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Original Research

ORIGINAL RESEARCH

Topical ATP Application in the Peripheral Swallowing-Related Regions Facilitates Triggering of the Swallowing Reflex Involving P2X3 Receptors

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Abstract

The swallowing reflex is a critical component of the digestive process, triggered when food or liquids pass from the oral cavity to the oesophagus. Although adenosine triphosphate (ATP) is involved in various physiological processes, its potential to trigger the swallowing reflex has not been fully explored. This study investigated the ability of ATP to induce the swallowing reflex and examined the involvement of the purinoreceptor P2X3 in this process. We observed that the topical application of exogenous ATP to the superior laryngeal nerve (SLN)-innervated swallowing-related regions dose-dependently facilitated the triggering of the swallowing reflex. P2X3 receptors were predominantly localized on nerve fibres within these regions, including intraepithelial and subepithelial nerves and those associated with taste-bud-like structures. In the nodose–petrosal–jugular ganglionic complex, approximately 40% of retrogradely traced SLN-afferent neurons expressed P2X3, with 59% being medium-sized, 30% small, and 11% large. Prior topical application of a P2X3 antagonist in SLN-innervated, swallowing-related regions significantly reduced the number of ATP-induced swallowing reflexes. Furthermore, topical application of a P2X3 receptor agonist more selective than ATP facilitated reflex triggering in a dose-dependent manner. These findings suggest that exogenous ATP facilitates the triggering of the swallowing reflex through the activation of P2X3 receptors. This activation excites afferent neurons that supply peripheral swallowing-related regions, stimulating the swallowing central pattern generator to facilitate the reflex. The current findings suggest the therapeutic potential of ATP or P2X3 agonists for dysphagia treatment and provide valuable physiological insights into the involvement of purinergic signaling in triggering the swallowing reflex.

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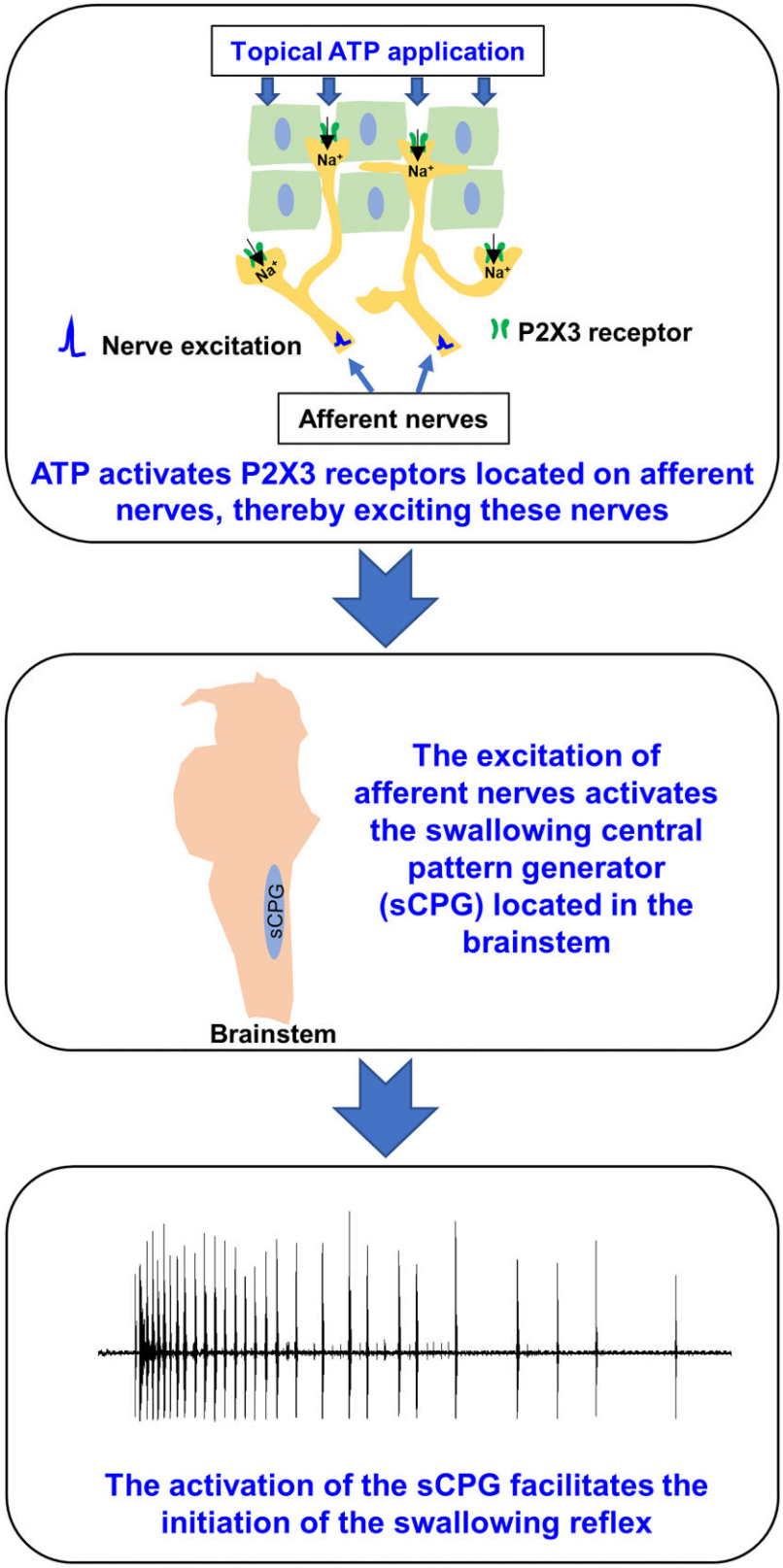


Key words: adenosine triphosphate; swallowing reflex; P2X3 receptors; superior laryngeal nerve-afferents; P2X3 agonists; nodose–petrosal–jugular ganglionic complex

Introduction

The swallowing reflex is crucial for transporting ingested materials like food, liquids, and saliva to the oesophagus while preventing their entry into the respiratory tract.[1–3] The laryngopharynx and associated laryngeal regions supplied by the superior laryngeal nerve (SLN) (a branch of the vagus nerve) play a pivotal role in triggering the swallowing reflex.[1,4–6] Applying mechanical and chemical stimuli to the regions or direct electrical stimulation to the SLN triggers the swallowing reflex, indicating the importance of these regions.[1,5–8] These stimuli potentially induce the release of signaling molecules that excite nerves, initiating the swallowing reflex.[5,6]

While adenosine triphosphate (ATP) is renowned as a cellular energy source, it also serves as a signaling molecule in various physiological and pathological processes across multiple systems, including cardiovascular, [9] respiratory,[10] immune,[11] renal,[12] gastrointestinal,[13–15] and central and peripheral nervous systems.[16–21] Although previous studies have indicated the involvement of ATP in triggering the swallowing reflex,[5,6,22,23] investigations into the effects of exogenous ATP in this context are lacking. Neurological disorders (eg, stroke) and aging often result in abnormality in the swallowing process or delayed initiation of the swallowing reflex, termed oropharyngeal dysphagia, leading to aspiration pneumonia caused by food particles entering the airways.[24–29] Understanding the ability of ATP to elicit the swallowing reflex could aid the development of therapeutic approaches for oropharyngeal dysphagia.

The action of ATP relies on purinergic receptor 2 (P2 receptors), which can be categorized into P2X and P2Y receptor classes.[16,17,30] P2Y receptors, belonging to the G protein-coupled receptor family, activate intracellular signaling pathways through second messengers. Conversely, P2X receptors function as ligand-gated ion channels, rapidly responding to ATP by facilitating cation influx (eg, Na + and Ca++), impacting cellular excitability. This receptor class includes 7 subtypes (P2X1-7), each characterized by specific distributions and functions. Among the P2 receptors, P2X3 is predominantly expressed in peripheral sensory neurons and plays a pivotal role in sensory transmission.[16,17,30] Hence, it is plausible that P2X3 receptors are involved in ATP-induced swallowing reflexes.

Our previous studies have shown that activation of various transient receptor potential (TRP) channels (eg, TRPV1, TRPV4, TRPA1)[31–33] in the peripheral swallowing-related regions facilitated the triggering of swallowing reflexes. Although these receptors are known for transducing various sensations (eg, heat, pain) from the oral cavity and craniofacial regions, our research, along with studies from other groups, has indicated that these receptors in the pharyngeal and laryngeal regions play a role in triggering the swallowing reflex.[5,6,31–35] On the basis of these findings, we hypothesize that purinergic receptors may also be involved in triggering the swallowing reflex, similar to TRP channels.

The current study aimed to determine whether exogenous ATP can trigger the swallowing reflex and investigate the potential involvement of P2X3 receptors in mediating this response.

Materials and Methods

Fifty-seven male Sprague Dawley rats, weighing approximately 300-450 g, were used in this study. The rats were used for immunohistochemistry ($n = 8$), PCR ($n = 2$), and the assessment of the swallowing reflex ($n = 47$). All experimental procedures were approved by the Matsumoto Dental University Intramural Animal Care and Veterinary Science Committee (Ref. No. 394). Animals were housed at the Matsumoto Dental University animal facility under controlled conditions, maintaining a temperature of 22 ± 2°C, 40 ± 5% relative humidity, and a 12-h light/dark cycle. Food and water were provided ad libitum. Our protocols prioritized minimizing animal suffering and reducing the number of animals used. All experimental procedures adhered to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines established by the National Centre for the Replacement, Refinement, and Reduction of Animals in Research.

Surgery

After inducing urethane anaesthesia (1.0-1.5 g/kg, administered intraperitoneally), the rats were positioned supine, and a midline incision was made on the ventral surface of the neck to expose the trachea. To sustain respiration, a custom-made cannula was delicately inserted into the trachea. A small segment of the ventral trachea was surgically removed to create a delivery window for solutions. The bilateral SLNs were preserved, while the pharyngeal (IX-ph) and lingual (IX-li) branches of the glossopharyngeal nerve, as well as the pharyngeal (X-ph) and recurrent laryngeal (RLN) branches of the vagus nerve, were bilaterally transected. This meticulous dissection was designed to focus on the SLNs and prevent any influence of non-SLN components on the triggering of the swallowing reflex. In a separate experiment, the bilateral SLNs were also transected, along with the aforementioned nerves, to confirm whether the SLNs are involved in triggering the swallowing reflex in this study's experimental setup.

