Abstract: Orexins (Oxs) are multifunctional neuropeptides, secreted from the lateral hypothalamus, that stimulate feeding behavior and energy expenditure. In this study, the direct effects of Oxs on the membrane properties of trigeminal motoneurons (TMNs) were examined, which critically participate in the genesis of rhythmical oral motor activities underlying sucking and mastication. Sprague-Dawley rats (3-6 day-old) were used to obtain whole-cell patch-clamp recordings from TMNs. Bath application of Ox-A depolarized the membrane potential and induced inward current, to obtain whole-cell patch-clamp recordings from TMNs. Bath application of Ox-A increased the peak amplitude and duration at half-amplitude of the medium-duration after hyperpolarization following the action potential. The interspike frequency of steady-state firings during repetitive discharge was increased, along with a shift in the frequency-current relationship occurring toward the left. Extracellular and intracellular Ca2+ were involved in regulating modulatory effects, but a requisite level of intracellular Ca2+ was not essential for Ox-induced upregulation of the interspike frequency. Ox-A also enhanced conditional bursting induced by N-methyl-D-aspartate and 5-HT, suggesting it participates in modulating TMNs’ discharge patterns during various oral motor activities.

Keywords; conditional bursting, neuropeptide, orexin, transient receptor potential channel, trigeminal motoneuron

Introduction
Feeding behavior, crucial for maintaining energy homeostasis, is executed by a combination of cognitive function and emotional changes [1-3]. Rhythmical oral motor activities, such as chewing and sucking, are critical components of the feeding behavior needed to convert a bolus of food into pieces appropriate for deglutition. These activities are carried out by integrating peripheral signals and regulated by functional linkages between higher brain regions, including the hypothalamus and lower brainstem, involving trigeminal neural circuitry [4-9].

The hypothalamus functions as a control center for many autonomic nervous system processes. The satiety and feeding centers are located in the ventromedial and lateral hypothalamus, respectively. Upon being alerted by various external stimuli, these centers monitor blood glucose levels and initiate or suppress feeding behavior, as appropriate [1]. In addition, neurons in the hypothalamus synthesize and release various kinds of neuropeptides, such as agouti-related peptide, neuropeptide-Y, and melanin-concentrating hormone [10-12], thus regulating behavior related to energy balance.

Orexins (Oxs), first identified as endogenous ligands for two orphan G-protein-coupled receptors in 1998, are powerful orexigenic agents produced by neurons in the lateral hypothalamic area that regulate feeding behavior [5,13,14]. Previous studies have revealed that Oxs also help to regulate sleep-wake patterns and related disorders, such as narcolepsy [15-17]. Ox-producing neurons in the lateral hypothalamus and its periphery are found widely throughout the brain, including areas of the monoamine nervous system involving the locus coeruleus, tuberomammillary nucleus, and raphe nucleus, as well as parts of the parasympathetic nervous system involving the lateroposterior tegmental and pedunculopontine tegmental nuclei [6,18,19]. Endogenous Ox ligands bind Ox receptors 1/2 (Ox1/2R), which are G-protein-coupled receptors characterized by their pharmacological characteristics [13,20]. They are widely distributed throughout the central nervous system [21,22].

Trigeminal motoneurons (TMNs) integrate many peripheral and central synaptic inputs from neurons, such as mesencephalic trigeminal and other premotor neurons, and generate oral motor outputs underlying rhythmical oral motor activities, including mastication, swallowing, and suckling [7,23,24]. Previous electrophysiological studies have revealed membrane properties in TMNs involving discharge pattern characteristics and their serotonergic modulation [8,25-27], while immunohistochemical studies indicate orexigenic neurons directly project to the TMNs [18,28,29] that specifically express both Ox1R and Ox2R [30,31]. In addition, a previous study demonstrated that, in rats, the paraventricular injection of Ox shortened the latency period prior to beginning feeding and increased the total amount of food intake [32]. Increased Ox levels in the brain also amplify the electromyographic activities of masticatory muscles, especially jaw-closing muscles, and alter the rhythmicity of the grinding and chewing phases [32].

Based on these findings, it was hypothesized that Ox receptors on TMNs are involved in regulating the TMNs’ burst generation and intrinsic properties underlying rhythmical masticatory muscle activities. However, little is known about Oxs’ direct role in regulating TMNs’ cellular excitability. Therefore, the present study examined the direct modulatory effects of Ox-A, one of two closely related Oxs, on TMN membrane properties involving various firing activity patterns by whole-cell patch-clamp recording.

