal group tested for the MO application only (MO group, already described, n=13), the MO application induced immediately appearing and long-lasting suppression of the TP-evoked JOR in bilateral Digs in the MO and naloxone group (Figure 4). Actually, for each point in time before the naloxone administration, no significant difference in the strength of the suppressive effect on the reflex amplitude was noted in bilateral Digs between these animal groups. At the point in time when the naloxone was administered (i.e., 30 min after the MO application), the reflex amplitude had been decreased to 24.2 +/- 14.3% of control for the Dig in the MO injection side or to 39.6 +/- 18.9 of control for the Dig in the MO non-injection side in the MO and naloxone group

(n=10). This suppressive effect was antagonized by the naloxone. The decrease in reflex amplitude was reversed to 104.5 +/- 63.5% of the control level (Dig in the MO injection side) or 86.1 +/- 44.7% of the control level (Dig in the MO non-injection side) 15 min after the systemic administration of naloxone (arrows a in Figures 4A and B). In addition, naloxone not only reversed the MO-induced suppressive effect on the TP-evoked JOR but also facilitated the reflex. The reflex amplitude was increased to 140.4 +/- 71.5% in the Dig in the MO injection side or to 109.8 +/- 23.1% in the Dig in the MO non-injection side 25 min after the naloxone administration (n=10, arrows b in Figures 4A and B), and this facilitation lasted for about 20 min. Peak facilitation was noted 30 min after the naloxone administration (141.0 +/- 35.6% for the Dig in the MO injection side or 113.9 +/- 27.4% for the Dig in the MO non-injection side, n=10). After the facilitatory effect reached its peak, it gradually declined with time. Although this facilitation was not statistically significant when compared with the control, significant differences were noted in the amplitude of the TP-evoked JOR between the MO group and the MO and naloxone group from 10 min to 90 min (Dig in the MO injection side) or from 10 min to 30 min and 60 min (Dig in the MO non-injection side) after the naloxone administration ($p<0.05$, Mann-Whitney Rank Sum Test).

Consistent with the MO group, the duration of the TP-evoked JOR also decreased after the MO application in the MO and naloxone group. Significant decrease in the reflex duration was noted from 11 to 26 min (Dig in the MO injection side) or 15 to 29 min (Dig in the MO non-injection side) after the MO application ($p<0.05$, Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's Method). This suppressive effect was also antagonized and facilitated by the naloxone. The facilitation was not statistically significant when compared with the control, a significant difference was noted in the duration of the

TP-evoked JOR between the MO group (n=13) and the MO and naloxone group (n=10) 10 min after the naloxone administration in the Dig in the MO injection side ($p<0.05$, Mann-Whitney Rank Sum Test).

3.3.3 Effect on MO induced modulatory effect on IAN-evoked JOR

Consistent with the MO group (n=13), the MO application induced suppression of the IAN-evoked JOR in bilateral Digs, in the MO and naloxone group (n=10)(Figure 5A). No significant difference in the suppressive effect on the reflex amplitude was noted in bilateral Digs between the MO group and MO and naloxone group for each time point before the naloxone administration. At the time point when the naloxone was administered, the reflex amplitude had been decreased to 79.4 +/- 31.1% of the control for the Dig in the MO injection side (Figure 5A) or to 86.6 +/- 22.7 of the control for the Dig in the MO non-injection side (n=10). The systemic administration of naloxone did not affect this suppressive effect. No significant difference was noted in the amplitude of the IAN-evoked JOR between the MO group and the MO and naloxone group after the naloxone administration. The MO application and the naloxone administration did not affect the latency and the duration of the reflex.

3.3.4 Effect on the MO induced modulatory effect on MesV-evoked JCR

Consistent with the MO group (n=13), the MO application induced suppression of the MesV-evoked JCR in the MO and naloxone group (n=8)(Figure 5B). No significant difference in the suppressive effect on the reflex amplitude was noted between the MO group and MO and naloxone group for each time point before the naloxone administration. At the time point when the naloxone was administered, the reflex amplitude had been decreased to 57.9 +/- 30.6% of control (n=8, Figure 5B). The systemic administration of naloxone did not affect this suppressive effect. No significant difference was noted in the

amplitude of the MO evoked JCR between the MO group and the MO and naloxone group throughout the recording period after the naloxone administration. Both the MO application and the naloxone administration did not affect the latency or the duration in the MO and naloxone group.

4. Discussion

The present study demonstrated that experimental jaw-muscle pain induced by the application of MO into the temporal muscle modulated jaw reflexes, and the modulatory effect was generally suppression. However, considerable differences were noted in the features of modulatory effect between the three reflexes tested.

MO application into the temporal muscle reflexly evoked a sustained increase in the baseline EMG activity both in the Dig (jaw-opening muscle) and Mas (jaw-closing muscle). Such sustained EMG increase in the orofacial muscles has also been reported (Ro et al., 2002; Yu et al., 1995). However, the increase in EMG activities was generally smaller and lasted for a shorter time than the previous observations, in that MO was injected into TMJ regions in rats anesthetized with halothane (Yu et al., 1995) and systemic administration of naloxone did not induce the so-called rekindling, i.e., a recurrence of the increase in EMG activity, which was observed in the previous study (Yu et al., 1994). The appearance of the rekindling after the naloxone administration indicates that long-lasting excitatory mechanisms in nociceptive transmission were driven by the noxious conditioning stimuli to the TMJ regions, and the effect was masked by the endogenous opioid mechanisms (Yu et al., 1994). This in turn suggests that such long-lasting excitatory mechanisms were not driven in the present experimental condition. This may explain the present results demonstrating shorter duration of the sustained increase in the EMG activity.

