Efferent and afferent connections of supratrigeminal neurons conveying orofacial muscle proprioception in rats

Atsushi Yoshida1 · Misaki Inoue1 · Fumihiko Sato1 · Yayoi Morita1 · Yumi Tsutsumi1 · Takahiro Furuta1 · Katsuro Uchino1,2 · Fatema Akhter3 · Yong Chul Bae4 · Yoshihisa Tachibana5 · Tomio Inoue6

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Abstract
The supratrigeminal nucleus (Su5) is a key structure for controlling jaw movements; it receives proprioceptive sensation from jaw-closing muscle spindles (JCMSs) and sends projections to the trigeminal motor nucleus (Mo5). However, the central projections and regulation of JCMS proprioceptive sensation are not yet fully understood. Therefore, we aimed to reveal the efferent and afferent connections of the Su5 using neuronal tract tracings. Anterograde tracer injections into the Su5 revealed that the Su5 sends contralateral projections (or bilateral projections with a contralateral predominance) to the Su5, basilar pontine nuclei, pontine reticular nucleus, deep mesencephalic nucleus, superior colliculus, caudo-ventromedial edge of the ventral posteromedial thalamic nucleus, parafascicular thalamic nucleus, zona incerta, and lateral hypothalamus, and ipsilateral projections (or bilateral projections with an ipsilateral predominance) to the intertrigeminal region, trigeminal oral subnucleus, dorsal medullary reticular formation, and hypoglossal nucleus as well as the Mo5. Retrograde tracer injections into the Su5 demonstrated that the Su5 receives bilateral projections with a contralateral predominance (or contralateral projections) from the primary and secondary somatosensory cortices, granular insular cortex, and Su5, and ipsilateral projections (or bilateral projections with an ipsilateral predominance) from the dorsal peduncular cortex, bed nuclei of stria terminalis, central amygdaloid nucleus, lateral hypothalamus, paraventricular thalamic nucleus, trigeminal mesencephalic nucleus, parabrachial nucleus, juxtaganglionic region, trigeminal oral and caudal subnuclei, and dorsal medullary reticular formation. These findings suggest that the Su5, which receives JCMS proprioception, has efferent and afferent connections with multiple brain regions that are involved in emotional and autonomic functions as well as orofacial motor functions.

Keywords Muscle spindle · Mastication · Swallowing · Neuronal tracer · BDA · CTb

Abbreviations
3  Oculomotor nucleus
5C  Caudal subnucleus of the trigeminal spinal nucleus
5I  Interpolar subnucleus of the trigeminal spinal nucleus
5O  Oral subnucleus of the trigeminal spinal nucleus
5Or  Rostro-dorsomedial part of the 5O
7n  Facial nerve
10  Dorsal motor nucleus of the vagus
ac  Anterior commissure
Acb  Accumbens nucleus

Atsushi Yoshida
yoshida@dent.osaka-u.ac.jp

Yoshihisa Tachibana
yoshi@med.kobe-u.ac.jp

1 Department of Oral Anatomy and Neurobiology, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan
2 Department of Acupuncture, Takarazuka University of Medical and Health Care, Takarazuka, Hyogo 666-0162, Japan
3 College of Dentistry, Dar Al Uloom University, Riyadh 11512, Saudi Arabia
4 Department of Anatomy and Neurobiology, School of Dentistry, Kyungpook National University, Daegu 700-412, Korea
5 Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki, Chuo, Kobe, Hyogo 650-0017, Japan
6 Department of Oral Physiology, Showa University School of Dentistry, Shinagawa-ku, Tokyo 142-8555, Japan
| Acronym | Description |
|---------|-------------|
| ACg     | Anterior cingulate cortex |
| AD      | Anterodorsal thalamic nucleus |
| Agl     | Lateral agranular cortex |
| Agm     | Medial agranular cortex |
| AI      | Agranular insular cortex |
| Amb     | Ambiguous nucleus |
| AmBl    | Basolateral amygdaloid nucleus |
| AmC     | Central amygdaloid nucleus |
| AmL     | Lateral amygdaloid nucleus |
| AP      | Area postrema |
| Aq      | Aqueduct |
| Au      | Auditory cortex |
| AV      | Anteroventral thalamic nucleus |
| BDA     | Biotinylated dextranamine |
| BPn     | Basilar pontine nuclei |
| BST     | Bed nucleus of stria terminalis |
| BSTl    | Lateral division of the BST |
| BSTm    | Medial division of the BST |
| CC      | Central canal |
| cc      | Corpus callosum |
| CL      | Centrolateral thalamic nucleus |
| Cl      | Claustrum |
| CM      | Central medial thalamic nucleus |
| CPu     | Caudate putamen |
| CTb     | Cholera toxin B subunit |
| Cu      | Cuneate nucleus |
| cu      | Cuneate fasciculus |
| dGIrvs2 | Dorsal part of the GI rostroventrally adjacent to the rostralmost part of the S2 |
| DI      | Dysgranular insular cortex |
| dmRf    | Dorsal medullary reticular formation |
| DP      | Dorsal peduncular cortex |
| DpMe    | Deep mesencephalic nucleus |
| DR      | Dorsal raphe nucleus |
| DTg     | Dorsal tegmental nucleus |
| Ect     | Ectorhinal cortex |
| ECu     | External cuneate nucleus |
| f       | Fornix |
| fr      | Fasciculus retroflexus |
| GI      | Granular insular cortex |
| Gr      | Gracile nucleus |
| I5      | Intertrigeminal region |
| IC      | Inferior colliculus |
| ic      | Internal capsule |
| IL      | Infrahinal cortex |
| IO      | Inferior olive |
| IPAC    | Interstitial nucleus of the posterior limb of the anterior comissure |
| J5      | Juxtratrigeminal region |
| JCM     | Jaw-closing motor nucleus |
| JCMS    | Jaw-closing muscle spindle |
| JOM     | Jaw-opening motor nucleus |
| KF      | Kölliker-Fuse nucleus |
| LEnt    | Lateral entorhinal cortex |
| If      | Longitudinal fasciculus |
| LGP     | Lateral globus pallidus |
| LH      | Lateral hypothalamus |
| LO      | Lateral orbital cortex |
| MD      | Mediodorsal thalamic nucleus |
| Me5     | Trigeminal mesencephalic nucleus |
| MGP     | Medial globus pallidus |
| ml      | Medial lemniscus |
| MO      | Medial orbital cortex |
| Mo5     | Trigeminal motor nucleus |
| OPC     | Oval paracentral thalamic nucleus |
| ox      | Optic chiasm |
| PA      | Parietal association cortex |
| Pa5     | Paratrigeminal nucleus |
| PC      | Paracentral thalamic nucleus |
| Pf      | Parafascicular thalamic nucleus |
| PnR     | Pontine reticular nucleus |
| Po      | Posterior thalamic nucleus |
| Pr      | Prepositus nucleus |
| Pr5     | Trigeminal thalamic nucleus |
| Prh     | Perirhinal cortex |
| PrL     | Prelimbic cortex |
| PBS     | Phosphate-buffered saline |
| Py      | Pyramidal tract |
| pyx     | Pyramidal decussation |
| R       | Red nucleus |
| Rfvm    | Reticular formation ventral to the Mo5 |
| RRF     | Retrorubral field |
| Rt      | Reticular thalamic nucleus |
| Rtg     | Reticulotegmental nucleus |
| S1      | Primary somatosensory cortex |
| S2      | Secondary somatosensory cortex |
| SC      | Superior colliculus |
| scp     | Superior cerebellar peduncle |
| Sm      | Submedial thalamic nucleus |
| SNc     | Substantia nigra pars compacta |
| SNr     | Substantia nigra pars reticulata |
| Sol     | Solitary tract nucleus |
| sp5     | Spinal trigeminal tract |
| st      | Stria terminalis |
| Sth     | Subthalamic nucleus |
| Su5     | Supratrigeminal nucleus |
| TA      | Temporal association cortex |
| tth     | Trigeminothalamic tract |
| VA      | Ventral anterior thalamic nucleus |
| Ve      | Vestibular nucleus |
| VII     | Facial nucleus |
Introduction

