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In Search of a Longitudinal Animal Model of Evoked Swallow Function

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Background: A malfunction or impairment of swallow function can potentiate aspiration events and interfere with both quality of life and survival. Establishing an animal model for swallow research would provide a better understanding of its pathophysiology and would also allow for the development and validation of physiologically based clinical interventions to improve swallow function. Two requirements define the ideal model for longitudinal exploration: 1) identification of species similar to human in form and function; and 2) provision for reliable and reproducible evoked swallow under general anesthesia and one that would also support a longitudinal study design.

Objective: We hypothesize that an anesthetized porcine model under dexmedetomidine-based or ketamine-based anesthesia will support a reproducible and stable evoked swallow response.

Methods: Seven neutered male Yorkshire pigs were anesthetized using combinations of dexmedetomidine-based or ketamine-based anesthesia for induction and maintenance of anesthesia during the experimental portion of our study. Single stimulation of iSLN or vagus nerve, bilateral simultaneous single stimulation of iSLN or vagus nerve, and stimulus trains applied to afferent nerves were performed.

Results: None of the seven pigs demonstrated evoked swallow events, both during inhalational anesthesia (1.0 MAC) or during post-washout intravenous anesthesia (dexmedetomidine, ketamine/fentanyl or ketamine alone).

Conclusion: Our results support a high degree of organizational neurophysiologic complexity characterizing the swallow reflex and highlight the challenges and limitations of intraoperative study in survival models.

Key Words: swallow, deglutition, animal model, porcine.

Level of Evidence: NA

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INTRODUCTION

A malfunction or impairment of swallow function can potentiate aspiration events and interfere with both quality of life and survival.[1,2] Establishing an animal model for swallow research would provide a better understanding of its pathophysiology and would also allow for the development and validation of physiologically based clinical interventions to improve swallow function. Two requirements define the ideal model for longitudinal exploration[3]: 1) identification of species similar to human in form and function; and 2) provision for reliable and reproducible evoked swallow under general anesthesia and one that would also support a longitudinal study design.

The majority of prior studies on this subject focus on the physiologic understanding of swallow reflexes in anesthetized rats, rabbits, dogs, and cats.[4,5] Others have suggested that pig is the most appropriate animal for the study of laryngeal function.[6,7] A comparison study, by Jiang et al., examining the laryngeal anatomy and function in human, dog, deer, and pig species, suggested pig as the animal with the most structural and functional similarity to human.[6]

Development of such a model also requires identification of an anesthetic regimen that provides adequate sedation and analgesia throughout operative experimental procedures, but one that also preserves functioning brainstem reflexes during experimental neurophysiology. Most intravenous or inhalational anesthetic agents commonly increase the responsiveness of gamma amino butyric acid (GABA) receptors, enhancing their inhibitory neural behavior.[8] Intravenous dexmedetomidine has been previously used for the study of glottic closure reflex[9] while intravenous ketamine is a dissociative agent that seems to preserve the swallow reflex in human subjects.[10] It has also been used successfully in porcine models for the study of laryngeal reflexes.[11,12]

We have decided to examine whether a porcine model satisfying the above requirements could be established for the study of evoked swallow events in a manner supportive of explorations to validate possible therapeutic interventions. We hypothesize that an anesthetized porcine model under dexmedetomidine-based or ketamine-based anesthesia will support a reproducible and stable evoked swallow response.

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TABLE I.
Anesthetic Table.

