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RESEARCH ARTICLE

Atropine facilitates water-evoked swallows via central muscarinic receptors in anesthetized rats

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Abstract

Anticholinergic medication causes impaired swallowing with hyposalivation. However, the underlying mechanisms by which these drugs modulate the swallowing reflex remain unclear. This study investigated the effects of the muscarinic acetylcholine receptor (mAChR) nonspecific antagonist atropine on the initiation of swallowing. Experiments were performed on 124 urethane-anesthetized rats. A swallow was evoked by either topical laryngeal application of a small amount of distilled water (DW), saline, citric acid, or capsaicin; upper airway distention with a continuous airflow; electrical stimulation of the superior laryngeal nerve (SLN); or focal microinjection of *N*-methyl-D-aspartate (NMDA) into the lateral region of the nucleus of the solitary tract (L-nTS). Swallows were identified by electromyographic bursts of the digastric and thyrohyoid muscles. Either atropine, the peripheral mAChR antagonist methylatropine, or antagonists of mAChR subtypes M1–M5 were intravenously delivered. Atropine at a dose of 1 mg/kg increased the number of DW-evoked swallows compared with baseline and did not affect the number of swallows evoked by saline, citric acid, capsaicin, or upper airway distention. Methylatropine and M1–M5 antagonists did not significantly change the number of DW-evoked swallows. Bilateral SLN transection completely abolished DW-evoked swallows, and atropine decreased the swallowing threshold of SLN electrical stimulation. Finally, microinjection of NMDA receptor antagonist AP-5 into the L-nTS inhibited DW-evoked swallows, and atropine facilitated the initiation of swallowing evoked by NMDA microinjection into this region. These results suggest that atropine facilitates DW-evoked swallows via central mAChR actions.

NEW & NOTEWORTHY Atropine facilitated the distilled water (DW)-evoked swallows in anesthetized rats. Atropine decreased the swallowing threshold evoked by electrical stimulation of the superior laryngeal nerve, which is a primary sensory nerve for the initiation of DW-evoked swallows. Atropine facilitated the swallows evoked by *N*-methyl-D-aspartate microinjection into the lateral region of the nucleus of the solitary tract, which is involved in the DW-evoked swallows. We speculate that atropine facilitates the DW-evoked swallows via central muscarinic receptor actions.

atropine; distilled water; nucleus of the solitary tract; superior laryngeal nerve; swallowing

INTRODUCTION

Swallowing is an airway defensive reflex. The swallowing impairment called dysphagia is associated with pneumonia, choking, malnutrition, and dehydration (1–3). Dysphagia is caused by neurological and structural damage, which can result from, for example, stroke, Parkinson's disease, and head and neck cancer surgery (4–6). Some drugs used for managing neurological conditions also induce dysphagia as an adverse effect (7, 8). Among them, effects of anticholinergic medications, which are widely prescribed in the older population, receive much attention. Castejon-Hernandez et al. (9) reported that hospitalized patients with more than three points on the anticholinergic cognitive burden scale (ACBs), which is an index for quantifying anticholinergic action, are fourfold more likely to develop oropharyngeal dysphagia compared with those with low or no ACBs. Furthermore, Takata et al. (10) reported that ACBs was significantly higher in patients who did not recover from tubal to oral feeding than those in patients who recovered.

Anticholinergic drugs can cause xerostomia due to the inhibitory effect of the M3 muscarinic acetylcholine receptor (mAChR), which mediates parasympathetic cholinergic neurotransmission to salivary glands (11). Xerostomia triggers impaired chewing and swallowing. For instance, xerostomic patients after cancer radiation or chemoradiation therapy exhibit a prolonged duration of chewing and oral manipulation of a dry cookie before swallowing compared with before therapy (12). Furthermore, patients with xerostomic with either Sjogren's syndrome or who underwent irradiation for head and neck cancer exhibit prolonged pharyngeal transit time during dry and water-assisted swallowing compared with age- and sex-matched control participants (13). Based on these clinical observations, one can assume that oral and

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pharyngeal dry conditions cause dysphagia in patients taking anticholinergic medications.

On the other hand, the results of animal studies on the effects of anticholinergic drugs on swallowing function are inconsistent. Intravenous administration of atropine (a nonspecific antagonist of mAChRs) decreased the swallowing rate at rest in ovine fetuses (14). In support of these findings, intracerebroventricular injection of carbachol, which is a dual muscarinic and nicotinic receptor agonist, increased the swallowing rate during low-voltage electrocorticogram activity in ovine fetuses (15). Conversely, Tsubouchi et al. (16) reported that intravenous administration of atropine increased the number of distilled water (DW)-evoked swallows and shortened the onset of the first DW-evoked swallow in conscious dogs. Although there is no doubt that anticholinergic medications affect chewing and swallowing functions, the underlying mechanisms by which these drugs modulate the swallowing reflex remain unclear.

Acetylcholine binds to two different types of receptors, i.e., nicotinic and muscarinic acetylcholine receptors. These receptors are widely distributed across the body. Muscarinic receptors are divided into five G protein-coupled receptors (M1–M5). Among them, M1, M3, and M5 couple to Gq protein to activate phospholipase C, which results in the production of inositol triphosphate and diacylglycerol, whereas M2 and M4 couple to Gi/o protein to inhibit adenylyl cyclase and modulate ion channels (17). Although atropine is used for patients with hypersalivation, bronchial secretions, or bradycardia, atropine is classified into a high-score category of ACBs with adverse reactions, including tachycardia, tachypnoea, and dry skin and mouth (18, 19).

The experiments in our study were designed to explore the effect of systemic atropine administration on swallowing function in anaesthetized rats. The swallowing reflex was evoked by mechanical, chemical, and electrical stimulation. We observed that atropine facilitated the initiation of DW-evoked swallows. Subsequent experiments revealed that atropine decreased the swallowing threshold evoked by electrical stimulation of the peripheral sensory nerve, related to DW-evoked swallows, and increased the number of swallows evoked by *N*-methyl-D-aspartate (NMDA) microinjection into the medulla, which is involved in the DW-evoked swallows. We speculate that atropine facilitates DW-evoked swallows via central mAChR actions.

MATERIALS AND METHODS

Ethical Approval

This study was reviewed and approved by the Niigata University Intramural Animal Care and Use Committee (SA01069) and was performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD).

Animals

Experiments were performed on 124 Sprague-Dawley male rats (7- to 9-wk old, Charles River Laboratories Japan, Inc., Yokohama, Japan). We housed two or three rats in each cage in the vivarium, which had controlled temperature and 12-h light/dark cycles. All experiments involved nonsurvival surgical procedures. The rats were anesthetized with urethane (1.3 g/kg ip), which was supplemented as necessary to maintain anesthesia at a level at which neither a corneal reflex nor spontaneous eye movements occurred. The rectal temperature was maintained at ~37.5°C by a thermostatically controlled heating pad. When experiments were completed, the animals were euthanized with an overdose of urethane.

Preparations

With the rat in a supine position, the bilateral digastric (Dig) muscles were exposed following a midline incision from the pogonion to the caudal portion of the neck on the ventral side. After cutting the sternohyoid, sternothyroid, and omohyoid muscles near the hyoid bone, the thoracic trachea was cannulated at its caudal-most end with a luer stub adaptor, and a pressure transducer was attached to a side port of the tracheal cannula to monitor respiratory efforts. Bipolar enamel-coated copper wire electrodes (0.18 mm in diameter, with an interpolar distance of 2 mm) were inserted into the left side of the digastric and thyrohyoid (TH) muscles for electromyographic (EMG) recording. The heart rate and arterial pressure were continuously monitored with a right femoral artery cannula.

Initiation and Recording of Swallowing

To deliver the drug topically, the larynx was incised laterally just above the vocal folds from the ventral side. Initially, we confirmed that the punctate mechanical stimulation of the interarytenoid fold using the smallest von Frey filament (0.008 g) succeeded in evoking the swallowing reflex in all rats. A swallow was identified by EMG bursts in the Dig and TH muscles and by visually observing laryngeal elevation.