Proprioceptive signals arising from muscles in the entire body play a crucial role in sensorimotor reflexes through reflex arcs, which are located at the levels of the lower brainstem or spinal cord. For example, proprioceptive signals arising from jaw-closing muscle spindles (JCMSs) are conveyed by primary afferents, whose neuronal cell bodies are located in the trigeminal mesencephalic nucleus (Me5), to jaw-closing motoneurons in the trigeminal motor nucleus (Mo5) in the rostral pons (Luo et al. 1995, 2001; Fujio et al. 2016; for review, see Dubner et al. 1978; Taylor 1990). This monosynaptic reflex arc induces the jaw-jerk reflex (a kind of stretch reflex). Proprioceptive signals from JCMSs are also transmitted by Me5 primary afferents to the supratrigeminal nucleus (Su5) in the rostral pons (Jerge 1963; Takata and Kawamura 1970; Miyazaki and Luschei 1963; Takata and Kawamura 1970). The Su5 contains excitatory or inhibitory premotor neurons that project to jaw-closing or -opening motoneurons in the Mo5 (Ohta and Moriyama 1986; Nakamura et al. 2008; Paik et al. 2009; Nonaka et al. 2012). Accordingly, JCMS proprioceptive sensation conveyed by Me5 primary afferents can induce reflexive jaw closing via the direct pathway to the Mo5, and facilitate (or suppress) reflexive jaw closing or opening via the indirect Su5–Mo5 pathways (Goldberg and Nakamura 1968; Kidokoro et al. 1968; Ohta and Moriyama 1986; Shigenaga et al. 1988a, 1990). Although functional connectivity via the Me5–Su5–Mo5 pathways has been well studied in the jaw reflex arc, the central processing and regulation of orofacial proprioception arising from masticatory muscle spindles remain unknown. We have previously reported that Me5 afferents in the rat transmit sensory signals almost exclusively from JCMSs, and rarely transmit sensory signals from periodontal ligaments around the upper and lower teeth (Fujio et al. 2016). This finding indicates that efferent projections from the rat Su5 specifically signal JCMS proprioception. Moreover, Me5 neurons transmitting JCMS proprioceptive signals do not send direct projections to the thalamus (e.g. Shigenaga et al. 1988a, 1989, 1990; Luo et al. 1995, 2001; Fujio et al. 2016), whereas the Su5 conveys JCMS proprioceptive signals to the thalamus (Yoshida et al. 2017). However, we should note that the thalamic projection site of the Su5 is restricted to a small area of the ventral posteros medial thalamic nucleus (VPM), the caudo-ventromedial edge of the VPM (VPMcvm), which is different from the “traditional” core VPM that conveys orofacial cutaneous/mucosal sensation (Yoshida et al. 2017). The Su5 also projects to the oval paracentral nucleus (OPC) in the intralaminar thalamic nuclei, albeit to a lesser extent (Yoshida et al. 2017; Sato et al. 2020). JCMS proprioceptive signals that travel via the VPMcvm are subsequently conveyed to the dorsal part (dGIRv2) of the granular insular cortex (GI) rostroventrally adjacent to the rostralmost part of the secondary somatosensory cortex (S2), whereas signals that travel via the OPC are conveyed to the rostral part of the primary somatosensory cortex (S1), rostral S2, and rostral GI (Sato et al. 2017; Tsutsumi et al. 2021). The existence of these cortical projection pathways strongly suggests that JCMS proprioceptive signals are more involved in sensory integrative functions than in sensory discriminative and motor functions, because the GI is historically considered to be involved in the integration of multimodal sensations—such as somatic sensations that include nociception, visceral sensation, gustation, olfaction, and hearing—in humans (Augustine 1985, 1996), monkeys (Mesulam and Mufson 1982) and rats (Yamamoto et al. 1981, 1988, 1989; Ito 1992; Hanamori et al. 1998a, b; Ogawa and Wang 2002; Gauriau and Bernard 2004). However, aside from the thalamus, it remains unknown which higher brain regions receive JCMS proprioceptive sensation via the Su5. This information is necessary to fully understand the brain networks that are required for the neuronal processing of orofacial proprioception. Therefore, in the first experiment of the present study, we sought to reveal the efferent projections of the Su5 in the entire brain using anterograde tracer injections into the rat Su5.

