THE EFFECT OF LARYNGEAL NERVE TRANSECTION ON SWALLOWING
FUNCTION IN A MOUSE MODEL

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the thesis entitled

THE EFFECT OF LARYNGEAL NERVE TRANSECTION ON SWALLOWING
FUNCTION IN A MOUSE MODEL

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ABSTRACT

Dysphagia is a common postoperative complication of cervical and thoracic surgical procedures, presumably caused by iatrogenic laryngeal nerve injury. It is unknown which laryngeal nerve contributes most to dysphagia and poor medical outcomes after injury. To address this clinically relevant question, we used our established Videofluoroscopic Swallow Study (VFSS) assay to objectively assess swallow function and our established laryngoscopy assay to assess vocal fold (VF) mobility after surgically-induced (iatrogenic) laryngeal nerve injury in a mouse model. C57BL/6J mice (n=31) underwent unilateral transection of the superior or recurrent laryngeal nerves (SLN or RLN) or a sham surgery. Swallowing was assessed through VFSS and VF mobility was assessed through laryngoscopy pre-surgery and several timepoints post-surgery. We validated our surgical procedure by confirming that our surgical technique itself was not negatively impacting swallow function or VF mobility. Unilateral SLN transection did not result in acute or chronic dysphagia or VF immobility, whereas unilateral RLN transection resulted in acute dysphagia and ipsilateral VF paralysis; dysphagia did not persist long-term, whereas VF paralysis did. SLN versus RLN transection produced different dysphagia profiles in our mouse model. In the future, we plan to use this model as a platform to investigate the pathophysiology of post-surgical dysphagia and to explore potential treatments.

CHAPTER I: BACKGROUND

Introduction

The typical swallowing pattern requires the coordinated movement of oral structures, the pharynx, the larynx, and the esophagus. During the oral phase of swallowing, the bolus is prepared by the oral structures of the mouth, including the teeth, lips, tongue, etc. The bolus then enters the pharyngeal phase of swallowing in which the bolus is pushed into the pharynx by the movement of the posterior portion of the tongue against the posterior pharyngeal wall followed by pharyngeal constriction in a superior to inferior direction towards the esophagus. Beginning the esophageal phase of swallowing, the upper esophageal sphincter relaxes which allows for the bolus to be squeezed by the pharyngeal muscles and tongue base into the upper esophagus (Sebastian, Nair, Thomas, & Tyagi, 2015).

Dysphagia is the medical term for difficulty or discomfort of swallowing in either the oral, pharyngeal, or esophageal phase of swallowing. In the oral phase, the preparation or positioning of the bolus may be affected due to reduced strength or poor coordination of the oral musculature. During the pharyngeal stage, dysphagia may be apparent in the absence or delay in the triggering of swallow reflexes, as well as entry of the bolus into the airway (aspiration). Esophageal dysphagia is caused by dysfunction of the esophagus or esophageal sphincters (upper or lower) (Sebastian et al., 2015).

Dysphagia is a common postoperative complication of surgical interventions targeting the cervical (neck) and thoracic (chest) regions, including thyroid surgery, skull base surgery to remove tumors, and coronary artery bypass graft (CABG). Dysphagia in these cases is inadvertently caused by the surgical procedure itself, which is commonly referred to as an **iatrogenic** complication. One of the most common spinal procedures is anterior cervical discectomy and fusion (ACDF) (Marawar et al., 2010), with more than 200,000 of these surgeries performed each year (Gaudinez et al., 2000; Papavero et al., 2007; Yue, Brodner, & Highland, 2005). Nearly 80% of these cases result in postoperative dysphagia (Anderson & Arnold, 2013; Cho, Lu, & Lee, 2013; Rihn, Kane, Albert, Vaccaro, & Hilibrand, 2011) that is frequently lifelong (Bazaz, Lee, & Yoo, 2002; Lee, Bazaz, Furey, & Yoo, 2007). These postoperative complications typically affect the pharynx, larynx, and esophagus, resulting in impaired sensation and motor function that puts airway protection at risk. This may further lead to aspiration causing other health problems, such as pneumonia. Current therapies include diet modifications (thick liquids), behavioral adaptations (chin tuck), and feeding tubes. These therapies are not curative and focus on alleviating the symptom rather than targeting the underlying cause, which remains largely unknown and is the focus of this thesis proposal.

Although the pathophysiology of dysphagia following surgery is poorly understood, surgical injury to the laryngeal nerves is suspected to be a leading cause (Chaw, Shem, Castillo, Wong, & Chang, 2012). Laryngeal nerves branching from the vagus nerve (the 10th cranial nerve or CNX) include the

superior laryngeal nerve (SLN) and the **recurrent laryngeal nerve (RLN)**.

These branches of the vagus nerve provide innervation of the striated muscles of pharynx and larynx that are crucial for motor functions. For instance, the SLN provides motor innervation of the inferior pharyngeal constrictor and cricothyroid muscles. The RLN provides motor innervation to the intrinsic muscles of the larynx (with the exception of the cricothyroid which is innervated by the SLN) and the cervical esophagus, including the upper esophageal sphincter. The SLN and RLN also provide sensory function to these regions. For example, the SLN transmits sensations from the mucous membrane of the larynx (vocal folds, epiglottis, aryepiglottic folds) and base of the tongue to the brain and transmits information from the muscle spindles and other stretch receptors in the larynx to the brain. The RLN transmits sensations from the mucous membranes of the larynx below the level of the vocal folds and the proximal striated portion of the esophagus to the brain and transmits information from the muscle spindles and other stretch receptors in these same anatomical regions to the brain. As a group, the SLN and RLN maintain all laryngeal motor and sensory activities necessary for swallowing (Duffy, 2013).

