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TITLE:

An Implantable System for Chronic In Vivo Electromyography

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KEYWORDS:

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SUMMARY:

Presented here is a protocol for the manufacturing of an implantable system for in vivo chronological recording of evoked and spontaneous electromyographic potentials. The system is applied to the investigation of reinnervation of laryngeal muscles following nerve injury.

ABSTRACT:

Electromyography (EMG) measures the muscle response to electrical stimulation or spontaneous activity of motor units and plays an important role in assessing neuromuscular function. Chronic recording of EMG activity reflecting a muscle's reinnervation status after nerve injury has been limited, due to the invasive nature of traditional EMG recording techniques. In this regard, an implantable system is designed for long-term, in vivo EMG recording and nerve stimulation. It has been applied and tested in a study on reinnervation of laryngeal muscles. This system consists of 1) two bipolar electrode nerve cuffs and leads for stimulating each of two nerves: the recurrent laryngeal nerve (RLN) and internal branch of the superior laryngeal nerve (SLN); 2) two EMG recording electrodes and leads for each of the two laryngeal muscles: posterior cricoarytenoid (PCA) muscle and thyroarytenoid-lateral cricoarytenoid (TA-LCA) muscle complex; and 3) a skin receptacle interfacing all implanted lead terminals to an external recording preamplifier and stimulator using a connection cable. The wire leads are Teflon-coated, multi-filament, type 316 stainless steel. They are coiled and can stretch during body movement of the awake animal to prevent lead breakage and electrode migration. This system is implanted during an aseptic surgery. Afterwards, baseline EMG recordings are performed before the RLN is transected in the second surgery to study muscle reinnervation. Throughout the study, multiple physiological sessions are conducted in the anesthetized animal to obtain evoked and spontaneous EMG activity that reflects the reinnervation status of laryngeal muscles. The system is compact, free of infection over the

course of the study, and highly durable. This implantable system can provide a reliable platform for research in which long-term recording or nerve stimulation is required in an anesthetized or freely moving animal.

INTRODUCTION:

EMG recording is a useful technique for measuring electrical activity produced by a skeletal muscle when activated by electrical stimulation of its nerve or spontaneous firing of its motor units. Monitoring EMG signals can be used for assessment of neuromuscular transmission and muscle biomechanics[1]. EMG recording also plays an important role in characterizing the quality and magnitude of muscle reinnervation following nerve injury[2–5]. However, multiple EMG recordings over the entire period of reinnervation cannot be achieved by an invasive approach. Therefore, implantable devices have been designed and developed for repeated, chronic stimulation and recording in neuromuscular systems[6–13]. The aim of this paper is to describe a protocol for the manufacturing and implantation of a stable system for obtaining reliable chronological EMG data from the larynx.

This system is applied here to the study of laryngeal muscle reinnervation. A brief overview of the larynx is provided for orientation (**Figure 1**). A precise coordination between sensory and motor components is essential for proper muscular movement during respiration, voicing, and airway protection. The PCA muscle, located in the posterior larynx, is the sole abductor of the vocal fold. This muscle is spontaneously activated during inspiration to increase glottal area for inhalation. The TA-LCA complex is the major adductor of the vocal fold. Activation of this muscle complex along with another adductor (i.e., the interarytenoid muscle) medialize the fold for vibration and sound production and close the fold for airway protection during swallowing.

Additionally, motor neuron fibers innervate both abductor and adductor muscles in the RLN. The abductor and adductor muscles can be distinguished based on motor unit composition[14,15]. The PCA muscle exhibits increased firing during hypercapnic and/or hypoxic conditions[16] due to the presence of inspiratory motor units. In contrast, reflex glottic closure (RGC) motor units, which close the glottis reflexively through activation of sensory receptors within the laryngeal mucosa, is present in the TA-LCA muscle complex. The internal branch of the superior laryngeal nerve (SLN) carries the afferent fibers of sensory receptors in the larynx[17]. Although voicing is primarily an adductor function, both abductor and adductor motor units are involved in this highly evolved laryngeal behavior.

[Place **Figure 1** here]

Injury to the RLN can result in vocal fold paralysis (VFP), which compromises both abducting and adducting functions due to laryngeal muscle denervation[14,18,19]. Subsequently, regeneration of RLN nerve fibers and reinnervation of muscles commonly occurs. However, reinnervation is a random process and results in misdirected, inappropriate muscle reconnection in most cases. This is referred to as synkinesis, in which spontaneous activation of abductor and adductor antagonists is faulty and produces ineffective or even paradoxical movement of the vocal

folds[14,19–21]. With synkinesis, the critical function that is lost is vocal fold abduction, resulting in inadequate ventilation. Although there are ongoing attempts to treat laryngeal synkinesis by either 1) blocking glottic closure with Botox[22,23] or 2) electrically stimulating the glottic opening with an implantable pacemaker[24,25], there is no clinical intervention that reliably prevents synkinesis[26]. However, there is evidence that electrical conditioning of the PCA muscle during reinnervation at a low frequency promotes appropriate neuromuscular reconnection and minimizes synkinesis from happening. Studies are currently being conducted to elucidate the underlying mechanisms[2].