The following muscarinic drugs were delivered via left femoral vein cannulation at a rate of 7 mL/h for ~10 min using an infusion pump (Terumo, Tokyo, Japan): atropine (a nonspecific antagonist of mAChRs, 0.01–10 mg/kg), methylatropine [a central nervous system (CNS)-impermeant form of atropine, 1 mg/kg], VU0255035 (a M1 muscarinic receptor antagonist, 3.3 μg/kg); AF-DX 116 (a M2 muscarinic receptor antagonist, 13.5 μg/kg), 1,1-dimethyl-4 diphenylacetoxypiperidinium iodide (4-DAMP, a M3 muscarinic receptor antagonist, 1 mg/kg) tropicamide (a M4 muscarinic receptor antagonist, 1 mg/kg), ML381 fumarate (a M5 muscarinic receptor antagonist, 84.2 μg/kg), or saline (vehicle for atropine). We selected the doses of 4-DAMP and tropicamide based on previous studies (20). The doses of VU0255035, AF-DX, and ML381 fumarate were set at twice the value of the inhibition constant (K_i). Effects of muscarinic drugs on the swallowing initiation were investigated.

To evoke a swallow by chemical stimulation, aliquots (3 μL) of either DW, saline, 10^{-2} M citric acid (pH 2.5), or 10^{-9}–10^{-5} M capsaicin were applied to the vocal folds, and the number of swallows was measured for 60 s. Each drug was evaluated in a different animal. Capsaicin was applied in ascending concentrations from 10^{-9} to 10^{-5} M. To evoke a swallow by mechanical stimulation, the swallowing reflex was evoked by upper airway (UA) distention with continuous airflow (8 mL/s) delivered through the rostral tracheal cannula following an incision below the vocal folds. The number of swallows was measured for 10 s during airflow application. To evoke a swallow by electrical stimulation, two enamel-coated silver wire electrodes (0.2 mm in diameter) were

placed on the right side of the superior laryngeal nerve (SLN), and the swallowing reflex was evoked by electrical stimulation of the SLN (30 Hz, 0.2-ms pulse duration, 10-s train). The stimulus threshold was defined as the minimum stimulus intensity that could evoke at least one swallow during 10 s of electrical stimulation. At 15 min after atropine or saline intravenous administration, the stimulus intensity at 100% of the initial threshold value as control was first tested. If a swallow was evoked at control intensity, the stimulus intensity was decreased in 5% decrements of the control value. Once the swallow failed to be evoked, the minimum current intensity that evoked swallows was recorded as the threshold. If no swallow was evoked at control intensity, the stimulus intensity was increased in 5% increments of the control value. Once the swallow was evoked, the intensity was recorded as the threshold (21, 22). To evoke a swallow by focal microinjection into medulla, microinjections (100 nL) were performed in 1 min using a manipulator (IMS-10; Narishige Scientific Instruments, Tokyo, Japan) through borosilicate micropipettes (1.0 mm in diameter) pulled sharp using a puller (PE-22; Narishige Scientific Instruments, Tokyo, Japan). The micropipette with a tip diameter of ~100 μm was advanced through the skull into the medulla via drilled access points using a stainless steel bar (1.0 mm in diameter). The position was on either side of the lateral region of the nucleus of the solitary tract (L-nTS; 7.0 mm posterior and 1.2 mm lateral to lambda, and 7.5 mm below the surface of skull), which was suggested to be involved in the initiation of DW-evoked swallows and includes ventral, lateral, ventrolateral, interstitial, and intermediate nTS subnuclei (23, 24). To determine the stereotactic coordinate position of the L-nTS based on the reference position (25), we conducted a few preliminary experiments with two microinjections into the L-nTS using fast blue (100 nL, 2.5%) and DiI (100 nL, 1%) in anesthetized rats. Because we made a small pin hole on the skull to just pass a micropipette, first Evans blue- and second DiI-microinjected positions showed high reproducibility, with a large amount of microinjected areas overlapping, detected as purple color.

DW-evoked swallows were also evaluated in rats that underwent transection of the superior laryngeal nerve (SLNx) or decerebration (Cx). SLN was carefully dissected from the surrounding tissues and cut bilaterally. For Cx, the dura mater was breached, and the brain was perpendicularly and coronally sectioned at the level between the superior and inferior colliculi. After Cx, a minimum recovery period of 30 min was ensured before data collection.

To elucidate the involvement of NMDA receptors in the L-nTS on DW-evoked swallows, we evaluated whether unilateral microinjection of NMDA receptor antagonist AP-5 modulates DW-evoked swallows. Once the AP-5 (100 nL, 0.2 nmol) microinjection was completed (the entire procedure takes <5 min), the swallowing reflex was evoked by the application of 3 μL of DW to the larynx. The number of DW-evoked swallows was measured before and 5 min and 60 min after AP-5 microinjection. The number of capsaicin-evoked swallows was measured only 60 min after AP-5 microinjection to avoid transient receptor potential vanilloid 1 (TRPV1) desensitization by repetitive capsaicin application (26). In other rats, NMDA (100 nL, 0.2 nmol) was microinjected into either side of the L-nTS, and the number of NMDA-evoked swallows was measured for 3 min before and 15 min and 60 min after intravenous administration of atropine (1 mg/kg).

After swallowing assay with AP-5 or NMDA microinjection was completed, DiI (100 nL) was microinjected into the same location to confirm the injection sites. The brainstems were removed from the euthanized rats, and DiI could be visualized in 50-μm-thick coronal sections of harvested brainstem. The injection sites in the brain were reconstructed according to the brain atlas (25).

Saliva Collection

To investigate the effect of atropine on salivation, we used pilocarpine, which induces salivary fluid via the M3 receptor (11). After the swallowing assay, the amount of salivary secretion elicited by intravenous administration of pilocarpine (0.25 mg/kg, 7 mL/h, for 30 s) using an infusion pump was measured with a cotton ball placed on the oral floor. The saliva volume was measured as the difference in the weight of the cotton ball before and 5 min after pilocarpine application.

Data Analyses

The EMG, blood pressure, and respiratory signals were amplified (AM-601G for EMG; AP-601G for blood pressure, Nihon Kohden, Japan; LDVF9 for respiration, LabDesign, Ibaraki, Japan) and digitized at a sampling rate of 10 kHz for EMG and 500 Hz for blood pressure and respiration. These parameters were analyzed using the Spike2 analysis package (Cambridge Electronic Design, Cambridge, UK). The EMG activity was defined as active when the rectified and smoothed EMG signal was more than the mean ± 3 standard deviations (SDs) of the background activity, which was estimated during the 10-s stable period at rest before each trial. To analyze the properties of swallow-related motor activity, the EMG burst duration and area of Dig and TH muscles, time interval between the peaks of the Dig and TH EMG bursts, and onset and offset lag time between the Dig and TH EMG bursts during first swallow were determined using filtered EMG signals. The respiratory rate, heart rate, and blood pressure were calculated from the traces before and 15 min after intravenous administration of saline and atropine (1 mg/kg). These vital values were averaged every 5 min.

Results are presented as means ± SD, median with interquartile range or point-to-point lines. Testing for normality and equality of variances was initially performed to determine whether parametric or nonparametric tests would be used. In multiple comparisons, parametric data were assessed by one-way analysis of variance (ANOVA), one-way repeated-measures ANOVA, or two-way repeated-measures ANOVA with one factor repetition, followed by Tukey's test. Nonparametric data were assessed by Friedman's repeated-measures ANOVA on ranks or Kruskal–Wallis test, followed by Dunn's test. In comparison of two samples, parametric data were assessed by paired t test or Student's t test. Nonparametric data were assessed by Wilcoxon signed rank test or Mann–Whitney U test. For the analyses of capsaicin- and citric acid-evoked swallows, we designed unpaired comparisons to avoid the influence of swallowing desensitization in repetitive trials. Statistical analyses were performed using Sigma Plot14.5

(Systat Software, Inpixon, Palo Alto, CA). Differences were considered significant at $P < 0.05$.

Reagents

Atropine (Fuso Pharmaceutical Industries, Osaka, Japan; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), methylatropine, DiI (Sigma-Aldrich, St. Louis, MO), citric acid, AP-5 (Wako, Osaka, Japan), and fast blue (Polysciences, Inc., Warrington, PA) were diluted in saline (Nisshin Seiyaku, Yamagata, Japan). Capsaicin (Wako, Japan) was dissolved in ethanol (Wako, Japan) before dilution in saline. VU0255035, AF-DX 116, tropicamide (Abcam, Cambridge, UK), 4-DAMP (Sigma-Aldrich), and ML381 (AOBIOUS, Inc., Gloucester, MA) were dissolved in dimethyl sulfoxide (Sigma-Aldrich) before dilution in saline.