Subject	Induction (mg/kg) (IM)	Inhalational anesthetic	Intravenous anesthetic
1	Ketamine 2.2, dexmedetomidine 0.02, tiletamine/zolazepam 4.4, atropine 0.05	Isoflurane	None
2	Ketamine 2.2, dexmedetomidine 0.02, tiletamine/zolazepam 4.4, atropine 0.05	Isoflurane	Ketamine (2.2mg/kg) and dexmedetomidine (0.02 mg/kg) bolus, followed by continuous infusion of dexmedetomidine (0.02 mg/kg/hr)
3	Ketamine 2.2, dexmedetomidine 0.02, tiletamine/zolazepam 4.4, atropine 0.05	Isoflurane	Dexmedetomidine (0.02mg/kg/hr) continuous infusion
4	Ketamine 2.2, dexmedetomidine 0.02, tiletamine/zolazepam 4.4, atropine 0.05	Isoflurane	Dexmedetomidine (0.02mg/kg/hr) continuous infusion
5	Ketamine 2.2, dexmedetomidine 0.02, tiletamine/zolazepam 4.4, atropine 0.05	Isoflurane	Fentanyl loading dose (0.05mg/kg), followed by ketamine (10–35 mg/kg/hr) and fentanyl (0.03-0.1mg/kg/ /hr) continuous infusion
6	Ketamine 16, midazolam 1, atropine 0.05	Sevoflurane	Ketamine continuous infusion (8 mg/kg/hr)
7	Ketamine 16 midazolam 1 atropine 0.05	Sevoflurane	Ketamine infusion (6 mg/kg/hr)

MATERIALS AND METHODS

Seven neutered male Yorkshire pigs, with an average weight of 32 kg each, were used in this study. Pigs were supplied by Earle M. Parsons & Sons, Inc. of Hadley, MA and were free of common swine pathogens. Each pig was conditioned in our animal resources center (YARC) for seven or more days. The animal care program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal use procedures were approved in advance by the Institutional Animal Care and Use Committee (IACUC). Different anesthetic combinations were trialed to elicit the swallow reflex (Table I). In five pigs, an intramuscular injection of ketamine (2.2 mg/kg), dexmedetomidine (0.02 mg/kg), tiletamine/zolazepam (4.4 mg/kg), and atropine 0.05 mg/kg was used for induction of anesthesia. In the two remaining pigs, intramuscular injection of a combination of ketamine (16 mg/kg) plus midazolam (1 mg/kg), and atropine (0.05 mg/kg) was used instead. No long-term muscle relaxants were used. Inhalational isoflurane was used for maintenance of anesthesia in the first five pigs, while sevoflurane was used for the remaining two pigs. Sevoflurane was chosen because it has shorter induction and recovery properties than isoflurane thus expediting titration of anesthetic depth and transition to ketamine-based intravenous anesthesia during the study phase of the procedure.[13,14] The local subcutaneous space beneath the incision for tracheotomy and pharyngeal exposure was infiltrated with 5 ml of 1% lidocaine/epinephrine 1:100,000 immediately after betadine/alcohol scrub of the skin. After a vertical skin incision, a midline tracheotomy between the third and fourth tracheal rings was performed and a 6-mm endotracheal tube was inserted. Inhaled isoflurane or sevoflurane anesthesia was administered via pressure controlled ventilation (GE Healthcare Aespire View) and maintained throughout the process of neck dissection for nerve identification.

The external divisions of the superior laryngeal nerve (eSLN) were identified and exposed bilaterally along their course to the cricothyroid (CT) muscles and their identity was confirmed by direct electrostimulation and observation of CT muscle contraction. The internal divisions of the superior laryngeal nerve (iSLNs) were identified and exposed bilaterally. The vagus nerves were identified and exposed after careful dissection of the carotid sheath (Fig. 1). Bipolar 200 mΩ platinum-iridium eletrodes were used to stimulate the nerves sequentially. Square-wave electrical stimuli of 0.1 ms duration were provided by Nicolet EDX EMG machine (Natus, Pleasanton, CA) starting at 0.1 mA and incrementally increasing by 0.1 mA steps to 10 mA. Each single stimulus was followed one minute later by a train of stimuli to generate the effect of temporal summation. A Nicolet EDX EMG machine was also used for recording EMG waveforms. A bipolar recording electrode was inserted into the mid-portion of strap muscles, CT muscles, and pharyngeal constrictor muscles. A ground electrode was positioned in the subcutaneous tissue at the skin incision. Adjacent muscles were carefully observed by the lead investigator in each dissection to ensure the absence of current spread and to isolate stimulation to target nerves. Thus, in stimulating the