In order to gain access to the surgical regions of interest during cervical and thoracic procedures, the pharynx, larynx, and/or esophagus are likely to be retracted from the midline for an extended period of time (Anderson & Arnold, 2013; Cho et al., 2013). This may result in the laryngeal nerves becoming stretched and/or crushed by the surgical retractors. In other instances, the nerves may accidentally become severed by surgical instruments (Mattsson, Hydman, &

Svensson, 2015). In addition, electrocautery and other energy-based devices used to control bleeding within the surgical site may result in heat/burn injury to the laryngeal nerves (Kwak et al., 2015; Lin et al., 2015). Thus, there are four main iatrogenic laryngeal nerve injuries: traction (stretching), compression (crushing), **transection (severing)**, and thermal (heat). It is currently unknown which of these injury types contribute most to dysphagia and poor medical outcomes. Similarly, it is unknown whether injury to the SLN or RLN results in poorer outcomes. Systematic investigations of this nature are impossible to conduct in humans; therefore, animal models may play an important role in this research. The limited animal research conducted thus far has included pigs and rats. Preliminary results suggest that unilateral RLN injury results in ipsilateral vocal fold (VF) paralysis (Hernández-Morato et al., 2013) and dysphagia affecting all three stages of swallowing (oral, pharyngeal, esophageal) (Gould et al., 2015). In contrast, unilateral SLN injury results in oral and pharyngeal dysphagia; assessment of VF paralysis was not included (Ding et al., 2013). However, VF paralysis after SLN injury is unlikely, given the RLN, not the SLN, provides motor innervation of the VFs (Duffy, 2013). We proposed to expand upon this preliminary animal research by systematically studying these injuries in C57BL/6 mice.

The C57BL/6J mouse, commonly referred to as B6, is the most widely studied laboratory rodent for understanding normal developmental biology and neurobiology (Bult, Eppig, Blake, Kadin, & Richardson, 2013; Lambert, 2007). Our lab has studied the swallow function of B6 mice across the lifespan, which

remains stable from 3-17 months of age, after which age-related changes in swallow function become apparent (Lever, Brooks, et al., 2015). For this reason, we studied B6 mice between 3 and 17 months of age for this thesis proposal. This age range in mice equates to 20 to 70 years in humans (Dutta & Sengupta, 2015). Moreover, we focused only on transection of the SLN or RLN to create a surgical model of iatrogenic laryngeal nerve injury that will serve as a platform for studying the other injury types in the future. Bilateral injury to the SLN or RLN are rare in human medicine, therefore we chose to focus only on unilateral transection to optimize the translational potential of our findings. Further, unilateral transection was chosen as a starting point because it is the easiest and most replicable injury type to perform experimentally and we expected it would produce the worst outcomes relative to dysphagia. Our future work will expand on this surgical model to include other iatrogenic laryngeal nerve injury types (traction, compression, thermal) that may occur more frequently, but produce less severe outcomes than transection injury.

To establish our translational surgical mouse model of laryngeal nerve injury, we used two behavioral assays previously developed in our lab: **videofluoroscopic swallow study (VFSS)** (Lever, Braun, et al., 2015; Lever, Brooks, et al., 2015) and **laryngeal function testing via laryngoscopy** (Shock et al., 2015). These tests are routinely used to assess swallow and laryngeal function in people, and our lab has successfully adapted both for use with mice. Our VFSS assay for mice (**Figure 1**) is an *x-ray* procedure that permits evaluation of the structure and function of the oral cavity, pharynx, larynx, and

esophagus of mice. A customized low energy fluoroscopy system called The LabScope (Glenbrook Technologies, Randolph, NJ) was used for this purpose. The LabScope is essentially a miniature X-ray microscope that can zoom in and out in real time to view and digitally record very small regions of interest, such as the swallowing mechanism of a mouse. During VFSS testing, unanesthetized, freely-behaving mice were enclosed in a custom test chamber that minimized behavioral distractions and facilitated voluntary drinking of a radiopaque contrast agent from a bowl. The procedure was video recorded at 30 frames per second (fps) for subsequent frame-by-frame analysis to objectively quantify several outcome measures (i.e., metrics). Examples include lick rate, swallow rate, and pharyngeal and esophageal transit times.

Figure 1. Videofluoroscopic Swallow Study (VFSS) Assay. VFSS is a radiographic (X-ray) procedure that permits evaluation of swallowing in awake, unanesthetized mice. **Left:** A miniature low energy fluoroscopy system (The LabScope, Glenbrook Technologies) was customized for VFSS testing of mice. **Right:** Mouse in a custom test chamber positioned in the fluoroscope beam.

Our laryngeal function assay for mice (**Figure 2**) is an *endoscopic* procedure that uses a miniature fiberoptic endoscope (Karl Storz Endoscopy, Germany) to view the larynx during rest breathing in lightly anesthetized mice. Our lab designed a miniature endoscopy suite for this purpose. During testing, the endoscope was inserted transorally to visualize both vocal folds in the camera field of view (FOV). The procedure was video recorded at 30 fps and subsequently analyzed frame-by-frame to objectively measure vocal fold paralysis.

Figure 2. Laryngeal Function Assay. **Left:** A miniature endoscope is inserted transorally to view the larynx of lightly anesthetized mice. **Right:** Endoscopic view of the bilateral vocal folds during rest breathing. Asterisk=glottis.