The focus of this paper is to describe a simple and inexpensive implantable system for chronic nerve stimulation and EMG recording. This system can be used to investigate the effects of low frequency electrical conditioning of the PCA muscle on the specificity of its subsequent reinnervation. EMG signals obtained by this system can reflect the quality and quantity of laryngeal muscle reinnervation over time.

PROTOCOL:

This study has been approved by the institutional animal care and use committee (IACUC) of Vanderbilt University and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, Maryland). This system includes five implantable components and one external cable.

1. Two bipolar RLN stimulus electrode cuffs, each with pair of coiled lead wires and terminal pins

1.1. Use Teflon-coated, multi-filament, type 316 stainless steel wire (with insulated diameter of 0.0078" or 0.198 mm) for each cuff lead wire. Cut a 70 cm length of wire and coil it into a 12 cm long spring using a coiling device or procure prefabricated coiled leads. If necessary, stretch the spring to increase its length for each implant site. Leave the ends of the coiled leads straight at 3 mm and 10 mm lengths and deinsulate them.

1.2. Solder a gold-plated copper female pin onto the 3 mm end of the coiled lead.

1.3. To prepare the nerve cuff, cut a 5 mm segment of silicone tube (OD = 0.156", ID = 0.094"; or OD = 3.96 mm, ID = 2.39 mm) from a roll of the tubing.

1.4. To insert a lead into the tube, use a 25 G hypodermic needle to pierce through the tubing wall 1.5 mm from the end and off-center close to the inner wall. Backfill the 10 mm end of the lead into the tip of the needle. Withdraw the needle to deposit the deinsulated portion into the tube. Bend back the bare wire end outside the tube and twist onto the lead at its point of entry into the tube.

NOTE: Use an operating microscope to perform these steps. A probe can be placed into the tube to curve the wire against the inner wall. The goal is to position the bare portion of the wire

so that stimuli can be delivered to the nerve without risking mechanical damage to the nerve.

1.5. Insert the second lead 1.5 mm from the opposite end of the tube using the same procedure. Align the point of entry to that of the first lead. Pierce the wall with the needle so that the bare portion of the wire is deposited near the inner wall opposite to the first lead.

NOTE: Looking down the tube, the two stimulus electrodes should form a 45° "V" shape, which will straddle the nerve once in place and assure current delivery through the nerve from anode to cathode.

1.6. Make an S-shaped slit in the tube wall opposite the electrode points of entry using a pair of curved scissors.

NOTE: The spiral lips of the cuff can then be opened to situate the nerve inside between the electrodes during surgery.

1.7. Insert a length of 6-0 monofilament, nonabsorbable suture into the cuff wall at each end using a curved microsurgical needle for eventual securement of the cuff around the nerve.

1.8. Apply medical grade type-A silicone gel to reinsulate all exposed bare wire outside the cuff.

2. Two bipolar SLN stimulus electrode cuffs, each with pair of coiled lead wires and terminal pins

2.1. Assemble the SLN stimulus electrode cuff in the same way as the RLN stimulus electrode cuff. However, use the smaller diameter (OD = 0.125", ID = 0.062"; or OD = 3.18 mm, ID = 1.57 mm) tube, because the nerve is smaller in diameter.

3. Two PCA muscle EMG recording electrodes, each with coiled lead wire and terminal pin

3.1. Assemble a coiled lead for the PCA muscle electrode as done in step 1.1.

3.2. Solder a female pin onto the lead as done in step 1.2.

3.3. Insert the 10 mm end of the PCA muscle lead into the tip of a deep brain stimulation (DBS) electrode using the same strategy for needle-lead insertion into a cuff (step 1.4). Bend the end of the lead to form a hook and clip it to provide a total of 5 mm recording length.

NOTE: In this application, the PCA muscle and its reinnervating nerve terminals are exposed to electrical conditioning. Stimuli are generated by an implantable pulse generator (IPG) and delivered to the laryngeal muscle through a DBS electrode (**Figure 1**, inset). This system is adapted from therapeutic brain stimulation (e.g., Parkinson's disease). The DBS electrode will be inserted into a submuscular pocket and anchored in place. If technology for electrical conditioning of the muscle is not required, the PCA EMG electrode can be directly inserted into

the muscle and anchored by its hook.

4. Two TA-LCA muscle complex EMG recording electrodes, each with coiled lead wire and terminal pin

4.1. Assemble a coiled lead for the TA-LCA muscle electrode as done in step 1.1.

4.2. Solder a female pin onto the lead as done in step 1.2.

4.3. Excise a 5 mm x 10 mm rectangular piece of knitted polyester graft. Make a hole in the center of the mesh with a 20 G hypodermic needle. Introduce the 10 mm end of the lead into the hole with an additional 3 mm of coil protruding beyond the hole. Affix the lead to the mesh using 6-0 monofilament, nonabsorbable suture.

NOTE: This piece of mesh will be used to anchor the electrode lead to the thyroid cartilage overlying the muscle complex.