Materials and Methods
Animals and brain slice preparation
Three- to six-day-old Sprague-Dawley rats were used according to the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (publication no. 85-23, updated 2011), and approved by the Osaka University Graduate School of Dentistry (Osaka, Japan) Animal Care and Use Committee (approval no. Doha-20-005-0). Brainstem slices were prepared as previously reported [27]. Briefly, after the rats were euthanized by halothane inhalation, the brainstems were carefully removed and immersed in oxygenated ice-cold cutting solution (126 mM of NaCl, 3 mM of KCl, 1.25 mM of NaH2PO4, 26 mM of NaHCO3, 10 mM of glucose, 5 mM of MgCl2, and 4 mM of lactic acid). Coronal brain slices (thickness of 300 µm) containing the trigeminal motor nucleus were prepared with a microslicer (Linear slicer PRO 7; Dosaka EM Co., Ltd., Kyoto, Japan) and incubated for 40 min at 37°C before recordings began. The recording solution (normal ACSF) contained 124 mM of NaCl, 3 mM of KCl, 1.25 mM of NaH2PO4, 26 mM of NaHCO3, 10 mM of glucose, 2 mM of CaCl2, and 2 mM of MgCl2. All the solutions were equilibrated with 95% O2 and 5% CO2, and the pH was adjusted to 7.3 (22-24°C). The intrapipette solution was composed of 115 mM of K-glucuronate, 25 mM of KCl, 9 mM of NaCl, 10 mM HEPES, 0.2 mM EGTA, 1 mM of MgCl2, 3 mM of K2-ATP, and 1 mM of Na-GTP. The pH and osmolality (Osm) were adjusted to 7.3 and 280 to 290 mOsm, respectively.
All drugs were dissolved in distilled water or dimethyl sulfoxide and added to the recording solution via rapid perfusion at the following concentrations: 100 nM of Ox-A (Peptide Institute Inc., Ibaraki, Japan), 1 µM of tetrodotoxin (TTX; Wako, Osaka, Japan), 10 µM of SKF96365 (Wako), 50 µM of N-methyl-D-aspartate (NMDA; Sigma-Aldrich Japan, Tokyo, Japan), 10 µM 5-HT (Sigma-Aldrich Japan). Choline-Cl was substituted for NaCl (reduces external Na+ concentration) in the TTX-dissolved recording solution tended to decrease both the membrane depolarization and inward current; raised external Ca2+ (3.5 mM) increased both the membrane depolarization and inward current. *P < 0.05

**Patch-clamp recordings and data analysis**

Whole-cell recordings from TMNs, identified by visual control with infrared differential interference contrast video microscopy, were obtained using a Multiclamp 700B patch-clamp amplifier and pCLAMP acquisition software program, version 9.0 (Axon Instruments, Foster City, CA, USA). Thick-walled borosilicate fire-polished glass (OD, 1.5 mm; ID, 0.86 mm) and a programmable puller (P-97; Sutter Instruments, Novato, CA, USA) were used. All signals were grounded using a 3 M KCl-agar bridge electrode (Ag-AgCl wire) and were filtered using a low-pass Bessel filter at 5 kHz. An uncompensated series resistance ~15 MΩ was compensated by 40–80% and monitored periodically during the experiments. Liquid junction potentials between normal bath and pipette solutions were not corrected offline. Voltage and current signals were digitized and recorded using the pCLAMP acquisition software (Axon Instruments) for subsequent analysis. To determine Ox-A’s effects on membrane properties in current-clamp experiments, the membrane potential was maintained at the control condition value by the application of extrinsic current via the recording pipette.

Data analysis was performed using a combination of software as follows: pCLAMP (Axon Instruments), Sigmaplot 4.0 (Jandel Scientific, San Rafael, CA, USA), and Microsoft Excel. Numeric results were expressed as means ± standard errors. Statistical analysis was performed using SPSS 24.0 statistics program (SPSS Inc, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to evaluate normality and Levene’s test to determine the equality of variances, respectively. Depending upon the normality of variable distribution and the homogeneity of variances, the means of two groups, recorded from the same subset of neurons or independent subset of neurons, were compared with a dependent or independent t-test, respectively. In addition, the means of multiple datasets, measured from the independent or same subset of neurons under different conditions with normality and homoscedasticity, were compared using one-way analysis of variance (ANOVA) or repeated-measures ANOVA, respectively. In repeated-measures ANOVA, Mauchly’s test of sphericity was used. A P value of <0.05 indicated statistical significance unless otherwise stated.