Research Questions and Hypotheses

Using VFSS and laryngeal function testing, I addressed the following research questions and hypotheses:

1. Does our surgical technique cause vocal fold paralysis and/or dysphagia?
 - a. I hypothesized that our surgical technique would not result in impaired ipsilateral vocal fold mobility and/or dysphagia. For valid and reliable results, it was imperative that our surgical technique

did not cause any adverse outcomes that may have confounded our study. Dr. Lever's lab has been perfecting its surgical technique for the past year to minimize post-surgical edema that may contribute to vocal fold immobility and/or dysphagia. Therefore, we did not expect any adverse outcomes from the sham surgical procedure, which underwent all aspects of surgery, with the exception of nerve transection.

2. Does unilateral transection of the SLN or RLN in mice cause acute vocal fold paralysis and/or dysphagia? For this study, we considered acute to be within the first week after surgery, which equates to less than one year in humans (Dutta & Sengupta, 2015).
 - a. I hypothesized that unilateral SLN transection would not result in VF paralysis because the SLN does not provide motor innervation of the VFs (Duffy, 2013). However, I expected that unilateral SLN transection would result in oropharyngeal dysphagia as was reported for an infant pig model (Ding et al., 2013).
 - b. I hypothesized that unilateral RLN transection would result in ipsilateral VF paralysis as was shown for rats (Hernández-Morato et al., 2013). I also expected that unilateral RLN transection would result in oropharyngeal and esophageal dysphagia as was reported for an infant pig model (Gould et al., 2015).

3. Does unilateral transection of the SLN or RLN in mice cause chronic vocal fold paralysis and/or dysphagia? For this study, we used 14-weeks post-surgery as our chronic (permanent deficit) timepoint, which equates to over a decade in human years (Dutta & Sengupta, 2015).
 - a. I hypothesized that any vocal fold paralysis and/or dysphagia present at the acute stage of recovery would persist at 14-weeks post-surgery (i.e., study endpoint). A portion of each nerve was removed during the surgical procedure in order to prevent the nerve from regenerating across the transected site to reestablish motor and sensory connections. Therefore, any deficits were expected to remain chronic in this model.

CHAPTER II: METHODS

Animals

This study included B6 mice (n=31) of either sex ranging from 3 to 12 months of age at the time of baseline testing. All mice were randomly selected from our colony established at the University of Missouri from B6 sibling breeder pairs purchased at six-weeks of age from The Jackson Laboratory (Bar Harbor, ME). Offspring were weaned between 21 and 24 days of age and group housed based on sex, with 3-4 mice per cage. Mice are housed in a standard 12:12 light/dark cycle facility with controlled temperature and humidity conditions. All mice are provided free access to water and standard rodent food pellets, except during test procedures as described below. To minimize aggressive behaviors, mice were provided enrichment materials in the home cage, including a nestlet, dental treats, food treats, and running wheel. Research and veterinary staff perform daily checks to ensure mice remain healthy through this study. At the conclusion of this study (14-weeks post-surgery), mice were euthanized and the SLN and RLN were collected bilaterally to establish histological methods for use in our lab's ongoing studies. The Institutional Animal Care and Use Committee (IACUC) of the University of Missouri approved this study.

Surgical Groups

The 31 mice were randomly allocated into one of three surgery groups: sham surgery, unilateral right SLN transection, unilateral right RLN transection, as show in **Table 1**.

Table 1. Sample size and sex for each of the mouse surgical groups

Surgical Group	Sample Size
Unilateral right SLN transection	n=10 (5 males, 5 females)
Unilateral right RLN transection	n=11 (6 males, 5 females)
Sham surgery	n=10 (6 males, 4 females)
Total number of mice	n= 31

Videofluoroscopic Swallow Study (VFSS) Protocol

All mice underwent VFSS testing in accordance with our established protocol (Lever, Braun, et al., 2015; Lever, Brooks, et al., 2015) to assess baseline swallow function prior to surgery. The mice underwent a two-week behavioral conditioning period to establish familiarity and acceptance of the VFSS test solution (50% stock iohexol, flavored with chocolate syrup) and the VFSS testing environment. During this period, mice were repeatedly exposed to the test chamber and test solution without contrast agent added.

The night prior to VFSS, a test chamber was placed in each mouse cage to allow the mice to explore and acclimate to the test chambers. Water was

restricted overnight and mice were provided chewable treats (nuts and seeds) to induce thirst. On the day of testing, individual mice were placed into a test chamber and videofluoroscopic recordings were acquired at 30 frames per second (fps) while the mouse drank in the lateral (horizontal) plane. The VFSS test solution was administered through a custom delivery system into the bowl. A webcam was positioned above the test chamber to provide real-time viewing of the mice. This ensured that the fluoroscopic machine was only activated while the mice were drinking to minimize any unnecessary radiation exposure while the mice were not actively drinking from the bowl. Videos were obtained for mice in the oral-pharyngeal stage of swallowing (Position 1) and for the esophageal stage of swallowing (Position 2) as explained in **Figure 3**. VFSS was repeated at the following post-surgical timepoints: 4-days, 6-weeks, and 14-weeks. Four-days post-surgery was the earliest that mice could be tested once pain medication had been eliminated from their systems. Due to the unknown effects of transection on swallow function, the earliest possible post-surgical timepoint was selected in order to detect adverse events that may confound outcomes. Waiting one-week in accordance with endoscopy timepoints may have resulted in potentially severe dysphagia going unnoticed.

Figure 3. VFSS Positioning. Position 1: The head and proximal thoracic region are visible in the fluoroscopy field of view (FOV), with the swallow trigger point (black arrow; vallecular space) positioned in the center. The tongue (black asterisk) is visible as the mouse drinks from a bowl. With each successive lick, contrast agent accumulates in the vallecular space before triggering a swallow. **Position 2:** The FOV spans from the swallow trigger point (black arrow) to the stomach (white asterisk). Note the bolus (black asterisks) passing through the distal esophagus. White arrows: 2nd cervical vertebra.