RESULTS

Effect of Intravenous Administration of Atropine on Swallowing Evoked by Natural Stimulation

We examined the effect of atropine (1 mg/kg) on the initiation of swallowing evoked by various natural stimuli 15 min after intravenous administration. DW application to the larynx evoked a larger number of swallows after atropine administration than before administration (Fig. 1, *A* and *B*). The number of DW-evoked swallows was not affected by vehicle administration (Fig. 1*B*). Atropine and vehicle administration did not affect the number of swallows evoked by airflow and saline (Fig. 2, *A* and *B*). Moreover, the number of swallows evoked by citric acid (10 mM) and capsaicin (10^{-9} to 10^{-5} M) did not differ between atropine and vehicle (Fig. 2, *C* and *D*). Compared with different capsaicin doses, the number of swallows evoked by 10^{-5} M capsaicin was significantly larger than those evoked by the other four doses (10^{-9} to 10^{-6} M). These results suggest that atropine facilitates DW-evoked swallows and does not affect the swallowing evoked by mechanical force or other chemical stimulants, i.e., saline, citric acid, or capsaicin. In addition, we evaluated the effect of atropine on the swallowing-related muscle activity in DW-evoked swallows (Fig. 3). The EMG burst duration and area of Dig and TH, the onset and offset lag time between the Dig and TH EMG bursts, and time interval between the peaks of the Dig and TH EMG bursts did not significantly differ between before and after atropine administration. These results indicate that atropine does not affect the properties of swallow-related motor activity.

Next, we examined the time-course and dose-dependent effects of atropine on DW-evoked swallows (Fig. 4*A*). The number of DW-evoked swallows 15 min after administration of 1 mg/kg of atropine was significantly larger than that at baseline and with 0.01 and 0.1 mg/kg of atropine but did not differ compared with 10 mg/kg of atropine. Furthermore, the number of DW-evoked swallows 30 min after administration was significantly larger with 1 mg/kg than 0.01 mg/kg of atropine. DW-evoked swallows were most effectively facilitated 15 min after administration of 1 mg/kg of atropine. The spatial distribution of mAChR subtypes is characteristic of specific tissues and organs (27), and thus we investigated whether each muscarinic antagonist subtype modulates the initiation of DW-evoked swallows (Fig. 4*B*). Although some of data in M1 and M2 antagonists represented the large increase in the number of DW-evoked swallows, no antagonists significantly changed the number of DW-evoked swallows.

Role of Peripheral and Central mAChRs on the Atropine-Induced Facilitation of Swallowing

To elucidate whether either peripheral or central mAChRs play a primary role in the atropine-induced facilitation of swallowing, we initially evaluated the effect of a CNS-

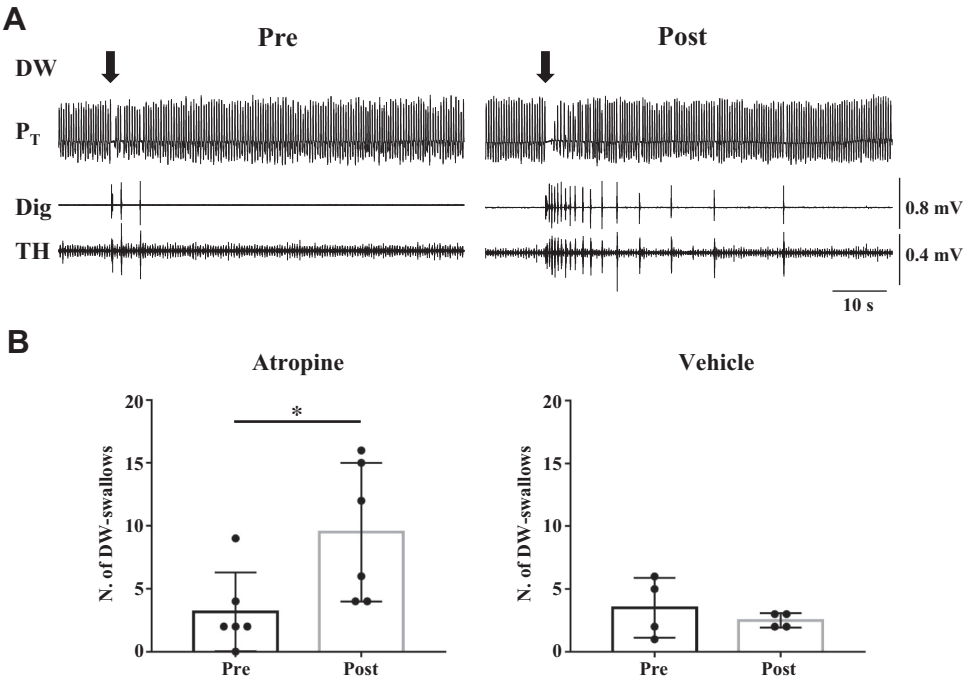

Figure 1. Atropine facilitates the initiation of swallowing evoked by distilled water (DW). *A*: traces illustrating the swallowing reflex evoked by DW application to the larynx (arrow). A swallow was typically characterized by electromyographic activation of the digastric (Dig) and thyrohyoid (TH) muscles, with short apnea identified by changes in tracheal pressures (P_T). The initiation of DW-evoked swallows was facilitated by intravenous atropine administration (Post) compared with before administration (Pre). *B*: atropine significantly increased the number of DW-evoked swallows (N. of DW-swallows, 9.50 ± 5.50), compared with before administration (3.17 ± 3.13). Vehicle (saline) did not change the number: 3.50 ± 2.38 (Pre) vs. 2.50 ± 0.58 (Post). Data are presented as means ± SD (n = 6 for atropine; n = 4 for vehicle); paired t test; *$P < 0.05$.