The JOR induced by noxious stimuli (i.e., TP-evoked JOR) was suppressed by the MO application, and the suppressive effect was more prominent than that on other reflexes; the rapid and strong suppressive effect occurred only on the TP-evoked JOR, and the suppressive effect lasted much longer than other reflexes. When considering the neuronal mechanisms of the MO induced suppressive effect on the TP-evoked JOR, several characteristic features of the effect should be noted. One was that the TP-evoked JOR was suppressed even during the time that sustained increase in the baseline EMG in Dig was observed after the MO application, i.e., during the Dig motoneuronal excitability increased. Another feature was that the reflex was also suppressed in the bilateral Dig. These facts indicate that suppression was the result of the decrease in the excitatory sensory inputs to the Dig motoneurons due to inhibition of the sensory transmission from the tooth pulp. The other characteristic feature of the suppressive effect was that the effect was reversed by the naloxone administration, indicating that the effect was opioid dependent. For this, the activation of the descending pain modulatory system, which apparently activates the endogenous opioid system (e.g., Basbaum and Fields, 1984; Fields and Basbaum, 1999), may be one of the possible neural mechanisms explaining the inhibition of sensory transmission from the tooth pulp. This possibility is supported by studies showing that electrical stimulation of periaqueductal gray matter (PAG) or the nucleus raphe magnus (NRM), major parts of the descending modulatory system, suppressed TP-evoked JOR (Iriki and Toda, 1982; Oliveras et al., 1974; Tanaka and Toda, 1982) or sensory transmissions to the nociceptive neurons located in the trigeminal spinal nuclei, (see Sessle, 1987, for review) and such suppressive effects were reversed by naloxone (Sessle and Hu, 1981; Sessle et al., 1981; Tanaka and Toda, 1982). Similar observations were also reported for the spinal nociceptive reflexes or sensory transmissions to the spinal nociceptive

neurons (see Basbaum and Fields, 1984; Fields and Basbaum, 1999, for review).

Another possible mechanism explaining the inhibition of sensory transmission from the tooth pulp could involve the nociceptive afferent input initiated by the MO application accessing inhibitory neurons within or adjacent to the trigeminal brainstem complex relatively directly, thereby inhibiting nociceptive transmission from the tooth pulp through a process known as segmental inhibition. Such a modulatory system suppressing nociceptive reflex was demonstrated in the spinalized animals in that major parts of descending modulatory system were dissociated from the reflex arc. Other studies have also demonstrated that the effect is reversed by the naloxone (Catley et al., 1984; Chung et al., 1983; Taylor et al., 1990).

It should be noted that the administration of naloxone not only reversed the suppressive effect on the TP-evoked JOR but also facilitated the reflex. This indicates that facilitatory effects on the TP-evoked JOR had been also induced by the MO application. The view that the facilitatory effects were "unmasked" by naloxone is supported by previous studies showing the long-lasting facilitation on the flexion reflex after MO application into the deep tissue (i.e., muscle or joint) in spinalized animals (Woolf and Wall, 1986).

The JOR induced by non-noxious stimuli (i.e., IAN-evoked JOR) was also suppressed by the MO application. However, the effect was not as strong as on the TP-evoked JOR, and it was not affected by naloxone. It was notable that a part of the stimulus-response curve of the TP-evoked JOR following the MO application was similar to that of the IAN-evoked JOR, suggesting that modulatory mechanisms common to these reflexes, i.e., relatively weak and short lasting and naloxone-insensitive suppression, exists. For this, previous studies showed that the responsiveness of the neurons in the trigeminal

spinal nuclei to both noxious and non-noxious stimuli was suppressed by the stimulation of PAG or NRM, and the effect was naloxone insensitive (Dostrovsky et al., 1983; Sessle and Hu, 1981; Sessle et al., 1981). The findings suggest that the MO induced suppressive effect on the IAN-evoked JOR and a part of the MO induced suppressive effect on the TP-evoked JOR, in that the neurons in the trigeminal spinal nuclei are involved in the reflex arc, were due to the activation of the descending modulatory system, but factors other than endogenous opioid (e.g., 5-HT, GABA, noradrenaline) may play crucial roles for these effects.

Proprioceptive jaw reflex (i.e., MesV-evoked JCR) was generally suppressed by the MO application. We need to be cautious to rationalize the present results directly to the functional significance, since possible changes in sensitivity of the jaw-closing muscle spindles after the MO application, which have been indicated by others (Capra and Ro, 2000; Ro and Capra, 2001), were not reflected in the MesV-evoked JCR. This may cause the difference in the effect of experimental muscle pain on the jaw-closing reflex between the present results (suppression) and that documented by others (facilitation)(Wang et al., 2002; Wang et al., 2000). Instead, the present results provide further insights into changes in the sensory transmission from muscle spindles to the jaw-closing motoneurons in the central nervous system following noxious conditioning stimuli to deep tissues.