4.4. Bend the end of the lead to form a hook and clip it to provide a total of 5 mm recording length.

5. Skin receptacle for interfacing connections between electrodes and external equipment

5.1. Utilize a single row female pin stripe connector to make the receptacle. Cut two pieces (each 17.5 mm in length) from the strip, each containing eight pin holes. First, roughen the external surfaces of each piece with sandpaper, then glue them together with phenol in a fume hood to make a double-row connector. Place the connector in 60–80 °C water in a fume hood for 30 min to allow for glue hardening.

NOTE: This double-row assembly format will provide convenience in the assignment of pinholes for left- vs. right-side electrodes.

5.2. Cut a 25.6 mm length piece from the strip to make the connector's faceplate (the portion that will protrude outside the implant site for skin anchoring). Cut a 5.4 mm x 17.4 mm rectangular hole in the middle of the faceplate with a scalpel.

5.3. Place the double-row connector inside the rectangular hole of the faceplate until it is flushed with the faceplate surface without protrusion. If the connector does not fit into the rectangular hole of the faceplate, the hole can be slightly enlarged with a file. Since the connector holes are not symmetrical, insert the connector edge with the larger diameter holes into the faceplate.

NOTE: As a result, a female pin inserted into the opposite edge of the connector with the smaller diameter hole will snap and lock into place.

5.4. Use phenol to glue the connector and the faceplate together. Place the assembly in 60–80 °C water in a fume hood for 30 min to allow for glue hardening.

5.5. Drill a 1.3 mm hole at each corner of the faceplate and on each side of the faceplate halfway from the ends for a total of six holes.

NOTE: These holes will be used to suture the final skin receptacle at the implant site.

5.6. Cut a 15 mm length tube of knitted polyester graft to surround the assembly below the faceplate, making the assembly biocompatible. To fix the tube to the assembly, use a hypodermic needle to thread stainless steel wires through the wall at three equally spaced positions (each 3.8 mm apart) along its length.

5.7. Place equally spaced notches in each corner of the connector to anchor the wires against the assembly surface. Twist the ends of each wire with a pair of pliers to cinch the tube to the assembly to form a skirt.

5.8. Make a permanent mark on the polyester patch at one end of the receptacle.

NOTE: Use this mark for orientation to identify the rostral end of the receptacle during implant surgery. In the rostral to caudal direction, the following pin electrode assignment for each of the two rows (left side and right side) should be as follows: PCA EMG, TA-LCA EMG, empty hole, empty hole, RLN anode, RLN cathode, SLN anode, and SLN cathode.

6. External connection cable to recording pre-amplifier and stimulator

NOTE: A cable is used for making connections between the implanted skin receptacle and external equipment during nerve stimulation-EMG recording sessions (sections 8 and 10). It is composed of 12 insulated wires terminating with male pins to insert into female pins in the skin receptacle. This cable consists of two parts: an EMG recording plug and nerve stimulation wires. A recording plug is necessary to isolate low voltage EMG signals from higher voltage stimulus artifacts radiating from stimulus pins. For the same reason, two holes in each row of the skin receptacle are left unoccupied to separate recording pins from stimulation pins.

6.1. To make the EMG recording plug, use a male strip connector (same length and width, but one-half the height of a female connector). Cut it into two pieces, each containing only two holes. Affix the two pieces using phenol adhesive using the same approach to make the double-row connector in the skin receptacle (step 5.1). Take the four EMG recording wires in the cable and insert their terminal male pins into each of the four holes until they lock in place with the tips protruding beyond the strip edge.

6.2. Use bone cement to seal the top of the plug to insulate wire-pin junctions.

6.3. Use the remaining eight wires in the cable terminating in male pins to make individual

connections to the nerve stimulation cuffs via their female pins.

7. First implant surgery

7.1. Obtain a 1–2 year-old, 20–25 kg canine of either sex from a licensed farm. Acclimate the animal before aseptic implant surgery. Autoclave all equipment before surgery. Withhold food for 10–12 h before the surgery.

7.2. Prepare the animal for surgery.

7.2.1. Shave the animal's head and neck and clean the skin with alcohol and betadine scrub solution. Anesthetize the animal by intravenous injection of 2–4 mg/kg tiletamine and zolazepam combination, followed by 3% isoflurane in oxygen through intubation.

7.2.2. Place the animal on an operating table with a heating pad in supine position and surgically drape the animal. Monitor animal's heart rate, respiratory rate, body temperature, and oxygen saturation at least every 15 min throughout the surgery to ensure physiological stability at a moderate plane of anesthesia.

7.3. Make a midline neck incision from the thyroid notch to manubrium. Dissect the trachea free from the esophagus and expose the inferior border of the cricoid cartilage.

7.4. Position the stimulus cuff onto each of the bilateral SLNs and RLNs. Close the lips of each cuff using the enclosed sutures.

7.5. Make a cartilage window with a biopsy punch (4 mm in diameter) at the anterior surface of the thyroid cartilage on each side. Expose the lateral aspects of both TA-LCA muscle complexes. Insert the EMG recording electrodes into the TA-LCA muscle complexes using a 23 G needle by inserting the barb into the tip of the needle. Suture the electrode polyester patch onto cartilage.