As well as the relay neurons that transmit JCMS proprioceptive sensation to higher brain regions (e.g. the VPMcvm and OPC), the Su5 also contains premotoneurons for reflexive jaw movements (Li et al. 1995; Ohta and Moriyama 1986; Nakamura et al. 2008; Paik et al. 2009; Yoshida et al. 2009; Nonaka et al. 2012). Therefore, an important question remains: which brain regions project to the Su5 for modulation of reflexive jaw movements? Previous studies have reported that the Su5 receives projections from cortical regions, such as the GI (Sato et al. 2013; Ikenoue et al. 2018), S1 (Hattox et al. 2002; Chang et al. 2009; Yoshida et al. 2009; Tomita et al. 2012), and lateral agranular cortex (AgI; Yoshida et al. 2009), and from subcortical regions, such as the bed nucleus of stria terminalis (BST; Dong and Swanson 2003), parvocellular reticular formation (Ter Horst et al. 1991), and solitary tract nucleus (Sol; Oka et al. 2013). However, it remains unclear whether other brain regions also send projections to the Su5. It is also unknown which brain

| Code | Name                          |
|------|-------------------------------|
| VL   | Ventrolateral thalamic nucleus |
| VM   | Ventromedial thalamic nucleus  |
| VO   | Ventral orbital cortex         |
| VPL  | Ventral postero lateral thalamic nucleus |
| VPM  | Ventral postero medial thalamic nucleus |
| VPMcvm | Caudo-ventromedial edge of the VPM |
| VPPC | Parvicular part of the ventral posterior thalamic nucleus |
| XII  | Hypoglossal nucleus            |
| ZI   | Zona incerta                   |

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regions have reciprocal connections with the Su5, which can form regulatory feedback loops. Thus, in the second experiment of the present study, we sought to reveal the afferent projections of the Su5 in the entire brain using retrograde tracer injections into the rat Su5.

Materials and methods

Animals

The experiments described here were performed on 19 male Wistar rats weighing 250–340 g. All experimental procedures were approved by the animal ethics committees of the Osaka University Graduate School of Dentistry for the care and use of laboratory animals, and were performed in accordance with the relevant guidelines. Efforts were made to minimize animal suffering and the number of animals used.

Surgery, recordings, and tracer injections

Each animal was anesthetized by intraperitoneal administration of ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (10 mg/kg) dissolved in saline, with supplementary doses administered such that neither spontaneous eye movements nor corneal reflexes were apparent. If necessary, a local injection of lidocaine hydrochloride was also administered before making a skin incision. Rectal temperature was maintained between 37 °C and 38 °C with a heating pad, and electrocardiography was performed continuously. Rat brainatlases published by Swanson (2004) or Paxinos and Watson (1998, 2014) were used to determine the coordinates for stereotaxic micropipette insertion.

We aimed to extracellularly inject an anterograde tracer (in the first experiment) and a retrograde tracer (in the second experiment) into the left Su5, which was identified electrophysiologically. To this end, an incision was made in the buccal skin to expose the left masseter muscle, and the left masseter nerve (which innervates JCMSs) was dissected so that it was free from the surrounding masseter muscle. Next, silver bipolar stimulation hook electrodes were positioned on the masseter nerve to enable electrical stimulation (single pulse, 200 μs duration, 1 Hz). After the initial surgery, the head of the animal was placed in a stereotaxic apparatus.

As in our previous study (Yoshida et al. 2017; Sato et al. 2020), after craniotomy, a glass micropipette filled with 2.0 M potassium citrate was inserted obliquely into the left Su5 with an 18° rostral-to-caudal inclination to the coronal plane. To precisely locate the left Su5, field potentials responding to both electrical stimulation of the left masseter nerve and passive, sustained jaw-opening movements were recorded. The glass micropipette was then changed to either one filled with the anterograde tracer biotinylated dextranamine (BDA, 10,000 MW, Molecular Probes, Eugene, OR, USA) dissolved in 0.01 M phosphate buffer (PB, pH 7.4) (in the first experiment), or one filled with the retrograde tracer 1% cholera toxin B subunit (CTb; List Biological Laboratories, Campbell, CA, USA) dissolved in 0.02 M phosphate-buffered saline (PBS, pH 7.4; in the second experiment). This was then reinserted into the Su5. Signals recorded from the microelectrode were amplified, filtered (300 Hz to 3 kHz), and stored in a computer at a sampling rate of 20 kHz (field potentials) or 10 kHz (unit activity). Subsequently, BDA or CTb was extracellularly injected into the Su5 using electrophoresis (delivering 2.0 μA positive, 300 ms duration pulses at 2 Hz for 3–7 min). After the tracer injection, the glass microelectrode was carefully withdrawn, and the stimulation electrodes were detached from the masseter nerve. All wounds were then sutured. Next, an analgesic (flurbiprofen axetil, 3.3 mg/kg) and an antibiotic (cefotiam hydrochloride, 66 mg/kg) were administered intraperitoneally, and the animals were allowed to recover from anesthesia in their cages. During postinjection survival, the rats were monitored on a daily basis to assess their general behaviors, body weight, and any postoperative complications such as bleeding or inflammation.

Histology

After a postinjection survival of 5–7 days, rats were reanesthetized deeply with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) and perfused with 100 ml of saline followed by 300 ml of a fixative containing 4% paraformaldehyde in 0.1 M PB through the ascending aorta. Next, the entire brain was removed and placed in 25% sucrose in 0.1 M PB at 4 °C for a few days, until the brain had sunk. The brain was then cut coronally at 60 μm thick on a freezing microtome, and the serial sections were alternately divided into three sets.

For the detection of BDA in the first experiment, all sets of alternate serial sections were washed in 0.02 M PBS (pH 7.4) and preincubated in 0.02 M PBS containing 0.01% H2O2 and 0.75% Triton X-100, as described previously (Sato et al. 2013; Akhter et al. 2014). For the visualization of CTb in the second experiment, all sets of sections were preincubated in 0.02 M PBS containing 3% normal goat serum, 0.2% Triton X-100, and polyclonal rabbit anti-CTb primary antibody (GeneTex, Alton Pkwy Irvine, CA, USA) diluted to 1:20,000, as described previously (Uemura et al. 2020). The sections were then incubated in 0.02 M PBS containing biotinylated goat anti-rabbit immunoglobulin G diluted to 1:400. Subsequently, all sections from all brains with BDA or CTb injections were incubated in 0.02 M PBS containing avidin–biotin–peroxidase complex diluted at 1:100, and were then placed in a diaminobenzidine solution (0.1 M PB...
[pH 7.4] containing 0.04% diaminobenzidine, 0.006% H₂O₂ and 0.08% nickel ammonium sulfate). The sections were then mounted on gelatin-coated slides and dried, and one set of sections was counterstained with Thionin or Neutral Red. Finally, all sections were dehydrated in graded alcohols, cleared in xylene, and coverslipped.