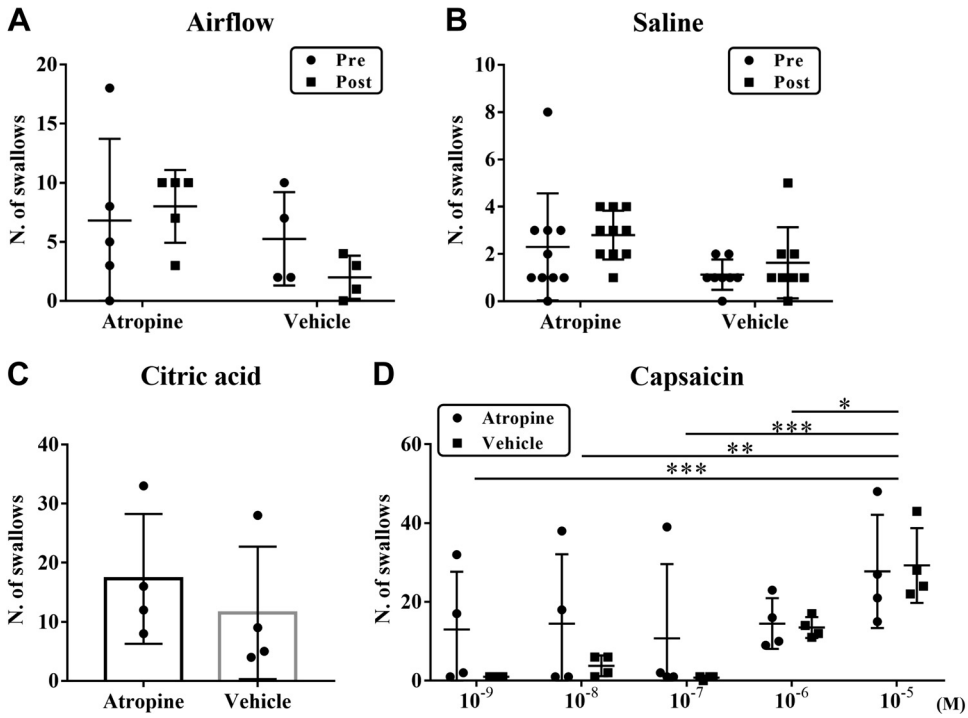

Figure 2. Atropine does not modulate the initiation of swallowing evoked by mechanical force, saline, acid, and capsaicin. Number of swallows (N. of swallows) evoked by airflow (*A*) and saline (*B*) was not altered by atropine or vehicle administration: airflow [6.80 ± 6.18 (Pre-atropine) vs. 8.00 ± 2.76 (Post-atropine); 5.25 ± 3.42 (Pre-vehicle) vs. 2.00 ± 1.58 (Post-vehicle)]; saline [2.30 ± 2.15 (Pre-atropine) vs. 2.80 ± 0.98 (Post-atropine); 1.13 ± 0.60 (Pre-vehicle) vs. 1.63 ± 1.41 (Post-vehicle)]. Number of swallows evoked by citric acid (*C*) and capsaicin (*D*) was not altered between atropine and vehicle administration: citric acid [17.3 ± 9.5 (atropine) vs. 11.5 ± 9.7 (vehicle)]; 10^{-9} M capsaicin [13.0 ± 12.7 (atropine) vs. 1.00 ± 0.00 (vehicle)]; 10^{-8} M capsaicin [14.5 ± 15.2 (atropine) vs. 3.75 ± 2.28 (vehicle)]; 10^{-7} M capsaicin [10.8 ± 16.3 (atropine) vs. 0.75 ± 0.43 (vehicle)]; 10^{-6} M capsaicin [14.5 ± 5.6 (atropine) vs. 13.5 ± 2.3 (vehicle)]; 10^{-5} M capsaicin [27.8 ± 12.4 (atropine) vs. 29.3 ± 8.2 (vehicle)]. Data are presented as means ± SD [n = 4 and 5 (airflow); n = 8 and 10 (saline); n = 4 (citric acid and capsaicin)]. Paired t test was used for airflow and saline, and the Mann–Whitney U test was used for citric acid. Two-way repeated-measures ANOVA with one factor repetition [main effect for drug, $F(1,24) = 1.331$, $P = 0.293$; main effect for dose, $F(4,24) = 9.108$, $P < 0.001$; interaction effects, $F(4,24) = 1.022$, $P = 0.416$] followed by Tukey's test was used for capsaicin. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

impermeant form of atropine, methylatropine, on the initiation of DW-evoked swallows (Fig. 5). Methylatropine (1 mg/kg) did not change the number of DW-evoked swallows 15–60 min after administration. The number of DW-evoked swallows was significantly smaller 15 and 30 min after administration of methylatropine (1 mg/kg) compared with atropine (1 mg/kg). These results suggest that peripheral mAChRs are less involved in the atropine-induced facilitation of swallowing.

Next, we investigated the effect of atropine on the swallowing threshold evoked by electrical stimulation of the peripheral sensory nerve related to DW-evoked swallows (Fig. 6). Bilateral SLNx completely abolished DW-evoked swallows, suggesting that SLN plays an essential role in the initiation of DW-evoked swallows. In addition, atropine did not recover the initiation of DW-evoked swallows in SLNx rats. Subsequent experiments revealed the change of the swallowing threshold evoked by SLN electrical stimulation. The initial stimulus threshold of the SLN electrical stimulation was 4.8–78 μA. The swallowing threshold evoked by SLN electrical stimulation was slightly but significantly decreased 15 min after administration of atropine (1 mg/kg). This was not the case after administration of vehicle. The ratio of number of animals that showed the decrease in swallowing threshold was largely different, i.e., 100% for atropine group (7 of 7 rats) versus 25% for vehicle group (1 of 4 rats). These results suggest that central mAChRs are involved in the atropine-induced facilitation of swallowing.

Because our results suggested that atropine facilitate both SLN- and DW-evoked swallows, and the initiation of SLN-evoked swallows was not affected by Cx in our previous study (28), we hypothesized that atropine facilitates DW-evoked swallows in decerebrated rats. However, atropine did not affect the number of DW-evoked swallows in decerebrated rats (Fig. 7*A*). Unlike the smaller effect of Cx on the initiation of SLN-evoked swallows, the number of DW-evoked swallows before atropine administration was significantly lower in decerebrated rats than that in intact rats (Fig. 7*B*).

Effect of Atropine on Medullary NMDA-Evoked Swallows

Initially, we investigated the generator region of DW-evoked swallows. Based on previous studies, we focused on the L-nTS as a candidate (23, 24). The number of DW-evoked swallows was significantly lower 5 min and 60 min after unilateral AP-5 microinjection compared with before microinjection (Fig. 8, *A* and *B*), suggesting that NMDA receptors in the L-nTS are involved in the initiation of DW-evoked swallows. On the other hand, capsaicin-evoked swallows were unaltered 60 min after AP-5 microinjection (Fig. 8*C*). The microinjection sites were located within the L-nTS in 7 of 9

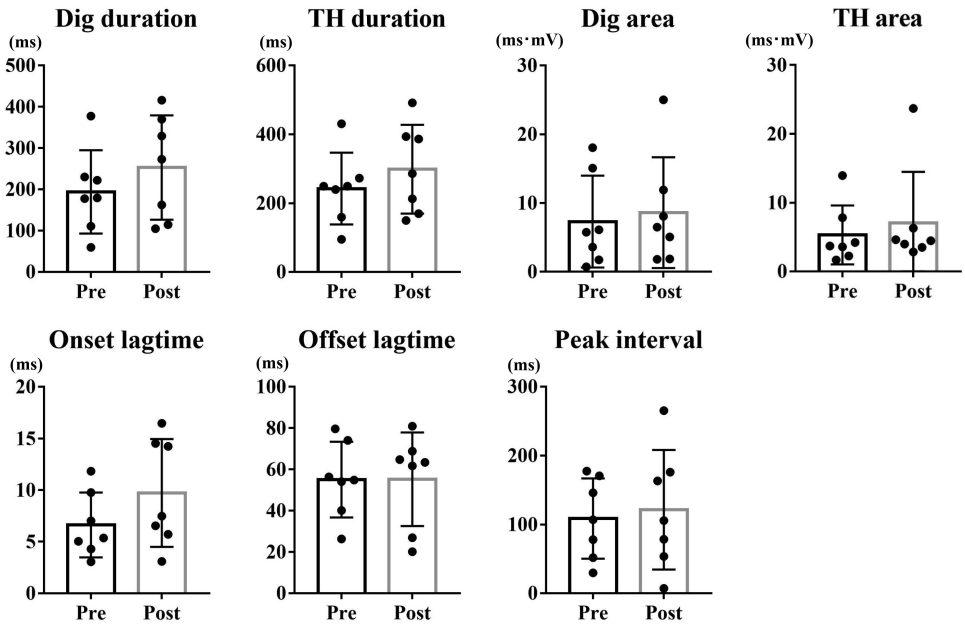

Figure 3. Atropine does not affect digastric (Dig) and thyrohyoid (TH) muscle activities and the temporal relationship between these activities in distilled water (DW)-evoked swallows. The properties of swallow-related motor activity were not altered by atropine administration (Post) compared with before administration (Pre). (Pre vs. Post): Dig duration (194 ± 110 ms vs. 253 ± 126 ms), TH duration (242 ± 104 ms vs. 298 ± 129 ms), Dig area (7.30 ± 6.68 ms·mV vs. 8.60 ± 8.05 ms·mV), TH area (5.31 ± 4.28 ms·mV vs. 7.05 ± 7.41 ms·mV), onset lag time between the Dig and TH electromyographic (EMG) bursts (6.62 ± 3.15 ms vs. 9.72 ± 5.23 ms), offset lag time between the Dig and TH EMG bursts (55.0 ± 18.4 ms vs. 55.2 ± 22.7 ms), time interval between the peaks of the Dig and TH EMG bursts (peak interval, 109 ± 58 ms vs. 121 ± 87 ms). Data are presented as means ± SD (n = 7 in each group). Paired t test for Dig duration, TH duration, Dig area, onset lag time, and offset lag time; Wilcoxon signed-rank test for TH area and peak interval.

rats (78%) (Fig. 8*D*). In two rats that were microinjected into outside the L-nTS, the inhibitory effect of AP-5 on the DW-evoked swallows was not observed.

Finally, we evaluated the effect of atropine on medullary NMDA-evoked swallows. The number of NMDA-evoked swallows was significantly increased 15 min but not 60 min after atropine administration compared with before administration (Fig. 9, *A* and *B*), suggesting that atropine facilitates swallowing evoked by activation of NMDA receptors in the L-nTS, which is involved in the DW-evoked swallows. The microinjection sites were located within the L-nTS in 6 of 8 rats (75%) (Fig. 9*C*). In two rats that were microinjected into outside the L-nTS, the effect of atropine on the NMDA-evoked swallows was inconsistent.