Surgical Procedure

A separate group of students in the Lever Lab performed all aspects of the surgical procedure for this project. The night before surgery, mice were restricted from food overnight to prevent residual food in the pharynx under anesthesia. Anesthesia entailed a single subcutaneous injection of ketamine-xylazine (KX) anesthetic mixture (10/80 mg/kg). Maintenance doses of ketamine (half the original dose) were administered every 20 minutes or as needed to maintain a surgical plane. Using aseptic surgery guidelines, the surgical field of the anterior neck was shaved and prepared for surgery. Eyes were lubricated to prevent drying. The head was stabilized in ear bars with the mouse in dorsal recumbency

on a custom surgical platform. Core body temperature was maintained at 37 °C using a homeothermic heating system.

A 2 cm midline skin incision was made from the suprasternal notch to the mandible. The large salivary glands were gently retracted from midline with forceps to visualize the larynx and surrounding musculature. The right SLN or RLN was identified and visually inspected prior to isolation and transection (**Figure 4**). The SLN was identified at the side of the larynx. The RLN was identified in the tracheoesophageal groove between the 4th and 6th tracheal rings. Transection of either the SLN or RLN was performed using microscissors and removing a 2 mm section to prevent re-attachment over time. The wound was closed with 6-0 vicryl suture and Dermabond surgical glue. A control group of sham surgery mice underwent all aspects of the procedure, with the exception that the SLN or RLN was not transected. Postsurgical analgesics (banamine and buprenorphine) and saline were subcutaneously administered to each mouse prior to being placed in a monitored, temperature-controlled surgical recovery station. Mice were returned to their home cage after becoming fully ambulatory. Pain management with analgesic medications were provided as needed up to 72 hours post-surgery.

Figure 4. Schematic of Laryngeal Nerve Innervation. Our surgical procedure entails transection of either the right SLN or RLN at the anatomical locations indicated with an X (red=SLN, blue=RLN).

Endoscopic Laryngeal Function Testing

Endoscopic laryngeal function testing was conducted during the surgical procedure and several timepoints thereafter. Therefore, this component of this study was performed by the same group of students performing the surgical procedure. Immediately before making the surgical neck incision during the surgical procedure, transoral laryngoscopy was performed to assess baseline VF mobility during rest breathing. For approximately 10 seconds, VF movement during rest breathing was video recorded at 30 fps using a Storz Tele Pack X

System. Laryngoscopy was repeated immediately after either laryngeal nerve transection or visualization to re-assess VF mobility. Laryngoscopy was repeated at 1-, 6-, and 14-weeks post-surgery for longitudinal assessment of laryngeal function. Mice cannot be subjected to anesthesia more than once per week due to risk of mortality. Therefore, one-week post-surgery was the soonest that testing could be performed. A three-day time window was allotted between VFSS and endoscopy at all timepoints so that effects of anesthesia did not interfere with VFSS testing and to allow for scheduling convenience. Laryngoscopy videos were viewed frame-by-frame to assess mobility of the injured (right) VF relative to the uninjured (left) side using a subjective rating scale: 0=no movement, 1=partial movement, and 2=normal movement.

Video Analysis

Videos from both VFSS and endoscopic examinations were analyzed on a computer using video editing software (Pinnacle Studio 14; Pinnacle Systems, Inc.). VFSS videos were analyzed to include a variety of swallow metrics, as described below. Endoscopic videos were analyzed using a Likert scale ranging from 0 to 2, where 0=immobile (paralyzed) and 2=normal mobility. All videos were independently reviewed and analyzed by 2 reviewers in a blinded fashion, with the VFSS start frames identified by the first reviewer. Three to 5 measures for each metric were obtained for each mouse for use in statistical analysis. All value discrepancies were subjected to group consensus to resolve reviewer errors.

Quantitative Swallow Metrics

Lick Rate: During VFSS testing in Position 1, the tongue was not always visible. The number of jaw open/close (excursion) cycles was used as a proxy to the number of tongue protrusion/retraction cycles per second to allow quantification of lick rate (Lever, Brooks, et al., 2015). Lick rate was calculated by counting the number of jaw open/close cycles during 1 second (30 frames) of uninterrupted drinking. Each cycle began with the jaw maximally opened and each subsequent maximal jaw excursion was counted as an individual jaw cycle (Lever, Braun, et al., 2015).

Swallow Rate: This metric is the number of swallows that took place during 2-second episodes of uninterrupted drinking (Lever, Braun, et al., 2015; Lever, Brooks, et al., 2015). This metric is also an indicator of oral transit time and pharyngeal swallow delay.

Lick-Swallow Ratio: This measure was calculated by counting the number of jaw open/close cycles (licks) that occurred between two successive, uninterrupted swallows. It is an indicator of oral transit time and pharyngeal swallow delay (Lever, Braun, et al., 2015; Lever, Brooks, et al., 2015).

Inter-Swallow Interval: This metric is the time (ms) between two successive, uninterrupted swallows during sequential drinking (Lever, Braun, et al., 2015; Lever, Brooks, et al., 2015). The initial frame, or “rest frame”, was the frame that immediately preceded triggering of the pharyngeal swallow. The end frame is defined as the “rest frame” of the following sequential swallow. The number of frames between the two swallows was then divided by 30 frames per

second (fps) to convert to time (ms). This VFSS metric is an indicator of oral transit time and pharyngeal swallow delay.

