



OPEN Optogenetic activation of serotonergic neurons changes masticatory movement in freely moving mice

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The serotonergic system modulates the neural circuits involved in jaw movement; however, the role of serotonin (5-HT) neurons in masticatory movement remains unclear. Here, we investigated the effect of selective activation of 5-HT neurons in the dorsal raphe nucleus (DRN), or the raphe obscurus nucleus (ROb), on voluntary masticatory movement using transgenic mice expressing the channelrhodopsin-2 (ChR2) mutant (C128S) in central 5-HT neurons. During voluntary mastication, DRN blue light illumination increased masticatory frequency and decreased the root mean square peak amplitude of electromyography (EMG) in the masseter muscles. DRN blue light illumination also decreased EMG burst duration in the masseter and digastric muscles. These changes were blocked by a 5-HT_{2A} receptor antagonist. Conversely, ROb blue light illumination during voluntary mastication did not affect masticatory frequency and EMG bursts in the masseter and digastric muscles. DRN or ROb blue light illumination during the resting state did not induce rhythmic jaw movement such as mastication but induced an increase in EMG activity in masseter and digastric muscles. These results suggest that both DRN and ROb 5-HT neurons may facilitate jaw movement. Furthermore, DRN 5-HT neuron may contribute to changes in masticatory patterns during the masticatory sequence.

Keywords Mastication, Serotonin, Dorsal raphe, Channelrhodopsins, Optogenetics, Electromyography

Masticatory jaw movements—characterized by rhythmic alternating contractions of jaw-closing and -opening muscles—are known to be modulated by a number of neuromodulators (e.g., serotonin (5-HT), orexin, histamine, and neuropeptide Y)^{1–4}. 5-HT neurons, originating in the raphe nuclei, innervate almost the entire brain region and play a critical role in various behaviors such as social⁵, sleep-awake⁶, and feeding behaviors⁷. In addition, 5-HT is involved in oral motor functions. 5-HT neuron activity in the dorsal raphe nucleus (DRN)^{8,9} and caudal raphe—including the raphe obscurus nucleus (ROb)¹⁰—is increased during rhythmic oral motor behavior (e.g., chewing, licking, and grooming). Systemic injection of the 5-HT agonist quipazine decreases the electromyography (EMG) activity burst amplitude in the masseter and digastric muscles. Quipazine also raises the electrical threshold for inducing rhythmic jaw movements elicited by repetitive stimulation of the masticatory area of the cerebral cortex in anesthetized guinea pigs¹¹. These results indicate that 5-HT affects masticatory jaw movements. Additionally, 5-HT has an influence on the neural circuits containing trigeminal motor neurons which transmit coordinated motor outputs to the masticatory muscles and receive 5-HT immunoreactive boutons from the ROb and raphe pallidus nucleus (RPa)¹². Our previous study using in vitro brainstem slice preparations showed that 5-HT increases the excitability of jaw-closing motoneurons by inducing membrane depolarization and enhancing glutamate receptor-mediated postsynaptic potentials^{13–15}. Therefore, we hypothesized that the serotonergic system, which is involved in controlling jaw movement, plays an important role in mastication.

Optogenetic techniques using a combination of genetic and optic approaches can control the activity of specific neuronal types at any time in freely moving animals and are used in various motor behavior experiments^{16–18}.

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Here, we analyzed EMG activity in the masseter and digastric muscles, a well-established method in the analysis of masticatory patterns^{3,19–21}, using double transgenic mice expressing the channelrhodopsin-2 (ChR2) mutant (C128S) only in central serotonergic neurons^{22,23} to ascertain the role of 5-HT neurons in voluntary mastication.

Results

Effect of optogenetic activation of ChR2-expressing 5-HT neurons in DRN on masticatory movements

To determine whether DRN 5-HT neuron activation affects masticatory movements, we investigated the effects of DRN blue light illumination on EMG activity during voluntary mastication. To activate DRN 5-HT neurons, we used transgenic mice expressing specifically the ChR2 (C128S) in central 5-HT neurons^{22,23} and implanted an optic fiber near the DRN (Fig. 1a,b). To quantify masticatory muscle activity, we recorded EMG signals from both the masseter and digastric muscles. Optogenetic activation of DRN 5-HT neurons by blue light (470 nm) illumination during voluntary mastication changed rhythmic masticatory activity to a higher frequency and decreased masseter activity (Fig. 1c).

To verify that this masticatory movement change was indeed due to ChR2 blue light activation and no other non-specific light stimulation, we substituted yellow light (590 nm) in the DRN as a control (Fig. 1d). Subsequently, we analyzed and compared 10 consecutive masticatory cycles after applying blue light (activation) and yellow light (control) (Fig. 2a). The DRN blue light illumination during voluntary mastication increased the masticatory rhythm frequency (control: 4.7 ± 0.9 Hz; activation: 8.7 ± 0.7 Hz, $n=8$; $P<0.001$; Fig. 2b). Furthermore, DRN blue light illumination inhibited pellet biting, and a high masticatory cycle frequency period (>7 Hz) persisted for 12.8 ± 4.1 s ($n=8$; Fig. S2a).

Regarding the EMG bursts, DRN blue light illumination during voluntary mastication significantly decreased the root mean square (RMS) peak amplitude of the masseter muscles (control: 0.60 ± 0.26 mV; activation: 0.22 ± 0.10 mV, $n=8$, $P<0.01$; Fig. 2c) and duration of the masseter EMG bursts (control: 102.8 ± 16.8 ms; activation: 65.5 ± 14.0 ms, $n=8$, $P<0.01$; Fig. 2d). Conversely, DRN blue light illumination during voluntary mastication did not significantly change the RMS peak amplitude of the digastric muscles (control: 0.29 ± 0.13 mV; activation: 0.22 ± 0.10 mV, $n=7$, $P=0.45$; Fig. 2e), but significantly decreased the duration of the digastric EMG bursts (control: 112.5 ± 20.1 ms; activation: 85.7 ± 27.2 ms, $n=7$, $P<0.01$; Fig. 2f). The phase relationships indicated by circular statistics did not differ between yellow and blue light illumination of the DRN for masseter/digastric muscle coordination (Fig. 3; Table 1).

These results indicate that activation of DRN 5-HT neurons during voluntary mastication drastically alters the characteristics of masticatory muscle activity without alternation of masseter/digastric muscle coordination.

Effects of optogenetic activation of DRN 5-HT neurons on masticatory movements at different masticatory stages

To determine whether DRN 5-HT neuron activation induced the initiation of rhythmic EMG activity, we examined the effects of optogenetic activation of DRN 5-HT neurons while the mice were at rest. We defined a state in which masseter and digastric muscle activity did not exceed 2 S.D. for 30 s as “resting.” Unlike during voluntary mastication, DRN blue light illumination during the resting state did not induce mastication-like rhythmic EMG activity in the masseter or digastric muscles (Fig. 5a). However, DRN blue light illumination during the resting state significantly increased EMG activity in masseter (control: $90.8 \pm 27.8\%$; activation: $214.0 \pm 48.9\%$, $n=5$, $P<0.01$; Fig. 5b) and digastric muscles (control: $113.4 \pm 40.1\%$; activation: $296.0 \pm 61.1\%$, $n=5$, $P<0.05$; Fig. 5b).