Effect of Atropine Administration on Vital Parameters and Salivation

We evaluated the effect of intravenous administration of atropine (1 mg/kg) on blood pressure, heart rate, respiratory rate, and saliva secretion (Table 1 and Fig. 10). The heart rate and respiratory rate significantly increased 15 min after atropine administration compared with before administration. These values were not affected by vehicle administration. At baseline, these rates did not differ between atropine and vehicle groups, whereas these rates were significantly higher after atropine administration compared with vehicle administration. Atropine did not affect blood pressure. Furthermore, compared with vehicle administration, pilocarpine-induced salivary fluid volume was significantly smaller after the administration of atropine (0.01–10 mg/kg) and methylatropine (1 mg/kg).

DISCUSSION

We elucidated the effect of atropine on swallowing. Atropine facilitated the initiation of swallowing evoked by DW but not that evoked by saline, citric acid, capsaicin, or UA distention. Methylatropine did not affect DW-evoked swallows. Bilateral SLNx completely abolished DW-evoked swallows, and atropine decreased the swallowing threshold evoked by SLN electrical stimulation. Finally, AP-5 microinjection into the L-nTS inhibited DW-evoked swallows, and atropine facilitated the initiation of swallowing evoked by NMDA microinjection into this region. These results suggest that atropine facilitates DW-evoked swallows via central mAChR actions. In this study, the anticholinergic effect of atropine was confirmed by altered vital parameters and pilocarpine induced salivation. Consistent with previous studies, systemic atropine increased heart rate (29) and respiratory rate (30). All doses of atropine (0.01-10 mg/kg) significantly reduced the amount of saliva compared with vehicle. Furthermore, the inhibitory effect of methylatropine (1 mg/kg) on salivation was similar to that of the same dose of atropine.

Facilitation of DW-Evoked Swallows by Atropine

We discovered that atropine at a dose of 1 mg/kg facilitated the initiation of swallowing in anesthetized rats. Conversely, the same dose of methylatropine failed to affect the number of DW-evoked swallows, suggesting that peripheral anticholinergic effects, including dry mouth, exert a smaller effect on the initiation of swallowing. In addition, atropine decreased the swallowing threshold evoked by electrical stimulation of the SLN, which is the primary

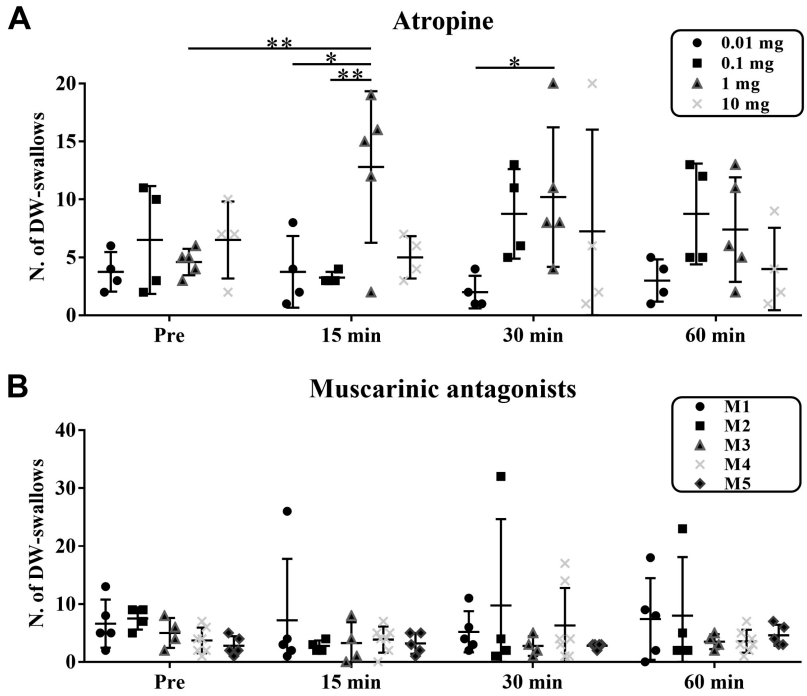

Figure 4. Time-course changes of different atropine doses and effects of specific muscarinic subtypes antagonists on distilled water (DW)-evoked swallows. *A*: the number of DW-evoked swallows 15 min after 1 mg/kg of atropine was significantly larger than baseline (Pre) or 0.01 or 0.1 mg/kg of atropine. Furthermore, the number of swallows 30 min after 1 mg/kg of atropine was significantly larger than that after 0.01 of mg/kg atropine. *B*: M1–M5 muscarinic subtype antagonist did not significantly change the number of DW-evoked swallows. M1, M1 antagonist VU0255035; M2, M2 antagonist AF-DX 116; M3, M3 antagonist 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP); M4, M4 antagonist tropicamide; M5, M5 antagonist ML381 fumarate. Data are presented as means ± SD (n = 3–5 for 0.01–10 mg/kg of atropine; n = 4–7 for M1–M5 antagonists). Two-way repeated-measures ANOVA with one factor repetition [main effect for dose, $F(3,36) = 2.634$, $P = 0.098$; main effect for time, $F(3,36) = 0.624$, $P = 0.604$; interaction effects, $F(9,36) = 2.157$, $P = 0.05$] followed by Tukey's test was used for time-course change of different atropine doses. One-way repeated-measures ANOVA was used: [$F(3,12) = 0.125$, $P = 0.943$] for M1; [$F(3,9) = 0.68$, $p = 0.586$] for M2; [$F(3,9) = 1.134$, $P = 0.386$] for M3; and [$F(3,12) = 1.604$, $P = 0.24$] for M5. Friedman's repeated-measures ANOVA on ranks was used for M4 ($P = 0.728$). $^{**}P < 0.01$, $^{*}P < 0.05$.

sensory nerve for the initiation of DW-evoked swallows. Furthermore, atropine facilitated the initiation of swallowing evoked by NMDA microinjection into the L-nTS, which is involved in the DW-evoked swallows. Although a previous study reported that atropine increased the number of DW-evoked swallows in conscious dogs (16), conscious animals might voluntarily initiate swallowing due to the thirst sensation caused by atropine (31). In this study, we provided the first scientific evidence that atropine facilitates DW-evoked swallows via central mAChR actions.

Atropine did not affect the initiation of swallowing evoked by saline, capsaicin, citric acid, or UA distention. Although

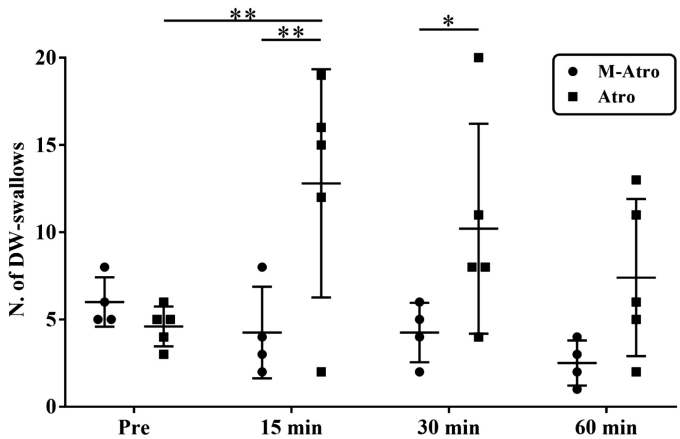

Figure 5. Methylatropine does not affect the initiation of swallowing evoked by distilled water (DW). Number of DW-evoked swallows was not altered 15–60 min after the administration of 1 mg/kg of methylatropine (a form of atropine that is impermeant to the central nervous system, M-Atro). Numbers were significantly smaller (after 15 and 30 min) than those after the same dose of atropine (Atro). Data are presented as means ± SD (n = 4 for methylatropine; n = 5 for atropine). Two-way repeated-measures ANOVA with one factor repetition [main effect for drug, $F(1,21) = 5.767$, $P = 0.047$; main effect for time, $F(3,21) = 2.352$, $P = 0.101$; interaction effects, $F(3,21) = 3.714$, $P = 0.028$] followed by Tukey's test was used. $^{**}P < 0.01$, $^{*}P < 0.05$.