Data analysis

The field potentials recorded in and around the Su5 were stored on a computer, and offline analysis was performed with computer assistance (PowerLab 8/30, ADInstruments, Sydney, Australia). Responses to six to nine successive peripheral stimuli were averaged at each recording site. Using a camera lucida attached to a light microscope, we drew the brain structures, BDA injection sites, anterogradely BDA-labeled axonal fibers and terminals, CTb injection sites, and retrogradely CTb-labeled neuronal cell bodies, all of which were found in the brain except for the cerebellum.

Results

Tracer injections into the Su5

In the first experiment, we sought to examine the detailed distribution of anterograde projections from the Su5 to the entire brain except for the cerebellum. First, to identify the Su5 (as in our previous studies; Fujio et al. 2016; Yoshida et al. 2017; Sato et al. 2020), we recorded the field potentials with short latencies that responded to electrical stimulation of the masseter nerve (innervating JCMS receptors) ipsilateral to the targeted Su5 (e.g. Fig. 1a). At the same recording site, we also recorded unit discharges during passive, sustained jaw opening (e.g. Fig. 1b). We then injected the anterograde tracer BDA into the recording site in the Su5 in ten rats via an electrophoretic procedure (Fig. 1c, d).

In the second experiment, we aimed to analyze the detailed distribution of origin neurons projecting to the Su5 from the entire brain except for the cerebellum. After the electrophysiological identification of the Su5 (e.g. Fig. 1a, b) as in the first experiment, we injected the retrograde tracer CTb into the recording site in the Su5 in nine rats via an electrophoretic procedure (Fig. 1e, f).

In both the first and second experiments, we then histologically confirmed the exact locations of the BDA and CTb deposits. We cytoarchitectonically delineated the Su5, as in our previous studies (Fujio et al. 2016; Yoshida et al. 2017; Sato et al. 2020); the center of the Su5 was located approximately 2.4 mm lateral, 9.7 mm caudal, and 7.6 mm ventral to the bregma. Note that the location of the Su5 was generally equivalent to that described by Torvik (1956) or by Swanson (2004), but seemed to be located more caudolaterally than that described in the atlases by Paxinos and Watson (1986, 1998, 2014) (see the Discussion section for a more detailed explanation). In the present study, the BDA and CTb deposits were successfully confined to the Su5, without extending into the structures surrounding the Su5, in three rats each (Figs. 1c–f, 4i).

Distribution of BDA-labeled terminals

The anterogradely BDA-labeled axons showed similar distribution patterns in all three rats (R116, R621, and R810) in the first experiment (Figs. 2, 3, 4). Hereafter, we describe the distribution patterns in the rostral sections followed by the caudal sections of a representative rat (R621). The relative densities of labeled axon terminals in each nucleus or region are also presented in Table 1. In the mesencephalic region immediately rostral to the injection site (Fig. 3h), on the side contralateral to the BDA injection site, a large number of BDA-labeled axon terminals originating from stem axons in the trigeminothalamic tract (tth) were observed in a small area of the basalar pontine nuclei (BPn) ventral to the longitudinal fasciculus (lf) (also Fig. 5d). In contrast, only a small number of terminals were detected in the contralateral reticulotegmental nucleus (RtTg) dorsal to the tth. A moderate number of axon terminals were also observed in the contralateral pontine reticular nucleus (PnR), whereas a few terminals in the ipsilateral PnR. In addition, a small number of axon terminals were labeled bilaterally in the rostral parabrachial nucleus (Pb).

In the more rostral mesencephalon (Fig. 3g) on the contralateral side, labeled axon fibers extended dorsolaterally into the deep mesencephalic nucleus (DpMe), and a moderate number of labeled terminals appeared in the DpMe. A moderate number of terminals were also labeled in the ventrolateral part of the contralateral superior colliculus (SC), mainly in its intermediate layer (also Fig. 5c). Few terminals were labeled in the contralateral red nucleus (R) and the bilateral periaqueductal gray (PAG).

In the caudal diencephalon (Fig. 3e, f), there were a large number of labeled axon terminals in the contralateral VPMcvm (also Fig. 5b), whereas rare terminals in the ipsilateral VPMcvm. A moderate number of labeled axon terminals were observed contralaterally in the parafascicular thalamic nucleus (Pf), caudo-ventromedial part of the zona incerta (ZI) (Figs. 3e, f, 5a), and caudo-dorsolateral part of the lateral hypothalamus (LH) medial to the ZI (Fig. 3e, f). There were also a small number of labeled terminals in the contralateral OPC in the intralaminar thalamic nuclei, whereas a few terminals in the ipsilateral OPC (Fig. 3e). No terminals were labeled in any sections rostral to this level, which included the amygdaloid complex (Figs. 2a–d, 3e, f).

At the level of the Su5 injection site (Fig. 4i), a large number of BDA-labeled axon terminals were observed in the
Fig. 1 Field potentials recorded in the left supratrigeminal nucleus (Su5) (a, b) and injection sites made at the recording sites with the anterograde tracer biotinylated dextran amine (BDA) (c, d) or the retrograde tracer cholera toxin B subunit (CTb) (e, f). a: Field potentials evoked by electrical stimulation of the left masseter nerve in a representative rat (R621). b: Extracellular multi-unit discharges recorded during a sustained jaw opening (for 5.4 s, indicated by the horizontal line) in the same representative rat (R621). c-f: Locations of three BDA injection sites (c) and three CTb injection sites (e), indicated by the respective rat numbers; the injection sites of rats R621 and R303 are denoted by a blue area in c and a red area in e, respectively, and also in Fig. 4i. These injection sites were well confined in the electrophysiologically and cytoarchitectonically identified left Su5. Boxed areas in c and e correspond to the photomicrographic images d and f of the injection sites in rats R621 and R303, respectively. Sections d and f were counterstained with Neutral Red. For abbreviations, see the abbreviations list.
contralateral Su5 (also Fig. 5e). A large number of axon terminals were also labeled in the ipsilateral Mo5. Importantly, the distribution of axon terminals in the ipsilateral Mo5 was concentrated in its dorsolateral division (the so-called jaw-closing motor nucleus [JCM]), but only a small number of axon terminals were observed in the caudal ventromedial

c.