**Results**

The present data are based on the recording from 80 TMNs in rat brainstem slices prepared from 45 postnatal day 3 to 6 animals. The mean values of basic membrane properties included a resting membrane potential of ~−60.5 ± 1.5 mV; 86.5 ± 4.3 MΩ input resistance, and 110.9 ± 8.3 pF membrane capacitance. The criteria for subsequent recordings were established with a resting potential greater than ~−55 mV, action potential amplitude exceeding 80 mV, and input resistance of at least 80 MΩ.

**Effect of Ox-A on TMN subthreshold membrane properties**

Bath application of Ox-A during current-clamp recording of depolarized membrane potentials peaked 5–7 min after application and produced an inward current from holding potentials of ~−60 mV under voltage-clamp recording (Fig. 1A). These voltage and current responses did not change in the presence of TTX and tended to decrease when choline-Cl was substituted for NaCl (reduces external Na+ concentration) in the TTX-dissolved recording solution (control: 6.9 ± 0.7 mV; TTX: 6.7 ± 0.8 mV; TTX + choline-Cl: 4.8 ± 0.6 mV for ΔV; control: 58.5 ± 10.7 pA; TTX: 54.5 ± 9.2 pA; TTX + choline-Cl: 41.4 ± 3.3 pA for ΔI, n = 5; Fig. 1B, C). The Kolmogorov-Smirnov test showed that all the data were normally distributed, and Levene’s test rejected the null hypothesis (ΔV: P = 0.35; ΔI: P = 0.14). There were no statistically significant differences between the values of membrane depolarization and inward current observed among the three groups (one-way ANOVA, ΔV: P = 0.06, ΔI: P = 0.25).

In another subset of neurons, the Ox-A-induced voltage, and current responses were also examined after altered extracellular Ca2+ concentrations. As shown in Fig. 1D and E, reduced external Ca2+ (0.5 mM) conditions decreased the membrane depolarization and inward current induced by Ox-A, whereas elevated concentration external Ca2+ (3.5 mM) conditions created a trend of increases in both values (control: 6.9 ± 0.7 mV; 0.5 mM Ca2+: 3.6 ± 0.5 mV; 3.5 mM Ca2+: 10.4 ± 0.7 mV for ΔV and control: 58.5 ± 10.7 pA; 0.5 mM Ca2+: 33.3 ± 3.3 pA; 3.5 mM Ca2+: 83.7 ± 7.6 pA for ΔI, n = 4). All data showed normality, and Levene’s test rejected the null hypothesis (ΔV: P = 0.59; ΔI: P = 0.57). There was a statistically significant difference between the values of membrane depolarization and inward current among the three groups (one-way ANOVA, P < 0.05). Meanwhile, the post-hoc analysis using Tukey’s test revealed that mean membrane depolarization values were significantly different in the comparison of all combinations among the three external Ca2+ conditions, while the mean values of the inward current only showed a significant difference between reduced (0.5 mM) and elevated (3.5 mM) Ca2+ conditions.

**Effect of transient receptor potential (TRP) channel blocker on Ox-A-induced subthreshold voltage and current response in TMNs**

Previous studies have revealed that TRP channels are Ca2+-permeable and modify receptor-activated Ca2+ influx [33, and, to date, several TRP channel subtypes have been found to be co-expressed and/or co-activated with Ox receptors [34,35]. Therefore, the effects of the potent TRP channel blocker SKF96365 (which blocks receptor-mediated Ca2+ entry) on Ox-A-induced current and voltage responses in TMNs were recorded from another subset of neurons. As shown, pre-incubating slices with SKF96365 remarkably suppressed the Ox-A-induced depolarization changes in membrane potential under current-clamp (control: 7.0 ± 0.8 mV; SKF96365: 3.3 ± 0.7 mV for ΔV; n = 5; Fig. 2A) and inward current under voltage-clamp (control: 65.4 ± 10.3 pA; SKF96365: 30.2 ± 8.1 pA for ΔI; n = 5; Fig. 2B). All the measurement values showed normal distribution and homoscedasticity (Levene’s test, ΔV: P = 0.42, ΔI: P = 0.89). Also, subsequent statistical analysis, conducted by the independent t-test for paired samples, revealed significant differences in both voltage and current responses between the control and SKF96365 groups (Student’s t-test, ΔV: P < 0.05; ΔI: P < 0.05).
Ox-A modulates spike-discharge characteristics in TMNs