Pharyngeal Transit Time (PTT): This metric is defined as the time (ms) it takes for the bolus to transfer entirely through the pharynx. It is based on bolus flow. The start frame was the “rest frame” as described above. The end frame was when the tail of the bolus completely transferred out of the pharynx into the esophagus. The number of frames between the start and end frames was then divided by 30 fps and converted to milliseconds (ms) (Lever, Braun, et al., 2015; Lever, Brooks, et al., 2015).

Bolus Area: A still frame photo of three separate swallows was captured using video editing software Pinnacle 14 to calculate the size (cm²) of the bolus. Each still photo was captured during the “rest frame”, or the frame that preceded the pharyngeal swallow, in the lateral view of Position 1 (Lever, Braun, et al., 2015; Lever, Brooks, et al., 2015). Next, these photos were viewed using NIH ImageJ software. Each photo’s measurements were calibrated to the size of the radiographic calibration marker visible during VFSS testing. The bolus was then traced using ImageJ, which calculated the area inside the outlined area on each image. This procedure was followed for each of the three photos obtained from one mouse and was completed by two reviewers. Reviewers independently averaged their three recorded measurements, and then their two averages were averaged together to obtain a final bolus area measurement.

Esophageal Transit Time (ETT): This metric is defined as the time (ms) it takes for the bolus to travel through the esophagus into the stomach (Lever,

Braun, et al., 2015; Lever, Brooks, et al., 2015). It was observed and quantified in Position 2 (esophageal view). The start frame was when the tail of the bolus first entered the esophagus (i.e., the PTT end frame). The end frame was identified as when the tail of the bolus completely entered the stomach with no remaining bolus in the esophagus. The number of frames between the start and end frames was then divided by 30 fps and converted to milliseconds (ms).

Effective Esophageal Swallows: This measure was quantified by observing uninterrupted swallowing in Position 2 (esophageal view) (Lever, Braun, et al., 2015; Lever, Brooks, et al., 2015). Reviewers determined if a second swallow was necessary to force the preceding swallow into the stomach. Reviewers recorded a 0 or 1. A score of 0 indicated that the initial swallow successfully reached the stomach prior to a second swallow being triggered. A score of 1 indicated that the primary swallow was not successful and did not reach the stomach prior to the next swallow trigger.

Number of Swallows to Clear the Esophagus: This measure is an extension of Effective Esophageal Swallows (Lever, Braun, et al., 2015; Lever, Brooks, et al., 2015). If the primary swallow was ineffective (i.e., it did not reach the stomach prior to the next swallow), reviewers then counted the number of pharyngeal swallows that were required for complete movement of the bolus tail through the esophagus and into the stomach.

Statistical Analysis

Summary and basic descriptive statistics for each outcome measure were calculated. Paired sample t-tests or analysis of variance (ANOVA) with post-hoc

tests (Tukey) were used for statistical analysis. Prior to ANOVA analysis, assumptions of normality and variance were verified. Two-sided significance levels were used with alpha set at 0.05. All analyses were completed using SPSS v23 (IBM).

CHAPTER III: Results

Thirty-one mice underwent the surgical procedure, divided into the following three groups: unilateral SLN transection (n=10), unilateral RLN transection (n=11), and sham surgery (n=10). Three mice did not survive the surgical procedure (one from the SLN group and two from the RLN group). This attrition rate of less than 10% (3/31) is consistent with other surgical studies in our lab. The remaining twenty-eight mice were included in statistical analyses for this study: SLN transection (n=9), RLN transection (n=9), and sham surgery (n=10).

All mice underwent serial VFSS testing and laryngoscopy assessment as planned through 14-weeks post-surgery. We initially intended to analyze nine VFSS swallow metrics to quantify swallow function. However, bolus area was excluded from analysis due to an unexpected calibration error in our VFSS system; this problem has been corrected for future studies in our lab. Pharyngeal transit time was also excluded from analysis because the rapid bolus movement through the pharynx could not be easily distinguished using our 30 fps camera. Laryngoscopy data were collected and analyzed as expected.

To answer Research Question 1 (Does our surgical technique cause vocal fold paralysis and/or dysphagia?), we used a paired samples T-test using two-sided significance to compare each swallow metric from the sham surgical group at baseline and 4-days post-surgery, using the null hypothesis that the difference is equal to 0. Results did not show a significant difference ($p>.05$) for any of the

swallow metrics, as shown in **Table 2**. For all sham-surgery mice, VF mobility remained normal (score=2) and did not change from pre- to post-surgery, thus rendering statistical analyses unnecessary. These findings validate that our surgical procedure itself does not cause VF paralysis or dysphagia, according to the seven reported outcome measures.

Table 2. Mean difference, standard deviation, and *p* value for VFSS metrics in sham surgery mice, baseline compared to 4-days post-surgery

VFSS Metrics	Mean difference	Std. Deviation	<i>p</i> value
Lick Rate	-.135	.507	.421
Swallow Rate	-.088	1.972	.891
Lick-Swallow Ratio	.665	1.214	.118
Inter-Swallow Interval	.020	.146	.681
Esophageal Transit Time	-.017	.107	.627
Effective Esophageal Swallows	.120	.527	.489
Swallows to Clear Esophagus	-.213	.612	.299

To answer Research Question 2 (Does unilateral transection of the SLN or RLN in mice cause acute vocal fold paralysis and/or dysphagia?), we focused only on the 4-days post-surgery timepoint. Our rationale was that this early timepoint would be the most clinically relevant to determine acute post-surgical effects on vocal fold mobility and swallow function.