Fig. 2 Semi-schematic drawings showing the distribution of retrogradely labeled neuronal cell bodies (large red dots) after a CTb injection into the left Su5 in rat R303. The CTb injection site is denoted by a red area in Figs. 1e and 4i, and its photomicrograph is shown in Fig. 1f. Coronal sections are arranged rostrocaudally from a to d. The left side of each drawing corresponds to the side that was ipsilateral to the injection sites. The upper-left number in each drawing denotes the distance in millimeters rostral or caudal (−) to bregma. The green boxed areas in b and d correspond to the areas presented in photomicrographs in Fig. 6a–d. Note that anterogradely labeled axon fibers and terminals were not observed at levels a–d after a BDA injection into the left Su5 in rat R621. For abbreviations, see the abbreviations list.
division of the ipsilateral Mo5 (the so-called jaw-opening motor nucleus [JOM]). In contrast, a small number of terminals were labeled in the dorsolateral part only of the contralateral JCm. A large number of axon terminals were also labeled in the ipsilateral intertrigeminal region (I5) between the Su5 and the Mo5, whereas a small number
of axon terminals were labeled in the contralateral region. Moreover, few terminals were labeled in other reticular formation around the Mo5, including the reticular formation ventral to the Mo5 (Rfvm).

At the pontine level caudal to the Mo5 (Fig. 4j), a moderate number of labeled axon terminals were observed in the rostro-dorsomedial part (5Or) of the ipsilateral oral subnucleus of the trigeminal spinal nucleus (5O). At the more caudal pontine level, where the facial nucleus (VII) appeared (Fig. 4k), a large number of axon terminals were labeled in the ipsilateral juxtaganglionic region (J5) in the lateral reticular formation medially adjacent to the 5O. A
moderate number of labeled axon terminals were also noted in the dorsomedial part of the ipsilateral 5O, whereas a small number of terminals were labeled in the contralateral 5O. Furthermore, a small number of terminals were labeled in the lateral part of the ipsilateral VII (also Fig. 5f).

At the level of the rostral medulla (Fig. 4l), on the side ipsilateral to the BDA injection site, a large number of labeled axons were observed in the dorsal medullary reticular formation (dmRf) ventral to the Sol (also Fig. 5g), and extended laterally to the J5 medial to the dorsomedial part of the interpolar subnucleus of the trigeminal spinal nucleus (5I), however, only a small number of terminals were detected contralaterally in the corresponding areas of the dmRf and J5. In addition, a moderate number of labeled terminals were also noted in the rostro-ventrolateral part of the ipsilateral Sol (Fig. 4l, m). A small number of labeled terminals were observed in the ipsilateral paratrigeminal nucleus (Pa5) (Figs. 4l, 5h), which denotes both the “paratrigeminal” and “dorsal paramarginal” interstitial nuclei as defined by Phelan and Falls (1989). More caudally, at the rostrocaudal mid-level of the medulla (including the obex; Fig. 4m), a large number of labeled axon terminals were noted in the ipsilateral dmRf ventromedial to the Sol and lateral to the hypoglossal nucleus (XII), whereas a few terminals in the contralateral dmRf; these continued to the J5 medial to the rostro-dorsomedial part of the caudal subnucleus of the trigeminal spinal nucleus (5C). This distribution pattern was bilateral with a clear ipsilateral predominance. A large number of axon terminals were also observed in the ipsilateral XII (also Fig. 5i), whereas a few terminals in the contralateral XII. In addition, a small number of terminals were labeled in the dorsomedial edge of the ipsilateral 5C. At the caudal 5C level (Fig. 4n),
a moderate number of terminals were mostly labeled in the ipsilateral reticular formation, ventral to the cuneate nucleus (Cu) and medial to the caudal 5C.

### Distribution of CTb-labeled neuronal cell bodies

In all three rats (R303, R310, and R214) in the second experiment, the retrogradely labeled neuronal cell bodies (hereafter termed “neurons”) after CTb injections into the Su5 showed similar distribution patterns in the entire brain. The relative densities of labeled neurons in each nucleus or region of a representative rat (R303) are presented in Table 1. In this representative rat, the distribution pattern of cortical labeling was bilateral with a contralateral predominance (except for in the dorsal peduncular cortex, DP) (Fig. 2a–c). Most rostrally (Fig. 2a), a small number of labeled neurons were observed in the rostralmost level of the Agl, and even fewer neurons were labeled in the medial agranular cortex (Agm) and the agranular insular cortex (AI). More caudally (Fig. 2b), a large number of labeled neurons were found in the ventral part of the rostral S1 (also Fig. 6a) and its ventral vicinity in the rostral GI (also Fig. 6b). At a more caudal level (Fig. 2c), a moderate number of neurons were also labeled in the rostral S2 and its ventral vicinity in the GI. Almost all the labeled cortical neurons were situated in the inner part of cortical layer V (layer Vb). In addition, a small number of neurons were labeled in layer VI of the DP, but this distribution was only observed on the ipsilateral side.

Most rostrally in subcortical structures (Fig. 2c), a moderate number of CTb-labeled neurons were observed ipsilaterally in the lateral part of the rostrocaudally middle level of the BST, which appeared to include the rhomboid subnucleus of the BST as denoted by Swanson (2004). At a slightly more caudal level (Fig. 2d), a large number of neurons were ipsilaterally labeled in the rostral part of the central amygdaloid nucleus (AmC), especially in its medial division (also Fig. 6c), whereas a small number of labeled neurons were observed in the paraventricular hypothalamic nucleus (PvH), especially in its medial parvicellular part (also Fig. 6d). More caudally (Fig. 3e, f), a moderate number of labeled neurons were noted ipsilaterally at the caudal level of the dorsolateral part of the
LH, which caudally included the parasubthalamic nucleus (Psth) (also Fig. 6e).