7.6. Place the DBS electrode along with its companion hook-wire EMG recording electrode underneath the PCA muscle on each side. Use an endoscope to confirm that stimulation produces vocal fold abduction for each channel. Anchor the DBS electrodes to the cricoid cartilage by 4-0 nonabsorbable sutures.

7.7. Insert all the wire leads of the nerve stimulation-EMG recording electrodes into the receptacle via their female pins. Press the pins into holes with an insertion tool fashioned from a hemostat. Seal the inferior surface of the receptacle to insulate lead-pin junctions using bone cement.

7.7.1. After the cement hardens, place the receptacle at the rostral end of the midline incision through the skin and suture it to subcutaneous tissues via its polyester skirt. Attach the skin edge to the receptacle by sutures passing through the holes in the faceplate.

NOTE: One jaw of the hemostat has an end slit leading to a counter-sink hole. The lead wire can be positioned through the slit into the hole and the countersink placed against the head of the pin. The second jaw is placed on the opposite side of the receptacle. Squeezing the hemostat presses the pin into its respective receptacle hole.

7.8. Make an incision on the left neck to expose the trapezius muscle. Perform dissection to make a submuscular pocket for placement of the implantable pulse generator. Tunnel each DBS lead subcutaneously to the neck incision for insertion into the IPG.

7.9. Close all surgical wounds with sutures. Monitor the animal closely until full recovery from the surgery.

7.10. Provide postoperative analgesics (e.g., buprenorphine: 0.01–0.02 mg/kg) routinely for up to 48 h. Administer antibiotics (e.g., cefpodoxime: 10 mg/kg) orally to the animal for at least 3 days. House the animal singly thereafter for throughout the study, and restrict exercise for a period of 10 days to allow normal wound healing and stabilization of the implanted device.

NOTE: The skin receptacle should be cleaned daily with tissue-compatible antiseptic solution. In addition, dummy male pins should be inserted into the female pins of the skin receptacle routinely except during the EMG recording sessions. This maneuver will avoid the accumulation of debris in the receptacle, allow effective connections to be made with the external cable, and prevent infection.

8. Nerve stimulation-EMG recording sessions at baseline

NOTE: Perform these sessions 2x–3x after implant surgery (section 7) and before nerve transection surgery (section 9) to obtain baseline EMG signals when the bilateral RLNs are intact. Apply the following protocol during a standard nerve stimulation-EMG recording session (sections 8 and 10).

8.1. Withhold food before the procedure for 10–12 h. Anesthetize the animal with tiletamine and zolazepam combination (initial loading dose 2–4 mg/kg by intravenous injection, then maintain with 0.4 mg/kg per hour via an i.v. line). Place the animal on a heating pad in supine position and maintain the animal in a moderate plane of anesthesia. Monitor the animal's vitals during the procedure as described in step 7.2.

8.2. Insert a zero-degree rigid endoscope with an attached CCD video camera through a laryngoscope to visualize vocal fold motion at the level of the glottis.

8.3. Interface the external cable that connects to the lab stimulator and EMG preamplifiers to the skin receptacle via its plug and pins. Connect the outputs from the preamplifiers to a data acquisition device and/or an oscilloscope to display, record, and measure EMG signals.

8.4. Deliver stimuli (single square-wave pulses, 0.1–0.5 ms duration, 0.5–2.0 mA amplitude) to the left and right RLNs, respectively, to record evoked EMG responses from bilateral TA-LCA complexes and PCA muscles under each condition.

8.5. Deliver stimuli (single square-wave pulses, 0.1–0.5 ms duration, 0.5–2.0 mA amplitude) to the left and right SLNs, respectively, to record evoked EMG responses from bilateral TA-LCA complexes and PCA muscles under each condition.

8.6. Deliver CO_2 mixed with room air through the mouth of the animal to induce hypercapnia and increase the animal's respiratory effort. Limit exposure to 1 min, during which the maximum inspiratory motor unit recruitment will occur. Record spontaneous EMG activities of TA-LCA complexes and PCA muscles under this hypercapnic condition.

8.7. Monitor the animal until full recovery from anesthesia and return the animal to the facility.

9. Second surgery for nerve transection and anastomosis

9.1. Perform the second surgery 10–14 days after the first surgery. Withhold food for 10–12 h before surgery.

9.2. Anesthetize the animal, drape and monitor vitals intraoperatively using the technique described in step 7.2.

9.3. Remove the sutures and reopen the midline incision by blunt dissection whenever possible. Avoid damage to the previous implantation during the dissection. Expose the bilateral RLNs through dissection. Isolate, transect and anastomose each nerve with 7-0 monofilament, nonabsorbable sutures to induce bilateral laryngeal paralysis.

9.4. Irrigate the neck incision with sterile saline and gentamycin antibiotic. Close the muscular and subcutaneous tissues using 3-0 absorbable sutures. Close skin with 3-0 nonabsorbable monofilament sutures.