Swallowing Reflex Recording

To identify the swallowing reflex, electromyogram (EMG) activity of the mylohyoid muscle was recorded using bipolar fine wire electrodes (stainless-steel and urethane-coated; Unique Medical Co., Ltd., Tokyo, Japan) .[31–33,36] During reflex triggering, high-amplitude EMG activity was observed in the mylohyoid muscle, with each high-amplitude event in the EMG signal corresponding to one instance of the swallowing reflex. Identification of triggered swallowing reflexes involved assessing the high-amplitude EMG signals of the mylohyoid muscle and visual checking of laryngeal movements associated with reflex initiation.[31–33,36] Examiners utilized a loudspeaker connected to the EMG signal to aid in observing mylohyoid muscle firing. In some rats, respiratory chest movements and the EMG activity of the rectus abdominis muscle were also recorded alongside the mylohyoid muscle activity. The rectus abdominis EMG activity was recorded using the same type of bipolar fine wire electrodes. The EMG signals were filtered and amplified using a bioelectric amplifier (MEG-5200, Nihon Kohden, Tokyo, Japan). Respiratory chest movements were measured using a piezoelectric transducer (ADInstruments, Inc., Colorado, USA). All signals were acquired and digitized using a Power 1401 data acquisition system (Cambridge Electronic Design Ltd., Cambridge, UK) and stored on a hard drive for subsequent analysis.

Recording the activity of one or more muscles associated with swallowing has been widely adopted as a standardized method for identifying the swallowing reflex. Previous studies, including those from our group, have utilized high-amplitude electromyographic (EMG) activity in the mylohyoid muscle,

along with visual observation of laryngeal movements, to reliably identify the reflex.[7,31–33,36–40] To further confirm that the high-amplitude EMG bursts observed in the mylohyoid muscle during our experiments represented swallowing reflexes rather than coughing or other airway defense reflexes, we recorded respiratory chest movements and EMG activity from both the mylohyoid and rectus abdominis muscles (the latter being associated with the cough reflex[41]) in certain rats (Figure S1A and B). Upon ATP administration, repetitive high-amplitude EMG bursts in the mylohyoid muscle were observed within a short timeframe, accompanied by a period of complete cessation of respiration (apnoea) (Figure S1B). Importantly, no EMG activity was detected in the rectus abdominis muscle. The absence of respiratory chest movements and rectus abdominis activity confirmed that the high-amplitude EMG bursts in the mylohyoid muscle were attributable to swallowing reflexes rather than coughing, as the cough reflex is typically associated with chest movements involving inspiration followed by forceful expiration and activation of abdominal muscles, including the rectus abdominis.[41,42] As respiration gradually resumed following the triggering of these repeated reflexes, subsequent swallowing reflexes occurred at progressively longer intervals, again without rectus abdominis activity. Following saline administration, a single swallowing reflex was induced, accompanied by a very brief cessation of respiration (Figure S1A). These observations further support that the high-amplitude EMG bursts in the mylohyoid muscle following ATP application were indicative of swallowing reflexes.

Stimulating Solutions

The stimulating solutions employed in the experiment included saline (NaCl 0.9%, Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) and various concentrations of ATP disodium salt (Tocris Bioscience, Bristol, UK; 0.5, 1, 10, 20, and 50 mM). A 50 mM stock solution of ATP disodium salt was prepared by dissolving it in saline. The pH of the stock solution was adjusted to match that of saline (approximately pH 6) by adding sodium hydroxide. Other concentrations of ATP were prepared by diluting the stock solution with saline. The tonicity of the solutions is unlikely to vary greatly, as they were diluted in saline, and the osmolarity of ATP at the aforementioned concentrations is relatively low, according to a previous study that measured the osmolarity of various ATP concentrations.[43] A syringe equipped with a 21-gauge needle (blunt tip) was utilized to administer the solutions to the SLN-innervated swallowing-related regions. Each solution was administered in a volume of 50 μL. Subsequently, reflex responses were recorded for a duration of 1 min following each delivery. A 2-3-min interval was observed between the administrations of different solutions. The administered solutions were aspirated during this interval, and the regions were rinsed with saline. All solutions were administered at room temperature (22-24°C).

P2X3 Antagonist

This study used Gefapixant (5-[(2,4-diamino-5-pyrimidinyl)oxy]-2-methoxy-4-(1-methylethyl)-benzenesulfonamide); MedChemExpress, New Jersey, USA) as a P2X3 antagonist. The efficacy of Gefapixant for this purpose has been previously confirmed.[44–48] Gefapixant was prepared by dissolving it in 3% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and 1% Tween 80 (Sigma-Aldrich, St. Louis, MO), followed by dilution in saline. Before administering ATP solutions, either the P2X3 antagonist or its vehicle was instilled into the SLN-innervated swallowing-related region for 15 min then aspirated. The vehicle for the P2X3 antagonist consisted of a solution of dimethyl sulfoxide, Tween 80, and saline.

P2X3 Agonist

To investigate whether activation of P2X3 receptors is sufficient to trigger swallowing reflexes, α,β-methylene ATP (α,β-Methyleneadenosine 5'-triphosphate trisodium salt; Cayman Chemical, Michigan, USA), a more selective agonist for P2X3 receptors compared to ATP, was used. Various concentrations (0.5, 1, 10, and 20 mM) were tested. The efficacy of α,β-methylene ATP in activating P2X3 receptors has been demonstrated in previous studies.[49–51] A 20 mM stock solution was prepared by dissolving the compound in saline, and the pH was adjusted to approximately 6 (matching that of saline) using sodium hydroxide. Lower concentrations were prepared by diluting the stock solution with saline.

Lidocaine

In an experiment, a local anaesthetic, 2% lidocaine (2-(Diethylamino)-N-(2,6-dimethylphenyl) acetamide Xylocaine; AstraZeneca Ltd., Osaka, Japan) was topically applied to assess whether the sensory nerves of the SLN-innervated, swallowing-related region are involved in triggering the ATP-induced swallowing reflex in the experimental setup of this study.

Immunohistochemistry of SLN-innervated Peripheral Swallowing-Related Regions

Deeply anaesthetized rats underwent transcardial perfusion with saline followed by 4% paraformaldehyde. The swallowing-related regions were dissected then immersed in 4% paraformaldehyde for 1 h. The specimens were subsequently incubated in 30% sucrose until fully submerged to ensure adequate cryoprotection. The samples were then embedded in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek, Tokyo, Japan) and sectioned in the sagittal plane (8 or 25 μm) before being mounted on glass slides.

The sections underwent incubation with 5% normal goat serum in 0.01 M phosphate-buffered saline containing 0.3% Triton X-100 for 1 h to minimize nonspecific binding. Subsequently, the sections were exposed to rabbit monoclonal anti-P2X3 antibodies (1:300 dilution; Cat# ab300493; RRID: AB_2 941 895; Abcam, Cambridge, UK) at 4°C overnight, followed by incubation with a fluorescent dye-conjugated secondary antibody (Goat anti-rabbit Alexa Fluor 594; Cat# A-11037; RRID: AB_2 534 095; Thermo Fisher Scientific, Waltham, MA, USA) for 2 h at room temperature. Sections were then treated with 4,6-diamidino-2-phenylindole (DAPI) for 10 min to visualize cell nuclei. Finally, coverslips were applied using a mounting medium (PermaFluor; Thermo Fisher Scientific), and the specimens were examined utilizing fluorescence microscopy (BZ-X700; Keyence Corp., Osaka, Japan).

Reverse Transcription Polymerase Chain Reaction

Total ribonucleic acid (RNA) was extracted from the nodose–petrosal–jugular ganglionic complex (NPJc) and trigeminal ganglia (TG) using a NucleoSpin® RNA kit (Macherey-Nagel; Takara Bio Inc., Shiga, Japan). Subsequently, cDNA was synthesized using SuperScript IV (VILO Master Mix with ezDNase™,

Table 1. Cell Size Distribution of Fluorogold-Stained and Non-Fluorogold-Stained P2X3 Immunoreactive Neurons in the NPJc.

	Small (<600 μm^2)		Medium (600-1200 μm^2)		Large (>1200 μm^2)	
	FG-stained	Non-FG-stained	FG-stained	Non-FG-stained	FG-stained	Non-FG-stained
NG	28.21% (101/358)	36.96% (119/322)	60.62% (217/358)	53.73% (173/322)	11.17% (40/358)	9.32% (30/322)
PG	29.63% (8/27)	46.16% (337/730)	40.74% (11/27)	40.74% (341/730)	29.63% (8/27)	7.12% (52/730)
JG	43.18% (19/44)	50.00% (185/370)	54.55% (24/44)	42.70% (158/370)	2.27% (1/44)	7.30% (27/370)
NPJc	29.84% (128/429)	45.08% (641/1422)	58.74% (252/429)	47.26% (672/1422)	11.42% (49/429)	7.67% (109/1422)

All data were obtained from 45 sections (3 sections/ganglion/rat) $n = 5$. The numbers in parentheses indicate the numbers of neurons analyzed. NG, nodose ganglion; PG, petrosal ganglion; JG, jugular ganglion; NPJc, nodose–petrosal–jugular ganglionic complex.

Thermo Fisher Scientific). PCR was conducted with specific primer sets employing TaKaRa Ex Taq® (Takara Bio Inc., Shiga, Japan). The primer sequences used were as follows: for P2X3, forward 5′-CTGGCTACAACTTCAGGTTT-3′, reverse 5′-TCACCTCCTCAAACTTCCTG-3′ (product size 258 bp) was used; for *β-actin*, forward 5′-AGACTTCGAGCAAGAGATGG-3′, reverse 5′-AGGAAGGAAGGCTGGAAGAG-3′ (product size 138 bp) was used. *β-actin* served as the reference gene. The PCR products were assessed by 2% agarose gel electrophoresis in 0.5% Tris-borate-ethylenediaminetetraacetic acid buffer and visualized post-staining with an ethidium bromide solution.