The masticatory sequence consists of two stages—incision and chewing stages^{2,19}. The transgenic mice used here also had an incision stage, with unstable but partially fast (>7 Hz), small bursts of the masseter muscles, and a chewing stage with slow, large bursts of these muscles (Fig. S1). To verify whether DRN blue light illumination induced a masticatory pattern change at any masticatory stage, we applied light at two points of the masticatory sequence—the incision (immediately after the start of feeding) and chewing (incision stage followed by large masseter muscle activity) stages. These two stages were confirmed by visual observation and EMG recordings. DRN blue light illumination during both stages induced rapid and small rhythmic EMG activity (Fig. 5c,d).

After DRN blue light illumination during the incision and chewing stages, there was no difference in the masticatory frequency (unpaired Student's *t* test, incision: 8.6 ± 0.7 Hz, $n=7$; chewing: 8.8 ± 1.6 Hz, $n=6$, $P=0.82$; Fig. 5e) and high frequency period of the masticatory cycle (unpaired Student's *t* test, incision: 13.0 ± 5.0 s, $n=7$; chewing: 10.2 ± 4.4 s, $n=6$, $P=0.76$; Fig. 5f). Similarly, in the incision and chewing stages, there was no significant difference in the properties of the induced small EMG bursts in the masseter (unpaired Student's *t* test, incision: $n=7$; chewing: $n=6$; RMS peak amplitude: $P=0.40$; duration: $P=0.67$; Fig. 5g,h) and digastric (unpaired Student's *t* test, incision: $n=7$; chewing: $n=5$; RMS peak amplitude: $P=0.86$; duration: $P=0.97$; Fig. 5i,j) muscles. These results suggest that DRN 5-HT neuron activation may not be responsible for masticatory movement initiation, but may modulate ongoing masticatory muscle activity, irrespective of the masticatory stage.

5-HT_{2A} receptor involvement in the modulation of masticatory movements by optogenetic activation of DRN 5-HT neurons

Because 5-HT_{2A/2C} and 5-HT_{1A} receptors contribute to the generation of trigeminal rhythmic activity²⁴, we examined whether these receptors were involved in increased masticatory frequency and decreased EMG bursts by the DRN blue light illumination. After systemic administration of the selective 5-HT_{2A} receptor antagonist MDL100907 (0.3 mg/kg, s.c.), DRN blue light illumination did not induce rapid masticatory frequency (control: 4.0 ± 0.2 Hz, activation: 4.4 ± 0.7 Hz, $n=5$; $P=0.29$; Fig. 6a,b). Furthermore, DRN blue light illumination did not change masseter EMG bursts (RMS peak amplitude: control: 0.47 ± 0.12 mV; activation: 0.44 ± 0.16 mV, $n=5$, $P=0.62$; duration: control: 111.8 ± 25.8 ms; activation: 102.2 ± 19.2 ms, $n=5$, $P=0.46$; Fig. 6a,c,d) and digastric

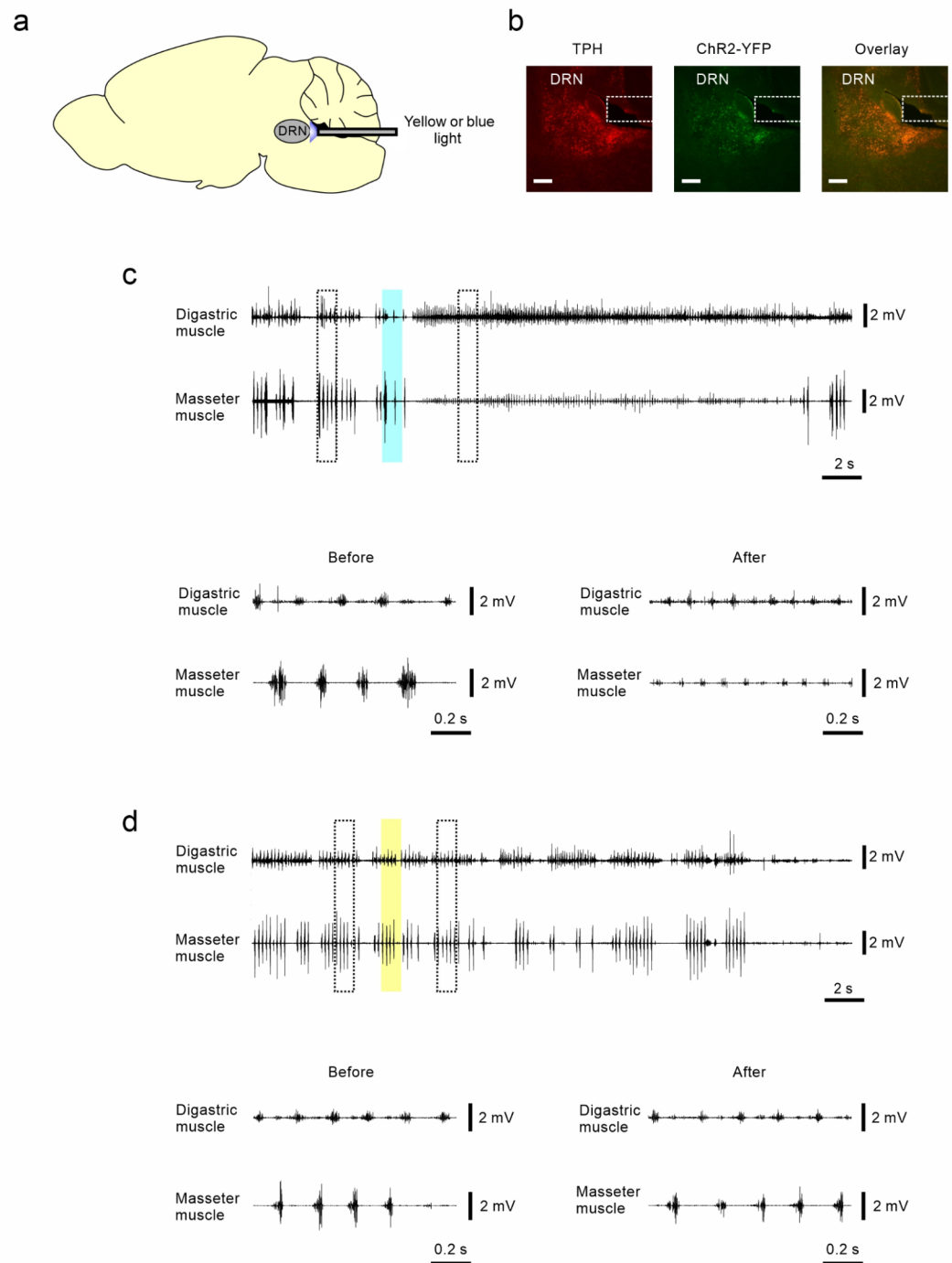


Fig. 1. Optogenetic activation of DRN 5-HT neurons during voluntary mastication alters muscle activity patterns. **(a)** Schematic illustration of the position of the optical fiber stereotactically inserted into the DRN of double transgenic mice expressing ChR2 in central 5-HT neurons for DRN 5-HT neuron activation. **(b)** Representative images showing Tph2-IR neurons (Alexa Fluor 594, red), YFP-IR neurons (Alexa Fluor 488, green) indicating ChR2 (C128S) expression, and the merged image in DRN. Scale bars = 300 μ m. The dashed white lines indicate optic fiber placement. **(c)** and **(d)** Representative EMG recordings of digastric and masseter muscles during voluntary mastication before and after blue light application (470 nm; blue bar) for optogenetic activation of DRN 5-HT neurons as activation **(c, top)**, or yellow light application (590 nm; yellow bar) as control **(d, top)**. Expanding EMG recordings picked from top EMG recordings (dotted box outlines) before and after light application are indicated at the bottom in **c** and **d**. DRN dorsal raphe nucleus, EMG electromyography, IR immunoreactive.