The time course and the strength of the MO induced suppressive effect on the JCR were similar to that of the IAN-evoked JOR, and the effect was also naloxone insensitive. However, a considerable difference was noted between the modulatory effects on these reflexes. Namely, the MesV-evoked JCR was facilitated, which was never observed in IAN-evoked JOR, in some animals after the sustained increase in the Mas EMG activity

had ceased. This suggests that the modulatory mechanism on these reflexes was different, at least partly. In addition, no facilitation was observed during the sustained increase in the Mas EMG activity following the MO application, whereas the reflex was quite often facilitated during a spontaneous Mas activity during the control period with its amplitude being equivalent to that evoked by the MO application. This indicates that changes in the reflex amplitude do not simply reflect the Mas motoneuronal excitability, and suggests the involvement of the presynaptic mechanisms (e.g., presynaptic inhibition) in the modulatory effect, which has been demonstrated in the spinal monosynaptic stretch reflex during voluntary movements (see Brooke et al., 1997, for review).

The present results have shown that MO-induced jaw-muscle pain induces suppression of all jaw reflexes tested. The suppression may prevent quick jaw movements responding to the sensory inputs, and may help "muscle splinting" with a synergistic increase in the baseline EMG activity in both jaw-opening and jaw-closing muscles (Sessle, 1995; Sessle, 1999). It is notable that a strong suppressive effect on the TP-evoked JOR, one of the nociceptive jaw reflexes having an important role in protecting the tissues from harmful stimuli, still remained even after the cessation of the suppressive effect on other reflexes, and this suppression was subject to the central opioid depressive effect. The pain modulatory system including the endogenous opioid systems is undoubtedly important for living animals to reduce pain sensations. However, the activation of such mechanisms may cause a potential risk that damage to deep tissues results in further damage to themselves and other tissues during movement. For example, the defense reflex to an excessive biting force may not be evoked properly during the long lasting suppression occurring on the nociceptive reflex. It should be emphasized that such a state may occur when the

nociceptive transmission is under the strong suppressive effect of the pain modulatory systems, i.e., when patients do not feel pain. This view may provide a key to understanding the changes in motor function following damage or inflammation of masticatory muscle and other deep tissues such as TMJ.

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Figure legends

Figure 1.

TP-evoked JOR evoked by different stimulus intensities. Right lower incisor was stimulated. Arrowheads indicate stimulus artifacts. The current strength was just above the reflex threshold (1.7 mA, A), 1.2T (B), 1.4T (C) and 1.6T (D). At the low intensity, only reflex responses with long latency (open triangles) were evoked (A and B). When the stimulus intensity was increased, reflex responses with short latency (filled triangles) were also evoked, and the long latency responses were suppressed (C and D). See text for details. L-Dig: left digastric muscle, R-Dig: right digastric muscle.

Figure 2.

An example of recordings before and after the MO application into the right temporal muscle. A shows the effect of the MO application on the baseline EMG activity. The period of MO application is indicated by the bar in the uppermost part. Sustained increases in the baseline EMG activity were induced by the MO application in the right digastric and right masseter muscles. B-E show examples of jaw reflexes during the control period (B), immediately after the MO application (C), 19 min after the MO application (D) and 60 min (E) after the MO application. Lower traces show the reflex responses of the selected time point in the upper traces with an expanded time base: TP-evoked JOR, IAN-evoked JOR and MesV-evoked JCR are indicated by the filled circles, open circles and filled triangles, respectively. Arrowheads in the lower traces indicate stimulus artifacts. Thresholds for each reflex were 0.8 mA for TP-evoked JOR, 120 μA for IAN-evoked JOR and 50 μA for MesV evoked JOR. The MO application induced suppressive effects on each reflex, but the effect was stronger, more rapidly appearing and longer lasting for the TP-evoked JOR than that

for other reflexes. See text for details.

IAN: right inferior alveolar nerve stimulation, TP: right tooth pulp stimulation, MesV: right trigeminal mesencephalic nucleus stimulation, L-Mas: EMG of the left masseter muscle, R-Mas: EMG of the right masseter muscle, L-Dig: EMG of the left digastric muscle, R-Dig: EMG of the right digastric muscle.

Figure 3.

Effect of MO application on jaw reflexes. Time course of the mean amplitude (area) of the TP-evoked JOR (A) IAN-evoked JOR (B) and MesV-evoked JCR (C) before and after the MO application are shown. Each point represents the mean + SD for the muscle in the MO injection side and mean – SD for the muscle in the MO non-injection side. The vertical broken line in each figure represents the onset of the MO application, and the horizontal dotted line represents the mean reflex amplitude during the control period. Arrows a, b and c indicate the time points immediately (a), 19 min (b) and 60 min (c) after the MO application. Note that the time courses in the TP-evoked JOR (A) IAN-evoked JOR (B) are similar except for the suppressive effect being induced immediately after the MO application and remaining 75 min after the MO application.

*: A significant difference was noted when the value was compared to the control ($P<0.05$, Kruskal-Wallis one-way ANOVA on Rank and Dunn's method). See text for details.

Ip Dig: the digastric muscle ipsilateral to the test stimulus and MO injection, Ct Dig: the digastric muscle contralateral to the test stimulus and MO injection, Ip Mas: the masseter muscle ipsilateral to the test stimulus and MO injection.

Figure 4.