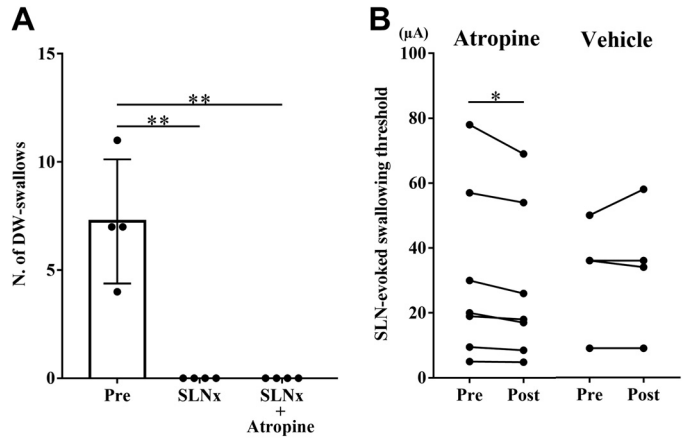

Figure 6. Atropine decreases the threshold for swallowing evoked by electrical stimulation of the superior laryngeal nerve (SLN), which is a primary sensory nerve for the initiation of swallowing evoked by distilled water (DW). *A*: number of DW-evoked swallows was completely abolished following bilateral superior laryngeal nerve transection (SLNx). Atropine did not recover the initiation of DW-evoked swallows in SLNx rats (SLNx + Atropine). Pre: intact rats before SLNx. *B*: swallowing threshold evoked by SLN electrical stimulation was significantly decreased 15 min after 1 mg/kg of atropine administration (Post: median, 18 μA and interquartile range (IQR), 12.8–40.0 μA) compared with before administration (Pre: median, 20 μA and IQR, 14.3–43.5 μA). This was not the case for vehicle administration (Pre: median, 36 μA and IQR, 29.3–39.5 μA; Post: median, 35 μA and IQR, 27.8–41.5 μA). Data are presented as means ± SD for SLNx (n = 4) and point-to-point lines for the SLN-evoked swallowing threshold (n = 7 for atropine; n = 4 for vehicle). One-way repeated-measures ANOVA [$F(2,6) = 25.485$, $P = 0.001$] was used for the effect of SLNx on DW-evoked swallows. Paired t test was used to compare the SLN-evoked swallowing threshold with and without atropine or vehicle. $^{**}P < 0.01$, $^{*}P < 0.05$.

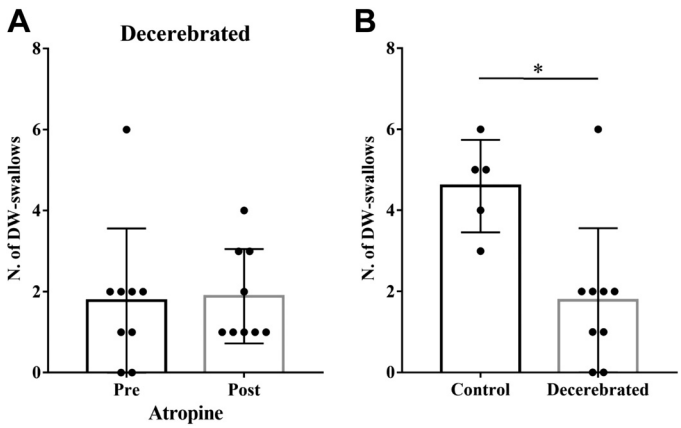

Figure 7. Atropine does not change the number of swallows evoked by distilled water (DW) in decerebrated rats. *A*: number of DW-evoked swallows did not differ before (Pre: 1.8 ± 1.7) and after atropine administration (Post: 1.9 ± 1.1) in decerebrated rats. *B*: number of DW-evoked swallows in decerebrated rats (1.8 ± 1.7) was significantly smaller than that in intact rats (Control: 4.6 ± 1.0). Data are presented as means ± SD (n = 5 for control; n = 9 for decerebrated). Paired t test was used to compare the effect of atropine in decerebrated rats. Mann–Whitney U test was used to compare the number of DW-evoked swallows between intact and decerebrated rats. *$P < 0.05$.

we do not know which receptors can initiate saline-evoked swallows, transient receptor potential vanilloid 1 (TRPV1) and epithelial sodium channel (ENaC) are supposed to be involved in capsaicin- and UA-evoked swallows, respectively (22). Furthermore, both TRPV1 and acid-sensing ion channel (ASIC) may be needed for citric acid to evoke swallows (22). The activation of TRPV1, ENaC, and ASIC receptors might be less involved in the atropine-induced facilitation of swallowing.

Neural Mechanisms of DW-Evoked Swallows

Consistent with a previous study (24), we showed that DW-evoked swallows were completely abolished following bilateral SLNx. Despite the fact that SLN plays a primary role in DW-evoked swallows, the glossopharyngeal nerve, pharyngeal branch of the vagus nerve, and recurrent laryngeal nerve can also initiate swallowing by laryngeal stimulation (22). We considered the possibility that these three sensory afferents are involved in the atropine-induced facilitation of swallowing and thus evaluated the effect of atropine on DW-evoked swallows in SLNx rats. However, atropine failed to initiate DW-evoked swallows in SLNx rats. These results indicate that these three nerves are less involved in the atropine-induced facilitation of swallowing.

SLN plays an important role in swallowing evoked by DW as well as carbonated water, capsaicin, and UA (22, 24, 26). In this regard, SLN electrical stimulation is a less specific swallow trigger than peripheral chemical application that may have implications for the neural mechanism of swallowing evoked by natural stimulation. Although most of the nerve cells in the SLN arise from jugular ganglia (32), we speculate that the SLN afferents minorly arising from the nodose ganglion play an important role in DW-evoked swallows. P2x2/

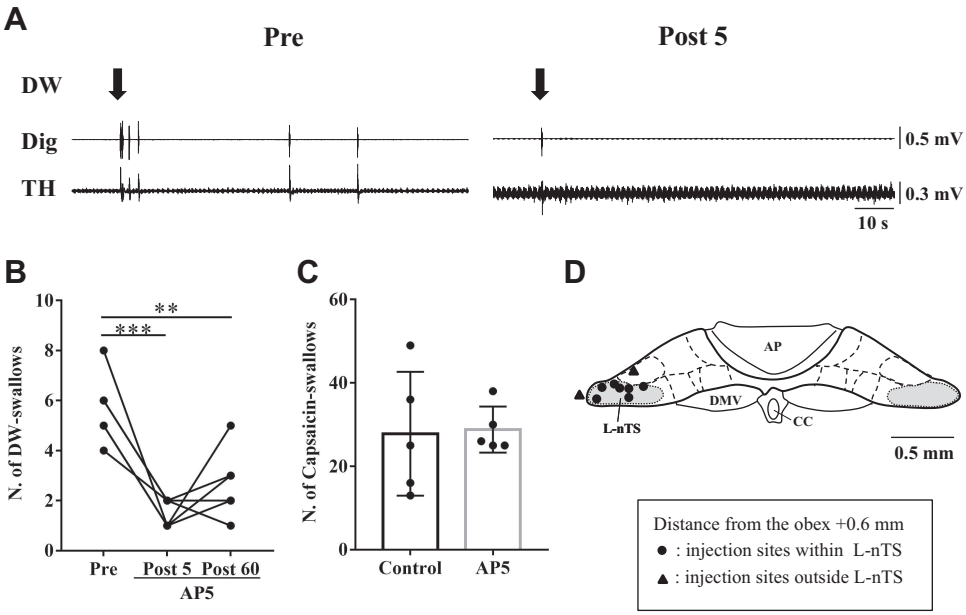

Figure 8. *N*-methyl-D-aspartate (NMDA) receptors in the lateral region of the nucleus of the solitary tract (L-nTS) are involved in the initiation of swallowing evoked by distilled water (DW). *A*: traces illustrating the swallowing reflex evoked by DW application to the larynx (arrow) before (Pre) and 5 min after (Post 5) NMDA receptor antagonist AP-5 microinjection into the L-nTS. *B*: number of DW-evoked swallows was significantly decreased 5 min (Post 5: 1.50 ± 0.50) and 60 min (Post 60: 2.67 ± 1.25) after AP-5 microinjection compared with before microinjection (Pre: 5.67 ± 1.25). *C*: number of capsaicin-evoked swallows was not altered 60 min after AP-5 microinjection compared with intact (Control) rats [27.8 ± 13.3 (Control) vs. 28.8 ± 5.0 (AP5)]. *D*: locations of AP-5 microinjection within the L-nTS. Schematic drawing adapted from the Paxinos brain atlas. Stimulation sites for both hemispheres are shown on the left side. L-nTS includes ventral, lateral, ventrolateral, interstitial, and intermediate nTS subnuclei and is shown in light gray color. AP, area postrema; CC, central canal; DMV, dorsal motor nucleus of vagus. Data are presented as point-to-point lines for the effect of AP-5 microinjection on DW-evoked swallows (n = 7) and means ± SD for the number of capsaicin-evoked swallows in intact and AP-5 microinjected rats (n = 5 in each group). One-way repeated-measures ANOVA [$F(2,10) = 17.448$, $P < 0.001$] followed by Tukey's test was used for the effect of AP-5 on DW-evoked swallows. Student's t test was used for the effect of AP-5 on capsaicin-evoked swallows. ***$P < 0.001$, **$P < 0.01$.