At the middle level of the mesencephalon (Fig. 3g), a small number of neurons were ipsilaterally labeled in the retrorubral field (RRF) (also Fig. 6f). At the levels from the middle mesencephalon to the rostral pons, a large number of labeled neurons were scattered rostrocaudally in the ipsilateral Me5 (Figs. 3g, h, 4i). At the same rostrocaudal level (Fig. 3h), a small number of neurons were scattered throughout the dorsal raphe nucleus (DR). Furthermore, in the ventrolateral part only of the rostral Pb and its ventral vicinity in the dorsolateral part of the PnR, a moderate number of neurons were ipsilaterally labeled.

At the rostral pontine level (Fig. 4i), a moderate number of labeled neurons were observed in the Su5 contralateral to the CTb injection site (also Fig. 6g). A moderate number of neurons were also labeled in the ipsilateral Rfvm, whereas only a small number of neurons in the contralateral Rfvm. In addition, a small number of labeled neurons were bilaterally detected in the I5 between the Su5 and Mo5. Labeled neurons in the ipsilateral trigeminal principal nucleus (Pr5) were occasionally observed. At the pontine level between the Mo5 and the VII (Fig. 4j), only a small number of neurons were bilaterally labeled in the 5Or and in the J5 medial to the 5Or. At the caudal pontine level where the VII appeared (Fig. 4k), a large number of labeled neurons were observed ipsilaterally in the dorsal part of the 5O and in the lateral reticular formation including the J5 medial to the 5O, whereas fewer labeled neurons in the contralateral regions.

At the level of the rostral medulla (Fig. 4l), a large number of neurons were ipsilaterally labeled in the dmRf ventral to the Sol (also Fig. 6h) and in the J5 medial to the dorsomedial part of the 5I, whereas only a small number of neurons were labeled contralaterally in the corresponding regions. In addition, a small number of neurons were ipsilaterally labeled in the rostro-ventrolateral part of the Sol, dorsomedial part of the 5I, and the Pa5 (also Fig. 6i). At the medullary level that included the obex (Fig. 4m), a large number of labeled neurons were observed in the dorsal part of ipsilateral 5C; however, we noted that these labeled neurons were not concentrated in the superficial layer of the 5C medially adjacent to the spinal trigeminal tract (sp5). In the contralateral 5C, few neurons were labeled. Bilaterally with an obvious ipsilateral predominance, a large number of

Fig. 6 Photomicrographs showing retrogradely CTb-labeled neuronal cell bodies after a CTb injection into the left Su5 in rat R303. CTb-labeled neuronal cell bodies were observed in the primary somatosensory cortex (S1) (a), granular insular cortex (GI) (b), central amygdaloid nucleus (AmC) (c), periventricular hypothalamic nucleus (PvH) (d), parasubthalamic nucleus (Psth) (e), retrorubral field (RRF) (f), Su5 (g), dmRf (h), and Pa5 (i). Areas in a–i correspond to the areas shown in the green boxes in Fig. 2b (upper box), b (lower box), d (left box), and d (right box); 3f and g; and 4i, l (lower right box), and l (upper left box), respectively. Scale bars = 50 μm
neurons were also labeled in the dmRf ventral to the caudal Sol and in the J5 medial to the 5C, whereas fewer neurons were labeled in the caudal Sol. At the caudal medullary level (Fig. 4n), a small number of neurons were ipsilaterally labeled in the 5C and caudal Sol.

Brain regions that both receive afferents from and send efferents to the Su5

Taken together, the data described in the previous sections indicate that the brain structures that both receive afferents from and send efferents to the Su5 are located at the rostrocaudal level of the Su5 and its more caudal levels only (Fig. 4i–m, Table 1). Such structures were the ipsilateral PhR and Pb (Fig. 3h), contralateral Su5 (Figs. 4i, 5e, 6g), bilateral J5 between the Su5 and Mo5 (Fig. 4i), ipsilateral 5Or (Fig. 4j), dorsomedial part of the bilateral 5O (Fig. 4k), bilateral J5 medial to the 5O or 5I (Fig. 4j–m), rostroventrolateral part of the ipsilateral Sol (Fig. 4l), bilateral dmRf ventral to the Sol (Figs. 4i, m, 5g, 6h), ipsilateral Pa5 (Figs. 4l, 5h, 6i), and rostro-dorsomedial part of the ipsilateral 5C (Fig. 4m).

Discussion

The present study demonstrated the detailed efferent and afferent projections of the Su5 in the entire brain (excluding the cerebellum). The rat Su5 sent outputs to and received inputs from multiple brain structures; some of which overlapped. In the rostral brain, the Su5 received cortical inputs from the sensorimotor and dorsal insular cortices and DP, and it also received subcortical inputs from limbic and autonomic structures such as the BST, LH, Pvh, and AmC. In the caudal brain, the Su5 has strong reciprocal connections with motor structures that control orofacial movements. Among all the peripheral signals, the rat Su5 almost exclusively receives proprioceptive signals arising from JCMSs (Fujio et al. 2016). Thus, it is highly likely that orofacial movements receive online feedback control of orofacial proprioception via the Su5, which is influenced by higher brain regions related to sensorimotor, emotional, and autonomic functions.

Location of the Su5

The Su5 was first identified as an interneuron (premotoneuron) pool in the trigeminal reflex arc by Lorente de Nó (1922, 1933). Åström (1953) confirmed that both the Su5 and the Mo5 receive axon collaterals of Me5 primary afferents that convey proprioceptive sensation from masticatory muscle spindles. Torvik (1956) defined the location of the rat Su5 as a dorsomedial extension of the rostro-dorsomedial part of the Pr5, but the Su5 is cytoarchitectonically distinguishable from the Pr5. In the present study, the Su5 was defined based on its electrophysiological responses to JCMS stimulation in addition to its cytoarchitectonic features, as in our previous studies (Fujio et al. 2016; Sato et al. 2017, 2020; Yoshida et al. 2017). In this respect, what we considered to be the Su5 was located more caudolaterally than the Su5 that is delineated in the popular atlases by Paxinos and Watson (1986, 1998, 2014), but corresponded almost completely to the Su5 as defined by Torvik (1956) and Swanson (2004) (see Fujio et al. 2016 for the detailed differences between these two Su5 definitions). The connections of the rat Su5, based on Paxinos and Watson’s atlases, have been investigated in many earlier studies (e.g. Rokx et al. 1986; Shammas-Lagnado et al. 2001; Hattox et al. 2002; Mascaro et al. 2009; Papp and Palkovits 2014). Therefore, in the current study, we aimed to reexamine the efferent and afferent connections of the electrophysiologically and cytoarchitectonically identified Su5, which receives JCMS proprioceptive sensation.