Single action potentials, followed by medium-duration after hyperpolarization (mAHP), were induced by a short (3-ms) depolarizing pulse from an adjusted same holding potential prior to, and after, Ox-A application. Ox-A caused a significant increase in mAHP amplitude and lengthened the half-amplitude duration of mAHP, whereas no significant changes were observed in the threshold, spike height, or spike half-amplitude duration (Fig. 3A, Table 1). A repetitive spike discharge, induced by a one-second depolarizing step-pulse, was also recorded. The discharge characteristics, before and after Ox-A application, were compared (Fig. 3B). Rheobasic currents to induce minimum train discharge were normally distributed and showed homoscedasticity between two conditions (Levene’s test, \( P = 0.23 \)). The mean value of the rheobasic current was significantly reduced by Ox-A (control: 355.5 ± 66.9 pA; Ox-A: 266.7 ± 37.3 pA; Student’s \( t \)-test, \( P = 0.05; n = 6 \)).

The first interspike interval (ISI), measured between the first and second spikes, and the interspike frequency for the steady-state period, as measured by the mean of the last five ISIs recorded from the same subset of neurons under different current stimuli, were examined before and after Ox-A application. The frequency-current (\( f-I \)) relationship for the first ISI showed Ox-A provoked little change (repeated-measures ANOVA, \( P = 0.23; n = 6 \); Fig. 3D). Although there was no significant difference, the interspike frequency for the steady-state period demonstrated a trend of increase, and the \( f-I \) relationship consistently shifted toward the left by Ox-A (repeated-measures ANOVA, \( P = 0.05; n = 6 \); Fig. 3D).

Extracellular Ca\(^{2+} \) is involved in the regulation of Ox-A-induced modulation of TMNs

Thereafter, it was examined whether the alternation of extracellular Ca\(^{2+} \) concentration helps to regulate spike-frequency control by Ox-A application. In repetitive spike discharge under decreased Ca\(^{2+} \) conditions (0.5 mM), Ox-A (100 nM) showed a trend toward increased spike frequency in the steady-state period (repeated-measures ANOVA, \( P = 0.13; n = 5 \)), and shifted f-I curves toward the left, as shown in the normal Ca\(^{2+} \) condition (Fig. 4A, B). In contrast, raising the external Ca\(^{2+} \) concentration to 3.5 mM augmented the amplitude of the post-spike mAHP after each spike during repetitive firing, which was enhanced by Ox-A (Fig. 4C). Ox-A shifted f-I curves toward the right in the steady-state period, with a modest decrease in interspike frequency (repeated-measures ANOVA, \( P = 0.21; n = 5 \); Fig. 4D).

Intracellular Ca\(^{2+} \) is involved in the regulation of Ox-A-induced modulation of TMNs

To determine whether the increased spike frequency by Ox-A depended on changes in intracellular Ca\(^{2+} \), BAPTA (10 mM) was added to the intrapipette solution to chelate free Ca\(^{2+} \). Fig. 5A and B show representative examples of action potential and repetitive firing activity prior to, and after, Ox-A application. As demonstrated, 20 to 30 min after whole-cell recordings, the mAHP after action potential was eliminated, and Ox-A promoted reduced change in the voltage trajectory, while the interspike frequency in steady-state firing increased but not significantly by Ox-A (repeated-measures ANOVA, \( P = 0.13; n = 5 \); Fig. 5C).

Effect of Ox-A on conditional bursting activity in TMNs

A previous study demonstrated that rat TMNs are capable of conditional bursting, induced by NMDA receptor activation, and the synergistic effect of 5-HT on the activity [26]. Therefore, the effects of Ox-A on bursting behavior via ligand-gated response by NMDA and 5-HT were examined. Bath application of NMDA and 5-HT depolarized the membrane potential and produced tonic firing activity, which was transformed into bursting activity by a hyperpolarized current injection (Fig. 6A, Ba). Subsequent Ox-A application further depolarized the membrane potential, and bursting activity was induced from an adjusted same holding potential. Herein, 10 consecutive bursts were analyzed and bursting characteristics were compared including burst duration, burst frequency, interspike frequency, and the interburst interval prior to, and after, Ox-A application. The Kolmogorov-Smirnov test revealed that all the collected data were normally distributed, and Levene’s test rejected the null hypothesis. A dependent