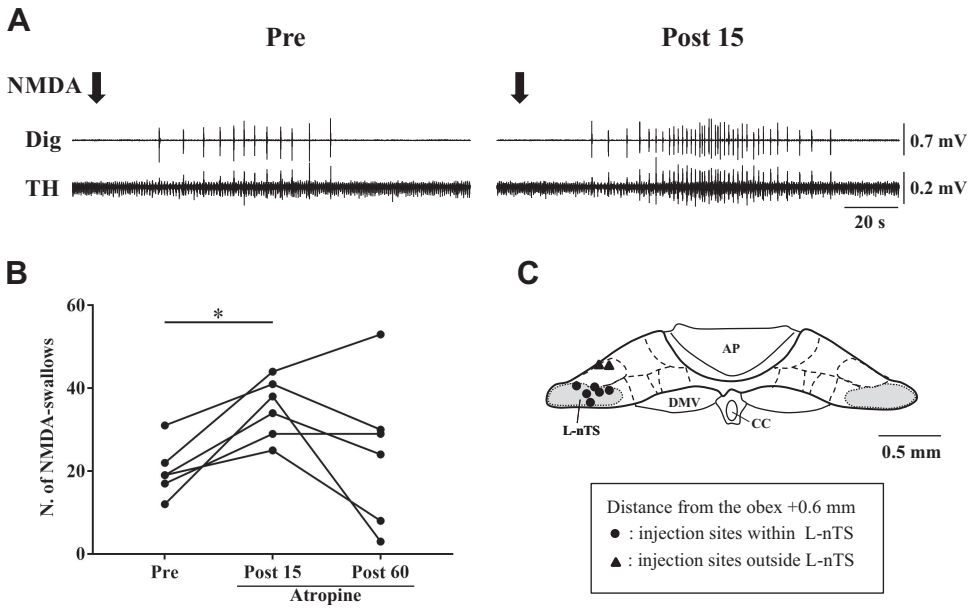

Figure 9. Atropine facilitates the initiation of swallowing evoked by *N*-methyl-D-aspartate (NMDA) microinjection into the lateral region of the nucleus of the solitary tract (L-nTS). *A*: traces illustrating the swallowing reflex evoked by NMDA microinjection (arrow) into the L-nTS before (Pre) and 15 min (Post 15) after 1 mg/kg of atropine administration. *B*: number of NMDA-evoked swallows significantly increased 15 min (Post 15: 35.2 ± 6.6) but not 60 min (Post 60: 24.5 ± 16.3) after atropine administration compared with before administration (Pre: 20.0 ± 5.8). *C*: locations of NMDA microinjection within the L-nTS. Schematic drawing adapted from the Paxinos brain atlas. L-nTS includes ventral, lateral, ventrolateral, interstitial, and intermediate nTS subnuclei and is shown in light gray color. AP, area postrema; CC, central canal; DMV, dorsal motor nucleus of vagus. Data are presented as point-to-point lines (n = 6). One-way repeated-measures ANOVA [$F(2,10) = 4.153$, $P = 0.049$] followed by Tukey's test was used. $^*P < 0.05$.

P2x3-knockout mice lose DW-evoked swallows (24), and vagal neurons express homomeric P2X3 and heteromeric P2X2/3 receptors (33), indicating that these receptors might participate in the initiation of DW-evoked swallows. However, when these receptors are activated by P2X agonist, P2X3 shows a transient and very rapidly inactivating current whereas P2X2/3 shows a long-lasting and slowly inactivating current (34), suggesting that only P2X2/3 receptors produce sustained currents. There are two vagal ganglia, i.e., jugular and nodose, and P2X2 was found in only nodose ganglion (35). Prescott et al. (24) reported that only ~50 nodose neurons/2,300 sensory neurons in nodose/jugular/petrosal superganglia control the initiation of DW-evoked swallows.

The nTS is located in the dorsomedial medulla and is considered to contain the generator neurons involved in swallowing (36). Although the nTS has no laminar structure, this nucleus can be classified into several subnuclei based on cytoarchitecture (37, 38). A recent study using genetic approaches revealed that P2RY1 vagal sensory neuron subsets play a critical role in DW-evoked swallows, and these afferents directly project to the L-nTS (23, 24). To our knowledge, the present study is the first to demonstrate the functional involvement of L-nTS on DW-evoked swallows. We also demonstrate that NMDA receptors in this region are involved in DW-evoked swallows, because AP-5 microinjection inhibited DW-evoked swallows but did not affect capsaicin-evoked swallows. An anatomical study described that capsaicin receptor TRPV1-expressing vagal afferents project to the dorsal/medial nTS subnuclei but not L-nTS (39). If the projection sites of capsaicin- and DW-sensitive vagal afferents differ, the mechanism of initiating DW-evoked and capsaicin-evoked swallows might also differ, as some drugs differently affect these swallowing (21, 26).

NMDA, non-NMDA, and GABA receptors in the nTS are suggested to regulate the initiation of swallowing (40, 41). Glutamate is a main excitatory neurotransmitter in the CNS, and glutamate receptors are classified into ionotropic or metabotropic types (42). Ionotropic receptors are further divided into NMDA and two non-NMDA receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. As efficient activation and ion permeation through NMDA receptors require a sufficiently strong depolarization and synaptic release of glutamate due to voltage-dependent block by Mg^{2+}, NMDA receptors are activated significantly more slowly than AMPA and kainate receptors (43). In other words, most synaptic pathways in the CNS utilize non-NMDA receptors for primary postsynaptic activation, and NMDA receptors are involved in subsequent manifestations of the response (42). This might mean that non-NMDA receptors mainly act during the initial response for evoking a swallow. GABA receptors might also play a critical role in the initiation of swallowing, because GABA neurons in the nTS tonically suppress swallowing (41). Nevertheless, our results indicate that NMDA receptors in the L-nTS are at least partially involved in DW-evoked swallows.

Possible Mechanism of Atropine-Induced Facilitation of Swallowing

Although we described that atropine facilitates the initiation of DW-evoked swallows via central mAChR actions, the underlying mechanisms remain unclear. Here, we discuss two possibilities. First, atropine might directly modulate nTS

Table 1. *The effect of intravenous administration of atropine (1 mg/kg) on blood pressure, heart rate, and respiratory rate*

Variable	Drug	Baseline	Time after Drug Administration		
			5 min	10 min	15 min
Blood pressure, mmHg	Atropine	69.52 ± 26.02	69.11 ± 27.96	67.32 ± 25.08	62.97 ± 23.09
	Vehicle	63.47 ± 9.04	66.64 ± 10.43	65.37 ± 9.41	62.93 ± 10.91
Heart rate, beats/min	Atropine	430.33 ± 138.31	504.27 ± 125.50***	525.80 ± 139.87***	530.47 ± 143.97***#
	Vehicle	360.56 ± 33.25	359.62 ± 28.45 +	369.29 ± 26.29 +	370.49 ± 30.50 +
Respiratory rate, cycles/min	Atropine	118.33 ± 9.01	127.96 ± 8.00*	132.50 ± 7.30**	137.03 ± 6.64***
	Vehicle	110.44 ± 22.62	105.20 ± 18.48 +	108.24 ± 20.42 +	109.91 ± 23.13 +