Afferent and efferent connections of the Su5 and their functional consideration

Connections with the pons and medulla. The present study revealed that the Su5 projects to the bilateral Mo5—mainly to the Jcm—with a clear ipsilateral predominance. This Su5–Mo5 pathway has been well examined (Mizuno 1970; Donga et al. 1990; Yamamoto et al. 2007; Chang et al. 2009; Yoshida et al. 2009). JCMS proprioceptive signals that travel via the Su5 are considered to activate or inhibit the jaw-closing or -opening motoneurons; this has been confirmed by both electrophysiological (Ohta and Moriyama 1986; Nakamura et al. 2008; Nonaka et al. 2012) and morphological (Paik et al. 2009) studies. In the current study, the Su5 also projected ipsilaterally to the VII and XII, suggesting that JCMS proprioceptive signals that travel via the Su5 also activate or inhibit facial and tongue muscle contractions. The Su5 bilaterally (with an ipsilateral predominance) projected to the five regions (the J5, dorsomedial 5O, J5 medially adjacent to the 5O and 5I, ventrolateral Sol, and dmRf) that are known to contain premotoneurons projecting not only to the Mo5, but also to the VII, XII, or ambiguous nucleus (which contains motoneurons innervating the pharyngeal muscles) (Travers and Norgren 1983; Li et al. 1995; Cunningham and Sawchenko 2000; Yoshida et al. 2009; Oka et al. 2013; Stanek et al. 2014). These findings suggest that JCMS proprioceptive signals that travel via the Su5 activate or inhibit facial, tongue, and pharyngeal muscle contractions as well as jaw muscle contractions. Notably, the present study also revealed that the Su5 receives afferents from the five premotoneuron regions. Therefore, the jaw, facial, tongue, and pharyngeal muscle contractions that are
regulated by JCMS proprioception via the Su5 may receive feedback control from premotoneurons in these five regions. Accordingly, it is highly likely that JCMS proprioceptive signals that travel via the Su5 are involved in the coordination of mastication and swallowing. In addition, we revealed reciprocal connections between the bilateral Su5. This commissural connection may contribute to the bilateral coordination of the contractions of several muscles during mastication and swallowing.

The present study also revealed reciprocal connections between the Su5 and the ipsilateral Pa5. The Pa5 receives sensory inputs through the trigeminal nerve (Takemura et al. 1991), glossopharyngeal nerve (Altschuler et al. 1989; Ma et al. 2007), and superior laryngeal nerve (Oka et al. 2013), andnoxious inputs from orofacial tissue (i.e., the tooth pulp and temporomandibular joint) (Zhou et al. 1999; Shimizu et al. 2006). Therefore, JCMS proprioceptive signaling via the Su5 may communicate either cutaneous or deep sensation (including nociception) arising from other orofacial, pharyngeal, and laryngeal tissue.

Connections with the mesencephalon. It is important to note that Me5 primary afferents that convey JCMS proprioceptive signals do not give off any axons that ascend towards the thalamus; moreover, of all the secondary sensory neurons, Su5 neurons are considered to receive the strongest projections from Me5 afferents (Shigenaga et al. 1988a, 1989, 1990; Luo et al. 1995, 2001). These findings suggest that JCMS proprioception might be principally transmitted to higher brain regions via the Su5.

At the level of the mesencephalon, we identified a moderate projection from the Su5 to the contralateral SC, and especially its intermediate layer. The Su5–SC pathway has been reported in an earlier study (Yasui et al. 1993), although the Su5 was not precisely identified. The intermediate layer of the SC is known to receive projections from the substantia nigra pars reticulata as well as from the trigeminal sensory nuclear complex that transmits orofacial sensation (including muscle sensation from the external ocular muscles) but not JCMS proprioception (Porter and Donaldson 1991; Bickford and Hall 1992; Yasui et al. 1995; VanderWerf et al. 1997). The intermediate layer of the SC also projects to head and eye movement-related regions (including the reticular formation around the Mo5) in the brainstem and spinal cord (Huerta and Harting 1984; Yasui et al. 1994). Thus, the Su5–SC pathway may influence head and eye movements.

In the current study, we also demonstrated that the Su5 sends very dense and restricted projections to the BPN. The BPN is a major relay site of cerebral cortical inputs to the cerebellar cortex (for review see Brodal 1982; Wiesendanger and Wiesendanger 1982). The BPN receives projections from the trigeminal sensory nuclear complex and spinal cord as well as from the dorsal column nuclei (including the external cuneate nucleus, which conveys proprioceptive signals from the neck and forelimb muscles) (Rosén and Sjölund 1973; Campbell et al. 1974; Swenson et al. 1984; Kosinski et al. 1986; Mihailoff et al. 1989). Therefore, the BPN may be the hub for conveying integrated sensory inputs from the entire body to the cerebellar cortex. It seems plausible that JCMS proprioception is included in these sensory inputs.

In regard to afferent projections, the Su5 received strong projections from the ipsilateral Me5 neurons, which were rostrocaudally scattered at levels from the midbrain to the rostral pons. In addition, the Su5 received weak projections from the ipsilateral RRF. Given that the RRF is involved in orofacial motor function (Arts et al. 1998; Uchida et al. 2005), this function may be mediated by the RRF–Su5 pathway. Furthermore, the Su5 received inputs from neurons in the DR, which is located at levels from the caudal midbrain to the rostral pons. DR neurons include serotonergic neurons, which are involved in mood, sleep, and modulating pain (Sanders et al. 1980; Graeff et al. 1996; Ito et al. 2013). The DR–Su5 pathway might be involved in stress-induced involuntary movements such as bruxism and clenching.