Laryngoscopy results showed SLN transection had no effect on VF mobility (score = 2 at baseline and 4-days post-surgery). RLN transection caused immediate, ipsilateral VF paralysis (i.e., immobility, score = 0) in all mice, which persisted at 1-week post-surgery. Thus, transection of only the RLN results in acute VF paralysis.

For VFSS data, we performed an analysis of variance [ANOVA with pairwise comparisons (Tukey HSD method, two-sided significance)] using only the 4-days post-surgery measures to compare each experimental group (SLN vs RLN transection) to the sham surgical group. As demonstrated, the sham surgery did not result in any post-surgical swallow dysfunction; therefore, this group serves as the normal control to permit detection of acute changes in swallow function in the experimental groups. Results showed that swallow function was not significantly different ($p > .05$) between the SLN transection and sham surgery groups for any of the VFSS swallow metrics, as shown in **Table 3**. However, after RLN transection, four of the seven swallow metrics were significantly different from the sham group (denoted by asterisks), as shown in **Table 3** and described below.

Table 3. Mean difference, standard deviation, and p value for VFSS metrics of experimental groups compared to sham surgery mice at 4-days post-surgery

VFSS Metrics	<i>SLN TRANSECTION</i>			<i>RLN TRANSECTION</i>		
	Mean diff.	Std. Error	p value	Mean diff.	Std. Error	p value
Lick Rate	.147	.102	.322	.766	.106	.000*
Swallow Rate	-.158	.254	.809	.246	.264	.623
Lick-Swallow Ratio	-.604	.362	.220	-.752	.376	.116
Inter-Swallow Interval	.032	.039	.680	-.087	.041	.085
Esophageal Transit Time	-.067	.067	.576	-.284	.070	.000*
Effective Esophageal Swallows	-.139	.096	.323	.263	.100	.026*
Swallows to Clear Esophagus	.085	.159	.855	-.450	.165	.020*

Note: asterisks denote statistical significance ($p < .05$)

Lick Rate: Statistically significant differences in lick rate were found between the RLN and sham surgical group ($p < .0001$), as shown in **Figure 5**. Specifically, the RLN group had significantly fewer licks per second than the control group. Lick rate was not statistically different between the SLN transection and control groups ($p > .05$).

Figure 5. Lick Rate at 4-days Post-Surgery. The number of licks per second is significantly lower for the RLN transected mice as compared to the sham surgery and SLN transection mice. Asterisk denotes statistical significance ($p < .05$); error bars = mean $\pm$ 1 standard error of the mean (SEM). n=sample size.

Esophageal Transit Time: A statistically significant difference in esophageal transit time was found between the RLN and sham surgical group ($p<.0001$), as shown in **Figure 6**. The RLN transected mice exhibited a significantly longer esophageal transit time when compared to controls, whereas esophageal transit time was similar between the SLN transection and control groups.

Figure 6. Esophageal Transit Time at 4-days Post-Surgery. Esophageal transit time is significantly longer for the RLN transected mice as compared to the sham surgery and SLN transection mice. Asterisk denotes statistical significance ($p<.05$); error bars = $\pm$ 1 SEM. n=sample size.

Effective Esophageal Swallows: A statistically significant difference in the percentage of effective esophageal swallows was found between groups ($p < .026$), as shown in **Figure 7**. The RLN surgical group had fewer effective esophageal swallows when compared to the SLN and control groups. Thus, the RLN transection group had more instances in which the primary swallow was considered unsuccessful (i.e., ineffective) because it did not reach the stomach prior to the next swallow trigger.

Figure 7. Effective Esophageal Swallows at 4-days Post-Surgery.

The RLN surgical group had fewer effective esophageal swallows when compared to the sham surgical group and SLN transected mice. Asterisk denotes statistical significance ($p < .05$); error bars = ± 1 SEM. n=sample size.

Number of Swallows to Clear the Esophagus: Statistically significant differences in the number of swallows required for esophageal clearance was found between groups ($p < .025$), as shown in **Figure 8**. The RLN surgical group required more swallows to completely transfer the bolus through the esophagus and into the stomach.

Figure 8. Swallows to Clear Esophagus at 4-days Post-Surgery. The RLN surgical group required more swallows to clear the esophagus as compared to the sham surgical group and SLN transected mice. Asterisk denotes statistical significance ($p < .05$); error bars = ± 1 SEM. n=sample size.

To answer research question 3 (Does unilateral transection of the SLN or RLN in mice cause chronic vocal fold paralysis and/or dysphagia?), we focused only on the 14-weeks post-surgery timepoint. Our rationale was that this late

timepoint would be the most clinically relevant to determine chronic post-surgical effects on VF mobility and swallow function.

Laryngoscopy results showed SLN transection had no chronic effects on VF mobility (score = 2, 14-weeks post-surgery). In contrast, VF paralysis resulting from RLN transection (i.e., immobility, score = 0) persisted at 14-weeks post-surgery in all mice. Statistical analysis was unnecessary because there was no change in VF mobility scores. Thus, transection of only the RLN results in chronic VF paralysis.

For VFSS data, we performed an analysis of variance (ANOVA) with pairwise comparisons (Tukey HSD method, two-sided significance) at only the 14-weeks post-surgery timepoint. All groups (SLN, RLN, Sham) were compared at this timepoint. Results showed that swallow function was not significantly different ($p > .05$) between groups for any of the VFSS swallow metrics, as shown in **Table 4**, indicating complete recovery of swallow metrics impaired in the RLN transection mice.