Effect of naloxone on MO induced suppressive effect on TP-evoked JOR. Time course of the mean amplitude (area) of the TP-evoked JOR in the MO injection side (A) and the MO non-injection side (B) before and after the MO application and/or the systemic administration of naloxone are shown. The data for the MO group are same as shown in Figure 3. Each point represents the mean + SD (MO and naloxone group) or mean – SD (MO group). The vertical broken line in each figure represents the onset of the MO application and the horizontal dotted line represents the mean reflex amplitude during the control period. The thick arrow indicates the time point of the naloxone administration in the MO and naloxone group. Small arrows (a) and (b) indicate the time points 15 min (a) and 25 min (b) after the naloxone administration. Note that the MO induced suppressive effect was reversed by naloxone, and the facilitatory effect on the reflex appeared. *: A significant difference was noted when the value was compared to the control ($P<0.05$, Kruskal-Wallis one-way ANOVA on Rank and Dunn's method). †: A significant difference was noted between the MO group and the MO and naloxone group ($p<0.05$, Mann-Whitney Rank Sum Test). See text for details. Abbreviations are the same as in Figure 3.

Figure 5.

Effect of naloxone on MO induced suppressive effect on IAN-evoked JOR and MesV-evoked JCR. Time course of the mean amplitude (area) of the IAN-evoked JOR in the MO injection side (A) and that of the MesV-evoked JCR in the MO injection side (B) before and after the MO application and/or the systemic administration of naloxone are shown. The data for the MO group are the same as in Figure 3. Each point represents the mean + SD (MO and naloxone group) or mean – SD (MO group). The vertical broken line in each figure represents the onset of the MO application and the horizontal dotted line

represents the mean reflex amplitude during the control period. The thick arrow indicates the time point of the naloxone administration in the MO and naloxone group. The small arrows (a) indicate the time points 30 min after the naloxone administration. The naloxone administration did not induce significant effects on the MO induced suppressive effect on these reflex. *: A significant difference was noted when the value was compared to the control ($P<0.05$, Kruskal-Wallis one-way ANOVA on Rank and Dunn's method). Abbreviations are the same as in Figure 3.

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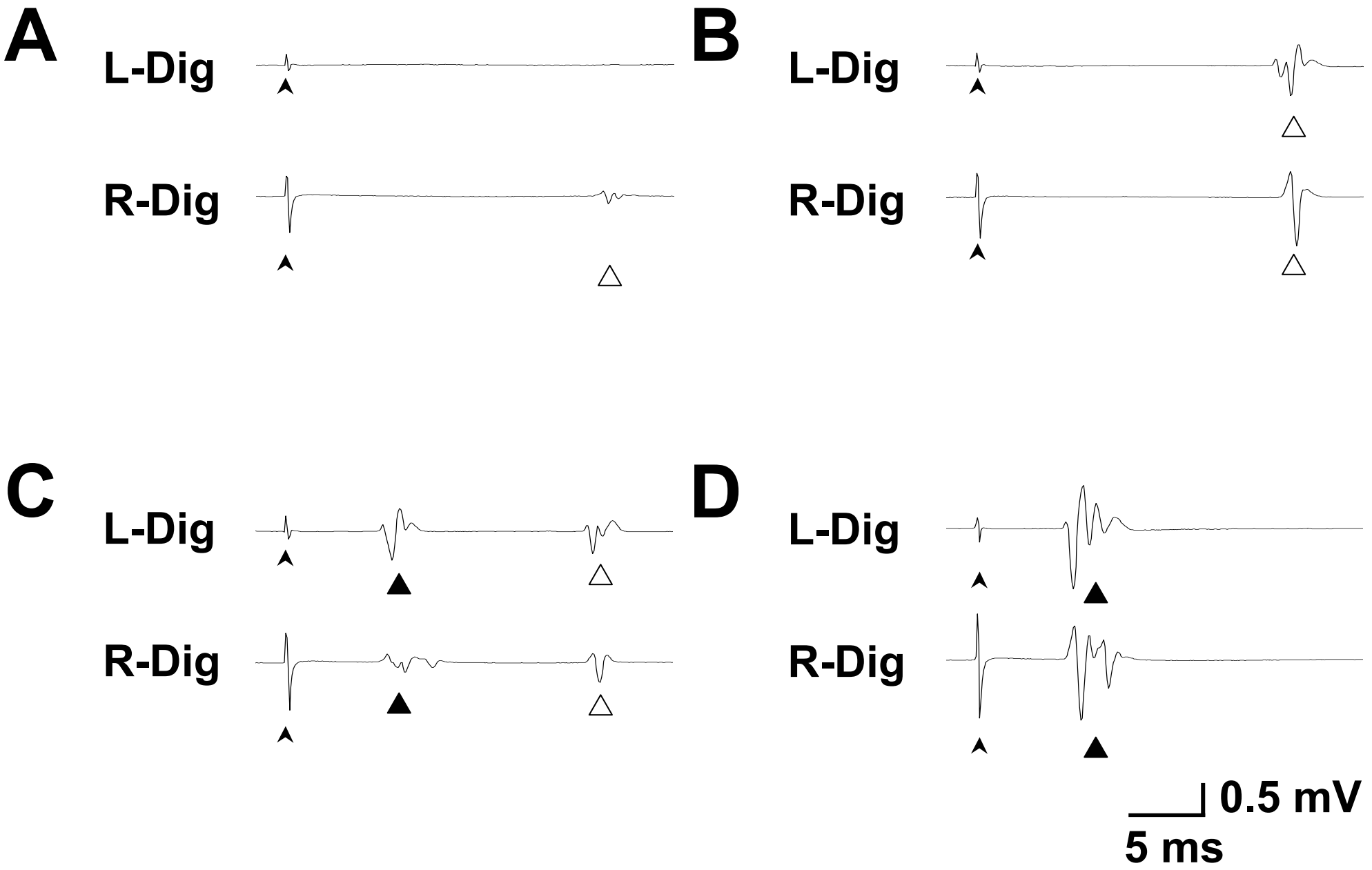
Table 1