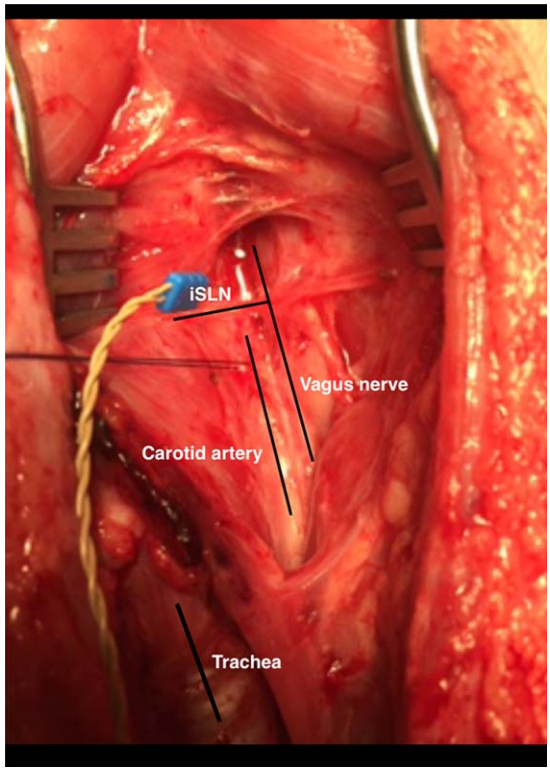

Fig. 1. Intraoperative dissection.

eSLN, positive confirmation was sought in each dissection by noting CT muscle twitch in the absence of adjacent strap muscle activity. This was a reproducible and reliable finding in each subject. A warming pad beneath each animal maintained the core body temperature at 38–39°C.

After the nerves of interest were surgically exposed, the inhaled anesthetic agent (isoflurane or sevoflurane) was decreased to 1.0 Minimum Alveolar Concentration (MAC) and nerve stimulation was attempted.

In four of the five pigs, inhaled isoflurane was used. If no evoked swallow events were observed, the inhaled agent was halted and an alternative combination of intravenous anesthetic was initiated to maintain general anesthesia. In one subject, intravenous ketamine (2.2 mg/kg) and dexmedetomidine (0.02 mg/kg) boluses were administered followed by a constant rate infusion (CRI) of dexmedetomidine (0.02 mg/kg/hr). In two other subjects, an intravenous dexmedetomidine (0.02 mg/kg) infusion was administered, while in a third subject an intravenous fentanyl loading dose (0.05 mg/kg) followed by intravenous ketamine (10–35 mg/kg/hr) and fentanyl (0.03–0.1 mg/kg/hr) infusion was used. If no response was observed in the subjects under sevoflurane, the inhaled agent was halted and ketamine CRI initiated to maintain general anesthesia. The ketamine infusion rate used in the first animal was 8 mg/kg/h while in the second 6 mg/kg/h was used. Heart rate, respiratory rate, and bispectral index (BIS), a processed electroencephalogram (EEG) variable (Mindray DPM/6 patient monitor), were continuously monitored to assess depth of anesthesia. After 15- to 120-minute washout periods, nerve stimulation protocols were completed. Nerves of interest were stimulated to evoke a swallow response as follows:

1. Sequential stimulation of each iSLN.
2. Combined simultaneous stimulation of both iSLNs (spatial summation).
3. Sequential stimulation of each vagus nerve.
4. Combined simultaneous stimulation of both vagus nerves (spatial summation).
5. A train of pulsed stimuli with incrementally increasing frequency (4–8 Hz) applied unilaterally and bilaterally to iSLN and vagus nerves (temporal summation).

RESULTS

None of the seven pigs demonstrated evoked swallow events, both during inhalational anesthesia (1.0 MAC) or during post-washout intravenous anesthesia (dexmedetomidine, ketamine/fentanyl, or ketamine alone). Mechanical stimulation of the pharyngeal mucosa did not elicit a swallow response. Neither temporally or spatially submitted protocols produced evoked swallow. However, spontaneous swallow events were observed in subject 6 during the ketamine CRI at 8 mg/kg/hr and subject 3 during the dexmedetomidine CRI. These events were not related to sensory nerve or mechanical stimulation but occurred infrequently in a random manner. Muscle EMG recordings showed no muscle response following stimulation of nerves of interest, but significant muscle activity was recorded during spontaneous swallow (Fig. 2).

