Application of oxytocin with low-level laser irradiation suppresses the facilitation of cerebrocortical excitatory propagation by partial ligation of the infraorbital nerve in rats: An optical imaging study

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This thesis is based on the following article and additional results in terms of the half duration of voltage-sensitive dye signals (Fig. 4):

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Abstract

The effects of current treatments for neuropathic pain are limited. Oxytocin is a novel candidate substance to relieve neuropathic pain, as demonstrated in various animal models with nerve injury. Low-level laser therapy (LLLT) is another option for the treatment of neuropathic pain. In this study, I quantified the effects of oxytocin or LLLT alone and the combination of oxytocin and LLLT on cortical excitation induced by electrical stimulation of the dental pulp using optical imaging with a voltage-sensitive dye in the neuropathic pain model with partial ligation of the infraorbital nerve (pl-ION). I applied oxytocin (OXT, 0.5 μmol) to the rat once on the day of pl-ION locally to the injured nerve. LLLT using a diode laser (810 nm, 0.1 W, 500 sec, continuous mode) was performed daily via the skin to the injured nerve from the day of pl-ION to 2 days after pl-ION. Cortical responses to electrical stimulation of the mandibular molar pulp under urethane anesthesia were recorded 3 days after pl-ION. Both the amplitude and area of excitation in the primary and secondary somatosensory and insular cortices in pl-ION rats were larger than those in sham rats. The larger amplitude of cortical excitation caused by pl-ION was suppressed following OXT or LLLT. The expanded area of cortical excitation caused by pl-ION was suppressed by OXT with LLLT but not by OXT or LLLT alone. These results suggest that the combined application of OXT and LLLT is effective in relieving the neuropathic pain induced by trigeminal nerve injury.

Introduction

Nerve injury often causes neuropathic pain, including allodynia and hyperalgesia (Renton and Yilmaz, 2011, Renton et al., 2012). However, treatments for neuropathic pain are not sufficient to restore normal somatosensation (Dworkin et al., 2007). Oxytocin is a 9-amino-acid neuropeptide synthesized in the paraventricular nucleus and supraoptic nucleus and is released from the posterior pituitary. Nerve injury upregulates the synthesis of oxytocin (Nishimura et al., 2019), and the application of oxytocin suppresses experimental nerve-injury-induced behavioral, electrophysiological, and molecular biological changes in the spinal cord of various neuropathic pain models (Boada et al., 2019, Chow et al., 2018, Condés-Lara et al., 2005, Sun et al., 2018). In the trigeminal nervous system, partial ligation of the infraorbital nerve (pl-ION) induces chronic pain in orofacial regions, which mimics human neuropathic pain syndromes well (Xu et al., 2008). Recently, Kubo et al. (2017) demonstrated that mechanical hypersensitivity in the whisker pad skin in pl-ION rats is suppressed by a direct injection of oxytocin into the trigeminal ganglion, indicating that oxytocin is a potential analgesic agent for abnormal pain-associated trigeminal nerve injury. However, in terms of clinical application, it is difficult to inject oxytocin directly into the trigeminal ganglion without adverse effects.

Receptors in the axon also modulate synaptic transmission. An AMPA receptor agonist and adenosine A_1 receptor antagonist in the axon shafts increase the width of action potentials, and as a result, broadened action potentials facilitate synaptic transmission (Sasaki et al., 2011). If the action site of oxytocin in the trigeminal ganglion was present not only in the soma but also in the axon, the local application of oxytocin on the injured nerve may contribute to relieve the hypersensitivity induced by nerve injury.

Low-level laser therapy (LLLT) could be an option in the treatment of neuropathic pain due to positive effects on the control of analgesia for abnormal pain in humans and experimental animals (de Andrade et al., 2016, de Pedro et al., 2019). It has been proposed that LLLT offers improvement in the quality of life of patients with neuropathic pain because drug application in combination with LLLT might reduce the dosage of drugs and reduce the side effects (Eckerdal and Bastian, 1996, Jameie et al., 2014). However, knowledge regarding the direct evidence of how LLLT exerts neural activities is limited.

The principal neural fibers involved in the dental pulp are Aδ and C fibers, which send nociceptive information to the central nervous system, including the somatosensory and insular cortices (Naftel et al., 1999, Nakamura et al., 2015). Therefore, the cortical response to dental pulp stimulation is likely to reflect the strength of nociception. Indeed, a previous study has demonstrated that cortical responses in the primary sensory cortex (S1) and in the secondary somatosensory and insular oral region (S2/IOR) depend on the voltage of the electrical stimulation of the dental pulp (Nakamura et al., 2016). Recently, it is reported that pl-ION enhanced responses to mandibular molar pulp stimulation in both the S1 and S2/IOR of rats (Zama et al., 2019). In this study, I performed *in vivo* optical imaging with a

voltage-sensitive dye to investigate whether local application of oxytocin (OXT) and LLLT on the injured nerve relieves cortical hyperexcitation induced by pl-ION. In addition, I estimated the summative effect of OXT in combination with LLLT.