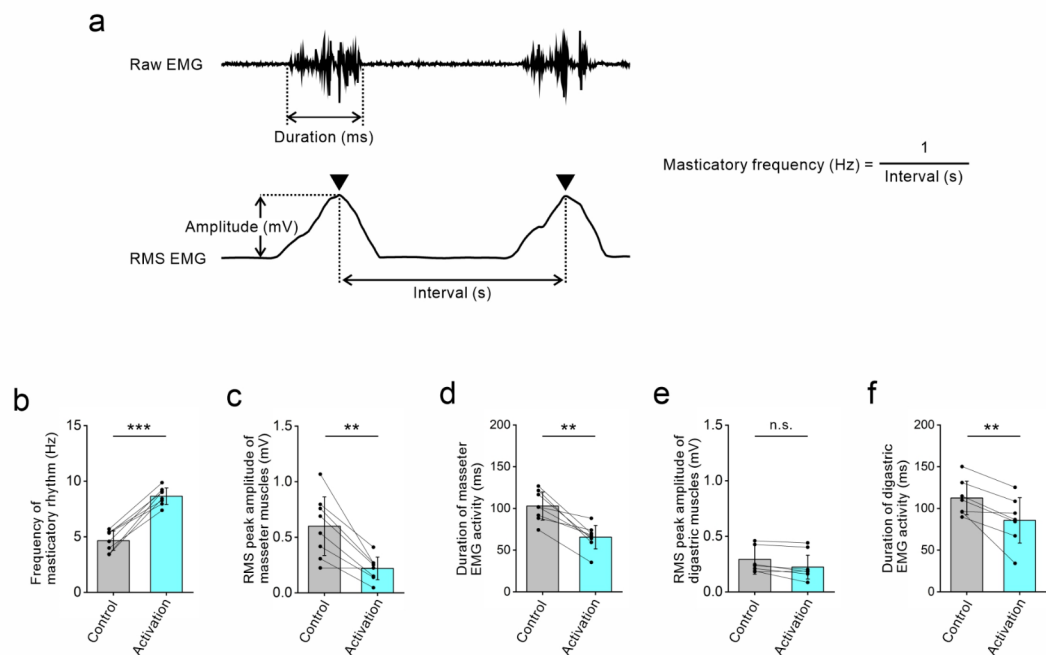


Fig. 2. Optogenetic activation of DRN 5-HT neurons during voluntary mastication increases masticatory frequency and decreases EMG bursts. **(a)** Representative of raw and RMS EMG of masseter muscles during mastication and measured parameters. The masticatory frequency was calculated from the interval between RMS peaks. **(b)** Pooled data for masticatory frequency after yellow (control) or blue light (activation) application. **(c)** and **(d)** Pooled data for RMS peak amplitude **(c)** and duration **(d)** in masseter EMG bursts after yellow or blue light application. **(e)** and **(f)** Pooled data for RMS peak amplitude **(e)** and duration **(f)** in digastric EMG bursts after yellow or blue light application. ** $P < 0.01$, *** $P < 0.001$ (paired Student's t test). Comparisons with n.s. have $P > 0.05$ and are considered not significant. Data are expressed as mean \pm SEM. RMS root mean square.

EMG bursts (RMS peak amplitude: control: 0.18 ± 0.04 mV; activation: 0.20 ± 0.05 mV, $n = 4$, $P = 0.38$; duration: control: 121.5 ± 32.7 ms; activation: 125.3 ± 23.7 ms, $n = 4$, $P = 0.38$, Fig. 6a,e,f) after MDL100907 application.

DRN blue light illumination induced rapid, small masticatory movements with systemic administration of the 5-HT_{2c} receptor antagonist SB242084 (1 mg/kg, i.p.) or the selective 5-HT_{1A} receptor antagonist WAY100635 (1 mg/kg, i.p.), similar to the results recorded in the absence of any antagonists (Fig. S2). These results suggest that 5-HT_{2A} receptors contribute to altered masticatory patterns evoked by DRN blue light illumination.

Effects of optogenetic activation of ROB 5-HT neurons on masticatory movements

5-HT neurons in the caudal raphe, including the ROB, innervate many brainstem regions including the trigeminal motor nucleus and central pattern generator (CPG) for mastication²⁵. We therefore investigated the effects of optogenetic activation of ROB 5-HT neurons using transgenic mice with implanted optical fibers above the ROB (Fig. 7a). To determine whether activation of ROB 5-HT neurons induced the initiation of rhythmic EMG activity, we examined the effect of optogenetic activation of ROB 5-HT neurons during the resting state. This activation did not induce rhythmic EMG activity such as mastication (Fig. 7b), but significantly increased EMG activity in masseter (control: $90.8 \pm 27.8\%$; activation: $214.0 \pm 48.9\%$, $n = 5$, $P < 0.01$; Fig. 7c) and digastric (control: $113.4 \pm 40.1\%$; activation: $296.0 \pm 61.1\%$, $n = 5$, $P < 0.05$; Fig. 7c) muscles. Subsequently, we examined the effects of optogenetic activation of ROB 5-HT neurons on EMG activity during voluntary mastication. ROB blue light illumination during voluntary mastication did not affect masticatory frequency (control: 4.1 ± 0.5 Hz; activation: 4.4 ± 0.6 Hz, $n = 5$, $P = 0.61$; Fig. 7d,e), masseter EMG bursts (RMS peak amplitude: control: 0.56 ± 0.21 mV; activation: 0.55 ± 0.17 mV, $n = 5$, $P = 0.98$, duration: control: 92.6 ± 9.1 ms; activation: 96.3 ± 12.7 ms, $n = 5$, $P = 0.66$; Fig. 7f,g) and digastric EMG bursts (RMS peak amplitude: control: 0.4 ± 0.1 mV; activation: 0.4 ± 0.1 mV, $n = 5$, $P = 0.95$; duration: control: 75.0 ± 12.9 ms; activation: 86.7 ± 14.4 ms, $n = 5$, $P = 0.09$; Fig. 7h,i). The phase relationships indicated by circular statistics did not differ between ROB yellow and blue light illumination for masseter/digastric muscle coordination ($n = 5$; Fig. 4; Table 1). These results indicate that the activation of