DISCUSSION

The target of our investigation was to activate peripheral neural projections to the Brainstem Swallowing Center as described in Figure 3, pathway A. Despite adequate washout of inhalational anesthetic agents, no evoked swallow pattern occurred despite a full range of stimulation amplitudes and the use of spatial and temporal summation protocols. Nevertheless, random automatic swallow events were captured, likely originating centrally as depicted in Figure 3, pathway B and consistent with current understanding. With respect to our anesthesia protocol, although total intravenous anesthesia was considered, in order to comply with humane standards for animal research during the neck dissection, it was necessary to add an inhalational agent. Thus, inhalational sevoflurane and isoflurane were used to achieve initial surgical anesthesia during neck dissection but allowing 15 to 120 minutes of washout before initiating experimental neurophysiology. Based on the

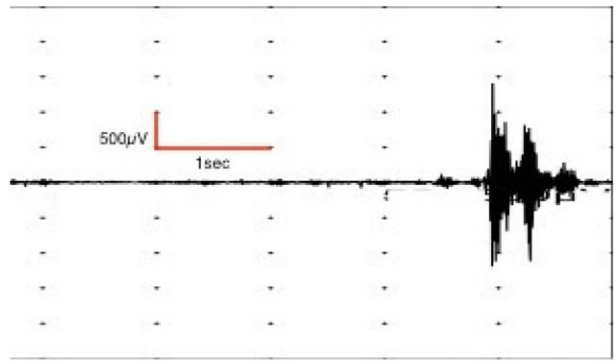

Fig. 2. Example of robust cricothyroid EMG activity during spontaneous swallow.

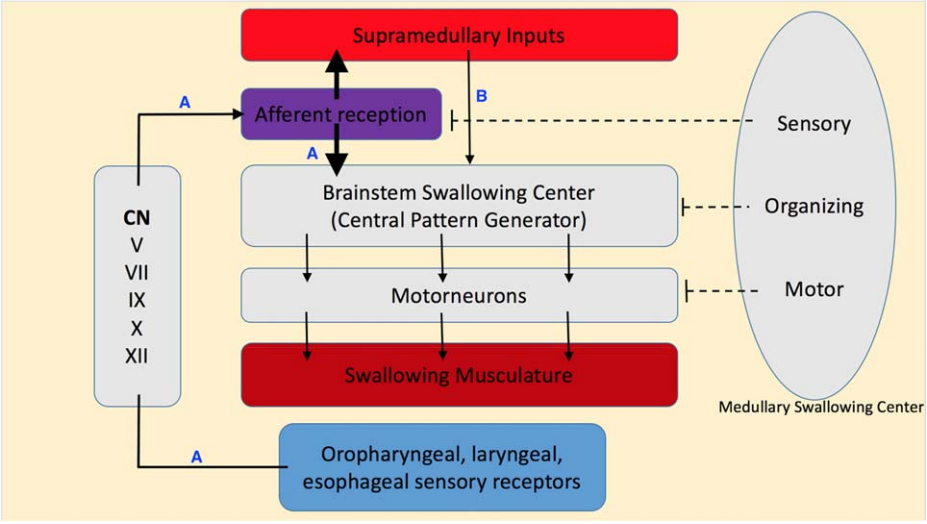

Fig. 3. Organizational model of the swallow network. Adapted from Mistry et al., 2008.[28] Note that peripheral afferent input (pathway A) and central supramedullary input (pathway B) converge separately on the central pattern generator.

known washout rate of sevoflurane and isoflurane in the pig model, we have reason to believe that blood levels of inhalation agents were insignificant during experimental neurophysiology.[14]