Immunohistochemistry of the NPJc

Fluorogold (FG) was employed to trace SLN-afferent neurons in the NPJc retrogradely. The right-sided SLN was isolated and transected at its entry to the thyrohyoid membrane under pentobarbital anaesthesia. Subsequently, the SLN was inserted into a small tube filled with 4% FG. Then, 5 to 7 days post-FG incorporation, deeply anaesthetized rats underwent transcardial perfusion with saline followed by 4% paraformaldehyde. Each ganglion in the right-sided NPJc was identified based on its anatomical location and relative position. The nodose ganglion was distinguished by its attachment to the main trunk of the vagus nerve, oriented toward the thorax. The jugular ganglion was located in the direction of the brainstem, opposite to the main vagus nerve trunk, while the petrosal ganglion was positioned between these 2 structures. To maintain proper anatomical orientation during embedding, the NPJc was excised along with a small segment of the main vagus nerve trunk. The excised tissue was immersed in 4% paraformaldehyde for fixation, transferred to 30% sucrose for cryoprotection until it sank, and subsequently embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek), preserving its anatomical position and orientation. The tissue was sectioned at a thickness of 16 μm, and the sections were mounted onto glass slides. These preparations enabled the identification of each ganglion after sectioning based on their relative anatomical positions within the NPJc.

Sections underwent incubation in 0.01 M phosphate-buffered saline containing 5% normal goat serum and 0.3% Triton X-100 for 30 min to minimize nonspecific binding. The sections were then incubated with rabbit monoclonal anti-P2X3 antibodies (1:2000 dilution; Cat# ab300493; RRID: AB_2 941 895; Abcam, Cambridge, UK) at room temperature, followed by incubation with a secondary antibody (Goat anti-rabbit Alexa Fluor 594; Cat# A-11037; RRID: AB_2 534 095; Thermo Fisher Scientific). The sections were then coverslipped and viewed by fluorescence microscopy (BZ-X700; Keyence Corp.). Immunoreactive cells were quantified using ImageJ software (NIH, Bethesda, MD), and only cells containing nuclei were included in the analysis. Cells were considered immuno-positive if the intensity of staining exceeded 2-fold the background level of the negative control. Positive and negative control slides (Figure S2) for P2X3 antibody were used as references to distinguish immuno-positive from immuno-negative neurons. The sections exhibiting the highest number of FG-stained cells were selected for counting. A total of nine sections were chosen from each rat (3 sections/ganglion). To prevent the same neuron from being counted twice across multiple sections, a minimum gap of 100 μm was maintained between the selected sections. Additionally, the researcher carefully examined the selected sections to ensure that the same neurons were not observed across different sections. On average, 212.20 ± 45.01 (mean ± SD) FG-stained cells were counted per animal in the NPJc.

ImageJ software was utilized to measure the cell body area of neurons expressing P2X3, both with and without FG staining. A cell area >1200 μm^2 was classified as large, that of 600-1200 μm^2 was classified as medium, and that of <600 μm^2 was classified as small (Table 1). This classification system was adopted from previous studies analyzing P2X3 expression in the human nodose ganglia,[52] rat dorsal root ganglia,[53] and TG.[54] Although neuronal sizes may vary across ganglia and species, the broad size ranges used for classification help mitigate the impact of inter-ganglionic and interspecies differences on categorization. The researcher measuring the area of the positive neurons was blinded to the final outcomes during measuring, ensuring unbiased measuring. Due to the varying shapes, sizes, and orientations of neurons within the ganglionic complex, it was not feasible to ensure that all neurons were sectioned precisely through their centers. Consequently, some measurement errors were unavoidable. However, to minimize inaccuracies, only neurons containing nuclei were included in the analysis. Additionally, potential measurement errors are unlikely to significantly affect neuronal classification, as categorization was based on broad size ranges (eg, 600-1200 μm^2 for medium-sized neurons).

Because P2X3 is localized in TG,[55–58] TG sections were utilized as a positive control for the anti-P2X3 antibody (Figure S2). As a negative control, the sections were incubated with a universal negative control reagent (Cat. # ADI-950-231-0025; Enzo Life Sciences, Inc., Farmingdale, NY) instead of the primary antibody (Figure S2).

Data and Statistical Analysis

Swallowing reflexes triggered by stimulating solutions were counted for 60 s. For statistical analysis, data underwent normality and equal variance tests to determine the appropriate test type, whether parametric or non-parametric. A one-way repeated measures analysis of variance (ANOVA), followed by Tukey's test, was used to compare the number of swallowing reflexes triggered by different concentrations of ATP, α,β-methylene ATP, and the reflexes with and without prior application of the P2X3 antagonist or its vehicle. A paired t-test was employed to compare the numbers of ATP-triggered swallowing reflexes with and without prior application of lidocaine or transection of SLNs. A P-value $< .05$ was considered statistically significant. The data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were conducted using Sigmaplot software (version 14.0; Systat Software Inc., San Jose, CA). Column graphs with individual data points were created using GraphPad Prism Software (version 10.2; San Diego, CA).

Results

Topical Application of ATP Dose-Dependently Facilitated Triggering of the Swallowing Reflex

We initiated our investigation by assessing the potential of exogenous ATP administration in peripheral swallowing-related regions to induce the swallowing reflex. Various concentrations of ATP disodium salt diluted in saline were topically applied to the SLN-innervated swallowing-related regions. Saline administration elicited only 1 or 2 swallowing reflexes per administration (Figure 1A and B). The administration of ATP resulted in the triggering of swallowing reflexes in a dose-dependent manner. Notably, the highest number of reflexes was observed with a concentration of 20 mM ATP (33.00 $\pm$ 7.57) (Figure 1B). Increasing the ATP concentration to 50 mM did not trigger more reflexes (31.86 $\pm$ 8.61). The numbers of swallowing reflexes triggered at 20 and 50 mM ATP concentrations were significantly higher than those triggered by 0.5, 1, 10 mM ATP, and saline (Figure 1B). Additionally, the number of swallowing reflexes triggered at 10 mM was significantly higher than the number triggered by 0.5, 1 mM ATP, and saline (Figure 1B).

An ATP Receptor, P2X3, Was Expressed on Nerves Innervating the Peripheral Swallowing-Related Regions

Next, we investigated the presence of an ATP receptor, P2X3, within the SLN-innervated peripheral swallowing-related regions. We observed P2X3 expression on nerve fibres within these regions (Figure 2A to J). P2X3-expressed nerve fibres were detected in both intra- and sub-epithelial areas. In the intraepithelial areas, these fibres traversed between epithelial cells, often extending near to the surface of the epithelial layer. Within the subepithelial areas, P2X3 expression was observed on both thin and thick nerve fibres. Moreover, P2X3 expression was noted on nerve fibres supplying taste-bud-like structures. Both intragemmal and subgemmal nerve fibres within these structures exhibited P2X3 expression. Additionally, P2X3 expression was observed on subgemmal neurogenous plaques associated with taste-bud-like structures.

P2X3 Expression on SLN-afferent Neurons in the NPJc

We also explored the localization of P2X3 in the NPJc, where the cell bodies of SLN-afferent neurons are situated. Utilizing FG, a retrograde tracer, we traced SLN-afferent neurons in the NPJc (Figure 3A-C). Our results showed that P2X3 was expressed in approximately 50% of FG-labelled SLN-afferent neurons in the nodose ganglion. In contrast, its expression was observed in about 20% of SLN-afferent neurons in the petrosal and jugular ganglia. Across the entire NPJc, P2X3 expression was approximately 40% (Figure 3C). Additionally, P2X3 mRNA was detected in the NPJc using reverse transcription polymerase chain reaction (RT-PCR) (Figure 3D). Analysis of the areas of FG-stained, P2X3-expressing SLN-afferent neurons (Figure 4A-D) revealed that 58.74% were medium-sized, 29.84% were small-sized, and 11.42% were large-sized across the entire NPJc (Table 1). In comparison, among non-FG-stained P2X3-expressing neurons (Figure 4A-D), 47.26% were medium-sized, 45.08% were small-sized, and 7.67% were large-sized (Table 1).

A P2X3 Antagonist Significantly Reduced the Number of ATP-induced Swallowing Reflexes

Given our identification of the ATP receptor P2X3 in the swallowing-related regions, our subsequent inquiry aimed to ascertain whether a P2X3 antagonist could mitigate the number of ATP-induced swallowing reflexes. Various concentrations of a P2X3 antagonist, Gefapixant, were administered into the swallowing-related regions 15 min before ATP administration. For this investigation, we employed ATP at a concentration of 20 mM, as this concentration elicited the highest number of triggered reflexes. Additionally, we assessed the effect of the vehicle for Gefapixant (vehicle for the highest concentration of Gefapixant used in this study). The findings demonstrated that the P2X3 antagonist at concentrations of 10, 20, and 50 mM significantly reduced the number of swallowing reflexes induced by 20 mM ATP (Figure 5A and B). Conversely, the vehicle for Gefapixant showed no significant effect on the number of ATP-induced swallowing reflexes (Figure 5A and B). Furthermore, the P2X3 antagonist at a concentration of 1 mM also exhibited no significant effect on the number of ATP-induced swallowing reflexes (Figure 5A and B).

Topical Application of a More Selective P2X3 Agonist, Compared to ATP, Facilitated the Triggering of Swallowing Reflex in a Dose-Dependent Manner

To investigate whether activation of P2X3 is sufficient to trigger the swallowing reflex, various concentrations of a more selective P2X3 agonist, α,β-methylene ATP, were topically administered to the SLN-innervated swallowing-related regions. Similar to ATP, α,β-methylene ATP induced swallowing reflexes in a dose-dependent manner (Figure 6A and B). The highest number of reflexes (21.60 $\pm$ 2.97) was observed at a concentration of 10 mM α,β-methylene ATP. The numbers of swallowing reflexes triggered at α,β-methylene ATP concentrations of 5, 10, and 20 mM were significantly higher than those triggered by 0.5, 1 mM ATP, and saline (Figure 6B).