Materials and Methods

The experiments performed in this study were approved by the Animal Experimentation Committee of Nihon University (AP17D017, AP18DEN011-1, AP19DEN014-1) and were conducted in accordance with institutional guidelines for the care and use of experimental animals as described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

Acute effects of LLLT and procedures for* in vivo *optical imaging

To establish conditions of LLLT, I started with an experiment regarding the acute effects of direct laser irradiation of the inferior alveolar nerve. Male Sprague-Dawley rats (Japan SLC; n = 10) were used. All rats were housed in clear polycarbonate cages (length × width × height = 48 × 26.5 × 21 cm) containing paper shavings as bedding and were kept in a temperature-controlled room (23 ± 2°C) on a 12 h light/dark cycle with adlibitum access to food and water. Rats received an atropine methyl bromide injection (0.5 mg/kg, i.p.) and were anesthetized with urethane (1.5 g/kg, i.p., Sigma-Aldrich, St. Louis, MO, USA). Additional urethane was administered depending on the toe pinch reflex. Body temperature was maintained at approximately 37°C using a rectal probe and a heating pad (BWT-100, Bio Research Center, Osaka, Japan). A part of the right mandibular bone above the angle of the mandible was removed to expose the inferior alveolar nerve. The mental nerve existing above the inferior alveolar nerve was transected to perform the direct application of laser irradiation of the inferior alveolar nerve (Fig. 1A). Laser irradiation (diode laser, 810 nm, continuous mode, Osada Lightsurge Square (OSL-S) with a modification to enable low-level laser irradiation, Osada, Tokyo, Japan) was performed via an optical fiber (diameter = 1 mm), and the distance between the tip and the inferior alveolar nerve was approximately 2 mm. Between laser irradiations (Fig. 1B), a cotton ball with 0.9% saline was applied to an opened part to moisten exposed soft tissues, including the inferior alveolar nerve.

Optical imaging using a voltage-sensitive dye (RH-1691, Optical Imaging, New York, NY, USA) was performed as previously described (Fujita et al., 2017, 2019a,b, Nakamura et al., 2015, 2016, Zama et al., 2019). Rats were fixed to a custom-made stereotaxic snout frame, which was tilted 60° laterally to image the surface of the S1 and S2/IOR processing nociceptive information from the right mandibular molar pulp using a CCD camera (MiCAM02, Brainvision, Tokyo, Japan; Nakamura et al., 2015). The left temporal muscle and zygomatic arch were carefully removed, and a craniotomy was performed to expose the cortical surface, including the S1 and S2/IOR, the middle cerebral artery (MCA), and the rhinal fissure (RF).

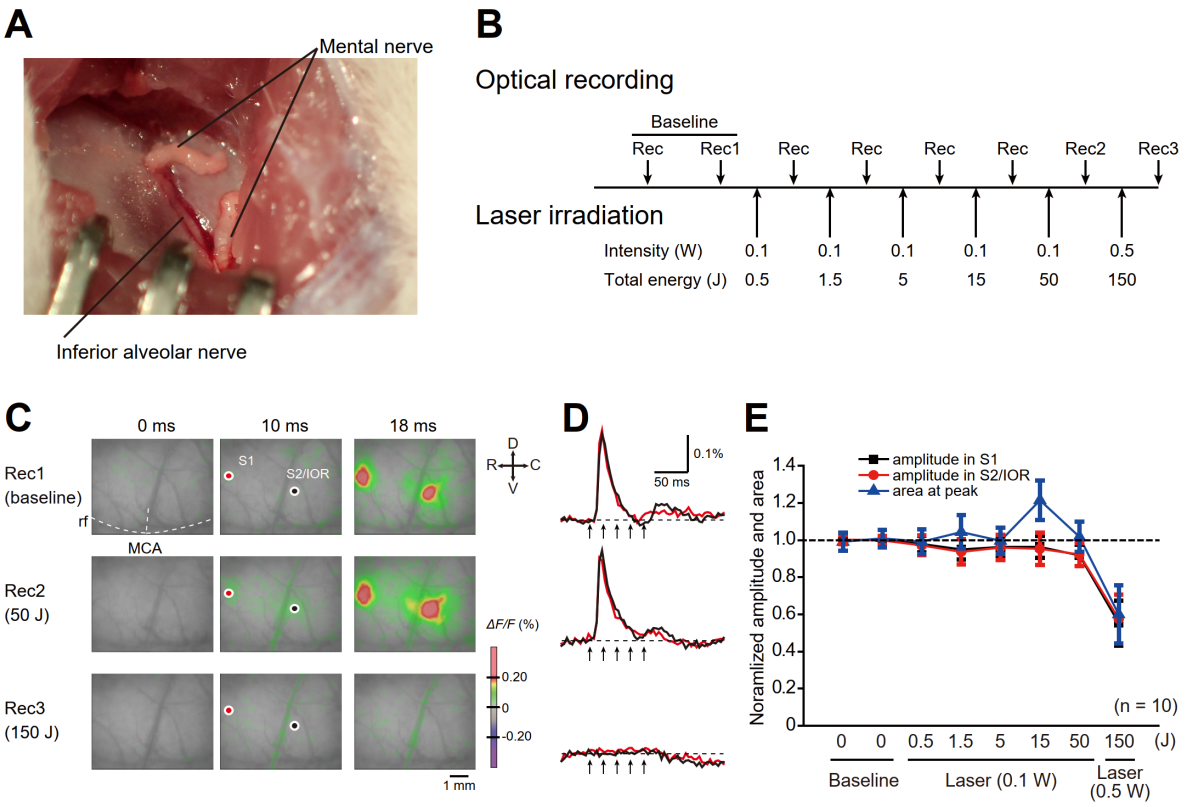

Figure 1. Low-level laser therapy (LLLT) on the inferior alveolar nerve innervating the mandibular molar pulps did not affect cortical responses to mandibular molar pulp stimulation. (A) Laser irradiation was directly applied to the inferior alveolar nerve. Note that the mental nerve existing above the inferior alveolar nerve was transected to perform the direct application of laser irradiation of the inferior alveolar nerve. (B) The experimental protocol. Laser irradiation (diode laser, 810 nm, continuous mode, Osada Lightsurge Square (OSL-S), Osada, Tokyo, Japan) was performed between the recordings. (C,D) An example of cortical responses (C) and traces of optical signals (D) to the electrical stimulation of the mandibular molar pulp (5 V, 5 voltage pulses at 50 Hz) in a rat. Note that the cortical response disappeared due to heat damage to the irradiated area, including the inferior alveolar nerve after laser irradiation with high intensity (Rec3, 0.5 W, 300 sec), but not low intensity (0.1 W, 500 sec). (E) Normalized peak amplitude and activated area. Note that laser irradiation with high intensity (0.5 W), but not low intensity (0.1 W) induced damage to irradiated areas, including the inferior alveolar nerve, in some cases.