9.5. Closely monitor the animal until full recovery from surgery.

9.6. Provide analgesics (e.g., buprenorphine: 0.01–0.02 mg/kg) routinely for up to 48 h postoperatively. Give antibiotics (e.g., cefpodoxime: 10 mg/kg) orally to the animal for at least 3 days. Restrict the animal from exercise for a period of 10 days to allow normal wound healing.

10. Nerve stimulation-EMG recording sessions following bilateral RLN injuries

10.1. Perform these sessions 1x per week during the first 3 months, then biweekly thereafter. Follow the protocol described in section 8 for these sessions.

REPRESENTATIVE RESULTS:

Examples of the components are shown in **Figure 2**. From left to right in **Figure 2A** are the nerve stimulus cuff, TA-LCA recording electrode, PCA recording electrode, and skin interface receptacle, respectively. The relative size of these components can be appreciated. The skin receptacle (**Figure 2B**) has two rows of holes into which the female pins at the end of each coiled wire (**Figure 2D**) are inserted. They are inserted opposite the faceplate (arrow) during the implantation surgery. The receptacle has a polyester skirt (**Figure 2C**) attached to its connector sidewalls. This skirt is designed to anchor the receptacle in position by connective tissue infiltration. Each Teflon-coated stainless-steel EMG lead (**Figure 2E**) is deinsulated (5 mm) at the tip to form a hook-shaped electrode for muscle recording. The stimulation cuff has two electrodes threaded against the inner cuff wall. They are separated by a distance of 2 mm (**Figure 2F**) and form a "V" shape (**Figure 2G**) to ensure current delivery across the nerve.

[Insert figure 2 here]

Figure 3 shows the implanted skin receptacle and how the cable from external equipment is interfaced to the receptacles. It should be noted that dummy male pins (not shown) are inserted into the female pins of the receptacle to keep them free of debris between recording sessions.

[Insert figure 3 here]

Figure 4 shows an EMG recording from one of the baseline sessions with the RLNs intact.

[Insert figure 4 here]

In a recording from the PCA muscle (**Figure 4A**), RLN stimulation produces a stimulus artifact (arrow) followed by a large evoked EMG potential. The maximum RLN-evoked responses provide a good index of the overall magnitude of normal innervation as well as the level of reinnervation following subsequent neurorrhaphy, irrespective of motor unit type. This is true because the RLN contains nerve fibers of both inspiratory and reflex glottic closure (RGC) motor units. RLN stimulation recruits both types of units. Evoked EMG motor unit activity is rectified and integrated over a 20 ms time period to obtain a quantitative measure of muscle innervation.

In a recording from the TA-LCA muscle complex (**Figure 4B**), SLN stimulation produces a stimulus artifact (arrow). This artifact is followed by a short-latency monosynaptic muscle response (a) and longer-latency polysynaptic RGC response (b). The potential (a) is a direct response from the cricothyroid muscle, because this muscle is innervated by the nearby external branch of the SLN. Stray activation of this branch commonly occurs during nerve cuff stimulation of the internal branch to activate the RGC response. The cricothyroid potential is recorded by the TA-LCA electrode, as this muscle is located near the complex. Previous studies have shown that the cricothyroid potential evoked by internal branch stimulation can be selectively abolished by sectioning the external branch of the SLN (Zealear, unpublished

observations). The maximum SLN-evoked EMG responses reflect the magnitude of natural innervation of the TA-LCA complex through its RGC sensory-motor pathway. Prior to RLN neurorrhaphy, there is no RGC innervation of the PCA muscle, so no SLN potential should be detected from this muscle. Following nerve transection and repair, SLN-evoked potentials reflect the amount of correct RGC reinnervation of the TA-LCA complex and incorrect RGC reinnervation of the PCA muscle. RGC activity is quantified by rectification and integration over a 20 ms time period to capture the entire RGC waveform.

In (**Figure 4C**), bursts (arrows) of spontaneous EMG activity are recorded from the PCA muscle during normal inspirations. This inspiratory EMG activity increases over the course of CO_2 delivery, as shown in (**Figure 4D**) at a slower sweep speed. Spontaneous PCA EMG activity provides a good estimate of the magnitude of normal innervation of this muscle by its original inspiratory motoneurons. There is no inspiratory innervation of the TA-LCA complex, so no inspiratory potentials should be detected from these muscles. This is because only inspiratory motor units are involved in abducting the vocal fold at maximal inspiratory effort in the anesthetized animal. Following nerve transection and repair, spontaneous inspiratory potentials reflect the magnitude of correct reinnervation of the PCA muscle and magnitude of incorrect reinnervation of the TA-LCA complex. Recordings of inspiratory EMG activity are amplified, rectified, and integrated over an 8 s time period.

FIGURE AND TABLE LEGENDS:

Figure 1: Anatomy of the larynx. The components of this implantable system are also displayed. SLN = superior laryngeal nerve; RLN = recurrent laryngeal nerve; PCA = posterior cricoarytenoid muscle; TA-LCA = thyroarytenoid–lateral cricoarytenoid muscle complex; DBS = deep brain stimulation. This figure has been reproduced with permission from Wiley [27].