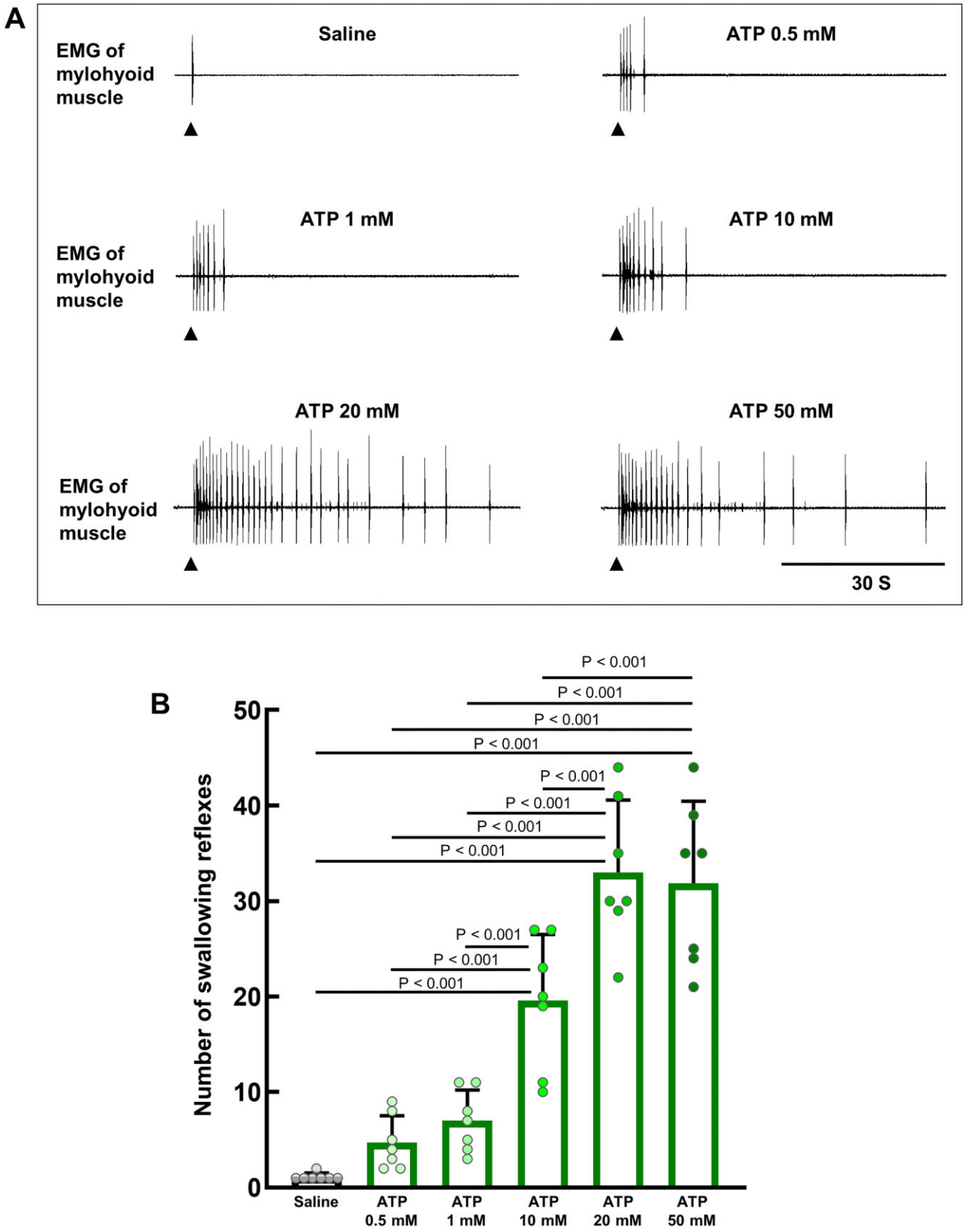


Figure 1. Dose-dependent facilitation of swallowing reflexes upon topical application of adenosine triphosphate (ATP). (A) Swallowing reflexes depicted by electromyogram (EMG) activities of mylohyoid muscle triggered by saline and various ATP concentrations, with black arrowheads denoting the onset of stimulating solution delivery. (B) Comparison of the number of swallowing reflexes triggered by saline and different ATP concentrations. Statistical analysis was conducted by one-way repeated measures ANOVA followed by Tukey's test ($n = 7$). The number of triggered swallowing reflexes was counted for 60 s post-application of the stimulating solutions. Data are represented as the mean ± SD, with individual data points depicted by circles in the column graph. S, seconds.

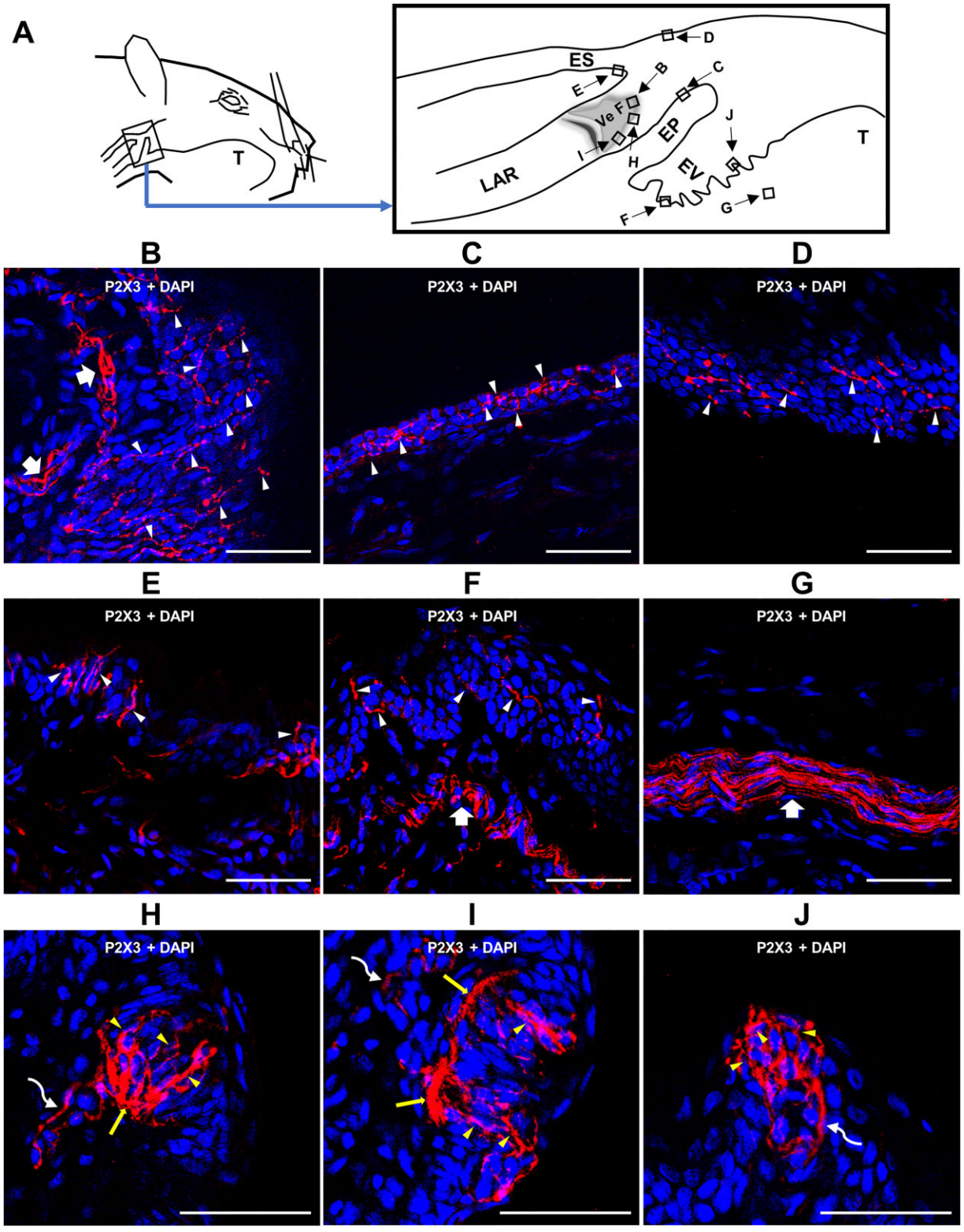


Figure 2. P2X3 expression was observed on nerve fibres within the swallowing-related regions. (A) A schematic illustrates the superior laryngeal nerve (SLN)-innervated peripheral swallowing-related regions. Rectangles with arrows and letters indicate the areas where the photomicrographs were captured. Photomicrographs depict P2X3 (red) localization in the (B) vestibular fold (Ve F), (C) epiglottis (EP), (D) laryngopharyngeal wall, (E) cervical oesophagus (ES), (F and G) epiglottic vallecula (EV), and (H-J) taste bud-like structures. In the images, small white arrowheads highlight examples of intraepithelial nerve fibres running between epithelial cells expressing P2X3, often reaching near the surface of the epithelium. Thick white arrows indicate examples of thick nerves expressing P2X3. Small yellow arrowheads denote examples of intragemmal nerve fibres in taste buds expressing P2X3, while curved white arrows illustrate subgemmal nerve fibres in taste buds expressing P2X3. Long yellow arrows demonstrate examples of subgemmal neurogenous plaques in taste buds expressing P2X3. P2X3 expression in peripheral swallowing-related regions was analyzed in 3 rats. The photomicrographs are representative images. Scale bars = 50 μm. LAR, larynx; T, tongue.