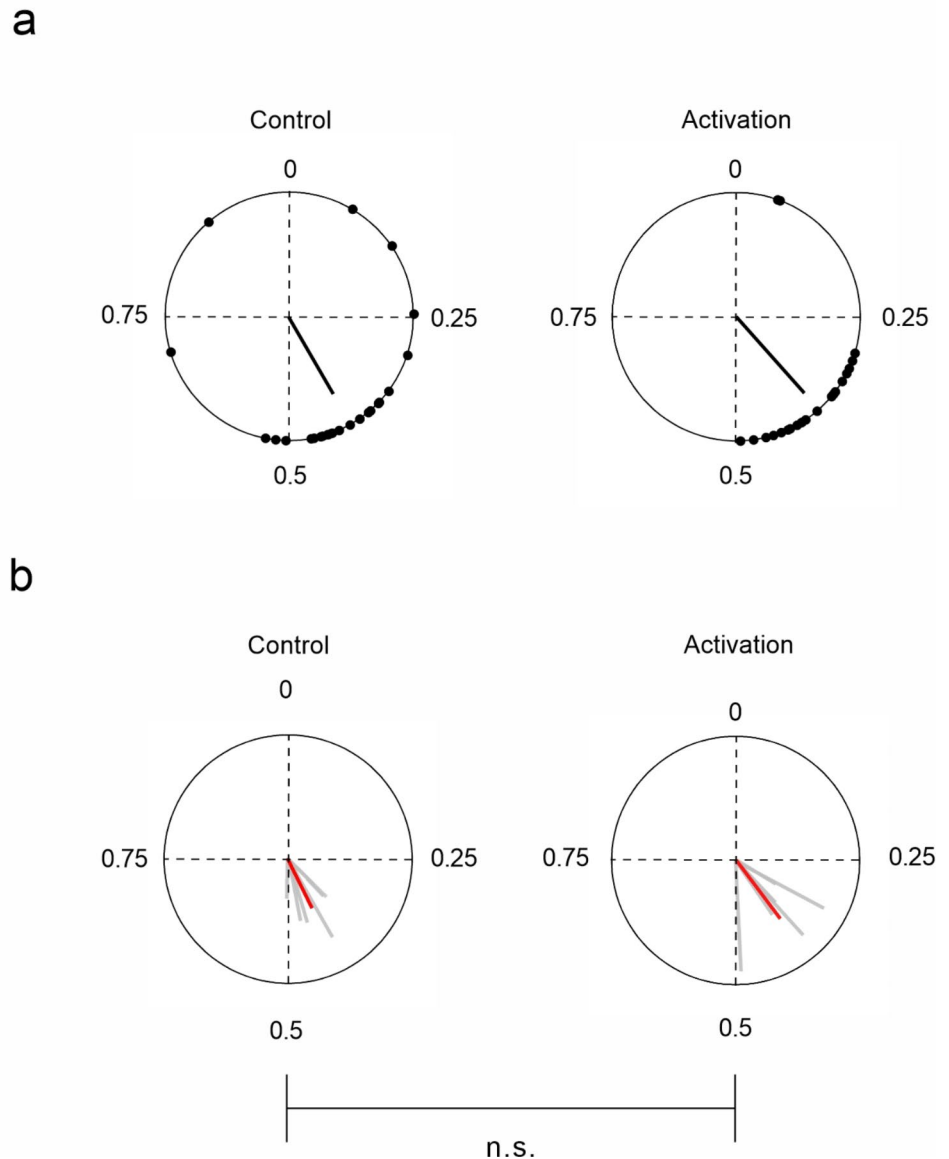


Fig. 3. Optogenetic activation of DRN 5-HT neurons does not involve coordination between masseter and digastric muscle activity during voluntary mastication. **(a)** Representative circular plots showing the relative burst phases recorded from the masseter muscles relative to the digastric muscles. The black dots on each circle indicate the phase shift between the corresponding RMS peak of the masseter-digastric muscle activity alternation after yellow (left) or blue (right) light application. Black straight lines indicate mean coordination r-vectors **(b)** Circular plot summary showing the phasing of all seven animals. Gray and straight red lines indicate the mean coordination r-vectors for individual animals and animal groups, respectively. Comparisons with n.s. have $P > 0.05$ and are considered not significant. The Watson–Williams test for phase comparisons did not show any statistically significant differences between the application of yellow and blue light for individual animals.

ROb 5-HT neurons increased EMG activity in masseter and digastric muscles during the resting state; however, no significant change in masticatory movement was observed after ROb blue light illumination during voluntary mastication.

Discussion

Here, we provide evidence for the involvement of the serotonergic system in voluntary masticatory movements. We found that DRN blue light illumination during voluntary mastication increased masticatory frequency (4.7 ± 0.9 to 8.7 ± 0.7 Hz) and decreased the RMS peak amplitude in masseter muscles and duration of EMG bursts in masseter and digastric muscles. The masticatory sequence is classified into two stages based on the

	DRN		ROb	
	Control	Activation	Control	Activation
<i>r</i> -value	0.45 ± 0.05	0.65 ± 0.08	0.61 ± 0.09	0.66 ± 0.09
Phase (°)	153.9 ± 7.2	139.9 ± 7.4	169.8 ± 6.2	185.2 ± 9.1

Table 1. Masseter/digastric coordination during mastication after application of yellow or blue light to DRN or ROb. *r*-value and phase indicate the length and direction of the vector in Figs. 3, 4, respectively. Data are expressed as mean ± SEM. DRN dorsal raphe nucleus, ROb raphe obscurus nucleus, Control yellow light application, Activation blue light application.

EMG activity of the constituent masticatory cycles: incision and chewing in mice^{19,20}. In these studies, EMG recordings revealed that, during the incision stage, rapid (7 Hz), small bursts of masseter muscles were evident, reflecting pellet biting. In the chewing stage, slow (5 Hz), large bursts of masseter muscles were evident, reflecting crushing and grinding.

Fast, small masticatory movements induced by DRN blue light illumination in the present study showed similar characteristics, in which the frequency of masticatory muscle activity during the incision stage of the masticatory cycle was > 7 Hz. DRN 5-HT neuron activation may be involved in the transition from the chewing to the incision stage during mastication. However, single application of the blue light induced rapid masticatory movements for 12.8 ± 4.1 s without pellet biting, while the incision stage duration was < 3 s in normal mastication in mice^{19,20}. This prolonged rapid masticatory movement may be due to ChR2 (C128S) characteristics, with a closing time constant of 100 s and causing vigorous, sustained spiking after short blue light application^{26,27}.

We found that the activation of 5-HT_{2A} receptors was involved in masticatory pattern modulation by DRN blue light illumination during voluntary mastication. 5-HT_{2A} receptors are widely expressed in the brain regions such as the cortex, striatum, amygdala, and hypothalamus to which DRN 5-HT neurons project^{28,29}. Furthermore, these regions are involved in the central control of masticatory movements^{3,30–32}. These pathways may be candidates for modulating masticatory patterns via the serotonergic system. However, future studies must clearly determine the projection sites that mediate the observed effects of DRN 5-HT neurons on masticatory movements.

Masticatory patterns—the bite force and duration of the masticatory cycle—are affected by sensory information from periodontal mechanoreceptors and muscle spindles based on the physical properties^{33,34}. The mesencephalic trigeminal nucleus (MesV) neurons, which innervate the periodontal ligament and muscle spindles, While electrophysiological evidence indicates that activation of 5-HT_{1A} receptors reduces hyperpolarization-activated inward current in MesV³⁵, and activation of 5-HT_{1B} receptors suppresses excitatory inputs from MesV to motoneurons³⁶, the role of 5-HT_{2A} receptors in MesV is not comprehensively understood. However, the MesV receives inputs from both DRN and ROb³⁷, and expresses 5-HT₂ receptors⁴, suggesting potential involvement in the alterations of voluntary masticatory patterns induced by activation of DRN 5-HT neurons.