The cortical surface was stained with RH-1691 (1 mg/ml) in 0.9% saline for 1 hour. Then, the cortical surface was rinsed with saline and covered with 1% agarose (Agarose Low EEO, Sigma-Aldrich) dissolved in Ringer's solution and affixed with a glass coverslip. RH-1691 fluorescence intensities were measured using the CCD camera system described above mounted on a stereomicroscope (Leica Microsystems, Wetzlar, Germany). The cortical surface was illuminated through a 632-nm excitation filter with a dichroic mirror using a tungsten-halogen lamp (CLS150XD, Leica Microsystems). Fluorescent emission was captured through an absorption filter (λ > 650-nm long-pass, Andover, Salem, MA, USA). The CCD camera had an imaging area of 6.4 × 4.8 mm^2 (184 × 124 pixels).

Changes in the fluorescence intensity in response to electrical stimulation (5 V, 5 voltage pulses for 100 μsec at 50 Hz, STG2008, Multi-Channel Systems, Reutlingen, Germany) were imaged at 250 Hz for 500 msec. To minimize signals induced by acute bleaching of the dye, the fluorescence intensity without stimulation was subtracted from each recording. Thus, each image was constructed from paired recordings with and without mandibular molar pulp stimulation. The stimulation was performed with an interstimulus interval of 20 sec, and 36 were averaged in the experiment of the acute effects of LLLT to improve the signal-to-noise ratio. In the following experiment in the pl-ION model, 48 images were averaged.

pl-ION model

Male Wistar rats (Japan SLC, Shizuoka, Japan) aged 5-6 weeks (n = 60) were used. The pl-ION model was constructed as previously described (Shinoda et al., 2007, Kubo et al., 2017). Briefly, rats were anesthetized with an intraperitoneal injection of butorphanol (2.5 mg/kg, Meiji Seika Pharma, Tokyo, Japan), medetomidine (0.375 mg/kg, Zenoaq, Koriyama, Japan) and midazolam (2.0 mg/kg, Sandoz, Tokyo, Japan) following a subcutaneous injection of the local anesthetic lidocaine (2%, Mylan, Pittsburgh, PA). A small incision was made in the right buccal mucosa, and the infraorbital nerve was freed from the surrounding connective tissue (Fig. 2A). A one-half to one-third thickness of the nerve was ligated tightly with 6-0 silk.

Oxytocin application to the pl-ION model

Rats participating in experiments with oxytocin application (Fig. 2A) were randomly assigned to 3 groups that received sham operation (n = 10), pl-ION (n = 11), or pl-ION-OXT (n = 10). A gelatin-based hydrogel, MedGel, which stably holds bioactive substances *in vivo* and sustainably releases the substances, was used to apply oxytocin (0.5 μmol/10 μl) or vehicle (0.01 M PBS, 10 μl) locally to the infraorbital nerve. The incisions were closed using 6-0 silk sutures.

As a control, I made sham-operated rats that received a sham operation identical to that described above without ligation. According to previous studies (Kubo et al., 2017, Shinoda et al., 2007, Zama et al., 2019), the decrease in the mechanical thresholds of whisker pad skin reaches a plateau approximately 3 days after pl-ION in rats. Therefore, optical imaging was performed 3 days after pl-ION in this study.

LLLT in pl-ION model

Rats participating in LLLT experiments (Fig. 2B) were randomly assigned to 3 groups that received (1) pl-ION with subsequent anesthesia once a day with an intraperitoneal injection of butorphanol, medetomidine, and midazolam in the same doses described above (pl-IONan; n = 9), (2) pl-ION-LLLT (n = 10), and (3) pl-ION-LLLT&OXT (n = 10). The low-level laser irradiation (0.1 W for 500 sec, continuous mode) to the inferior alveolar nerve had little effect on nerve conduction (Fig. 1), suggesting that LLLT does not induce neuroparalysis and heat