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**Table 1** Action potential properties before and after Ox-A

| Parameter          | Control       | Ox-A (\( n = 6 \)) |
|--------------------|---------------|-------------------|
| AP                 | Control       | Ox-A              |
| Threshold (mV)     | –44.0 ± 0.8   | –42.4 ± 0.8*      |
| Spike height (mV)  | 95.9 ± 1.4    | 95.0 ± 1.8        |
| Half width (ms)    | 1.55 ± 0.1    | 1.54 ± 0.2        |
| Slope (mV/ms)      | –65.3 ± 7.1   | –68.2 ± 8.2       |
| AHP                | –6.06 ± 0.9   | –8.27 ± 1.0*      |
| Duration at half amplitude (ms) | 58.2 ± 5.2 | 67.5 ± 8.0*      |

* \( P < 0.05 \), paired \( t \)-test

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### References

1. [26] Reference text.

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**Fig. 2** Effect of TRP channel antagonist SKF96365 on current and voltage responses in TMNs. In the presence of SKF96365 (10 \( \mu M \)), changes in membrane potential depolarization, under current-clamp (A) and inward current under voltage-clamp (B), were substantially decreased. *\( P < 0.05 \).

**Fig. 3** Action potential properties before and after Ox-A (100 nM). Averaged value are means ± SE.

**Fig. 4** Effect of Ox-A on spike-discharge characteristics in TMNs. A: Representative voltage responses evoked by a short (3-ms) depolarizing step-pulse prior to, and after, the application of Ox-A (100 nM) in postnatal day 5 TMN. Ox-A increased the peak amplitude and duration of mAHP (see summary in Table 1). B: Repetitive firing activities induced by long (one-second) step-pulse depolarization prior to, and after, Ox-A application (100 nM) in postnatal day 6 TMN. C: The frequency-time relationship was increased by Ox-A. D: The frequency-current relationships for steady-state firing shifted to the left following the application of Ox-A.
t-test for paired samples showed that Ox-A significantly increased burst duration (control: 1068 ± 115 ms; Ox-A: 1394 ± 88 ms; n = 5; P < 0.05), burst frequency (control: 0.073 ± 0.009 Hz; Ox-A: 0.144 ± 0.015 Hz; n = 5; P < 0.05), and interspike frequency (control: 9.94 ± 0.99 Hz; Ox-A: 10.6 ± 0.52 Hz; n = 5, P < 0.05). It also decreased the interburst interval (control: 12.0 ± 0.20 ms; Ox-A: 5.8 ± 0.06 ms; n = 5; P < 0.05, Fig. 6Bb, C).

**Discussion**

The present electrophysiological study first demonstrated that Ox-A could directly modulate, and show excitatory effects on, TMN membrane properties accompanied by inward currents and depolarized the resting membrane potential. Similar effects by Ox-A application have previously been reported in locus coeruleus [36], nucleus tractus solitarius [37,38], dorsal motor nucleus of vagus [39], and pedunculopontine tegmental [40] neurons.

In the present study, membrane depolarization and production of inward current by Ox-A persisted in the presence of TTX with blockade of peripheral synaptic inputs, indicating that Ox-A could directly act on TMNs similar to other neurons, as reported previously [36,37,39].

Immunohistochemical studies have revealed that orexigenic neurons directly project to TMNs [18,28,29] that express orexin receptor type 1 (Ox1R) and orexin receptor type 2 (Ox2R) [30,31]. Though the comparable expression of both Ox1R and Ox2R was shown in the TMNs of adult rats [30,31], Ox1R has a binding affinity for Ox-A that is 50 times greater than that of Ox-B. By contrast, Ox2R shows similar binding affinities for both Ox-A and Ox-B [5]. Another study, using patch-clamp experiments, demonstrated that OX1R facilitates Ca2+ entry, accompanied by an inward current and membrane depolarization [35]. Although further investigation is needed to reveal any postnatal changes in Ox1R and Ox2R expression, Ox1R may play a role in the Ox-A-induced modulation of TMNs during early postnatal development.