Under conditions in which EMG activity does not exceed 2 S.D. for 30 s (resting state), ROb blue light illumination increased EMG activity in the masseter and digastric muscles. ROb 5-HT neurons project primarily to the brainstem, including the trigeminal motor nucleus and regions in which jaw movement-related neurons are located²⁵. Trigeminal motoneurons receive 5-HT terminals, and electrical stimulus application to the RPa and ROb complex induces a monosynaptic excitatory postsynaptic potential in jaw-closing and -opening motoneurons in cats¹². Furthermore, 5-HT directly increases the excitability of jaw-closing motoneurons^{13–15}. ROb 5-HT neurons may be related to EMG activity regulation in these muscles by directly activating jaw-closing and -opening motoneurons in the brainstem. ROb 5-HT neurons also project to the spinal cord³⁸ and spinal motoneuron activity is modulated by serotonin³⁹. ROb 5-HT neurons may affect masticatory muscles as well as systemic activation of skeletal muscles. However, in the present study, ROb blue light illumination did not reveal any initiation of locomotion during the resting state. Our data also showed that DRN blue light illumination during the resting state increased EMG activity in the masseter and digastric muscles. DRN 5-HT neurons project mainly to the forebrain⁴⁰, and optogenetic activation of DRN 5-HT neurons during the sleep state increased the EMG power in trapezius muscles due to induced wakefulness¹⁶. Therefore, DRN 5-HT neurons may indirectly increase masticatory muscle activity via neural circuits involving the forebrain rather than directly activating trigeminal motoneurons as in ROb 5-HT neurons. In contrast, DRN blue light illumination during mastication decreased EMG bursts in the masseter and digastric muscles. The neural circuits that control rhythmic jaw movements, such as mastication, include inhibitory inputs from GABA and glycine neurons in the brainstem to MoV, which regulate masticatory muscle activity^{41–43}. The small pattern of masticatory movements induced by activation of DRN 5-HT neurons may be attributed to an increase in these inhibitory inputs.

Our present data show that ROb blue light illumination did not induce rhythmic jaw movements such as mastication during the resting state and did not affect rhythmic mastication activities during voluntary mastication. CPG activity may not be critically dependent upon ROb 5-HT neurons. Conversely, DRN blue light illumination altered masticatory movements but did not induce rhythmic jaw movements during the resting state. Optogenetic activation of DRN 5-HT neurons during free movement does not induce stereotypical behavior in mice—freezing, grooming, and rearing²⁷—and reduces spontaneous locomotion, as found in an open field test in mice⁴⁴. Therefore, DRN 5-HT neuron activation may not be involved in rhythmic movement initiation. DRN 5-HT neurons activation alone cannot activate the CPG for mastication, but it may be able to alter CPG activity patterns activated by other inputs.

Conclusion

Here, we demonstrated that both DRN and ROB blue light illumination increased the EMG activity in the masseter and digastric muscles during the resting state. These results suggest that DRN and ROB may facilitate jaw movement, possibly via different pathways. We showed also that the optogenetic activation of DRN 5-HT neurons, but not ROB 5-HT neurons, induces a fast, small pattern of masticatory movements similar to the incision stage characteristics during voluntary mastication. We also revealed that this modulation was mediated via 5-HT_{2A} receptor activation. Since masticatory movements are accomplished by alternating between the incision and chewing stages in mice, DRN 5-HT neurons may enable sustained masticatory movements by switching from the chewing to the incision stage. Changes in masticatory patterns aid in proper food processing and efficient nutrient absorption. Our experimental results suggest that drug therapies or behavioral therapies that modulate 5-HT levels may be useful in the treatment of masticatory dysfunction. Although further studies examining the effects of 5-HT neuron inhibition on masticatory movements are needed to more precisely determine the functional role of DRN 5-HT neurons on masticatory jaw movements, our study provides a better understanding of the neuro-moludatory mechanisms controlling voluntary mastication.

Methods

Animals

All experiments were conducted with the approval of the Institutional Animal Research Committee of Showa University (approval reference 14028), operating in accordance with Japanese Government Law No. 105 and the Animal Research: Reporting of In Vivo Experiments (ARRIVE 2.0) guidelines. All animals had ad libitum access to food and water and were housed in an air-conditioned room with 12–12 h dark-light cycles. Double-transgenic mice (tph2-tTA::tetO-ChR2(C128S)-EYFP, $n=29$) were obtained by crossing Tph2-tTA and tetO-ChR2(C128S)-EYFP mice. All lines were obtained from the RIKEN BioResource Center (Ibaraki, Japan). Genotyping was performed using polymerase chain reaction (PCR), as previously described^{22,23}. The following PCR primer sets were used in the mouse genotyping: ChR2 (5'-ACCGACCCCTTTGGCACAGTATG-3') for tetO-ChR2(C128S) and tTA (5'-CCAGGGTCTCGTACTGCTTC3') for Tph2-tTA mice. Mice with the transgene expression confirmed were randomly assigned to different experiments, and the same individuals were used across different experimental conditions for direct comparisons. Male mice aged 10–28 weeks were used for the experiments. Each experiment included 4–8 mice.

Surgical procedures

All surgical procedures were performed using a stereotaxic system (Model 940; David Kopf Instruments, Tujunga, CA, USA). All mice were anesthetized with intraperitoneal injection of a mixture of medetomidine hydrochloride (0.75 mg/kg, Domitor; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), midazolam hydrochloride (4.0 mg/kg, Dormicum; Sandoz K.K., Tokyo, Japan), and butorphanol tartrate (5.0 mg/kg, Vetorphale; Meiji Seika Pharma Co., Ltd., Tokyo, Japan).

For optogenetic stimulation at DRN, a craniotomy (−8.75 mm anteroposterior, 0 mm mediolateral, 3.0 mm dorsoventral from bregma) was prepared, and an optical fiber (400 μ m diameter, 0.39 NA, Core Multimode Optical Fiber, Thorlabs, Inc. Newton, NJ, USA) inserted 3.0 mm horizontally from the posterior to the anterior⁴⁵. For optogenetic stimulation at ROB, an optical fiber was inserted vertically above ROB (−4.2 mm anteroposterior, 0 mm mediolateral, −4.5 mm dorsoventral from bregma).