Number of animals used for the analysis

	Effective stimulation							
	TP only	IAN only	MesV only	TP and IAN	TP and MesV	IAN and MesV	TP and IAN and MesV	Total
Test stimuli only group	1	N.A.	2	3	1	2	N.A.	9
MO group	6	5	6	1	N.A.	1	6	25
Naloxone only group	2	N.A.	2	2	N.A.	2	1	9
MO and naloxone group	N.A.	2	5	7	2	N.A.	1	17
Total	9	7	15	13	3	5	8	60

N.A.: not applicable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R-Dig
B
L-Dig
R-Dig
C
L-Dig
R-Dig
D
L-Dig
R-Dig
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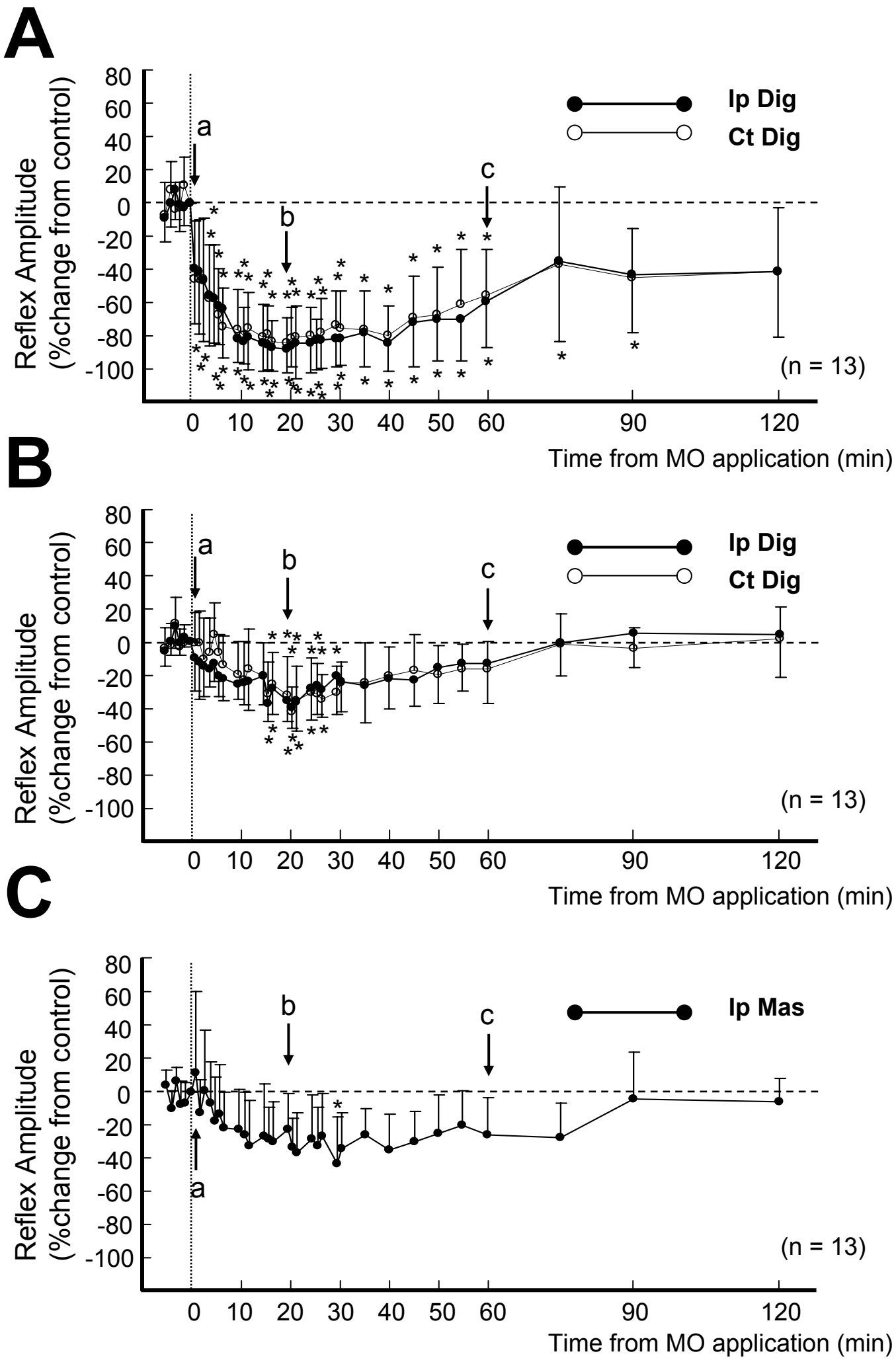
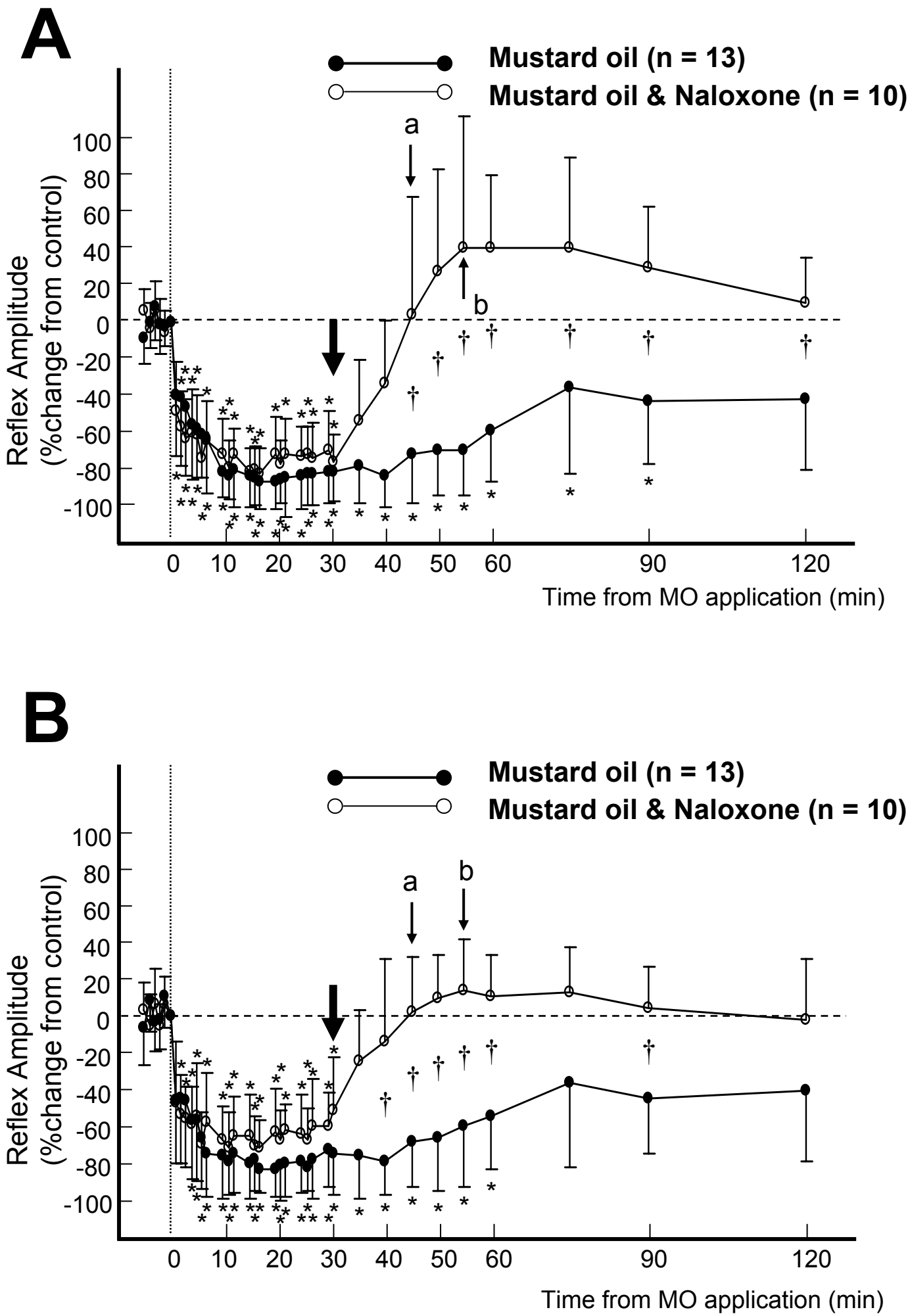
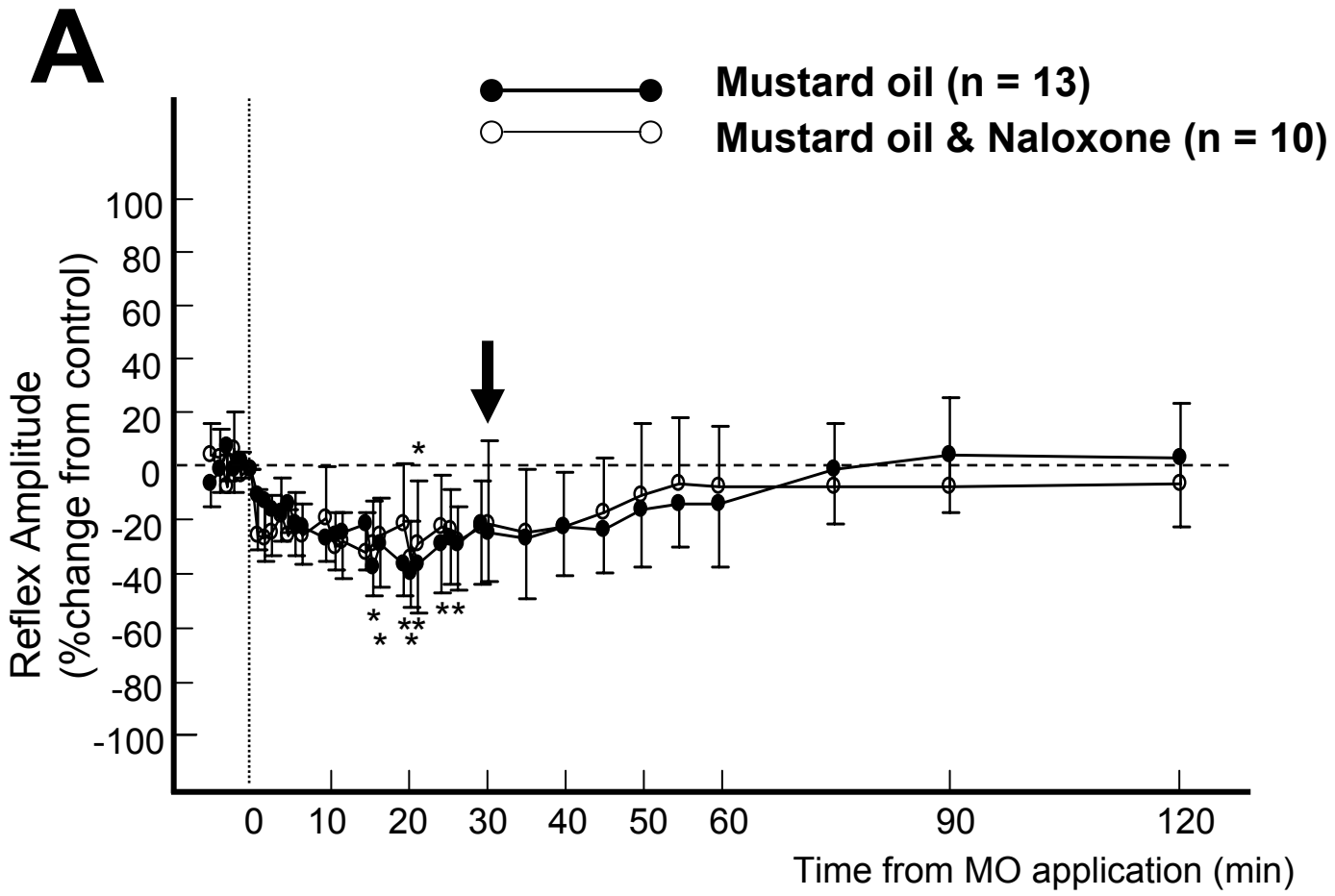
5 ms