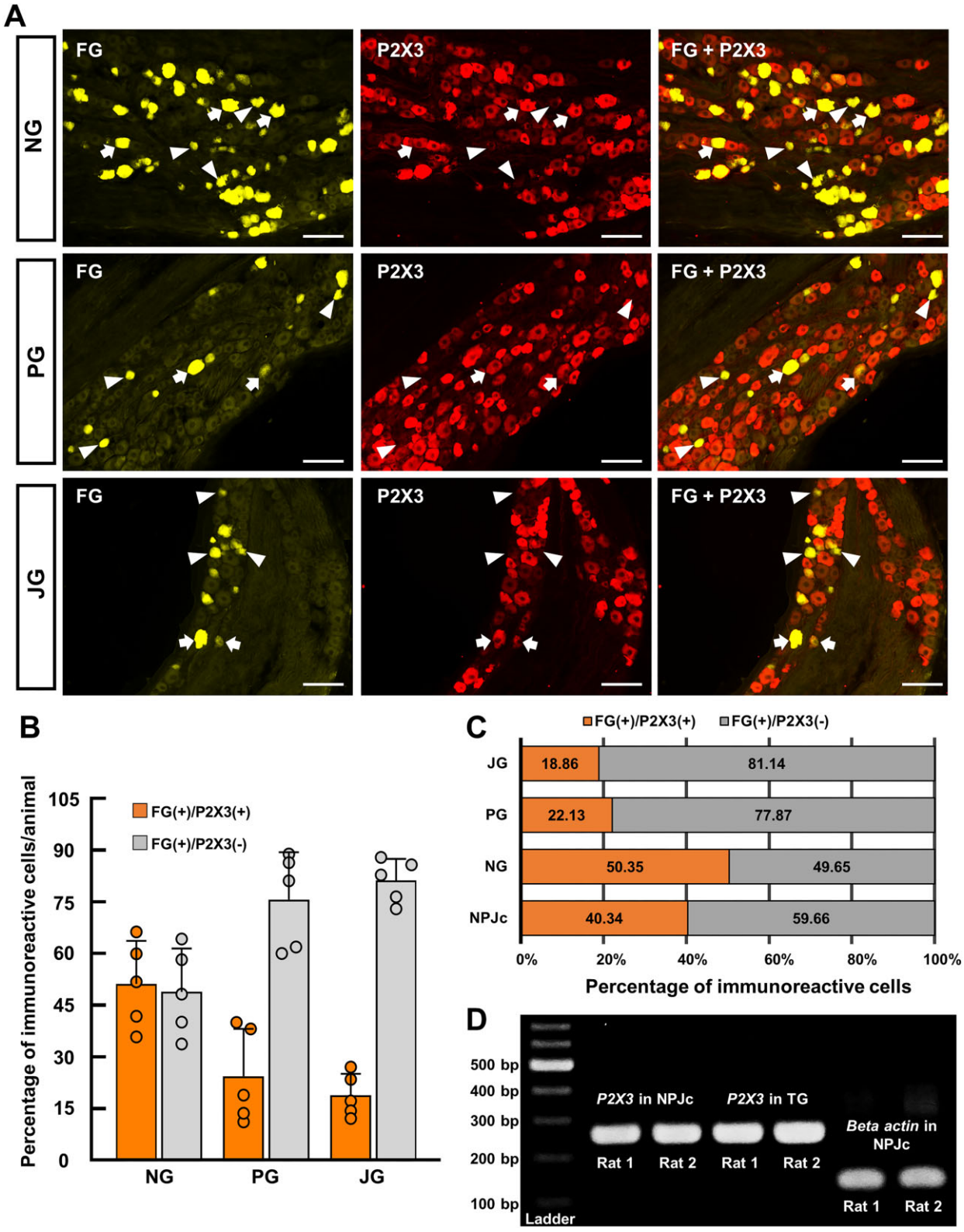


Figure 3. Localization of P2X3 on superior laryngeal nerve (SLN)-afferent neurons in the nodose–petrosal–jugular ganglionic complex (NPJc). (A) Expression of P2X3 in the nodose ganglion (NG), petrosal ganglion (PG), and jugular ganglion (JG). White arrows indicate cells that were positive for fluorogold (FG) and P2X3. White arrowheads indicate cells that were positive for FG but negative for P2X3. Scale bars = 100 μm. (B) The percentage of FG-stained, P2X3-positive, or P2X3-negative cells/animal. Data are presented as mean ± SD $n = 5$. Sections exhibiting the highest number of FG-stained cells were selected for cell counting, with 9 sections used from each rat (3 sections per ganglion). Circles in the column graph represent individual data points for each animal. (C) Percentage of FG-stained, P2X3-positive, or P2X3-negative cells in the NG, PG, JG, and NPJc. FG(+)/P2X3(+), FG-stained cells immunopositive for P2X3; FG(+)/P2X3(−), FG-stained cells immunonegative for P2X3. (D) P2X3 mRNA in NPJc and trigeminal ganglion (TG). Two rats were used for reverse transcription polymerase chain reaction (RT-PCR). TG was used as a positive control.

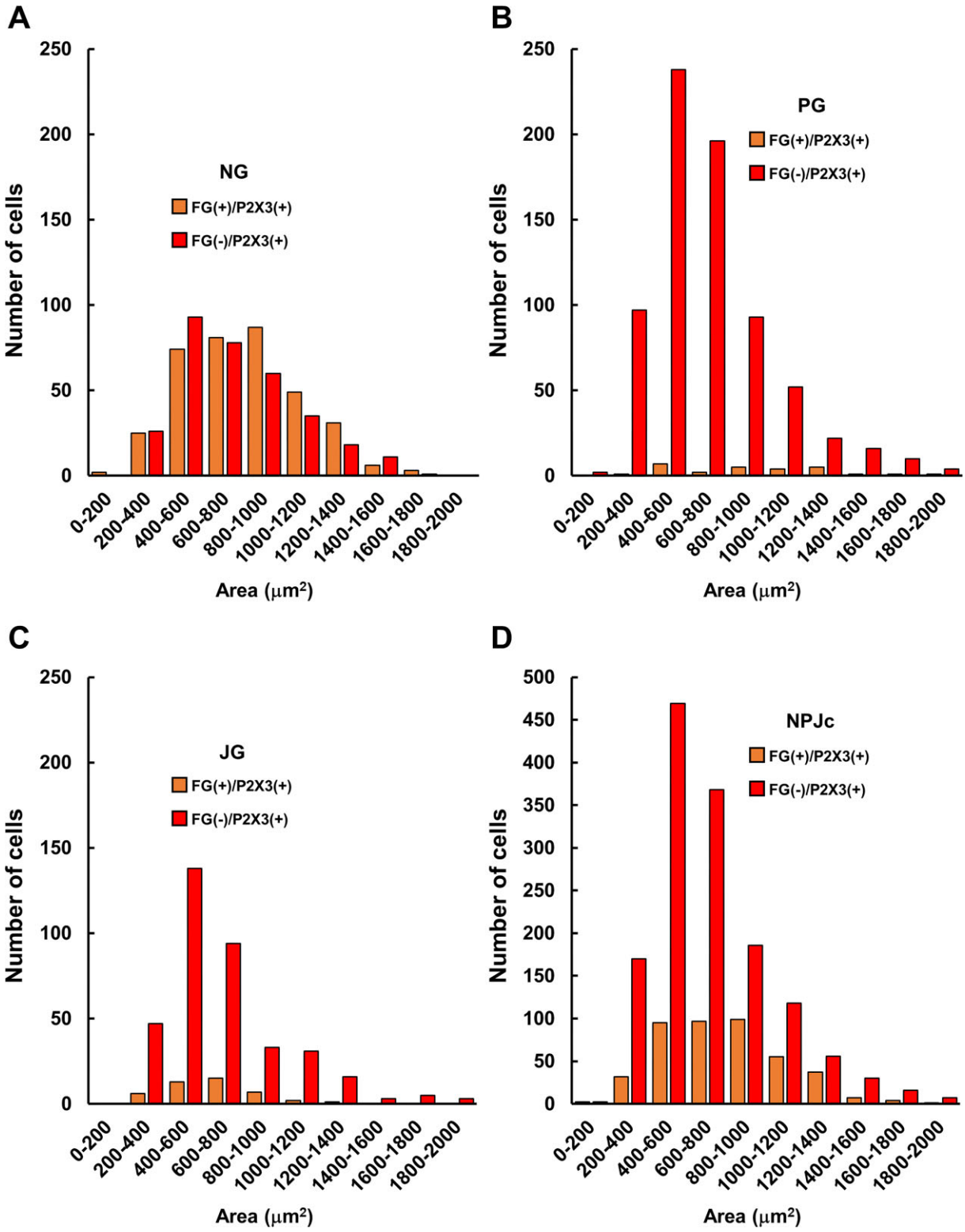
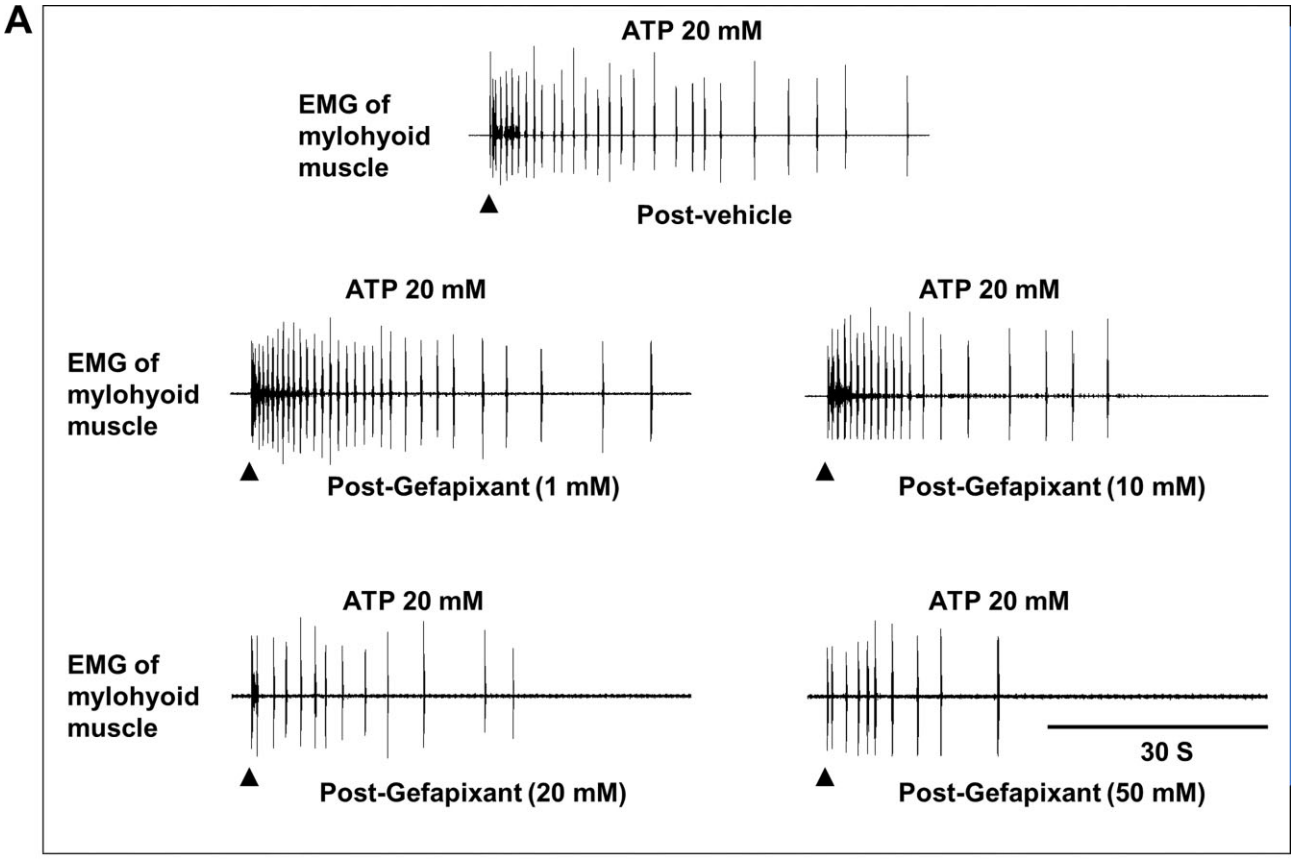