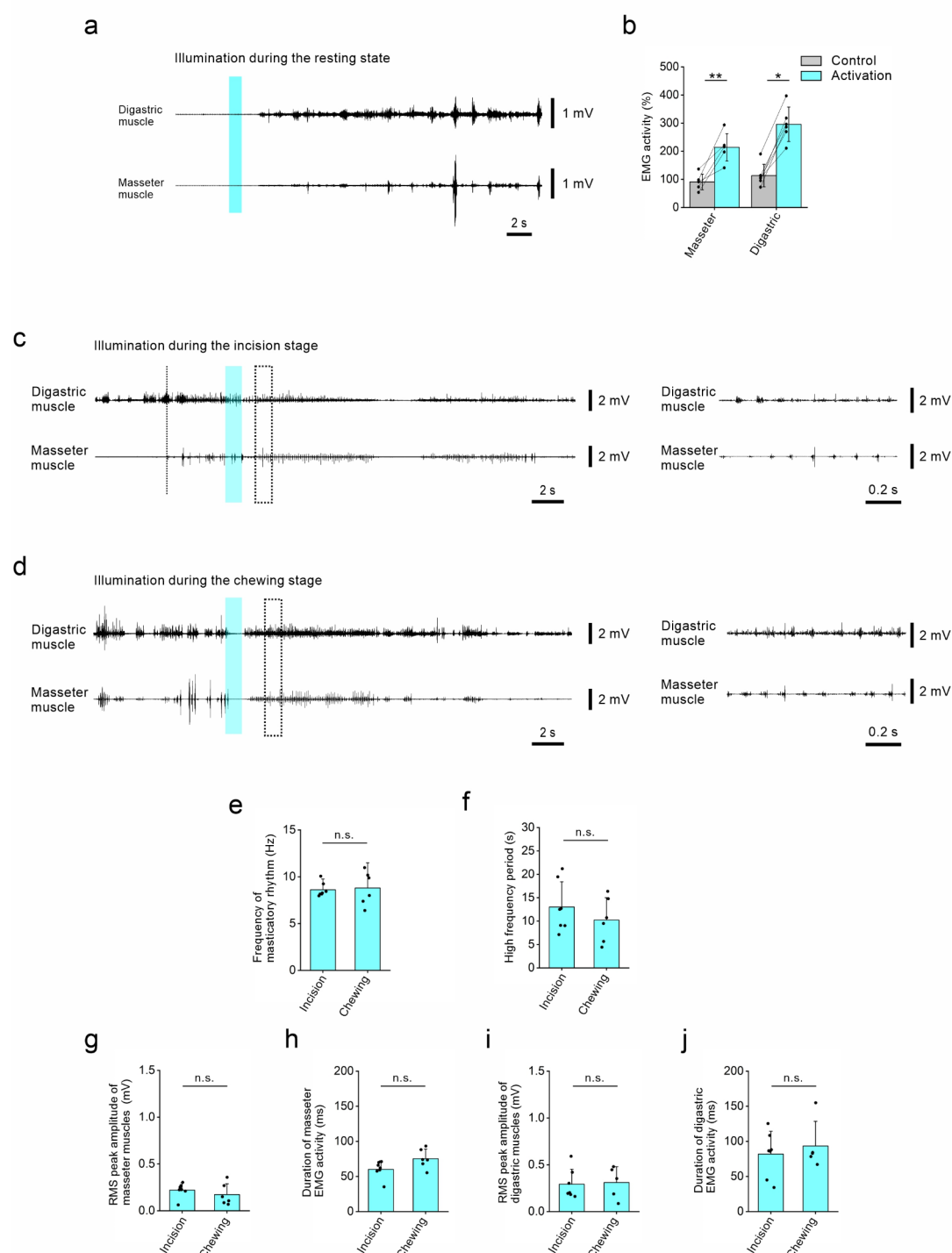
For the EMG recordings of the masseter and digastric muscles, optical fiber-implanted mice were used. The detailed protocol has been previously described⁴⁶. A pin connector to which a stainless-steel screw (M1-2, Unique Medical, Tokyo, Japan) for grounding and pairs of Teflon-coated stainless-steel wires (#AS631, Cooner Wire Co., Chatsworth, CA, USA) were soldered were attached to the skull using dental resin cement (56849; 3 M Dental Products, St Paul, MN, USA). A pair of wires was located in the left masseter and digastric muscles. Atipamezole (0.75 mg/kg, Antisedan, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) as an antagonist of medetomidine hydrochloride was administered postoperatively.

Rerecording procedures and EMG analysis

Mice were allowed a recovery period of at least 1 week after implant surgery. Before EMG recording, the mice were deprived of food for 24 h with free access to water. Following the food-deprivation period, EMG recordings were made, while the mice ate the uniform size of food (diameter: approx. 10 mm, weight: 0.2–0.3 g) to eliminate the effects of differences in size on masticatory movement.

The EMG signals were filtered (100–1000 Hz) and amplified (AB-611 J device, Nihon Kohden Co., Tokyo, Japan). The EMG data were digitized at 4 kHz using a PowerLab 8/35 analog-to-digital converter (PL3508, AD Instruments Inc., Dunedin, New Zealand) and stored on a computer using the Lab Chart 7 software (AD Instruments).

Individual bursts were then detected when the rectified EMG exceeded the mean level by 2 SD. To evaluate masticatory frequency and EMG masticatory movement bursts, values were obtained from consecutive gnaw 10 bursts of rectified EMG activity exceeding the mean level by 2 S.D after light application. Masseter muscles activity was measured to determine masticatory frequency because digastric muscles activity often occurs twice in a single cycle^{2,19}. EMG amplitude was estimated using the RMS EMG calculated over a 40-ms sliding window. EMG activities of the masseter and digastric muscles were rectified and integrated using Lab Chart 7 (AD Instruments). For EMG activity, the change in the mean integrated values for masseter and digastric EMG activities during 30 s after light illumination was measured using the mean integrated values for masseter and digastric EMG activities during 30 s before light illumination as 100%.



Optogenetic stimulation

Optogenetic stimulation was carried out using blue (470 nm) or yellow (590 nm) light with an optical fiber-coupled 470 nm light-emitting diode (LED) light source (M470F3, Thorlabs) or a 590 nm LED light source (M590F3, Thorlabs) controlled by an LED driver (DC2200, Thorlabs) and an electronic stimulator (SEN-8203MG, Nihon Kohden). The light power intensity of both blue and yellow at the fiber optic tip was 1–2 mW. For optogenetic stimulation, blue light (1 s duration) was used with yellow light (1 s duration) used as the control.

Drug application

The drugs used were the selective 5-HT_{2A} receptor antagonist (*R*)-(+)- α -(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol (MDL100907; Tocris Bioscience, Ellisville, MO, USA), the selective 5-HT_{2C} receptor antagonist 6-chloro-5methyl-1[2-(2-methylpyridyl-3-oxo)-pyri-5-yl carbonyl] indoline

Fig. 4. Optogenetic activation of DRN 5-HT neurons does not induce masticatory movement initiation but modulates ongoing mastication movement. **(a)** Representative EMG recordings of digastric and masseter muscles before and after blue light application (blue bar) during resting state. **(b)** Pooled data for the percentage of EMG activity evoked after yellow (control) or blue light (activation) illumination to that during the resting state before yellow or blue light illumination in the digastric and masseter muscles. **(c)** Representative EMG recording of digastric and masseter muscles before and after blue light application (blue bar) during incision (left). The dashed line indicates the start of feeding. Expanding EMG recordings in dotted box outlines of left EMG recordings (right). **(d)** Representative EMG recording of digastric and masseter muscles before and after blue light application (blue bar) during chewing stage (left). Expanding EMG recordings in dotted box outlines of left EMG recordings (right). **(e)** Pooled data for masticatory frequency after blue light application during incision and chewing stages. **(f)** Pooled data for periods showing high frequency (> 7 Hz) masticatory muscle activity after blue light application during incision and chewing. **(g)** and **(h)** Pooled data for RMS peak amplitude **(f)** and duration **(g)** in masseter EMG bursts after blue light application during incision and chewing. **(i)** and **(j)** Pooled data for RMS peak amplitude **(h)** and duration **(i)** in digastric EMG bursts after blue light application during incision and chewing. $**P < 0.01$, $***P < 0.001$ (unpaired Student's *t* test). Comparisons with n.s. have $P > 0.05$ and are considered not significant. Data are expressed as mean \pm SEM.