In addition, lowering either the external Na+ or Ca2+ concentrations caused a substantial decrease in inward current and membrane potential depolarization changes. Higher external Ca2+ increased both parameters, suggesting the involvement of Na+ and Ca2+ as charge carriers in voltage and current responses induced by Ox-A in TMNs. Likewise, increased excitability with membrane depolarization and intracellular Ca2+ elevation have been observed as typical responses to Oxs by other types of neurons [41,42]. Furthermore, significantly decreased Ox-A-induced neuronal excitation, by membrane depolarization and inward current in TMNs, caused by TRP channel blocker SKF96365 herein indicates a possible involvement of TRP channel-mediated Ca2+ entry into TMNs. Most TRP channels are nonselective cation channels that are permeable to Ca2+ [33]. Similar to the OxR family, the TRPC subfamily, which includes TRPC1-TRPC7, is known to be regulated by G-proteins and lipid signaling molecules, such as diacylglycerol. TRPC channels are widely distributed throughout different tissues, including the brain and central nervous system. Furthermore, the co-expression of OxsRs and TRPC channels occurs in several different areas of the brain [43]. Of note, these subtypes may exist as either homotetrameric or heterotetrameric ion channel complexes. Previous studies have indicated that channel complexes containing members of the TRPC3/6/7 subfamily contribute to the Ox1R-mediated Ca2+ influx in IMR-32 cells [34]. Also, TRPC1 and TRPC3 have been found to open after Ox1R activation in CHO-OX1-C1 cells, in which the functional interactions between the two channels were weak [35]. Among the subfamily of TRPC channels, TRPC3 channels behave as a Ca2+-activated nonselective cation channel and are strongly stimulated by intracellular Ca2+ [33,44]. Therefore, TRPC3-mediated cation entry might be involved with the Ox-A-mediated modulation of TMNs, although further investigation is needed to deter-

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**Fig. 4 Involvement of external Ca2+ in the regulation of Ox-A-induced modulation of repetitive spike-discharge characteristics in TMNs.**

A: Representative traces of repetitive spike discharge prior and after Ox-A application (100 nM) on postnatal day 6 TMN under reduced external Ca2+ (0.5 mM) conditions; mAHP following each spike was underdeveloped. B: Frequency-current relationships of repetitive spike discharge prior to, and after, Ox-A application (100 nM) on postnatal day 6 TMN under reduced external Ca2+ (0.5 mM) conditions; mAHP following each spike was less prominent than that with normal external Ca2+ (2.0 mM). D: Steady-state frequency-current traces of repetitive spike discharge prior to, and after, Ox-A application (100 nM) on postnatal day 6 TMN under reduced external Ca2+ (0.5 mM) conditions; mAHP following each spike was shifted to the left after application of Ox-A. C: Representative traces of repetitive spike discharge prior to, and after, Ox-A application on postnatal day 6 TMNs under reduced intracellular Ca2+ (3.5 mM) conditions; mAHP following each spike was more prominent than that with normal external Ca2+ (2.0 mM). D: Steady-state frequency-current relationships shifted to the right, with decreased spike frequency after Ox-A application.

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**Fig. 5 Requisite intracellular Ca2+ is not essential for the upregulation of spike frequency by Ox-A.** A: Intracellular BAPTA (10 mM) application eliminated mAHP following action potential, and Ox-A application thereafter showed less change. B: Representative traces of repetitive spike discharge prior to, and after, Ox-A application on postnatal day 6 TMNs under reduced intracellular Ca2+ conditions. C: Steady-state frequency-current shifted to the left, with increased spike frequency after Ox-A application.
Fig. 6 Effect of Ox-A on conditional bursting activity characteristics in TMNs. Bath application of NMDA and 5-HT depolarized membrane potential, and subsequently promoted bursting activities, in response to hyperpolarizing current injection (A, Ba). The additional Ox-A application (100 nM) further depolarized the membrane potential and transformed bursting into continuous spiking. Hyperpolarizing current injection recovered conditional bursting with enhanced duration (Bb). C: burst parameters. Ox-A increased burst duration (a), decreased interburst intervals (b), and increased burst frequency (BF) (c). The interspike frequency (ISF) of each burst was also increased by Ox-A (d). *P < 0.05

Because SKF96365 did not completely suppress the Ox-A-induced changes in TMNs, other mechanisms, such as the reduction in resting K+ conductance, might help modulate these changes. This would result in higher input resistance and provide a target for depolarizing the resting membrane potential. This would also be accompanied by a decrease in rheobase and increased spike frequency. This was previously discussed as a mechanism of 5-HT-induced modulation of the TMNs’ excitability [25].