Figure 4. P2X3-positive cell size in the nodose–petrosal–jugular ganglionic complex (NPJc). (A) Size (area) distribution of fluorogold (FG)-stained and non-FG-stained P2X3-positive cells in the nodose ganglion (NG). (B) Size (area) distribution of FG-stained and non-FG-stained P2X3-positive cells in the petrosal ganglion (PG). (C) Size (area) distribution of FG-stained and non-FG-stained P2X3-positive cells in the jugular ganglion (JG). (D) Size (area) distribution of FG-stained and non-FG-stained P2X3-positive cells in the whole NPJc. FG(+)/P2X3(+), FG-stained cells immunopositive for P2X3; FG(-)/P2X3(+), non-FG-stained cells immunopositive for P2X3; $n = 5$. Nine sections were used from each rat (3 sections/ganglion).



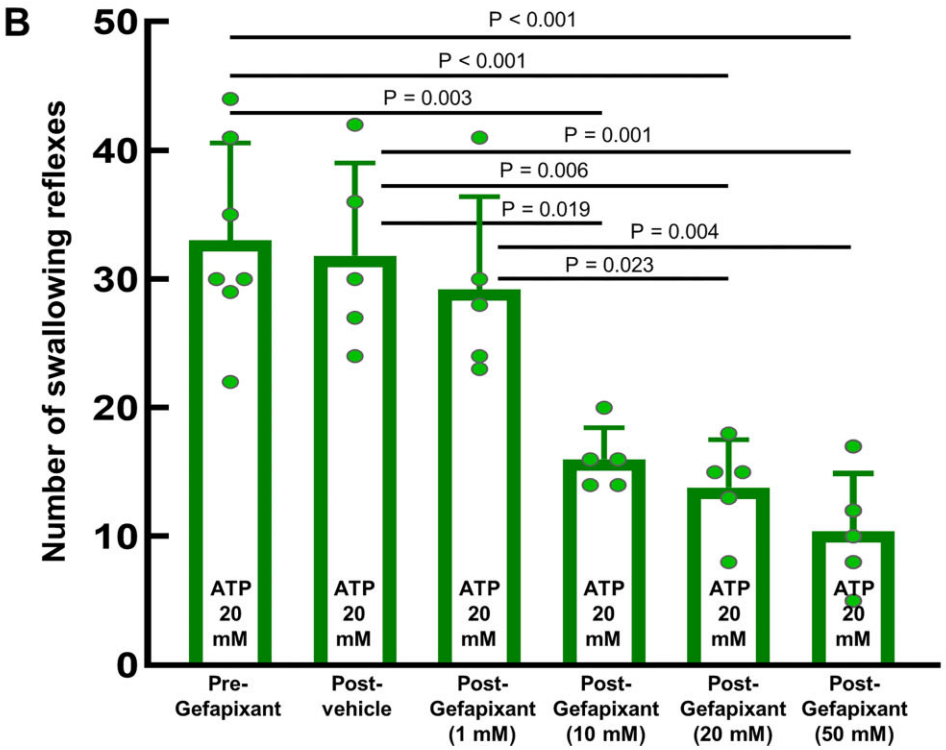


Figure 5. The impact of topical application of Gefapixant, a P2X3 antagonist, prior to adenosine triphosphate (ATP) application, on the number of ATP-triggered swallowing reflexes. (A) Swallowing reflexes triggered by ATP 20 mM after prior topical application of various concentrations of the P2X3 antagonist or vehicle for the antagonist, with black arrowheads indicating the onset of stimulating solution delivery. (B) Comparison of the numbers of swallowing reflexes triggered by ATP 20 mM with and without prior application of various concentrations of the P2X3 antagonist or vehicle. Statistical analysis was conducted by one-way repeated measures ANOVA followed by Tukey's test ($n = 5$-7 for each group). The number of triggered swallowing reflexes was counted for 60 s post-application of the stimulating solutions. Data are depicted as the mean ± SD, with individual data points represented by circles in the column graph. S, seconds.

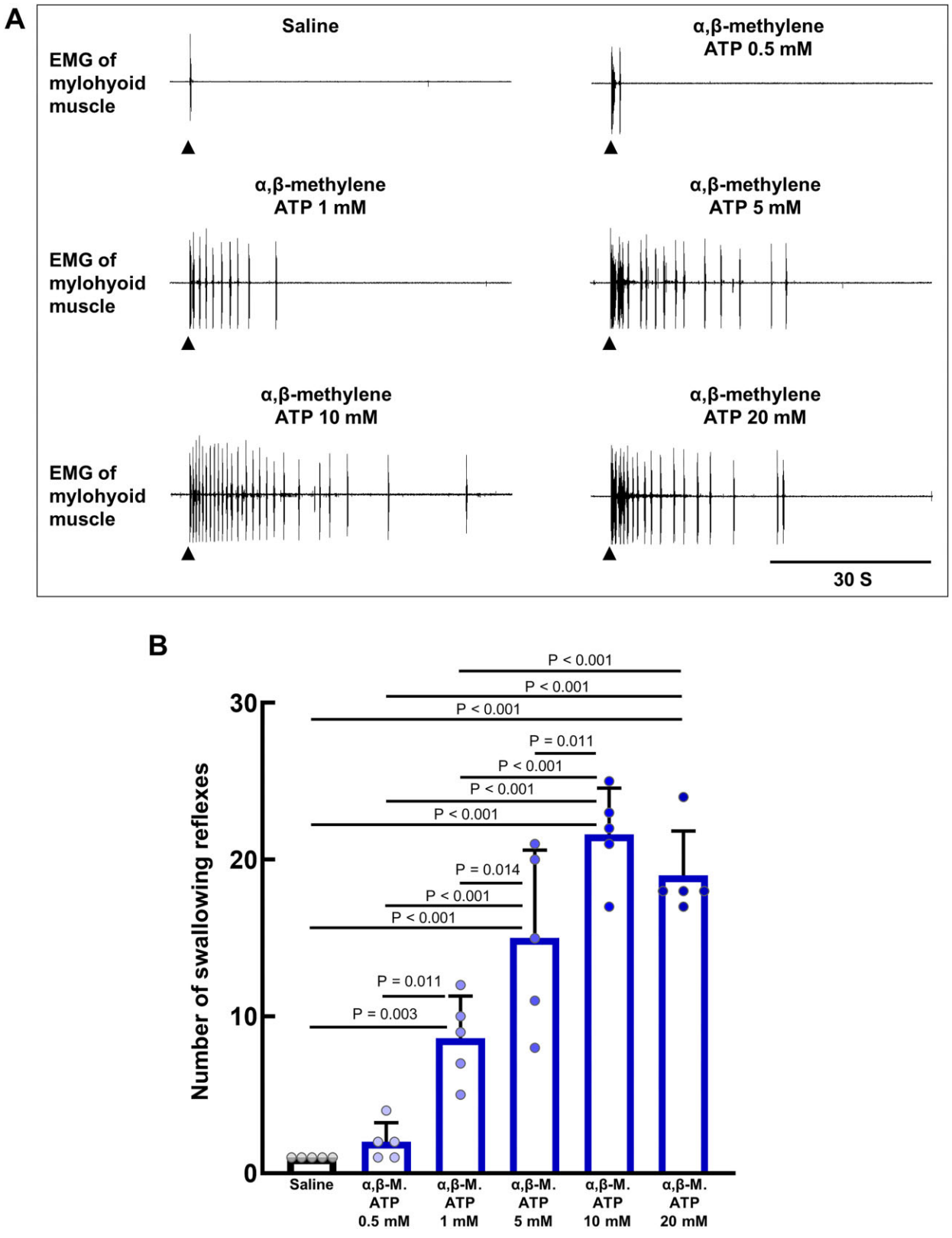


Figure 6. Topical application of α,β-methylene adenosine triphosphate (ATP) triggered swallowing reflexes in a dose-dependent manner. (A) Swallowing reflexes depicted by electromyogram (EMG) activities of mylohyoid muscle triggered by saline and various α,β-methylene ATP concentrations, with black arrowheads denoting the onset of stimulating solution delivery. (B) Comparison of the number of swallowing reflexes triggered by saline and different α,β-methylene ATP concentrations. Statistical analysis was conducted by one-way repeated measures ANOVA followed by Tukey's test ($n = 5$). The number of triggered swallowing reflexes was counted for 60 s post-application of the stimulating solutions. Data are represented as the mean ± SD, with individual data points depicted by circles in the column graph. S, seconds.