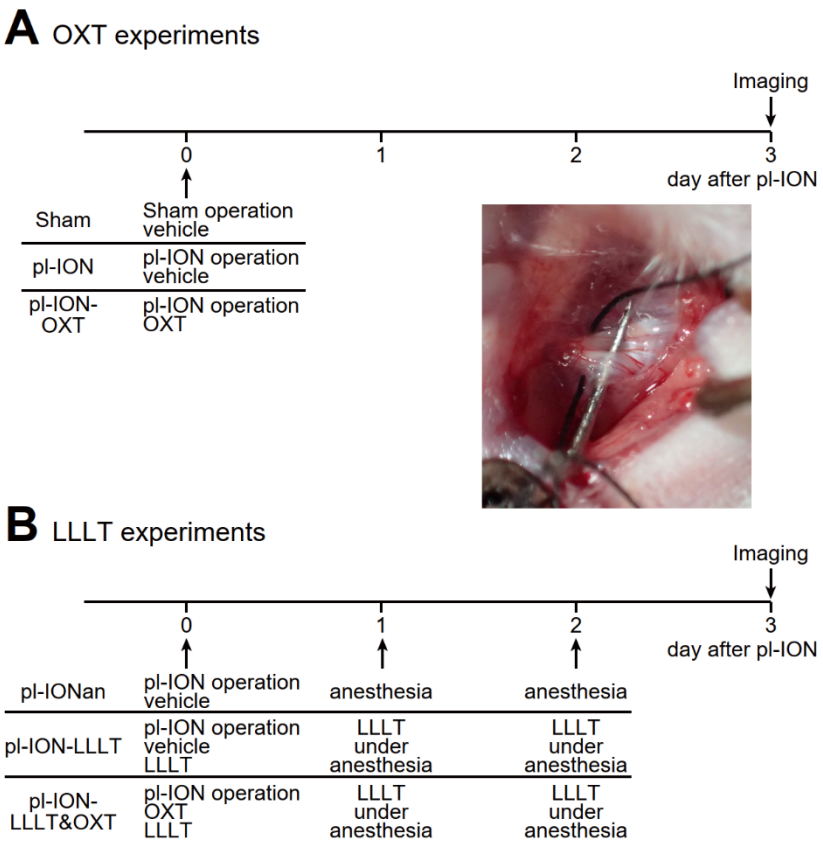

Figure 2. The experimental protocols of oxytocin or low-level laser therapy. (A) Protocol of the local application of oxytocin (OXT). The inset shows the partial ligation of the infraorbital nerve (pl-ION). The infraorbital nerve taken up by a needle was ligated in the pl-ION model but not in the sham operation. A black silk thread was placed under the whole infraorbital nerve to clearly show it. After pl-ION, MedGel with oxytocin or vehicle was applied to the injured nerve. (B) Protocol of LLLT experiments. LLLT with diode laser (0.1 W for 500 sec, continuous mode) was applied daily to the injured nerve under general anesthesia.

damage to the nerve. Thus, LLLT (0.1 W for 500 sec, continuous mode) was performed daily under anesthesia as described above from the day of pl-ION to 2 days after pl-ION operation with a distance between the tip and the skin was approximately 2 mm. After the completion of LLLT, atipamezole hydrochloride (0.75 mg/kg, Nippon Zenyaku Kogyo, Koriyama, Japan), an α_2-adrenergic receptor antagonist, was injected intraperitoneally to reverse the sedative and analgesic effects of medetomidine.

Data analysis

Optical signals were processed and analyzed using a software program (Brain Vision Analyzer; Brainvision). Changes in the intensity of fluorescence (ΔF) of each pixel relative to the initial intensity of fluorescence (F) were calculated ($\Delta F/F$), and the ratio was processed with a spatial filter (9 × 9 pixels). A region of interest (ROI) was a circle consisting of 77 pixels (~ 0.1 mm^2). Peak amplitude and half duration were defined as the maximum amplitude of an optical response and as the duration at half the maximum optical response, respectively. A significant response was defined as a signal exceeding 7 times the SD of the

baseline period. It is considered that S2/IOR plays a key role in nociceptive information processing of the oral structures (Kobayashi and Horinuki, 2017). To evaluate the activated area, we used the frames in which the optical signal was at peak in the amplitude in the center of the initial response of the S2/IOR. To overlap the outlines of the activated area across multiple rats, RF and MCA were used as landmarks.

In multiple comparison of peak amplitude and activated area, an ANOVA with Tukey's test following a normality test (Shapiro-Wilk test) and equal variance test (Brown-Forsythe test) was used. In multiple comparisons of half duration, an ANOVA on ranks was used because the data set did not pass the normality test and/or equal variance test. In comparisons between 2 groups, Student's *t* test following a normality test and equal variance test was used. SigmaStat software (ver. 4.0, Systat Software, San Jose, CA, USA) was used for statistical analyses, and $p < 0.05$ was considered significant. All data were expressed as the means ± SEM.

Results

Acute effects of LLLT on the inferior alveolar nerve

To establish conditions of LLLT, I investigated the acute effects of direct laser irradiation of the inferior alveolar nerve. A typical example is shown in Fig. 1C. The mandibular pulp stimulation-induced cortical excitation occurred in the S1 and S2/IOR simultaneously. The cortical responses were comparable between the recording at baseline (Rec1) and the recording after laser irradiation with low intensity (Rec2, 0.1 W, 500 sec, Fig. 1D). In this case, the cortical response disappeared after laser irradiation with high intensity (Rec3, 0.5 W, 300 sec) due to heat damage to the irradiated area, including the inferior alveolar nerve. The summary of normalized peak amplitude and activated area was shown in Fig. 1E. The laser irradiation with low intensity (0.1 W) did not affect the cortical responses to mandibular molar pulp stimulation in amplitude and activated area, whereas laser irradiation with high intensity (0.5 W) induced damage to irradiated areas, including the inferior alveolar nerve, in some cases. These results suggest that laser irradiation with low intensity (0.1 W) has no acute effects on spike conduction.

Effects of oxytocin on cortical responses to mandibular molar pulp stimulation

I observed cortical responses to mandibular molar pulp stimulation using RH-1691 to assess the effects of oxytocin on pl-ION-induced changes in nociceptive information processing in the cerebral cortex (Fig. 2A). In agreement with previous studies (Fujita et al., 2017, 2019a, Nakamura et al., 2015, 2016), electrical stimulation of the mandibular molar pulp elicited cortical excitation in the S1 and S2/IOR simultaneously in sham (Fig. 3A,B).

pl-ION increased the amplitude of cortical excitation in the S1 (0.29 ± 0.04% in sham, n = 10; 0.71 ± 0.06% in pl-ION, n = 11; $p < 0.001$, ANOVA with Tukey's test; Fig. 3C) and S2/IOR (0.28 ± 0.04% in sham, n = 10, 0.61 ± 0.06% in pl-ION, n = 11; $p < 0.001$, ANOVA with Tukey's test). In addition, the activated area was increased by pl-ION (9.1 ± 1.6 mm^2 in sham, n = 10; 18.5 ± 1.4 mm^2 in pl-ION, n = 11; $p < 0.001$, ANOVA with Tukey's test; Fig. 3D). The expanded area of excitatory propagation in pl-ION was primarily observed dorsally adjacent to that in sham (Fig. 3E). The excitation in the S1 and S2 frequently expanded beyond the field of view in pl-ION. On the other hand, the half duration of cortical excitation was comparable in the S1 (52 ± 18 ms in sham, n = 10; 23 ± 2 ms in pl-ION, n = 11, ANOVA on ranks; Fig. 4) and S2/IOR (74 ± 30 ms in sham, n = 10; 28 ± 2 ms in pl-ION, n = 11, ANOVA on ranks).