The present study further demonstrated that Ox-A increased the peak amplitude and duration of post-spike AHP in TMNs. Previous research has shown the critical contributions of Ca2+-dependent K+ conductance in shaping single-spike AHP and complete blockade of mAHP by apamin, a specific blocker of Ca2+-dependent K+ channels (i.e. SK channels) in guinea pig and rat TMNs [8,47]. In the present study, mAHP was Ca2+-dependent since raising extracellular Ca2+ levels enhanced intraspike AHP during repetitive firing, and Ox-A’s modulatory effect on mAHP amplitude was more prominent relative to normal extracellular Ca2+ concentrations, probably due to an increase in Ca2+ influx. These trends were also recognized in a previous study, in which 5-HT-induced suppression of mAHP was enhanced by elevated extracellular Ca2+ conditions [27]. Based on the findings of the present study, Ox-A possibly stimulates Ca2+ influx, thereby elevating intracellular Ca2+ levels, which most likely activates Ca2+-dependent K+ conductance at rest, leading to mAHP amplification, since depolarization could be altered by adjusting the external Ca2+ concentration.

mAHP is also known to be a candidate for adaptive control of firing properties, and AHP attenuation tends to enhance motoneuron excitability by increasing the input-output gain [25,27]. Moreover, AHP amplification quickens adaptation during repetitive discharge by lowering spike frequency. Here, the minimum current amplitude (rheobase) required to elicit a repetitive spike discharge, evoked from the same holding potential, was significantly decreased, while the frequency-current relationship of TMNs shifted left. These results suggest that TMNs require less synaptic current to depolarize the membrane potential to the spike threshold and produce a spike train with a higher frequency in the presence of Ox-A. Interestingly, although the rheobase, in the presence of Ox-A, was still smaller than the control, heightened extracellular Ca2+ shifted the I-I curve to the right, with decreased steady-state spike frequency. In contrast, nominal extracellular Ca2+ conditions or buffering of intracellular Ca2+ to lower levels by the intrappetite application of BAPTA caused a substantial decrease in mAHP, upon which Ox-A had little effect. The I-I curve shifted to the left in this context with increased steady-state spike frequency. These results suggest that other conductance may play a role in the facilitative modulation of intraspike frequency during repetitive spike discharge by Ox-A. Reduced levels of leak K+ conductance might increase membrane excitability and increase intraspike frequency, as discussed earlier. Since Ox-A-induced current persisted in the presence of both TTX and chloride substitute for NaCl, TTX-resistant Na+ conductance could be another candidate mechanism, which may be confirmed by future studies.

NMDA receptor activation is essential to the central pattern generation of rhythmical trigeminal motor activities in vitro [9,48,49] and oral motor activity in vivo [50,51]. NMDA receptor activation is also critically involved in conditional bursting production, with a synergistic effect brought on by 5-HT, in rat TMNs [26]. Endogenous serotonergic activation has not been reported to be critically involved in generating rhythmic jaw movements; rather, it exerts modulatory effects on masticatory movements [52]. Immunohistochemical studies have reported the expression of 5-HT1A receptors in trigeminal motor nuclei and surrounding areas during the neonatal period [53]. The colocalization of 5-HT1A subtypes and Ox has been analyzed in the hypothalamus, posterior tubercle, thalamus, and mesencephalic tectum using immunofluorescence [54,55], but colocalization has not yet been reported in trigeminal neurons. In the present study, Ox-A enhanced bursting activities, accompanied by increased burst duration and frequency. These results, together with those showing a declined Ca2+ entry by lowering external Ca2+ to eliminate the bursting and that internal Ca2+ is critically involved as an intracellular messenger in the genesis of bursting [26], suggest that Ox-A-induced Ca2+ influx enhances bursting activity in TMNs.

In conclusion, the present study revealed that Ox-A, a multifunctional neuropeptide with orexigenic effects, enhances TMN excitability by membrane depolarization and production of inward current. Specifically, Ox-A decreased the minimum stimulus, bringing the membrane potential to the spike threshold. In turn, this produced a spike discharge with a higher frequency and may modulate the final discharge pattern of TMNs during various oral motor activities, such as sucking and chewing.

Acknowledgments
This work was funded by KAKENHI Grants-in-Aid for Scientific Research (C) (15K11245) from the Japan Society for the Promotion of Science.

Conflict of interest
No conflict of interest, financial or otherwise, is declared by the authors.
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