Topical Administration of Local Anaesthetic or Bilateral SLN-transection Prior to ATP Completely Abolished the Triggering of the Swallowing Reflex

We administered a local anaesthetic (2% lidocaine) 3 min before ATP application in the swallowing-related regions to confirm that ATP triggered swallowing reflexes by excitation of the afferent nerves of the regions. The local anaesthetic application completely blocked the triggering of the swallowing reflexes by ATP (Figure 7A and B). Notably, no apnoea was observed following ATP application (Figure S3A).

Additionally, we transected bilateral SLNs before administering ATP into the swallowing-related regions to ascertain whether the swallowing reflexes triggered by ATP involved the SLN afferents. Following the transection of the SLNs, no swallowing reflexes were triggered by ATP administration (Figure 7A and C). Consistent with the lidocaine application, no apnoea was observed following ATP application (Figure S3B).

Discussion

To the best of our knowledge, this study presents the first evidence of the ability of exogenous ATP to facilitate the triggering of the swallowing reflex. We observed a dose-dependent induction of swallowing reflexes upon topical application of exogenous ATP into the SLN-innervated peripheral swallowing-related regions. To elucidate the receptors underlying ATP-induced facilitation of the swallowing reflex, we investigated the expression of an ionotropic ATP receptor, P2X3, in these regions. While ATP can activate various purinergic receptors, our focus on P2X3 stems from its reported predominant expression in peripheral sensory nerves.[16,17,30,59,60] Consistent with this focus, our observations revealed P2X3 expression on nerve fibres supplying the swallowing-related regions. Specifically, P2X3 expression was noted on nerve fibres innervating the epithelium of these regions, traversing between epithelial cells toward the epithelial surface. Sub-epithelial nerve fibres and those supplying taste bud-like structures also exhibited P2X3 expression. These findings align with earlier reports of P2X3 expression in the rat larynx[59] and trachea[60] where P2X3-immunoreactive ramified intraepithelial nerve endings, as well as subepithelial nerves were identified. The ramified endings in that study exhibited a beaded, rounded, or flat morphology, similar to our findings. Moreover, P2X3 immunoreactivity has been reported in nerve fibers within the pharyngeal mucosa of rats[61] and in nerve fibers supplying the laryngeal taste bud-like structures across multiple species, including rats, mice, monkeys, and humans.[59,62] Given the predominant localization of P2X3 on nerve fibers in regions related to the triggering of the swallowing reflex, this receptor is a plausible and compelling candidate for mediating ATP-induced facilitation of this reflex. Accordingly, our study specifically aimed to investigate whether P2X3 receptors contribute to ATP-induced swallowing reflex facilitation.

Additionally, we investigated P2X3 expression on SLN-afferents traced into the NPJc using a retrograde tracer fluorogold. Approximately 50% of the SLN-afferent neurons in the nodose ganglion were found to express P2X3, compared to around 20% in the petrosal and jugular ganglia. These findings suggest that P2X3-expressing SLN-afferent neurons in the nodose ganglion may play a more significant role in ATP-induced swallowing reflexes. To further characterize, we classified the P2X3-neurons based on their sizes, as neuron size may provide insights into their functional properties. Our analysis revealed that P2X3 was localized to cell bodies of various sizes within the NPJc, with a predominant distribution in medium- to small-sized neurons for both FG-stained and non-FG-stained P2X3-expressing populations (Table 1). These findings align with previous studies examining P2X3 expression in the nodose, jugular, trigeminal, and dorsal root ganglia.[52–54,58,63–65] For instance, a study on human cadavers investigating P2X3 expression in the entire nodose ganglion reported that most P2X3-expressing neurons were medium- to small-sized.[52] Similar results were observed in studies focusing on rat nodose,[65] trigeminal,[54,58] and dorsal root ganglia.[53,64] Furthermore, a study in rat pups analyzed P2X3 expression on retrogradely traced SLN-afferent neurons within the nodose and jugular ganglia.[63] While that study did not explicitly categorize neurons by size (eg, small, medium, or large), its size-distribution analysis demonstrated that most P2X3-expressing SLN-afferent neurons fall within the medium- to small-size range. Moreover, the same study reported that intralaryngeal ATP application induced a period of apnoea, which is consistent with our findings (Figure S1). However, unlike our observations, the study did not record concomitant swallowing muscle activity. In our experiments, this apnoea was accompanied by repeated triggering of the swallowing reflexes, as evidenced by high-amplitude bursts in a muscle associated with swallowing. Furthermore, the study demonstrated that P2X3 receptors mediate ATP-induced apnoea, as pre-treatment with a P2X3 antagonist significantly attenuated the response.

The observation of P2X3 expression in neurons of varying sizes suggests its presence in both myelinated and unmyelinated neurons within the NPJc that project to laryngopharyngeal regions. Previous studies have shown that neurons in the nodose[66,67] and jugular[67] ganglia projecting to the guinea pig trachea express markers for both myelinated and unmyelinated neurons. For example, retrograde tracing from the guinea pig trachea revealed that over 90% of nodose neurons projecting to the trachea are medium-sized and express neurofilament, a marker commonly associated with myelinated neurons.[66,67] In contrast, approximately half of the neurons in the jugular ganglion projecting to the trachea express neurofilament, while the other half express substance P, a marker commonly associated with unmyelinated neurons.[67] Conduction velocity analyses revealed that most nodose neurons projecting to the trachea are neurons of fast-conducting Aδ fibres, whereas the jugular ganglion contains equal proportions of neurons of C fibres and Aδ fibres.[67] In the rat larynx[68] and trachea,[60] parent axons of intraepithelial ramified nerve endings residing in subepithelial regions express markers for myelin sheaths (eg, myelin basic protein). In contrast, intraepithelial free nerve endings in the larynx[59] and trachea[60] express substance P and calcitonin gene-related peptide (CGRP), markers commonly associated with unmyelinated nerves. Notably, P2X3-expressing intraepithelial nerve endings in the larynx and trachea do not colocalize with substance P- or CGRP-immunoreactive nerve endings, indicating that they are non-peptidergic.[59,60]

Collectively, these findings underscore the need for comprehensive studies to characterize the neurochemical profile of P2X3-expressing SLN-afferent neurons in the NPJc, as well as P2X3-expressing nerve fibres in peripheral swallowing-related regions. Such studies should employ a variety of neurochemical markers (eg, markers for myelinated and unmyelinated neurons, neurotransmitters, and neuropeptides). In addition to neurochemical characterization, functional studies are crucial to elucidate the role of P2X3-expressing neurons in swallowing reflexes triggered by various stimuli. Previous research, including our own, has demonstrated that swallowing reflexes can be