The known literature presents many clues describing the high complexity of the swallow reflex as a limiting factor in achieving reproducible experimental results in large non-decerebrated animal models. In this regard coordinated swallow function demands the interaction of many levels of multi layered neural activity from the cerebral cortex to the brainstem medulla[4,15–18] (Fig. 3). It is believed that tactile or chemical stimulation of afferent fibers within the oropharynx and supraglottis are involved in the initiation of swallow, mediated through the maxillary branch of the trigeminal nerve and superior laryngeal nerve branch of the vagus.[4] Sensory inputs project to the brainstem nucleus tractus solitarius (NTS) while others project to cortical areas associated with the initiation of swallow. It has also been suggested that NTS receives descending cortical and subcortical input, associated with evoked swallow.[4,5,19]

Multileveled Brainstem Control

The sequential muscle activation and inhibition that is necessary for the coordinated swallow mechanism, is modulated by many other neural structures of the brainstem. It has been suggested that these structures are anatomically organized into three control levels: afferent, organizing, and efferent (Fig. 3). The peripheral and central afferent fibers constitute the ascending and/or descending inputs to the organizing control level, whereas motor nuclei that are involved in the innervation of swallow musculature constitute the third or efferent level. Between the afferent and efferent levels lies a complex network of interneurons constituting an organizing level known as Central Pattern Generator (CPG).[4,17,20–22] Such a pattern generator is located within the brainstem, controlling neural events from the point the pharyngeal reflex is triggered up to its esophageal phase. These interneurons are believed to be organized in the dorsal swallowing group (DSG) in and around the NTS and the ventral swallowing group (VSG), located dorsal to nucleus ambiguus (NA)[17,20,23–26] (Fig. 4). There is further evidence that there are two-hemi CPGs that are tightly connected allowing ipsilateral stimulation of the hemi-CPG to transfer premotoneuron signals to its contralateral CPG, facilitating synchronized and organized contraction of the bilateral muscles involved in swallowing.[17] In 2001, Jean et al. suggested that the DSG, located within the NTS, contained the generator neurons involved in the triggering, shaping, and timing of the swallow pattern, while the VSG located in the ventrolateral medulla, contained the switching neurons that distributed the swallow drive to associated motoneurons.[17]

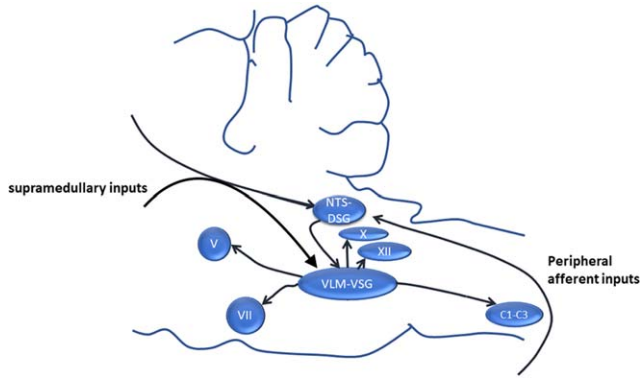

Fig. 4. **Central Pattern Generator (CPG)** CPG includes a dorsal swallow group (DSG) located within nucleus tractus solitarius (NTS) and ventral swallow group (VSG) located in the ventrolateral medulla (VLM) adjacent to the nucleus ambiguus (NA). The DSG neurons trigger, shape, time and sequence the swallowing pattern. VSG distributes the swallow drive to associated motoneurons. Note that central supramedullary and peripheral afferent inputs converge separately on DSG and VSG. Adapted and modified from Jean et al., 2001.17

Cortical Influence

The effect of cortical control on the bulbar CPG is even more highly complex.[17,21] Several animal models, including non-human primates, have been used to provide insight into the functional organization of the cortical pathways involved in swallowing.[26–28] The cortex, despite its complex connections to the brainstem, is not thought to be completely essential to swallowing. For example, a typical human fetus is able to swallow around the twelfth week of gestation, well before the development of cortical and subcortical structures.[17,29] Furthermore, swallowing can be elicited despite removal of the entire cortical and subcortical structures above the brainstem, and infants with severe central neural deficits rostral to the midbrain are capable of swallowing.[30]

More recent literature supports the cortical influence as triggering voluntary swallowing. This is supported by the fact that humans can voluntarily swallow without food ingestion or need to protect their upper airway.[17] Swallowing can be triggered by stimulation of anterolateral cortex fibers that descend subthalamically via the internal capsule to the substantia nigra and reticular formation.[30] Direct projections from the cortex to the NTS are also known to exist. Cortical stimuli travel through internal capsule, the pyramidal pathway, and the mesencephalic reticular formation ending within the NTS.[17] The DSG neurons in the NTS receive both peripheral and cortical inputs that can elicit swallowing.[17] It is this supramedullary voluntary swallow that we believe we witnessed in subjects 3 and 6 (Fig. 3, pathway B).