A gelatin-based hydrogel, MedGel (MedGel, Tokyo, Japan), which stably holds bioactive substances *in vivo* and sustainably releases the substances, was used to apply oxytocin locally to the partially ligated infraorbital nerve (pl-ION-OXT). OXT significantly suppressed the pl-ION-induced increase in the amplitude both in the S1 (0.46 ± 0.04% in pl-ION-OXT, n = 10; vs. pl-ION, $p = 0.006$, ANOVA with Tukey's test; Fig. 3C) and in the S2/IOR (0.42 ± 0.04% in pl-ION-OXT, n = 10; vs. pl-ION, $p = 0.023$, ANOVA with Tukey's

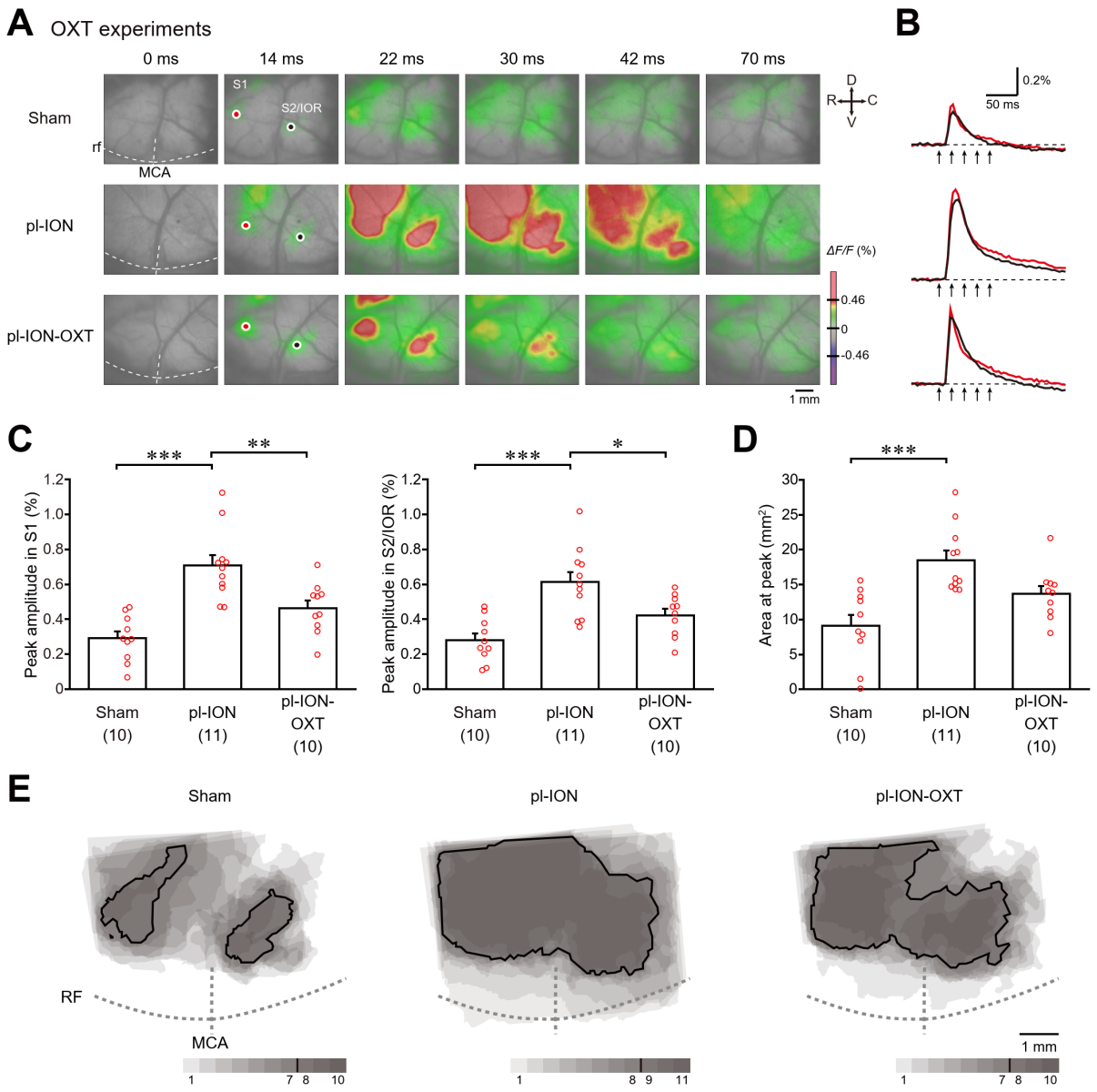

Figure 3. The application of oxytocin suppressed pl-ION-induced cortical hyperexcitation. (A) Examples of excitatory propagation. The amplitude of the optical signal (ΔF/F) is color-coded, and the time from electrical stimulation of the mandibular molar pulp is shown at the top of each panel. Note that the first excitation was simultaneously found in the primary somatosensory cortex (S1) and the border between the ventral secondary somatosensory cortex and insular oral region (S2/IOR), and it propagated to the surrounding regions. The cortical response in pl-ION was larger than that in sham. The pl-ION-enhanced cortical responses were suppressed in the pl-ION-OXT. (B) Traces of optical signals before and after electrical stimulation (5 V, 5 voltage pulses at 50 Hz). Red and black lines indicate optical signals in the S1 and S2/IOR, respectively, shown in (A). Arrows indicate 5 electrical stimuli. (C) Comparison of the peak amplitude of optical signals in the S1 and S2/IOR. The numbers of animals are shown in parentheses. Note that the peak amplitude in the S1 and S2/IOR of pl-ION-OXT was lower than that in pl-ION. (D) Comparison of the area activated by the stimulation. (E) Superimposed images of the activated area at the peak amplitude. The number of overlapping responses is represented by the gradation of the colors. The line outlines the area responding to stimulation in 80% of animals. C, caudal; D, dorsal; R, rostral; V, ventral; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA with Tukey's test.

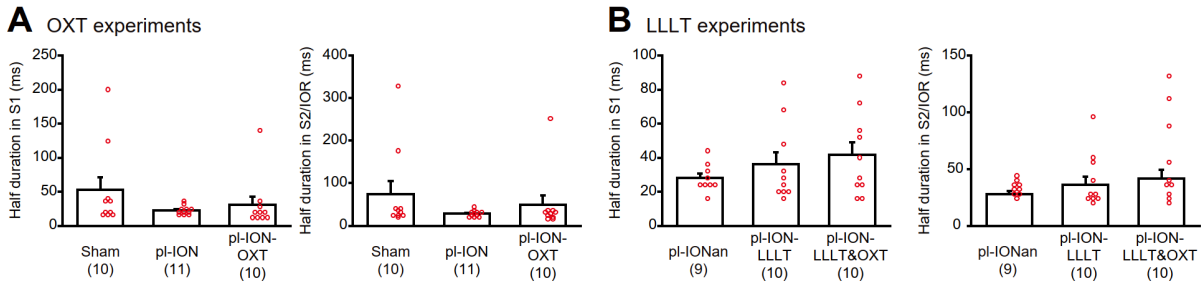

Figure 4. Half duration of voltage-sensitive dye signal was not changed by pl-ION, OXT, and LLLT. (A) Summary in OXT experiments. The half duration in the S1 and S2/IOR was comparable among sham, pl-ION, and pl-ION-OXT. (B) Summary in LLLT experiments. The half duration in the S1 and S2/IOR was comparable among pl-IONan, pl-ION-LLLT, and pl-ION-LLLT&OXT.