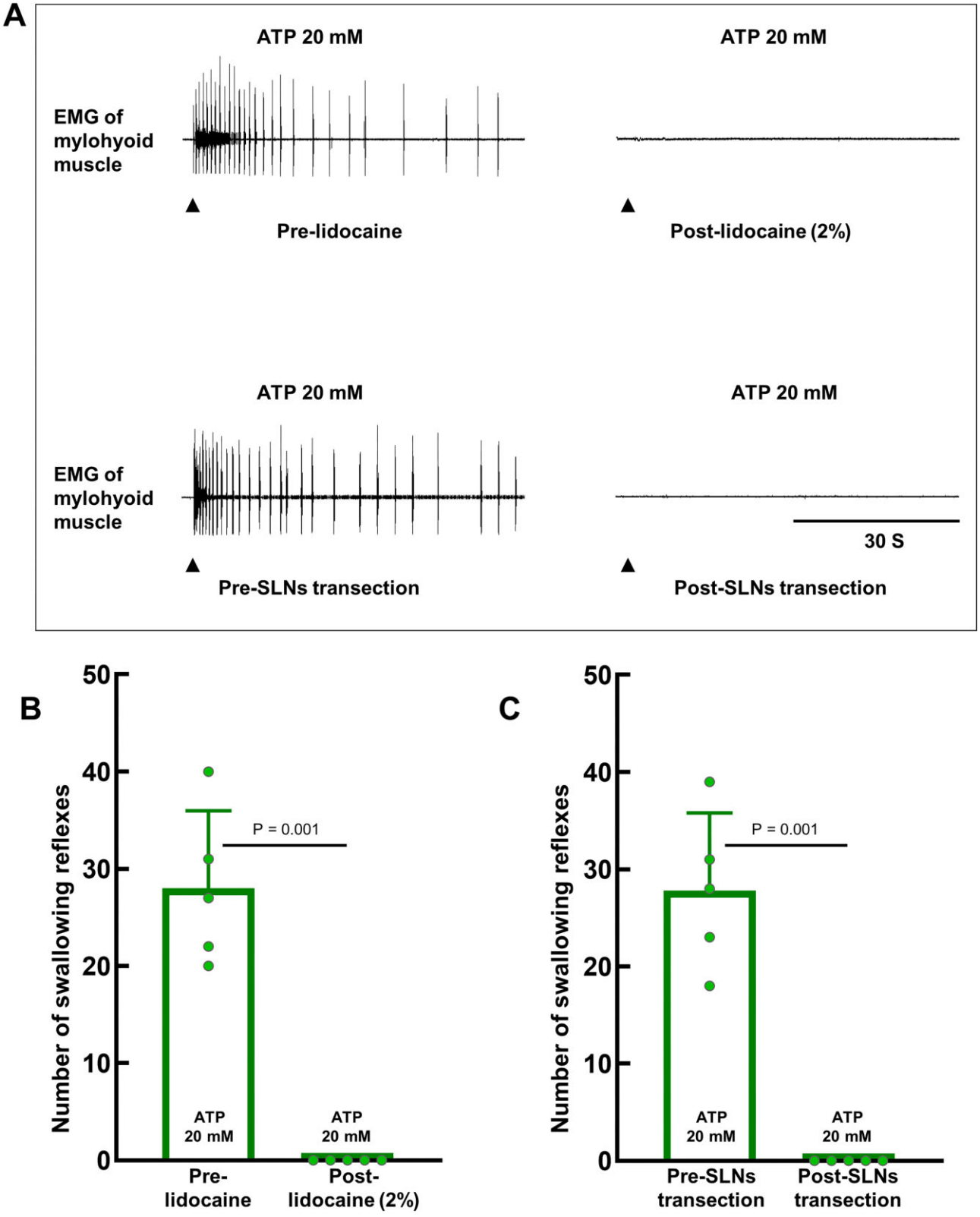


Figure 7. Topical lidocaine administration or transection of bilateral superior laryngeal nerve (SLNs) before adenosine triphosphate (ATP) application prevented the triggering of swallowing reflexes by ATP. (A) Swallowing reflexes triggered by ATP 20 mM with or without prior topical application of lidocaine (2%) or bilateral transection of SLNs, with black arrowheads indicating the onset of ATP delivery. (B) Comparisons of the number of swallowing reflexes triggered by ATP 20 mM with and without prior application of lidocaine. (C) Comparisons of the number of swallowing reflexes triggered by ATP 20 mM with and without prior bilateral transection of SLNs. Statistical analysis was conducted by paired t-test ($n = 5$). The number of triggered swallowing reflexes was counted for 60 s post-application of the stimulating solutions. Data are presented as the mean ± SD, with individual data points represented by circles in the column graph.

induced by both mechanical[33,37,69] and chemical[31,33,36] stimuli in SLN-innervated laryngopharyngeal regions. The localization of P2X3 in SLN-afferent neurons of varying sizes within the NPJc suggests their potential involvement in mediating swallowing reflexes induced by diverse stimuli.

Our findings of the dose-dependent induction of the swallowing reflex by exogenous ATP, along with the localization of P2X3 in nerve fibers within the laryngopharynx and SLN-afferent neurons in the NPJc, suggest that exogenous ATP may activate P2X3 receptors. This activation excites nerve fibres in peripheral swallowing-related regions, ultimately stimulating the central pattern generator for swallowing and facilitating the reflex. To further substantiate the involvement of P2X3 receptors in ATP-triggered swallowing reflexes, we topically applied a P2X3 antagonist to these regions. Prior application of the antagonist significantly reduced the number of ATP-triggered swallowing reflexes, underscoring the role of P2X3 receptors in this mechanism. Additionally, we tested a more selective P2X3 agonist compared to ATP (α,β-methylene ATP), which also dose-dependently triggered the swallowing reflex, further supporting the involvement of P2X3 receptors in this mechanism. However, while pre-treatment with the P2X3 antagonist significantly reduced the number of ATP-triggered swallowing reflexes, it did not completely abolish them, suggesting the contribution of other P2 receptors to ATP-triggered swallowing reflexes. Notably, α,β-methylene ATP is not only more selective for P2X3 receptor but also exhibits higher selectivity for P2X1 receptor compared to ATP.[49–51] Therefore, future studies investigating the effects of agonists and antagonists of other P2 receptors, as well as assessing their expression in swallowing-related regions and associated ganglia, will be crucial for comprehensively understanding their roles in this process.

Our previous studies have demonstrated that various TRP channels are localized in SLN-afferent neurons and play a role in triggering swallowing reflexes.[6,31–33] For instance, the application of a TRPV1 agonist, capsaicin, to SLN-innervated peripheral swallowing-related regions induced swallowing reflexes, and TRPV1 expression was detected in retrogradely traced SLN-afferent neurons within the NPJc.[31] It is plausible that the application of TRP channel agonists in peripheral swallowing-related regions not only activates TRP channels but also promotes the release of ATP, which in turn may activate P2X3 receptors. Thus, ATP and P2X3 receptors might also contribute to TRP channel agonist-induced swallowing reflexes, and P2X3 could potentially be co-localized with various TRP channels. To explore these possibilities, future studies should investigate the co-localization of different TRP channels with P2X3 in the NPJc and peripheral swallowing-related regions. Additionally, functional studies are needed to determine whether P2X3 antagonists can attenuate TRP channel agonist-induced swallowing reflexes.

Our findings regarding the ability of exogenous ATP to enhance the triggering of the swallowing reflex are promising in terms of clinical significance. The results suggest that exogenous ATP or P2X3 agonists could be used to develop a viable treatment option for managing oropharyngeal dysphagia, which is a significant health concern that can lead to severe complications, such as pneumonia, malnutrition, and dehydration.[24,25,27] In patients with oropharyngeal dysphagia, abnormality in the swallowing process or delayed triggering of the swallowing reflex often leads to the aspiration of food particles or liquids into the airway, culminating in aspiration pneumonia.[24–29,70] Current clinical management strategies for oropharyngeal dysphagia predominantly rely on compensatory techniques (eg, altering food bolus viscosity or texture) and swallowing exercises/manoeuvres (eg, chin tuck), which exhibit limited efficacy.[71–75] Hence, there is an imperative need to explore novel treatment avenues in the realm of swallowing/dysphagia-related research.[5,6,76] Recently, chemical neuro-stimulation, targeting various chemosensory ion channels in peripheral swallowing-related regions (eg, targeting TRP channels), has shown promising outcomes in both preclinical and clinical settings.[5,6,77–79] Chemical neuro-stimulation in these regions has demonstrated improvements in swallowing efficacy, safety, and physiology among patients with oropharyngeal dysphagia.[80,81,90,82–89] Our findings extend this line of inquiry by proposing the potential use of exogenous ATP or P2X3 agonists as chemical neuro-stimulants to augment swallowing function. Such interventions hold promise for enhancing the management of oropharyngeal dysphagia and warrant further investigation in clinical settings. Given that exogenous ATP has been used as a dietary supplement to improve physical performance,[91,92] researching ATP as a chemical neuro-stimulant to improve swallowing performance may be feasible and safe in clinical research contexts.

Our findings also provide significant insights into the physiological role of purinergic signaling in triggering the swallowing reflex. ATP is well-established as a neurotransmitter in various physiological processes.[17–20,93–96] The present findings and evidence from other recent studies indicate that ATP may also act as a neurotransmitter or neuroactive molecule in triggering the swallowing reflex. ATP release can occur in response to various stimuli in peripheral swallowing-related regions.[5,6,22,23] Previous studies in mice have suggested that ATP may be released from taste buds and neuroendocrine cells in the laryngeal regions upon applying water or acid.[22,23] The released ATP then acts on purinergic receptors on nerve fibres supplying these regions. Furthermore, knockout of P2X2/P2X3 receptors in a previous study resulted in attenuation of water and acid-induced swallowing reflexes, highlighting the pivotal role of these receptors in mediating ATP-induced responses.[23] Beyond water/acid stimuli, ATP may also be released in response to other stimuli, such as mechanical stimuli. In accord with this notion, previous studies reported that ATP is released from the airway and nasal epithelium upon the application of mechanical stimuli.[97,98] Future studies may reveal further insights into the involvement of ATP in various stimuli-induced swallowing reflexes, thereby enhancing our understanding of its role in regulating swallowing physiology.

We observed that topical application of a local anaesthetic to the peripheral swallowing-related regions completely abolished the swallowing reflex triggered by ATP. This observation highlights the critical role of sensory nerves in initiating the swallowing reflex in response to ATP. To precisely evaluate the contribution of SLNs, we recorded reflexes with intact SLNs while transecting other nerves, including bilateral IX-ph, X-ph, IX-li, and RLN. Additionally, we conducted an additional experiment to confirm the specific role of SLNs in transmitting sensory information to trigger the swallowing reflex under our experimental conditions. In this experiment, bilateral transection of SLNs resulted in the complete abolition of the swallowing reflex triggered by ATP applied into the SLN-innervated regions. This confirms the essential involvement of SLN-afferents in initiating the swallowing reflex under the experimental conditions of our study.

One limitation of this study is that it was conducted exclusively with male rats. Female rats were not included to avoid the oestrous cycle. Although a meta-analysis has shown that female rats are not more variable than male rats in neuroscience-related traits (including behavioral, electrophysiological, neurochemical, and histological measures),[99] there remains the possibility of different outcomes had female rats been included in this study. Consequently, the findings may not be directly applicable to females, as neurohumoral differences between the sexes could influence the results.

In conclusion, the current study demonstrated that exogenous ATP applied to the peripheral swallowing-related regions enhanced the triggering of the swallowing reflex. Additionally, we observed the expression of an ATP receptor, P2X3, on the afferent nerves innervating these regions. The presence of P2X3 on these nerves suggests a mechanism by which ATP facilitates this reflex. Activation of P2X3 receptors by ATP may excite these nerves, thereby promoting the initiation of the swallowing reflex. These findings underscore the potential of exogenous ATP and P2X3 agonists as therapeutic agents to improve swallowing function and manage oropharyngeal dysphagia. By targeting the sensory nerves involved in swallowing initiation, these agents may offer a promising approach for addressing this debilitating condition.

Author Contributions

M.Z.H. and J.K.: Conceptualize and design the work. M.Z.H., H.A., R.R.R., and J.K.: acquisition, analysis, or interpretation of data. M.Z.H., H.A., R.R.R., and J.K.: Draft or revise the manuscript critically. All authors have approved the final version of the manuscript. All persons designated as authors qualify for authorship.

Supplementary Material

Supplementary material is available at the *APS Function* online

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Conflict of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability

The data underlying this article will be made available to other researchers from the authors upon reasonable request.

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