Data are presented as means ± SD (n = 6 for atropine; n = 8 for vehicle). Two-way repeated-measures ANOVA with one factor repetition [main effect for drug, $F(1,36) = 0.0398$, $P = 0.845$; main effect for time, $F(3,36) = 4.324$, $P = 0.011$; interaction effects, $F(3,36) = 2.055$, $P = 0.123$; main effect for drug, $F(1,36) = 5.762$, $P = 0.033$; main effect for time, $F(3,36) = 43.018$, $P < 0.001$; interaction effects, $F(3,36) = 31.061$, $P < 0.001$ for heart rate, and main effect for drug, $F(1,36) = 6.251$, $P = 0.028$; main effect for time, $F(3,36) = 6.349$, $P = 0.001$; interaction effects, $F(3,36) = 6.587$, $P = 0.001$ for respiratory rate] followed by Tukey's test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. baseline, #$P < 0.05$ vs. 5 min, +$P < 0.05$ vs. atropine. For blood pressure, 5 min was significantly higher than 15 min ($P < 0.05$).

neurons. The mAChRs are expressed in the nTS (44), and mAChRs in this nucleus modulate many functions, such as cardiovascular, respiratory, and gastrointestinal activities (45–47). Furthermore, Cinelli et al. reported that mAChRs in the caudal nTS regulate the cough reflex in anesthetized rabbits (48). The same airway afferents probably regulate the initiation of both coughing and swallowing, and similar central regions, including nTS, might participate in these initiation (40, 49, 50). The mAChRs in the nTS may also be involved in atropine-induced facilitation of swallowing. Second, atropine might modulate DW-evoked swallows via interneurons in other brain regions. We found facilitated swallowing 15 min after atropine administration, indicating that complex neural circuits, including the cortical network, might facilitate DW-evoked swallows. One possible higher centers involved in this facilitation might be amygdala that is a part of the limbic system. Amygdala expresses M1–M5 receptors (51) and directly projects inhibitory neurons to the medial and L-nTS (52). Atropine might change the excitability of the inhibitory neurons in amygdala and modulate L-nTS neurons that are involved in DW-evoked swallows. Further research should focus on identifying the brain areas activated by atropine and evaluating the effect of systemic atropine on neuronal activities in the nTS and other brain regions involved in DW-evoked swallows. In addition, the effect of microinjecting atropine into the nTS on DW-evoked swallows should be assessed.

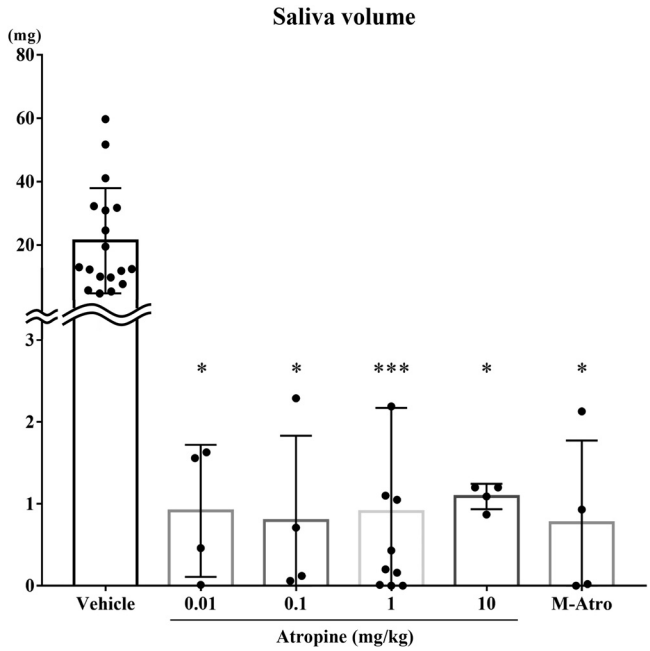

Figure 10. Atropine and methylatropine reduce pilocarpine-induced salivation. The salivary fluid volume induced by the M3 receptor agonist pilocarpine was significantly smaller after administration with atropine (0.01–10 mg/kg) and methylatropine (1 mg/kg) compared with vehicle. Data are presented as means ± SD (n = 4–18). Kruskal–Wallis tests ($P < 0.001$) followed by Dunn's test were used. ***$P < 0.001$, *$P < 0.05$.

It is difficult to interpret why we did not observe a dose-dependent effect of atropine or any effect of specific muscarinic subtype antagonists on DW-evoked swallows. We observed that 1 mg/kg, but not 10 mg/kg, atropine most effectively facilitated DW-evoked swallows 15 min after administration. A proper dose of atropine might be able to facilitate DW-evoked swallows. Although we used sufficiently high doses for each antagonist to activate the target receptors, we could not detect any effect on DW-evoked swallows. All M1–M5 receptors are expressed in the CNS (53), and thus a synergistic or additive effect among these receptors may be involved in the atropine-induced facilitation of swallowing. A limitation to pharmacological approaches using mAChR antagonists is poor pharmacological uniqueness. Although VU0255035 has relatively high selectivity for M1 (54), the selectivity of antagonists for mAChR subtypes rarely exceeds tenfold (55). As an alternative, transgenic approaches, including knockout mice for specific receptor subtypes, might be useful for delineating functions. Identifying the targeted receptors for atropine-induced facilitation of swallowing should clarify this mechanism.

Different Effects of Cx on DW- and SLN-Evoked Swallows

We observed that Cx inhibited DW-evoked swallows, although Cx exerted a smaller effect on SLN-evoked swallows in a previous study (28). One possibility for the discrepancy between DW- and SLN-evoked swallows might be the stimulus intensity. In the previous study, 1.2-fold the threshold for SLN was applied to evoke the swallowing reflex. This stimulus intensity evoked approximately ten swallows during 30 s, and there were no additive effects when combined

with electrical stimulation of the insula cortex, which can evoke swallowing; although, an additive effect was observed with the threshold for SLN combined with insula cortex stimulation (28, 56). Thus, 1.2-fold the threshold for SLN may be enough to strongly activate the swallowing central pattern generator. On the other hand, a small amount of DW was applied to reduce mechanical stimulation, and that evoked approximately five swallows during 60 s. These results indicate that the stimulus intensity of DW in this study was much weaker than that of SLN electrical stimulation in the previous study. This may explain why Cx negatively affected the initiation of DW-evoked swallows and atropine did not facilitate the DW-evoked swallows in decerebrated rats. However, we must consider the possibility of the involvement of mAChRs in the cortical regions for atropine-induced facilitation of swallowing.

Clinical Implications

We selected 1 mg/kg of atropine based on a previous study (20). In humans, 1 mg/kg of atropine shows central effects but is much lower than the lethal dose (31). Although this dose is considered relatively low in animal studies (57), it is much higher than the useful dose for clinical treatment (58). Nevertheless, we believe that the present study has clinical implications. In the sensory aspect, anticholinergic medications probably do not directly impair swallowing initiation, as high atropine doses did not inhibit swallowing evoked by mechanical force or four chemical stimuli in this study. In the motor aspect, anticholinergic medications might affect the esophageal but not the pharyngeal phase of swallowing. We showed that atropine did not affect Dig and TH muscle activities or the temporal relationship between these muscle activities during swallowing. Car et al. (59) also elucidated the effects of systemic atropine on the swallowing motor activity in anesthetized sheep, reporting that atropine suppressed primary esophageal contraction but did not affect geniohyoid muscle activity or secondary esophageal peristalsis. Based on our results, we speculate that the main cause of dysphagia due to anticholinergic medication is the oral and pharyngeal dry conditions. Difficulty in swallowing due to dry condition in the mouth would make sense. Ameliorating these symptoms might largely contribute to improving dysphagia in patients taking anticholinergic medication (60).

DATA AVAILABILITY

Data will be made available upon reasonable request.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.T. conceived and designed research; Y.N., T.T., Y.T., T.C., S.K., and N.D. performed experiments; Y.N., T.T., Y.T., T.C., S.K., and N.D. analyzed data; T.T., J.M., and M.I. interpreted results of experiments; Y.N., T.T., and M.I. prepared figures; T.T. drafted manuscript; Y.N., J.M., and M.I. edited and revised manuscript; Y.N., T.T., Y.T., T.C., S.K., N.D., J.M., and M.I. approved final version of manuscript.

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