A
B
C
Reflex Amplitude
(%change from control)
80
60
40
20
0
-20
-40
-60
-80
-100
a
b
c
Ip Dig
Ct Dig
Ip Mas
(n = 13)
0
10
20
30
40
50
60
90
120
Time from MO application (min)


A
Mustard oil (n = 13)
Mustard oil & Naloxone (n = 10)
a
b
Reflex Amplitude
(%change from control)
100
80
60
40
20
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
90
120
Time from MO application (min)
B
Mustard oil (n = 13)
Mustard oil & Naloxone (n = 10)
a
b
Reflex Amplitude
(%change from control)
100
80
60
40
20
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
90
120
Time from MO application (min)


A
Mustard oil (n = 13)
Mustard oil & Naloxone (n = 10)
Reflex Amplitude
(%change from control)
100
80
60
40
20
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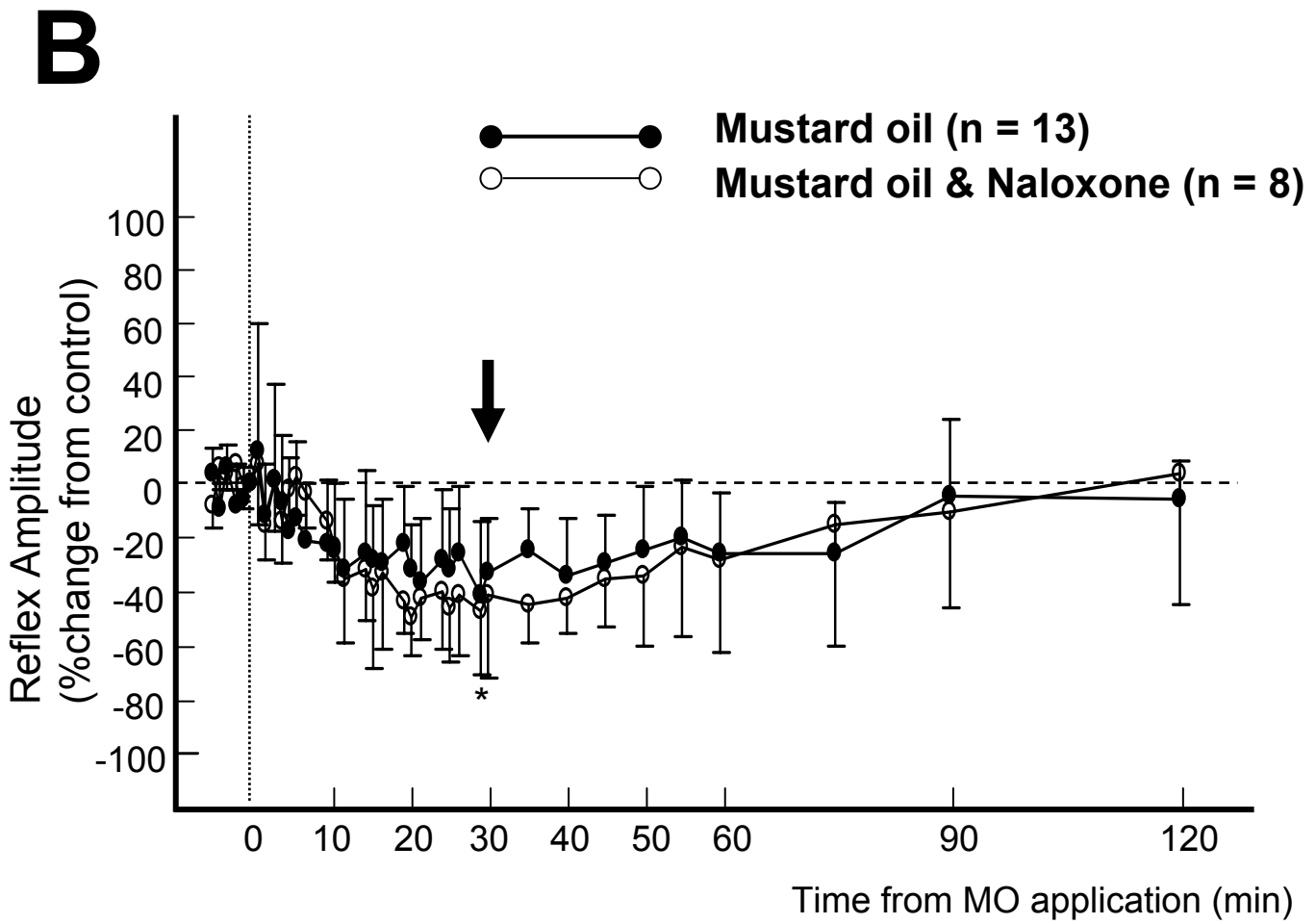
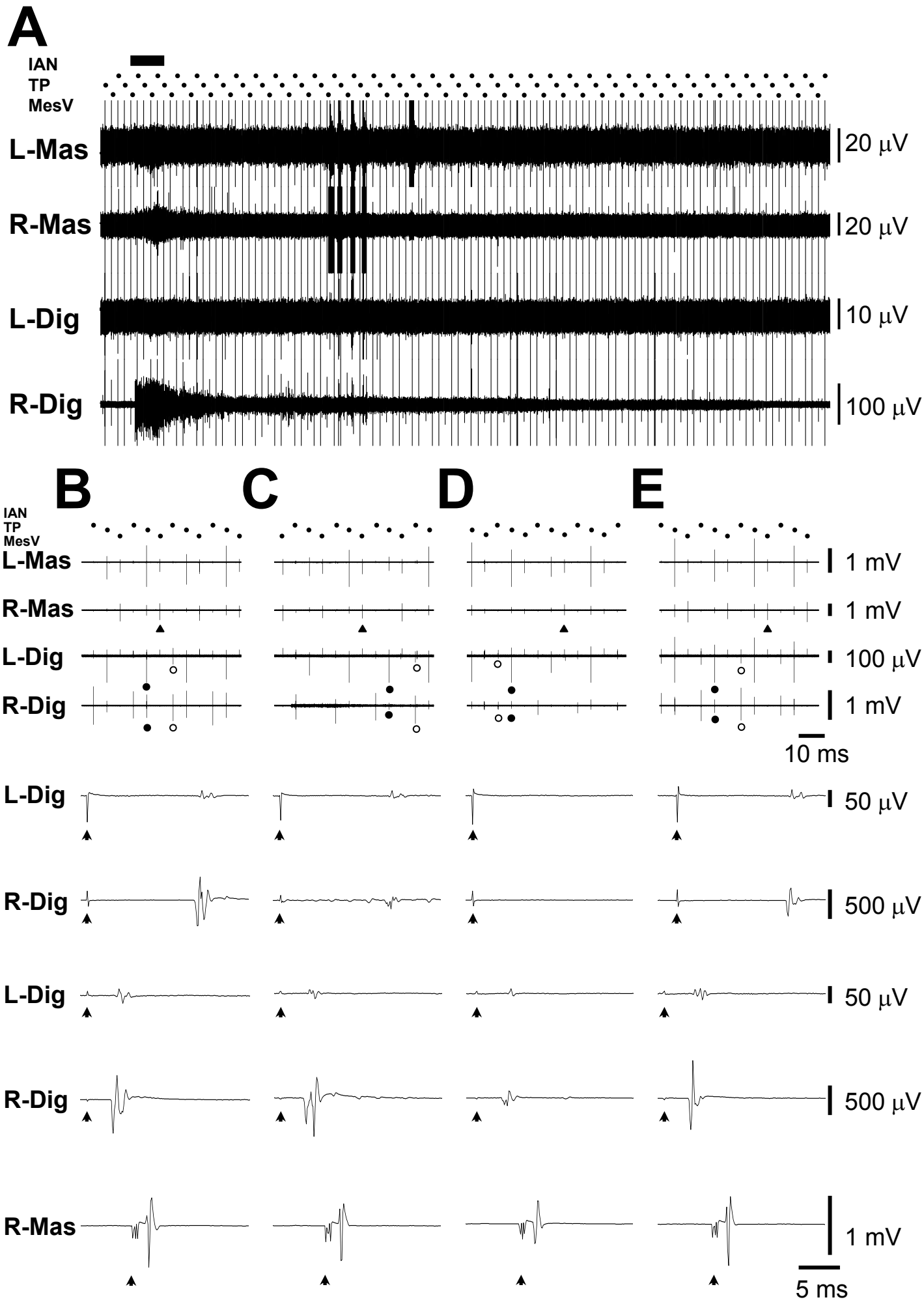
20
30
40
50
60
90
120
Time from MO application (min)


B
Mustard oil (n = 13)
Mustard oil & Naloxone (n = 8)
Reflex Amplitude
(%change from control)
100
80
60
40
20
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
90
120
Time from MO application (min)


A
IAN
TP
MesV
L-Mas
R-Mas
L-Dig
R-Dig
20 μV
20 μV
10 μV
100 μV
B
C
D
E
IAN
TP
MesV
L-Mas
R-Mas
L-Dig
R-Dig
1 mV
1 mV
100 μV
1 mV
10 ms
L-Dig
R-Dig
L-Dig
R-Dig
R-Mas
50 μV
500 μV
50 μV
500 μV
1 mV
5 ms