test). The activated area in pl-ION-OXT tended to be decreased compared to that in pl-ION (13.7 ± 1.1 mm^2 in pl-ION-OXT, n = 10; p = 0.060, ANOVA with Tukey's test; Fig. 3D), and there was no significant difference in activated area between sham and pl-ION-OXT (p = 0.083, ANOVA with Tukey's test).

Effect of LLLT with or without OXT on cortical excitation

I further addressed the effects of LLLT with or without OXT in pl-ION rats (Fig. 2B). In a series of LLLT experiments, LLLT using a diode laser (810 nm, 0.1 W, 500 sec, continuous mode) was performed daily under anesthesia with butorphanol, medetomidine, and midazolam, and then, atipamezole hydrochloride was administered after LLLT. Considering the influences of these chemicals, I prepared the group pl-IONan as a control, in which rats received pl-ION and a daily injection of chemicals described above in the same way without LLLT.

The amplitudes in pl-IONan were larger than those in sham in oxytocin experiments (Figs. 3 and 5) both in the S1 (0.29 ± 0.04% in sham, n = 10; 0.65 ± 0.08% in pl-IONan, n = 9; p = 0.001, Student's t test) and in the S2/IOR (0.28 ± 0.04% in sham, n = 10, 0.60 ± 0.08% in pl-IONan, n = 9; p = 0.003, Student's t test). Likewise, the activated area was increased by the pl-ION (9.1 ± 1.6 mm^2 in sham, n = 10; 20.4 ± 1.8 mm^2 in pl-IONan, n = 9; p < 0.001, Student's t test). These results indicated that pl-ION-induced hyperexcitation in the cerebral cortex remained in rats with daily injections of the anesthetics and the antagonists.

LLLT to the injured nerve via the skin (pl-ION-LLLT) significantly suppressed the pl-ION-induced increase in amplitude both in the S1 (0.39 ± 0.06% in pl-ION-LLLT, n = 10; vs. pl-IONan, p = 0.035, ANOVA with Tukey's test; Fig. 5A-C) and in the S2/IOR (0.37 ± 0.05% in pl-ION-LLLT, n = 10; vs. pl-IONan, p = 0.043, ANOVA with Tukey's test). The activated area in pl-ION-LLLT tended to decrease compared to that in pl-IONan (15.2 ± 1.8 mm^2 in pl-ION-LLLT, n = 10; p = 0.15, ANOVA with Tukey's test; Fig. 5D). LLLT with OXT (pl-ION-LLLT&OXT) significantly suppressed the pl-ION-induced increase in amplitude both in the S1 (0.38 ± 0.05% in pl-ION-LLLT&OXT, n = 10; vs. pl-IONan, p = 0.027,