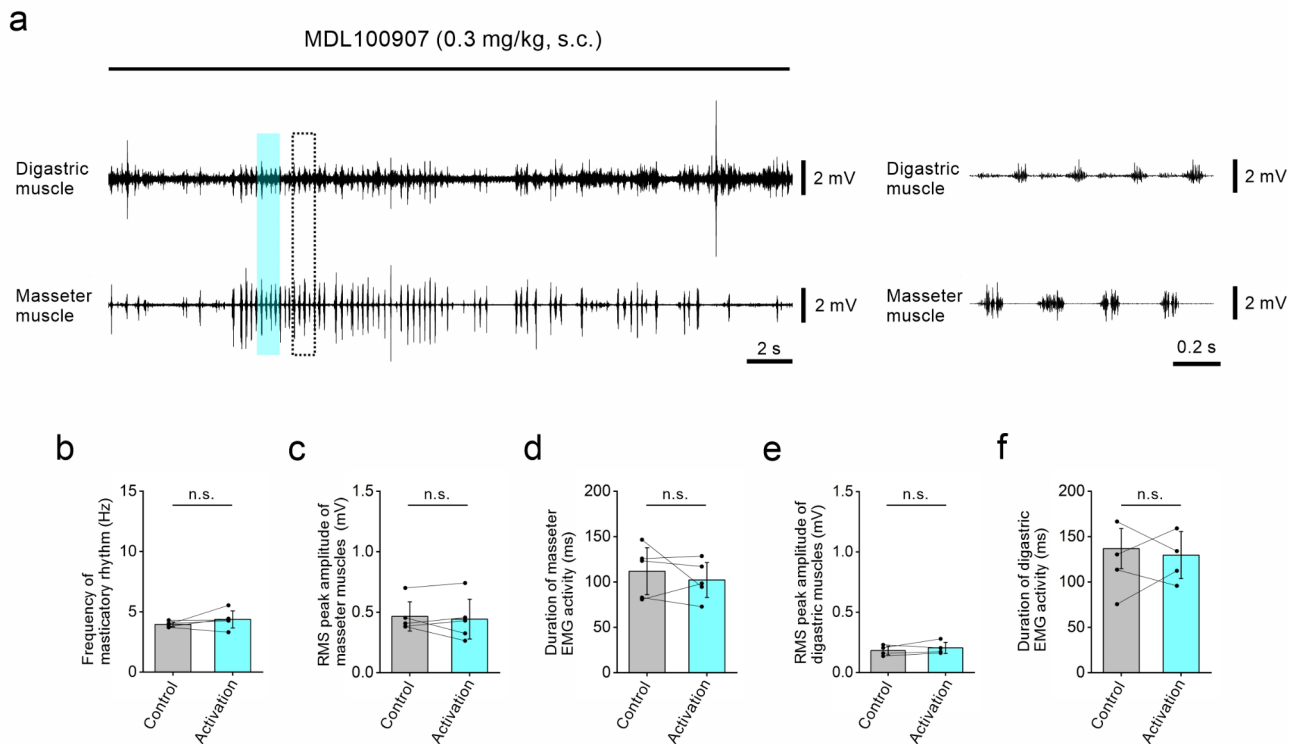


Fig. 5. 5-HT_{2A} receptors are involved in induced fast masticatory movement by optogenetic activation of DRN 5-HT neurons. **(a)** Representative EMG recording of digastric and masseter muscles during voluntary mastication at 30 min after the administration of 0.3 mg/kg MDL100907 (s.c.) (left). Expanding EMG recordings in dotted box outlines of left EMG recordings (right). **(b)** Pooled data for masticatory frequency after yellow (control) or blue light (activation) application with the administration of MDL 100,907. **(c)** and **(d)** Pooled data for RMS peak amplitude **(c)** and duration **(d)** in masseter EMG bursts after yellow or blue light application with the administration of MDL 10,090. **(e)** and **(f)** Pooled data for RMS peak amplitude **(e)** and duration **(f)** in digastric EMG bursts after yellow or blue light application with the administration of MDL 100,907. Comparisons with n.s. have $P > 0.05$ and are considered not significant. Data are expressed as mean \pm SEM.

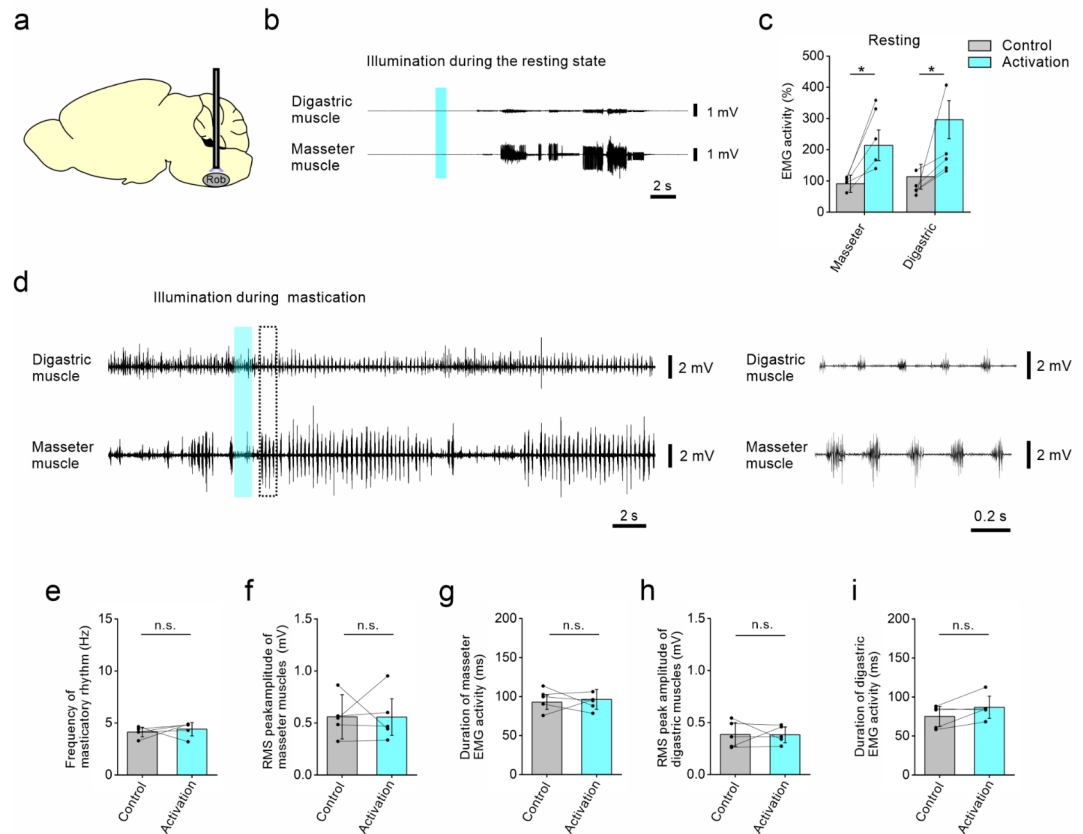


Fig. 6. Optogenetic activation of ROB 5-HT neurons during voluntary mastication does not affect masticatory movement patterns. **(a)** Schematic illustration of the position of the optical fiber stereotactically inserted into the ROB of double transgenic mice expressing ChR2 in central 5-HT neurons. **(b)** Representative EMG recordings of digastric and masseter muscles during the resting state before and after optogenetic activation of ROB 5-HT neurons by blue light application (blue bar). **(c)** Pooled data for the percentage of EMG activity evoked after yellow (control) or blue light (activation) illumination to that during the resting state before illumination in the digastric and masseter muscles. **(d)** Representative EMG recordings of digastric and masseter muscles during voluntary mastication before and after blue light application (blue bar) for optogenetic activation of ROB 5-HT neurons (left). Expanding EMG recordings in dotted box outlines of left EMG recordings (right). **(e)** Pooled data for masticatory frequency after yellow or blue light application. **(f)** and **(g)** Pooled data for RMS peak amplitude (f) and duration (g) in masseter EMG bursts after yellow or blue light application. **(h)** and **(i)** Pooled data for RMS peak amplitude (h) and duration (i) in digastric EMG bursts after yellow or blue light application. * $P < 0.05$ (paired Student's t -test). Comparisons with n.s. have $P > 0.05$ and are considered not significant. Data are expressed as mean \pm SEM. ROB raphe obscurus nucleus.

(SB 242084; Tocris Bioscience), and the selective 5-HT_{1A} receptor antagonist N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate salt (Way-100635; Merck KGaA, Darmstadt, Germany). MDL100907 was dissolved in water containing 5% Tween 80. SB242084 and Way-100,635 was dissolved in isotonic saline. Respective drugs were administered 30 min before the recording.