Figure 2: Components of the implant system. (**A**) From left to right is the nerve stimulus cuff, TA-LCA recording electrode, PCA recording electrode, and skin interface receptacle, respectively. (**B**) The skin receptacle showing two rows of holes. (**C**) The receptacle showing a polyester skirt attached to its connector sidewalls. (**D**) Coiled wire containing female pins to be inserted into B. (**E**) Teflon-coated stainless-steel EMG lead is deinsulated (5 mm) at the tip to form a hook-shaped electrode for muscle recording. (**F**) The stimulation cuff has two electrodes threaded against the inner cuff wall, which are separated by 2 mm. (**G**) “V” shape formation of electrodes to ensure current delivery across the nerve. This figure has been modified with permission[27].

Figure 3: Skin receptacle and interface cable. (**A**) The implanted skin receptacle on the anterior neck without dummy male pins is shown. (**B**) The image depicts how the stimulus pins and EMG recording plug (arrow) of the cable from external equipment is interfaced to the receptacle during a nerve stimulation-EMG recording session. This figure has been modified with permission[27].

Figure 4: EMG recordings from laryngeal muscles with normal innervation. (**A**) Example

recording from the PCA muscle where RLN stimulation produces a stimulus artifact (arrow) followed by a large evoked EMG potential. (**B**) Example recording of the TA-LCA muscle complex, in which SLN stimulation produces a stimulus artifact (arrow). Represented here is (**a**) a short latency monosynaptic muscle response and (**b**) a longer latency polysynaptic RGC response. (**C**) Bursts (arrows) of spontaneous EMG activity recorded from the PCA muscle during normal inspirations. (**D**) Increase of inspiratory EMG activity over the course of CO_2 delivery. This figure has been modified with permission[27].

Table 1: Troubleshooting guide.

DISCUSSION:

This paper describes the steps required in the manufacturing of a novel, economical, and implantable system for stimulation of laryngeal nerves and recording of EMG responses from laryngeal muscles over a long term. The protocol is uncomplicated and can produce an implant that is compact enough to be utilized in an animal as small as a rat. There are several critical steps that should be emphasized. First, lead wires should be coiled carefully and uniformly to prevent lead de-insulation, kinking or breakage. If a coiling machine is not available, prefabricated coiled leads can be obtained commercially. Second, the strategy of inserting lead wires into a silicone tube to form a "V" that straddles the nerve is critical to promote current delivery through the nerve inside the cuff. If both leads are placed on the same side of the tube, shunting of current between electrodes can occur. It is also important that the leads are positioned against the tube inner wall to avoid the possibility of slice injury to the nerve.

Third, during the implantation surgery, laryngeal nerves should be dissected carefully to prevent damage. At the later stage of implantation, when inserting pins into the receptacle, force should be applied to the pin in alignment to its hole to prevent sudden bending of the head of the pin. Subsequently, bone cement should be distributed thoroughly on the receptacle bottom for complete insulation and prevention of crosstalk between channels. Finally, prevention of infection is critical to ensure integrity of the implant system over time. It can be achieved by a combination of several maneuvers: addition of a skirt to the receptacle, administration of antibiotics, daily cleaning of the wound and receptacle with tissue-compatible antiseptic solution, and placement of dummy male pins into the female pins of the receptacle to keep them clean of debris between sessions.

The protocol has been proven successful in this dog laryngeal model. However, some modifications or alternative strategies may be considered for other applications. For example, the deinsulated sensing tips of the PCA and TA-LCA EMG electrodes are anchored in the muscles by an external means-either the polyester graft or the DBS electrode. In an application in which external anchoring is not needed or performed, the barb of the electrode alone can serve as the anchor. In such a case, Teflon-coated, stainless steel, monofilament wire may be preferable to multifilament wire in view of its greater tensile strength, providing a barb that is more stable in tissue. However, it should be noted that multifilament wires may be less prone to breakage. An alternative strategy to fabrication and assembly of the skin receptacle is to 3D-

print using biocompatible polymers (e.g., MED610 by Stratasys). This may simplify the manufacturing process.

Following implantation surgery and recovery of the animal, physiological sessions are conducted with the RLNs still intact to obtain baseline data. During a session, absence of EMG signals from a laryngeal muscle may occur following RLN stimulation. In order to troubleshoot the cause (**Table 1**), it should first be determined whether vocal fold movement is present. If it is present, this means that the nerve is effectively activated by the cuff, but there is a problem with the EMG lead. In this situation, users should further look at the EMG stimulus artifact. If the EMG artifact is absent, there is likely a discontinuity in the EMG input to the preamplifier. Sixty-cycle noise will also be present and large in amplitude. If the artifact is large, shunting from a stimulus pin to the recording pin may be responsible for saturating the channel preamplifier and obliterating the EMG response. If the artifact is normal, then the EMG lead has likely dislocated from the muscle and cannot detect its activity. On the other hand, if the vocal fold movement is absent, then the nerve is not being activated. If the artifact is absent, there may be a discontinuity in the stimulation circuit, preventing nerve activation. If the artifact appears normal, the nerve may have been injured during implant surgery or the cuff may have migrated off the nerve. A similar strategy can be applied to troubleshoot the cause of absent EMG signals during SLN stimulation.