The role of the cortex in initiation and propagation of automatic, reflex swallowing is less well understood.[31] Recent studies using functional magnetic resonance imaging (fMRI) highlighted the differences in cortical regulation between volitional, automatic, or reflexive swallowing in humans.[32] The most prominent and consistent cortical zone associated with swallowing in humans is considered to be the lateral precentral gyrus including the primary motor and premotor cortex. Volitional swallowing, compared to automatic swallowing, seems to be related to more extensive cortical activation involving both hemispheres. Interestingly, automatic swallow also involves activation of the precentral cortex (area M1) in 80% of the examined subjects.[32] These observations clearly support the idea that cortex actively participates in the control of automatic or reflexive swallowing in humans.

Not only is the cortex understood to provide excitatory influence on the neural periphery, it is also believed to provide inhibitory influences as well. Similarly, there are descending inhibitory drives from the cortex and subcortex in the swallow reflex.[33,34] One of the main cortical inhibitory areas involves the A-area of the orofacial motor cortex. Tsujimura et al. have demonstrated that the number of swallows and swallow intervals during SLN evoked swallowing in rats is strongly inhibited during simultaneous A-area activation.[35]

Decerebration

In an attempt to isolate and exclude the often confounding excitatory and inhibitory influences arising from the cortex, previous publications have utilized decerebration in order to bypass cortical control, successfully reproducing an evoked brainstem initiated swallow in non-human mammals.[19,36] Such models have additionally benefited from further isolation of brainstem reflexes by blocking inhibitory influences of the cerebral cortex and thereby broadly amplifying reflexes mediated by the brainstem.[19,36] Although extensively used in the study of swallow responses, decerebration clearly precludes a longitudinal study design.[19,36] Decerebration has been effective in reproducibly initiating swallow events after stimulation of the superior laryngeal nerve (SLN), in cats, dogs, pigs, monkeys,[37,38] and in non-decerebrated small animals, such as rabbits[39,40] under urethane anesthesia. However, the literature is noticeably lacking in detailed reports of evoked swallow in non-decerebrated, anesthetized large animals or primates.

The Challenge

Clearly decerebration does not favor survival surgery and our results support the fact that developing a model yielding stable and reproducible evoked swallow events without decerebration poses further significant challenges. On the one hand, general anesthesia enhances the inhibitory afferent cortical impulses to CPG, opposing the effect of afferent stimuli from iSLN and vagus stimulation in eliciting reflex swallow. Although inhalational anesthetics, such as isoflurane and sevoflurane, increase the reactivity of gamma-aminobutyric acid (GABA) receptors inhibiting cerebral function and thus suppressing levels of consciousness,[41] they also suppress brainstem activity.[42] Our studies support the observation that even small doses of inhaled sevoflurane at much less than 1.0 MAC can significantly attenuate the swallow reflex. Although ketamine does not activate GABA receptor activity, acting mainly on N-methyl-D-aspartate (NMDA) receptors, it theoretically increases vagal tone, and has therefore been successfully used as an anesthetic agent for the study of laryngeal reflexes in pigs.[11,12] In contrast to laryngeal reflexes, neither ketamine nor dexmedetomidine elicited evoked swallowing in our model even at low doses of 6 or 8 mg/kg CRI after washout of sevoflurane.