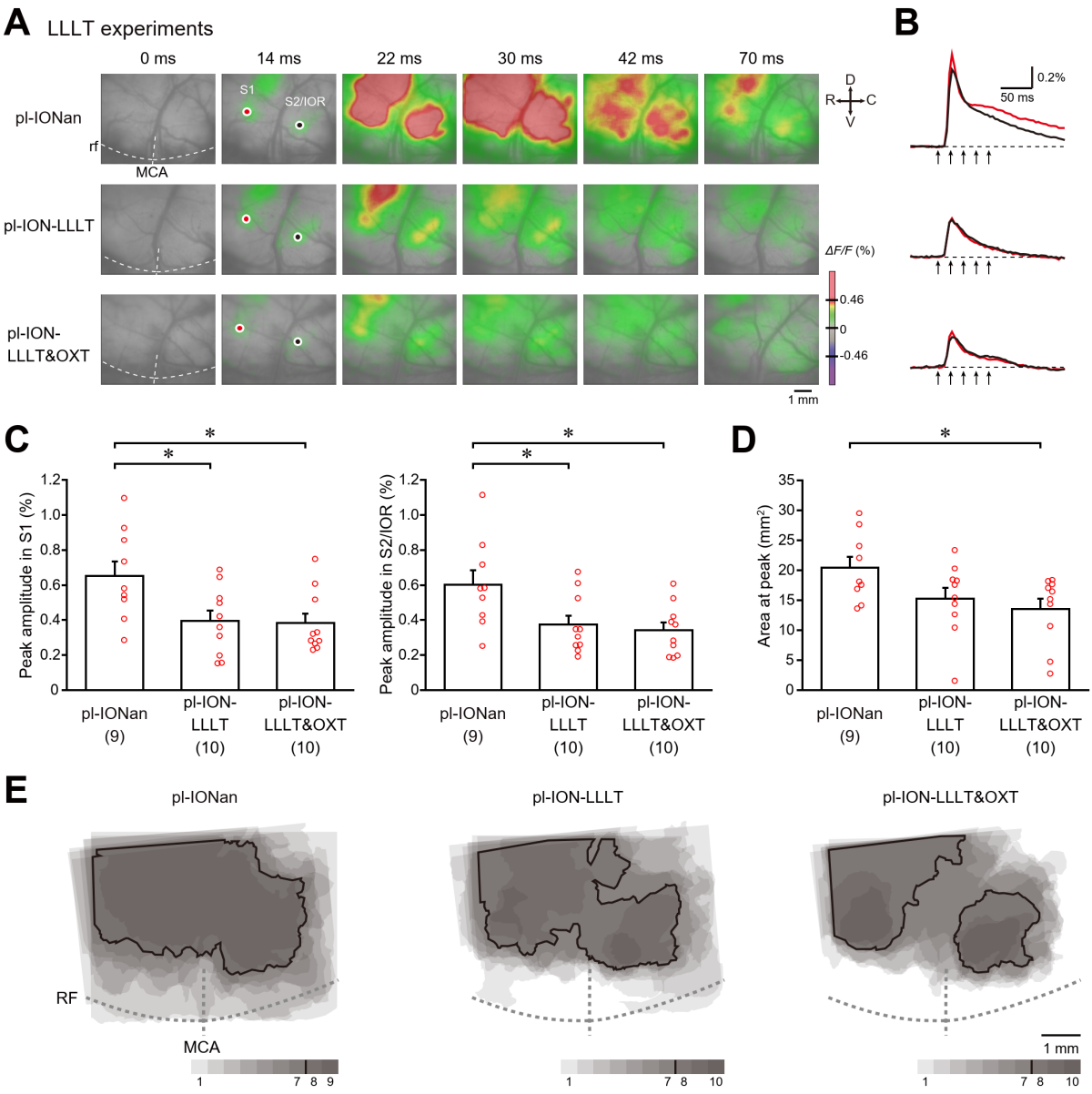

Figure 5. The application of LLLT with and without OXT suppressed pl-ION-induced cortical hyperexcitation. (A) Examples of excitatory propagation. The amplitude of the optical signal (ΔF/F) is color-coded, and the time from the electrical stimulation of the mandibular molar pulp is shown at the top of each panel. (B) Traces of optical signals before and after electrical stimulation (5 V, 5 voltage pulses at 50 Hz). Red and black lines indicate optical signals in the S1 and S2/IOR, respectively, shown in (A). (C) Comparison of the peak amplitude of optical signals in the S1 and S2/IOR. The numbers of animals are shown in parentheses. Note that the peak amplitudes in pl-ION-LLLT and pl-ION-LLLT&OXT were lower than those in pl-IONan. (D) Comparison of the area activated by the stimulation. Note that the activated area in pl-ION-LLLT&OXT was smaller than that in pl-IONan. (E) Superimposed images of the activated area at peak amplitude. The number of overlapping responses is represented by the gradation of the colors. The line outlines the area responding to stimulation in 80% of animals. C, caudal; D, dorsal; R, rostral; V, ventral; * $p < 0.05$, ANOVA with Tukey's test

ANOVA with Tukey's test; Fig. 5C) and in the S2/IOR (0.34 ± 0.04% in pl-ION-LLLT&OXT, n = 10; vs. pl-IONan, $p = 0.019$, ANOVA with Tukey's test). In addition, the activated area in

pl-ION-LLLT&OXT was significantly smaller than that in pl-IONan (13.5 ± 1.7 mm^2 in pl-ION-LLLT&OXT, n = 10; p = 0.042, ANOVA with Tukey's test; Fig. 5D,E).

Discussion

OXT suppresses pl-ION-induced cortical hyperexcitation

I demonstrated that OXT applied to the injured nerve reduced hyperexcitation in the cerebral cortex. In rats, oxytocin and vasopressin receptors are expressed in the trigeminal ganglion (Kai-Kai et al., 1985, Kubo et al., 2017, Tzabazis et al., 2016). Kubo et al. (2017) reported that the direct injection of oxytocin into the trigeminal ganglion reduced abnormal mechanical hypersensitivity in the whisker pad skin in pl-ION rats. In their experiments, the effects of oxytocin were antagonized with a selective vasopressin V1A receptor antagonist, SR49059. Taking the finding that axonal receptors contribute to the regulation of action potentials in nerve conduction (Sasaki et al., 2011), it is reasonable to postulate that V1A receptors in the axon might contribute to reduce cortical hyperexcitation in pl-ION-OXT. Indeed, Kubo et al. (2017) demonstrated that oxytocin hyperpolarizes the resting membrane potential, increases rheobase, and shortens the spike duration of trigeminal ganglion neurons. These effects of oxytocin are likely to decrease glutamate release from the axon terminals in the medullary dorsal horn (trigeminal spinal nucleus caudalis).