Connections with the diencephalon. The Su5 did not receive projections from the thalamus. In contrast, the Su5 sent projections to the dorsal and ventral thalamus. For example, the Su5 projected strongly to the contralateral VPMcvm and weakly to the OPC, paracentral nucleus, and posterior thalamic nucleus; these projection features are consistent with those reported in our previous studies (Yoshida et al. 2017; Sato et al. 2020). The VPMcvm projects principally to the dG1rvs2 and less strongly to the rostral S2, while the OPC projects to the rostral S1 and S2 as well as to the rostral GI (Sato et al. 2017; Tsutsumi et al. 2021). We also identified a weak projection from the Su5 to the Pf in the present study. Berendse and Groenewegen (1991) reported that the rat Pf projects chiefly to the rostral level of the Agl, which corresponds to the primate primary motor cortex (Donoghue and Wise 1982; Donoghue and Parham 1983), and less strongly to the rostral part of the Agl, which corresponds to the primate premotor and supplementary motor cortices (Donoghue and Parham 1983; Hicks and Huerta 1991; Van Eden et al. 1992). Notably, we revealed contralateral projections from the Su5 to the ZI, which is part of the ventral thalamus, in the current study. The ZI receives strong exteroceptive and interoceptive inputs from the spinal cord and subformical region as well as from many brainstem nuclei (for review, see Mitrofanis 2005). Thus, the ZI may be an integrative hub between exteroception and interoception from the entire body. Together, these findings suggest that JCMS proprioception via the Su5 may be useful for the neuronal processing of emotion, sensory integration and discrimination, and motor actions, depending on the distinctive thalamic projections.
In the current study, we also demonstrated that the Su5 sends moderate projections to the LH and receives inputs from the posterior part of the ipsilateral LH, which includes the Psth. The hypothalamus has previously been reported to receive strong projections from the trigeminal sensory nuclear complex, which receives almost all orofacial sensation except for JCMS proprioception (Malick and Burstein 1998; Malick et al. 2000). Electrical stimulation of the cat LH activates the masseter muscle and facilitates the jaw-closing reflex (Landgren and Olsson 1980; Weiner et al. 1993). Goto and Swanson (2004) and Notsu et al. (2008) have also suggested that the Psth plays specific roles in central parasympathetic control. Therefore, the LH/Psth–Su5 pathways may regulate mastication during feeding behavior.

The Su5 also received ipsilateral projections from the PvH, especially its medial parvicellular part, in the present study. Physical and psychological stressors are known to activate parvicellular PvH neurons (Sawchenko et al. 1996; Herman and Cullinan 1997; Thompson and Swanson 2003; Coote 2005). The PvH–Su5 pathway, triggered by multiple stressors, may activate premotoneurons for masticatory movements (for review, see Dubner et al. 1978; Taylor 1990). Thus, this pathway might be another route for the induction of stress-induced involuntary movements.

Connections with the basal telencephalon. In the present study, the Su5 received ipsilateral projections from the dorsal part of the lateral BST (BSTl), which appeared to partly include the rhomboid subnucleus of the BST as denoted by Swanson (2004). The rhomboid subnucleus of the BST projects ipsilaterally to the Su5, Me5, Sol, salivary nucleus, and ambiguous nucleus (Dong and Swanson 2003). The Su5 also received ipsilateral projections from the rostral level of the AmC in the current study. Because the BSTl and AmC have similar neuronal connections with other brain regions (Alden et al. 1994;Bienkowski and Rinaman 2013), it is plausible that both the BSTl and AmC project to the Su5. In fact, electrical stimulation of the amygdala can induce rhythmical jaw movements (Kawamura and Tsukamoto 1960; Nakamura and Kubo 1978; Sasamoto and Ohta 1982) and excite Su5 neurons (Ohta and Moriyama 1986). In addition, both the BSTl and AmC are thought to coordinate behavioral and physiological responses to internal and environmental stressors (Alden et al. 1994; Bienkowski and Rinaman 2013). Therefore, this BSTl/AmC–Su5 pathway, activated by stressors, may cause involuntary movements.

Connections with the cerebral cortex. The Su5 had no projections to the cerebral cortex in the present study, but it received strong bilateral projections with a contralateral predominance from the rostroventral S1, rostral S2, and the GI ventrally adjacent to the S1 and S2 areas. The Su5 also received weaker projections from the ipsilateral DP and contralateral rostralmost Agl. These corticofugal pathways to the Su5 are consistent with previous findings after the injection of anterograde tracers into these cortical regions (rostroventral S1, Chang et al. 2009; Yoshida et al. 2009; Tomita et al. 2012; rostral S2, Haque et al. 2012; GI, Sato et al. 2013; Ikenoue et al. 2018; rostralmost Agl, Yoshida et al. 2009; DP, Akhter et al. 2014). Projections from the cortical areas to the Su5 may regulate the activity of Su5 neurons, thus enabling the emotional, sensory, and motor cortices to control orofacial movements, including jaw movements. Our previous studies (Sato et al. 2017, 2020; Tsutsumi et al. 2021) have demonstrated that JCMS proprioceptive signals are transmitted from the VPMcvm and OPC to the GI, rostral S2, and rostroventral S1. Thus, the GI–Su5, rostral S2–Su5, and rostroventral S1–Su5 pathways may play important roles in the feedback control of coordinated orofacial movements. In fact, electrical stimulation of the rostroventral S1 and rostralmost Agl induces rhythmical jaw movements (Sasamoto et al. 1990; Satoh et al. 2007; Avivi-Arber et al. 2010; Uchino et al. 2015). In contrast, direct projections from the cerebral cortex to cranial motoneurons (including the jaw-closing and opening trigeminal motoneurons) are sparse in the rat (Valverde 1962; Zhang and Sasamoto 1990), suggesting that corticofugal projections to premotoneurons (i.e., rostroventral S1–Su5 and rostralmost Agl–Su5 projections) may serve to drive stimulation-induced rhythmical jaw movements. We note that electrical stimulation of the so-called P-area in the rat insular cortex induces rhythmical jaw movements (Sasamoto et al. 1990; Satoh et al. 2007); however, the effective stimulation sites are located in the agranular or dysgranular insular cortices, but not in the GI. In addition, the DP is located in the prefrontal cortex, which is related to emotional and autonomic functions, and drives the suppression of fear and drug seeking (Vidal-Gonzalez et al. 2006; Peters et al. 2009). Thus, some emotional or autonomic functions of the DP may affect Su5 neurons through the DP–Su5 route.

Author contributions All authors read and approved the final manuscript. AY and YTa conceptualized the hypothesis, designed and supervised the experiments and data analysis. MI, FS, YM and YTas carried out the experiments and data analysis. TF and KU helped with laboratory and histological procedures. MI, FS, YM, TF, KU, AY and YTa performed the statistical analysis. AY, MI, FS, YM and YTas wrote the manuscript. AY and YTa finalized the figures and text. AY and YTs provided funding support and critical comments upon request.

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