Table 4. Mean difference, standard deviation, and p value for VFSS metrics of experimental groups compared to sham surgery mice at 14-weeks post-surgery

VFSS Metrics	<i>SLN TRANSECTION</i>			<i>RLN TRANSECTION</i>		
	Mean diff.	Std. Error	p value	Mean diff.	Std. Error	p value
Lick Rate	-.049	.108	.891	.133	.109	.445
Swallow Rate	.275	.204	.372	.446	.206	.082
Lick-Swallow Ratio	-.608	.369	.229	-.568	.374	.284
Inter-Swallow Interval	-.065	.042	.279	-.060	.043	.341
Esophageal Transit Time	.090	.061	.301	.018	.060	.952
Effective Esophageal Swallows	-.084	.094	.647	.058	.093	.810
Swallows to Clear Esophagus	.136	.162	.679	-.067	.162	.910

CHAPTER IV: DISCUSSION

For this project, we set out to establish a translational surgical mouse model of laryngeal nerve injury. We tested three groups of B6 mice (unilateral SLN transection, unilateral RLN transection, and surgical sham), all targeting the right side of the neck. Mice were tested using two behavioral swallow assays developed in our lab for mice, specifically VFSS to assess dysphagia and laryngoscopy to assess VF mobility. Testing was conducted from pre-surgery (baseline) to 14-weeks post-surgery to identify acute versus chronic indications of laryngeal dysfunction and dysphagia.

A fundamental result of this study was that our sham surgery group did not develop VF immobility or dysphagia, thus validating that the surgical technique itself (without laryngeal nerve injury) was not confounding this study. For all sham-surgery mice, VF mobility remained normal (score=2) and did not change pre- to post- surgery. In addition, results failed to show a significant difference ($p>0.05$) for any of the swallow metrics analyzed for our sham surgery group. Prior published studies investigating this topic did not include a surgical sham group to validate their surgical skills, thus our study is the first to do so. Instead of a surgical sham group, previous studies have included unoperated animals (Hernández-Morato et al., 2013) or used each animal as its own control (in pre- to post- surgery comparisons) (Ding et al., 2013). Therefore, our explicit intent to include a sham surgical group in this study provides a needed and scientifically necessary perspective when interpreting our main research findings.

The primary goal of this study was to explore the acute and chronic effects of unilateral laryngeal nerve transection on VF mobility and swallow function. As we hypothesized, unilateral transection of the SLN does not impair VF mobility, likely because it does not innervate the intrinsic laryngeal muscles essential to normal VF movement (Duffy, 2013); however, the laryngeal innervation pattern has not yet been mapped for mice. Contrary to our hypothesis, unilateral transection of the SLN did not result in dysphagia, either acute or chronic. This finding is surprising because the SLN is known to innervate the pharyngeal and laryngeal mucosa to provide sensory input to the brainstem (specifically the nucleus tractus solitarius in the medulla) that is critical to evoking swallowing. In fact, electrical stimulation of the sensory (afferent) fibers of the SLN reliably evokes swallowing in all mammals studied to date, whereas stimulation of the motor (efferent) fibers of the SLN causes only local contractions of the corresponding muscles innervated by the SLN (i.e., cricothyroid and cricopharyngeus) (Corbin-Lewis et al., 2004). Based on this foundational information, we expected that injury to the SLN would have negative consequences on swallowing function, similar to findings reported for this same nerve injury type in infant pigs (Ding et al., 2013). However, our finding suggests that additional cranial nerves are involved in triggering the swallow reflex in mice, similar to humans. Further suspected reasons for this unexpected finding in our mouse model are discussed in the study limitations section below.

We also hypothesized that unilateral transection of the RLN would result in ipsilateral VF immobility and dysphagia at both the acute and chronic stages of

recovery. As expected, results of this study confirmed acute changes in laryngeal and swallowing function. Specifically, the ipsilateral (right) VF became immobile immediately following transection and persisted at the 4-days post-surgery timepoint. This finding suggests the motor innervation pattern of the RLN in mice is similar to humans. Unilateral RLN transection also resulted in significantly altered swallowing function at this same timepoint, characterized by slower lick rate, longer esophageal transit time, fewer effective esophageal swallows, and increased number of swallows to clear the esophagus while drinking thin liquid. These results are indicative of oral and esophageal stage dysphagia; there was no acute evidence of pharyngeal stage dysphagia using our VFSS assay. Our finding of oral stage dysphagia (impaired lick rate) following unilateral RLN transection was unexpected, given that oral feeding (suckling rate) was not impaired in a similar investigation of infant pigs (Gould et al., 2015). We suspect this difference may be due to licking (drinking) behaviors in mice requires marked tongue protrusion, whereas suckling in infant pigs does not. Furthermore, the tongue is anatomically coupled to the larynx via the hyoid (Duffy, 2013); thus, the resultant VF immobility after unilateral RLN transection may be causing an anchoring effect that hinders normal tongue protrusion. The larynx is indirectly connected to the tongue via the hyoid, therefore if the larynx has restricted range of motion, we would expect this reduced range of motion to translate to the tongue. Our finding of acute esophageal dysphagia after unilateral RLN transection is consistent with results from this same infant pig study (Gould et al., 2015), reflecting the typical innervation pattern of the esophagus by the RLN

(Duffy, 2013). The surprising lack of evidence for pharyngeal dysphagia in our mouse model is incongruent with findings reported for infant pigs (Gould et al., 2015); potential reasons are discussed in the study limitations section below.