Histology

At the experiment conclusion, the mice perfused with a 4% paraformaldehyde phosphate buffer solution (Wako, Osaka, Japan). The brain was removed and postfixed in the same fixative at 4 °C overnight. The specimens were then immersed in 0.1 M phosphate buffer containing 15% and 30% sucrose at 4 °C until submerged. Sagittal 40- μ m-thick serial sections were cut using a cryostat (Leica CM3050S; Leica Biosystems, Nussloch, Germany). The sections were incubated with Alexa 488-labeled anti-GFP antibody (1:500; Santa Cruz biotechnology, Dallas, TX, USA) overnight at 4 °C. The sections were then incubated with rabbit anti-tryptophan hydroxylase antibody (1:400; Merck KGaA) overnight at 4 °C and incubated with Alexa 594 donkey anti-rabbit IgG (1:500; Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature (24–26 °C). Sections were mounted and examined under a light microscope (BZ-X710; Keyence, Osaka, Japan).

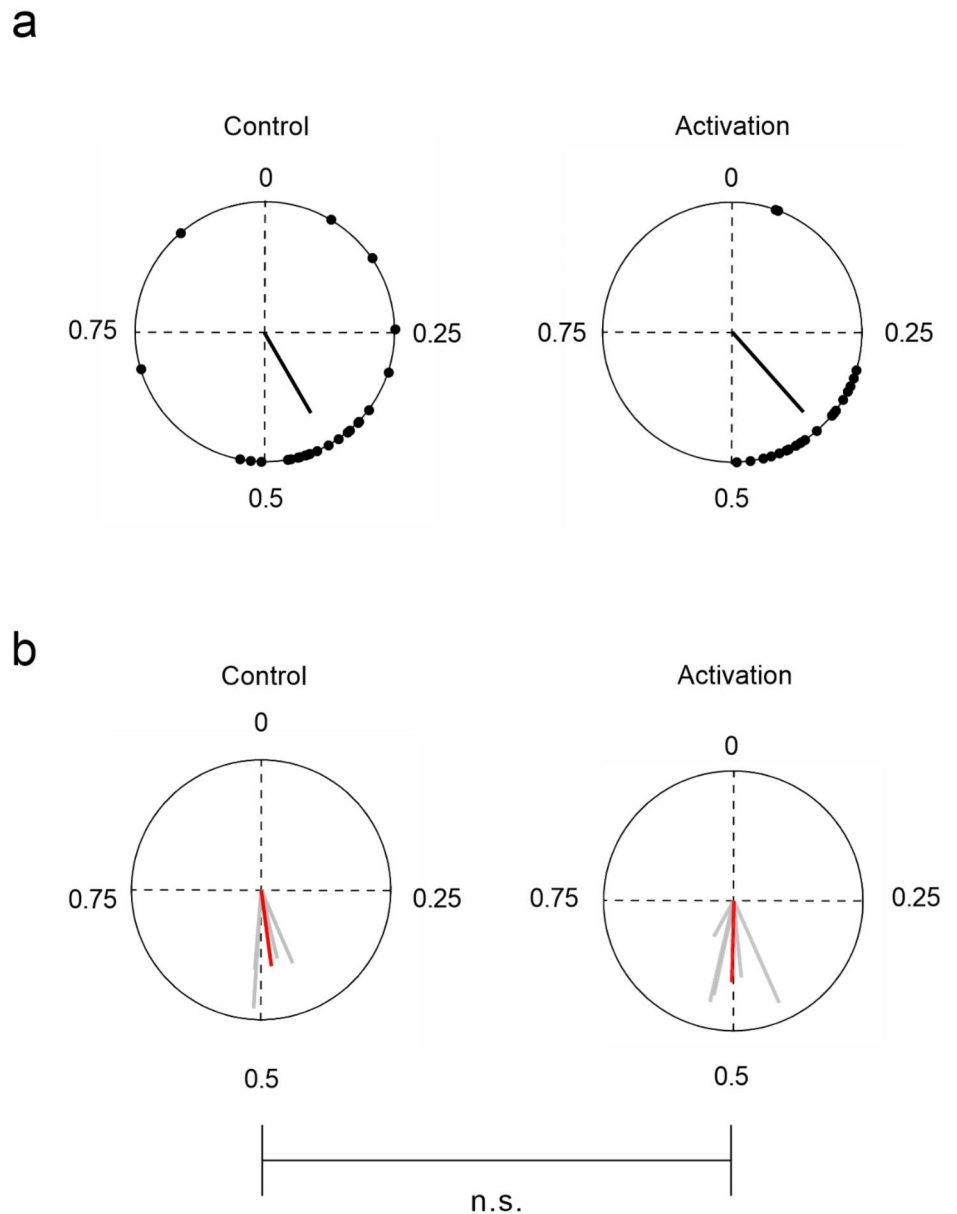


Fig. 7. Optogenetic activation of ROb 5-HT neurons does not involve coordination between masseter and digastric muscle activity during voluntary mastication. **(a)** Representative circular plots showing the relative burst phases recorded from the masseter muscles relative to the digastric muscles. The black dots on each circle indicate the phase shift between the corresponding RMS peak of the masseter-digastric muscle activity alternation after yellow (left) or blue (right) light application. Black straight lines indicate mean coordination r-vectors. **(b)** Circular plot summary showing the phasing of all five animals. Gray and straight red lines indicate the mean coordination r-vectors for individual animals and animal groups, respectively. Comparisons with n.s. have $P > 0.05$ and are considered not significant. The Watson–Williams test for phase comparisons did not show any statistically significant differences between the application of yellow and blue light for individual animals.

Circular statistics

Phase relationships between the masseter and digastric muscle activities were analyzed using circular plots⁴⁷. MATLAB (R2020b, MathWorks Inc., Natick, MA, USA) and Excel (Microsoft Co., Redmond, WA, USA) were used for data analysis. The 25 RMS peak phases of the masseter muscles from each animal were calculated in accordance with the RMS peak of the digastric muscles. The direction of an individual vector indicates the phase (0 or 360, synchrony; 180, alteration). The r -value (vector length) indicates the concentration of the phase-shift values around the mean, indicating the strength of coordination between the analyzed RMS peaks (dots

at the circle). Rayleigh's test was used to determine whether r -values associated with coupling were significant. Coupling was regarded as significant when the Rayleigh test result was $P < 0.05$. Multiple sample testing of the angles was performed using the Watson–Williams test to compare the resulting mean phase values.

Statistics

Statistical analyses were performed using SPSS software (version 22.0; IBM Co., Armonk, NY, USA). Pooled data are expressed as the mean \pm standard error of the mean (SEM). To compare EMG activity after blue light illumination with that after yellow light illumination, we used Student's paired t -tests. Differences between groups were analyzed using unpaired Student's t -tests or one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* multiple comparison test, when appropriate. Statistical significance was set at $P < 0.05$. The sample size was not calculated beforehand; instead we determined the sample size based on previous studies with similar experimental designs^{2,3,19,20,48}.

Data availability

All data and material are available from the corresponding author on reasonable request.

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Author contributions

M.D. contributed to the conception, design, data acquisition, analysis, and interpretation, and drafted and critically revised the manuscript; A.M., K.N., M.K., M.I., and K.T. contributed to data interpretation and critically revised the manuscript; T.I. contributed to the conception and design and critically revised the manuscript; S.N. contributed to the conception, design, data acquisition, analysis, and interpretation, and drafted and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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