Although further effort should be directed to determine the optimal parameters to evoke swallowing under general anesthesia, the observations obtained from our current study suggest the parallel exploration of surrogate markers for evoked swallow function to be used for the assessment of future therapeutic interventions.[9,43] The glottic closure reflex (GCR) is typically elicited during the pharyngeal phase of swallow and plays a pivotal role in the protection of lower airway from invasion of material[44] that may lead to aspiration pneumonia.[2] GCR may be a possible surrogate not only because it clinically serves as an essential component of the swallow reflex, but more essentially because it shares many of the same central nuclei in the generation of the more global swallow reflex response. GCR is by comparison a less complex, "elementary",[45] polysynaptic brainstem reflex that is considered to be elicited after stimulation of mechanoreceptors

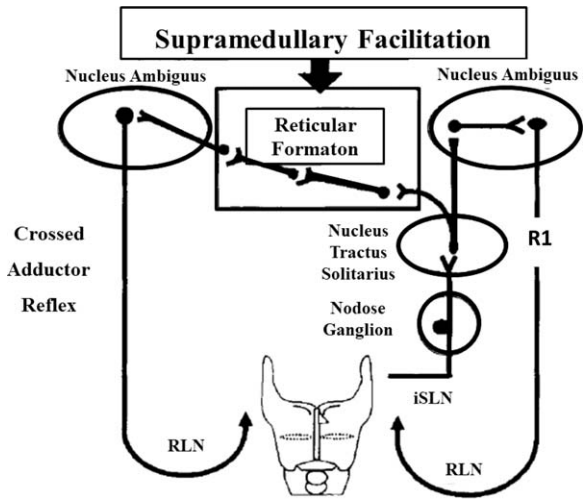

Fig. 5. **An organizational model of the glottic closure reflex**. The sensory input projects through the internal superior laryngeal nerve (iSLN) to ipsilateral nucleus tractus solitarius (NTS). Projection of the stimulus to ipsilateral nucleus ambiguus (NA) leads to recurrent laryngeal nerve (RLN) excitation eliciting an ipsilateral short-latency ipsilateral adduction of the vocal cord, known as R1 response. Contralateral R1 requires travel of the signal from the ipsilateral NTS to the contralateral NA, through the reticular formation. Adapted from Sasaki et al., 2003.[42]

and chemoreceptors of the laryngopharyngeal mucosa and subsequent transduction through afferent sensory fibers of the iSLN that synapse at the ipsilateral NTS (Fig. 5). Afferent signals then project to the ipsilateral and contralateral NA, and synapse with efferent motor neurons of the same nuclei. Finally, motor neurons project through the recurrent laryngeal nerve (RLN) to supply the thyroarytenoid (TA) muscle and other vocal fold adductors, leading to protective reflex glottis closure.[43,46,47] In previous studies, our laboratory has quantitatively characterized the glottic closure reflex in the form of stable and reproducible measures.[9]

Further estimation of the difference in complexity between GCR and swallow reflex can alternatively be expressed in a theoretical perspective by the difference in latency of both reflexes and the estimated number of interneurons involved in each reflex. We can turn to two historical sources for synthetic data to guide our calculations. In a human model, Sasaki et al.[48] elicited reflex glottic closure via iSLN stimulation with 16.5 ms latency. Furthermore, Kitigawa et al.[39] stimulated reflexive swallowing via SLN stimulation in a rat model with a 700 m/sec latency. In order to estimate the number of synapses involved in each pathway, synthetic calculations could be performed given the following considerations. Assuming nerve conduction velocity of 5 cm/ms, synaptic delay of approximately 1.5 ms and distance between braintstem and human larynx (20 cm)[43–45] versus rat (3 cm), the number of neural synapses involved in human GCR approximates 5 compared to 466 synapses in rat evoked swallows. Although the calculations are gross estimates, we can hypothesize large differences in organizational complexity that perhaps suggest greater vulnerability of the latter to central influences.

CONCLUSIONS

Our results support a high degree of organizational neurophysiologic complexity of the swallow reflex and highlight the limitations of intraoperative study in survival models. Although further study should be dedicated toward the investigation of the exact pathways that contribute to the multilevel regulation of the swallow reflex in mammals and human, as well as determination of necessary parameters for the reproduction of evoked swallow under general anesthesia. However, the measurable and reproducible components of swallowing function that have been previously studied, like GCR, may be useful for the evaluation of the effects of treatment interventions for swallow disorders in vivo.

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