[Insert **Table 1** here]

It should be mentioned that there are two minor limitations in the current application of this technology. First, sudden bending of the female pin during insertion into the receptacle has occurred in several instances. Fortunately, the pins can be straightened and inserted into their holes successfully. If pin damage is irreparable, the lead and its entire component need to be replaced. Therefore, backup components should be readily available before surgery. Second, the time required to complete the surgical implantation is long (~10 h). The long duration partially reflects the large number of stimulation and recoding components required for this study: four nerves, four muscles, a receptacle, and an IPG. If fewer components are required using this technology, the implantation time should be significantly reduced (e.g., the rat tongue model[28]).

This technological approach introduces several features that have advantage over existing methods. The coiling of lead wires is the most novel and important feature of this system. Coiled leads are not commonly available for non-commercial animal experimentation despite the many benefits they provide. A coiled lead can be expanded to the desired length during implantation. Further, it will stretch in the awake, moving animal to prevent dislocation of the electrode tip or wire breakage after implantation. This feature ensures longevity of the implant and stable nerve stimulation and muscle recording over the long term. Furthermore, adding a tissue compatible skirt around the receptacle prevents exposure of the wound to this foreign body and promotes normal fibrosis and wound healing in the absence of infection. Previous studies without this skirt resulted in early infection and premature termination of the experiment. Lastly, this implant system is compact and multi-channeled, allowing effective data

acquisition from numerous neuromuscular structures in animal models of various size.

This technical approach has been adapted and successfully translated to a rat model. This study was designed to investigate the effect of electrical conditioning in preventing tongue muscle atrophy and dysfunction in the aging rat. The hypoglossal nerves were implanted with the cuff electrodes for conditioning and the tongue implanted with the EMG recording electrodes[28]. This technology can also be utilized in other research applications. As an extension of the current protocol in the canine larynx, the effects of electrical conditioning on promoting selective reinnervation are currently being studied in rabbit facial muscles. This study may provide a foundation for the prevention of facial synkinesis in patients with Bell's palsy, a common and debilitating medical condition. A final potential use of this technology is to stimulate and record from awake, freely moving animals. At present, such data has been obtained via external cable from awake, unrestrained rats[28]. In the future, this economical system may also be combined with remote recording-stimulation technology (e.g., telemetry) to activate or probe neuromuscular systems wirelessly.

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DISCLOSURES:

Dr. David Zealear is marketing this implantable, nerve stimulation-EMG technology for a variety of neuromuscular systems and animal models.

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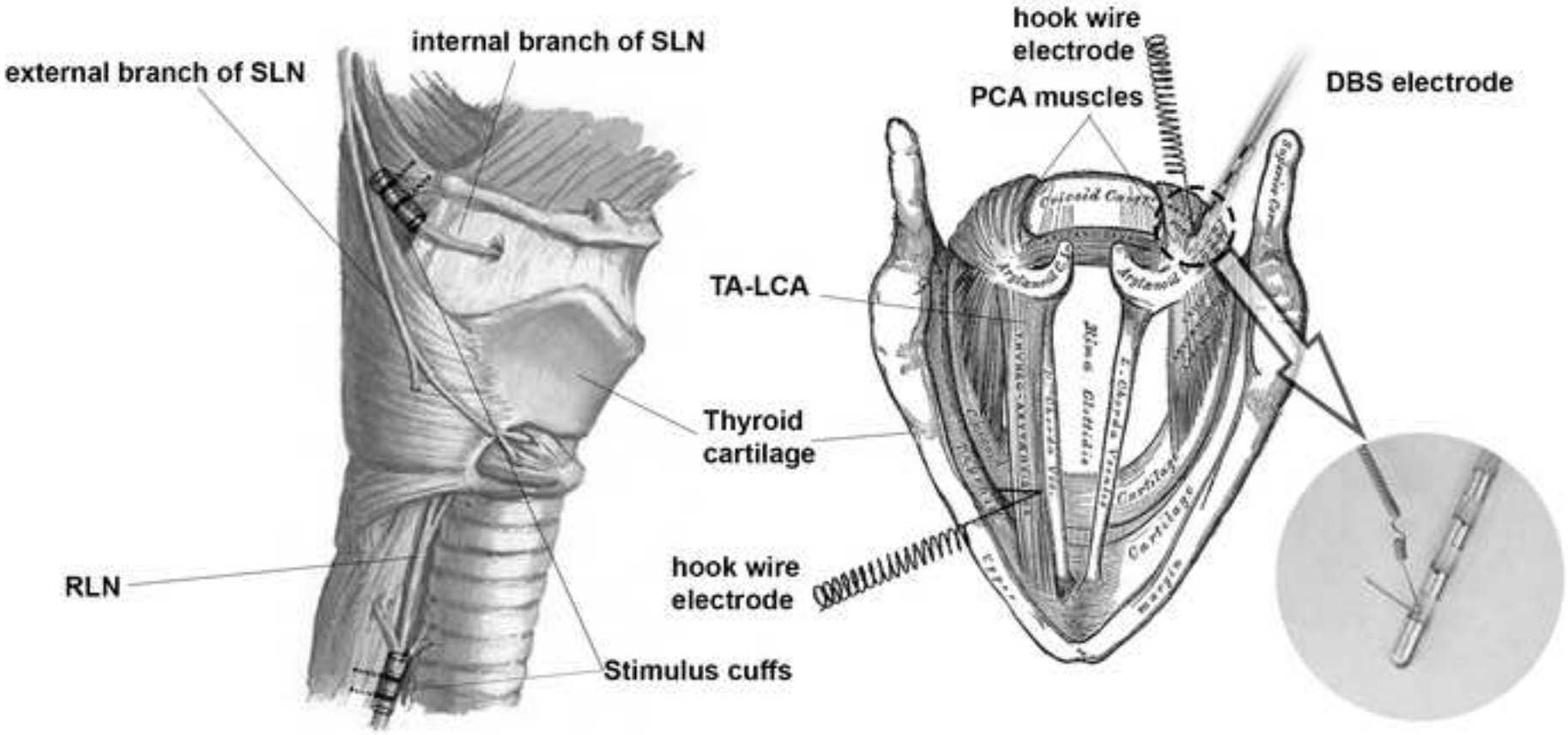