We hypothesized that the acute effects of unilateral RLN transection would persist at 14-weeks post-surgery, corresponding to the chronic recovery stage. Indeed, this was the case for VF immobility, as there was no change in VF mobility scores (score=0) at all timepoints. However, all swallow metrics that were impaired at 4-days post-surgery resolved by the 14-weeks post-surgery timepoint. While we have not begun to investigate the underlying mechanisms responsible for this unexpected recovery pattern, we speculate it may be due to the mice adopting compensatory behavioral strategies during feeding to maintain adequate nutrition and hydration, as is typically seen in people. In contrast to humans and other larger animal models of RLN injury, VF paralysis in mice does not result in aspiration during swallowing. Therefore, the persistent VF immobility in mice does not have the same extreme consequences as humans that require modifications to adequately protect the airway and prevent aspiration pneumonia. This point will be clarified in the study limitations section.

The combined results of this study provide novel evidence that unilateral RLN transection injury results in the most clinically adverse outcomes relative to laryngeal and swallowing function. This finding was made possible by concurrent investigation of swallowing and laryngeal function in an animal model of laryngeal nerve injury, targeting both the SLN and RLN injury in a single study. To date, the

most commonly studied models are rats and pigs. The studies with rats have utilized laryngoscopy, but not VFSS (Hernández-Morato et al., 2013), whereas the studies with pigs have utilized VFSS, but not laryngoscopy (Ding et al., 2013; Gould et al., 2015). Although laryngoscopy can directly assess the effects of laryngeal nerve injury on VF mobility, VFSS cannot. Thus, combining both behavioral tests in a single study overcomes the individual limitations of each study alone. Furthermore, we have used these combined tests to individually assess the effects of SLN versus RLN injury on laryngeal and swallowing function. Given that laryngoscopy and VFSS are considered gold-standard for diagnostic tests of laryngeal and swallowing dysfunction, our successful adaption of these tests for use with mice highlights the translation potential of this line of research. Although mice do not aspirate, aspiration is only one symptom of dysphagia; this mouse model develops several other laryngeal and swallow deficits that translate to the human population. Furthermore, mice are the model organism recognized by the NIH for biological studies, and they are the most widely used laboratory species. Thus, establishing our surgical model in mice is directly in line with current research trends.

Limitations

While this study has provided us valuable translational perspective on iatrogenic laryngeal nerve injuries in general, there are several limitations that warrant consideration. One limitation is that we only investigated a single injury type, specifically unilateral transection of either the right SLN or RLN. We are

uncertain if the outcomes would be consistent across different injury types (compression, thermal, traction) or a left-sided injury. Although a unilateral SLN injury did not result in VF immobility or dysphagia, we are uncertain if a simultaneous RLN and SLN injury would result in more severe outcomes, potentially due to a cumulative effect. Therefore, we plan to investigate these other injury types and combinations in future studies.

Another limitation is that mice are preferential nasal breathers whose larynx is naturally protected from the path of bolus flow during swallowing. As a result, we have found that healthy mice and mouse models of advanced aging and neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS), do not aspirate (Lever, Brooks, et al., 2015). While we did not specifically include a measurement of aspiration in this study, no instances of aspiration were observed during VFSS. Thus, our study was focused primarily on nine VFSS swallow metrics, two of which we were unable to quantify: bolus area and pharyngeal transit time.

For bolus area, calculations were inaccurate due to an unfortunate alteration of the calibration marker in the field of view during VFSS. This error can be overcome by retracing all of the bolus areas for every mouse to recalculate bolus area. While this is entirely our intent, it is an extremely labor intensive process that could not be completed within the time frame of this thesis project.

The issue with pharyngeal transit time was a limitation in our VFSS recording frame rate, which was only 30 fps. Typically, pharyngeal transit time lasts only 2-4 frames in mice in comparison to approximately 15-30 frames (1/2 – 1 second) for people; the rapid speed of the pharyngeal phase of the swallow cannot be appreciated with our limited frame rate. We suspect that pharyngeal dysphagia may exist; however, we are unable to detect it with our current VFSS technology. We expect a markedly higher framerate camera (over 100 fps) may enable detection of clinically relevant differences in pharyngeal transit time after SLN or RLN transection in our mouse model of laryngeal nerve injury (Lever, Brooks, et al., 2015).

New Directions Emanating from this Study

Our lab is currently conducting histological studies of the brain and laryngeal nerves to investigate the underlying mechanisms of persistent VF immobility in conjunction with recovery of dysphagia after unilateral RLN transection. We hypothesize that the mice have developed compensatory strategies to compensate for acute dysphagia allowing them a more efficient swallow. However, we want to investigate potential evidence of neuroplasticity in the regions of the brain involved in swallowing.

We are also currently investigating other more common nerve injury types, specifically a compression injury. In addition, we are investigating bilateral nerve injuries and a simultaneous RLN and SLN injury to investigate a potential cumulative effect that may result in more severe or chronic complications related

to VF mobility and swallow function. Thus, this initial model is already serving as a platform for studying other nerve injury types relative to the impact on laryngeal and swallowing dysfunction.

Due to VF immobility seen in the mice that underwent a unilateral right RLN transection, we hope to investigate impacts on vocal function. Mice communicate using ultrasonic vocalizations that are similar in many ways to human communication. Our lab has recently received funding to purchase an ultrasonic vocalization system, which will allow us to better assess laryngeal dysfunction in mice. We expect the addition of this behavioral test, in combination with laryngoscopy and VFSS, to enable us to establish a more translational model of laryngeal nerve injury.

Our most exciting new direction resulting from this study is our new ability to investigate potential therapeutic strategies to improve laryngeal and swallowing function after laryngeal nerve injuries. For example, we are currently investigating the use of electrical stimulation to promote laryngeal nerve regeneration following a crush injury, with the goal of significantly improving laryngeal and swallowing function. We also intend to investigate the impact of nerve growth factors in combination with electrical stimulation for optimal outcomes that may ultimately benefit people with laryngeal nerve injuries.

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