V1A receptors are coupled with $G_{q/11}$ (Liu and Wess, 1996); therefore, OXT is likely to activate phospholipase C and increase $[Ca^{2+}]_i$ via IP_3 production. It has been reported that an increase in $[Ca^{2+}]_i$ often activates Ca^{2+}-dependent K^+ channels, which increases the amplitude of the afterhyperpolarization (Kobayashi et al., 1997). As a result, repetitive firing frequency is reduced (Kobayashi et al., 1997). This is a possible explanation for the OXT-induced suppression of cortical activities via V1A receptors expressed in axons; however, the details of the underlying mechanisms should be addressed with electrophysiological techniques in future studies.

LLLT suppresses pl-ION-induced cortical hyperexcitation

In previous studies in human and experimental animals, LLLT has been reported to have positive effects on the control of analgesia for neuropathic pain (Bertolini et al., 2011, de Andrade et al., 2016, 2017, de Oliveira Martins et al., 2013, Jameie et al., 2014, Kobiela Ketz et al., 2017, Martins et al., 2017, Mojarad et al., 2018). Similarly, I found relieving effects of LLLT on pl-ION-induced cortical hyperexcitation in response to mandibular molar pulp stimulation (Fig. 5). However, the molecular mechanisms of LLLT are still argued. In previous studies in neuropathic pain animal models, several effects induced by LLLT have been reported: the enhancement of the expression of nerve growth factor and the suppression of the expression of brain-derived neurotrophic factor (de Oliveira Martins., 2013); the improvement in morphology of the injured nerve, accompanied by the suppression of myelin protein zero, laminin, and neurofilaments (Martins et al., 2017); the modulation of macrophage/microglial activation to an anti-inflammatory phenotype (Kobiela Ketz et al., 2017); the increase in β-endorphin blood dosage (de Andrade et al., 2017); and the suppression of interleukin-6 increased by spinal cord injury (Mojarad et a., 2018). On the

other hand, LLLT is considered to have few side effects (Chow et al., 2009, Cotler et al., 2015). In agreement with previous studies, present results support that LLLT has positive effects in the relief of neuropathic pain. It is well established that side effects in pharmacological therapy are general problems (Eckerdal and Bastian, 1996, Jameie et al., 2014). Therefore, the combined application of oxytocin with LLLT has the advantage of avoiding side effects by reducing the dosage of drugs.

Functional implication

A previously study demonstrated that the amplitude of cortical responses in the S1 and S2/IOR depends on the electrical stimulation intensity of the dental pulp (Nakamura et al., 2016). Therefore, the reduction of amplitude by OXT or LLLT in response to dental pulp stimulation may reflect a decrement of pain. In addition to the amplitude, pl-ION induced expansion of the activated area, which was significantly reduced by OXT in combination with LLLT. On the other hand, temporal kinetics in the half duration were comparable between sham and pl-ION, which indicates a possibility that pl-ION might not impact the duration of nociception in the S1 and S2/IOR. The symptoms of neuropathic pain can be present not only in the regions innervated by the injured nerve but also in the regions beyond the innervation territory (Finnerup et al., 2016). This indicates that responses to peripheral stimulation may arise not only in the originating cortical regions but also in other cortical regions in animals receiving nerve injury. Indeed, I found that pl-ION induced not only an increase in the amplitude of cortical excitation but also an expansion of the activated area (Fig. 3). The expanded area of excitatory propagation in pl-ION was primarily observed dorsally, in which somatosensory information from other orofacial regions is processed (Fujita et al., 2019b, Nakamura et al., 2015, Remple et al., 2003, Zama et al., 2018, 2019). The disturbance of somatotopy might reflect an aspect of neuropathic pain regarding the largeness of the receptive field of pain and/or other abnormal sensations. The combination of OXT and LLLT, but not either alone, significantly reduced the pl-ION-induced expansion of the activated area. Thus, the combined application of OXT and LLLT may not only reduce the strength of pain sensation but also shrink the expanded receptive field by pl-ION. These suppressive effects may contribute to reducing ectopic pain.

According to previous studies (Kubo et al., 2017, Shinoda et al., 2007, Zama et al., 2019), the decrease in the mechanical thresholds of whisker pad skin reaches a plateau approximately 3 days after pl-ION and persists for at least 14 days after pl-ION. Therefore, my experiments 3 days after pl-ION are at a relatively early stage in pl-ION-induced neuropathic pain. Nerve injury causes central sensitization, i.e., plastic changes in increases in synaptic efficacy and reductions in inhibition (Woolf, 2011). Such plastic changes in the somatosensory pathway, including the cerebral cortex, are considered to be difficult to recover, and the abnormal symptoms are long-lasting. To avoid the transition from acute to chronic pain, it is necessary to suppress abnormal neural activities in the early stage of recovery from peripheral nerve injury. A previous study has demonstrated that the administration of minocycline partially

inhibits hyperexcitation in the cerebral cortex induced by pl-ION, and this suppressive effect is seen only in the case of a preceding injection of minocycline but not the injection after pl-ION (Zama et al., 2019). From this view, the present results suggest that the combined application of OXT and LLLT has a large advantage: it is possible to apply the current method after nerve injury.

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