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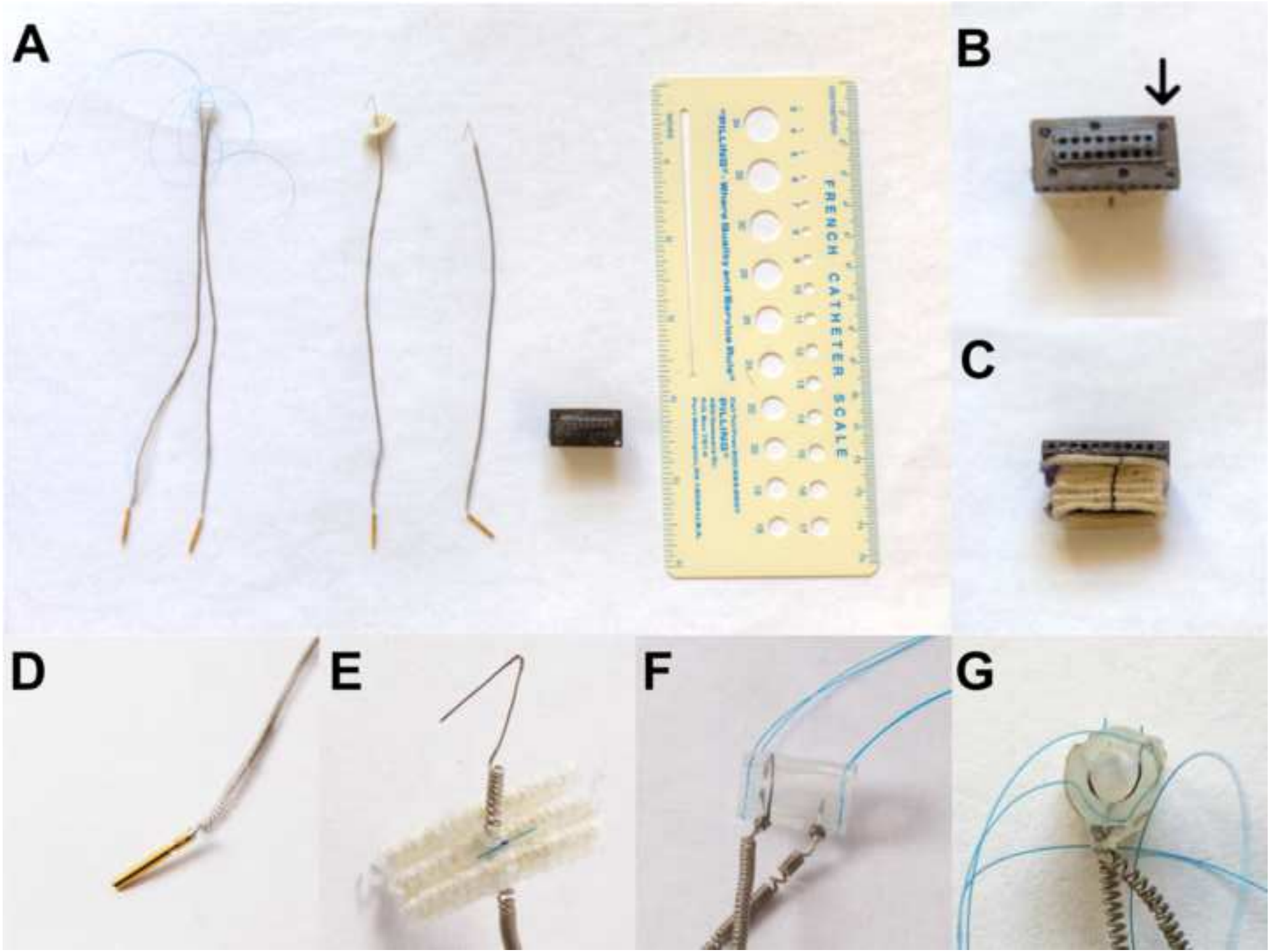

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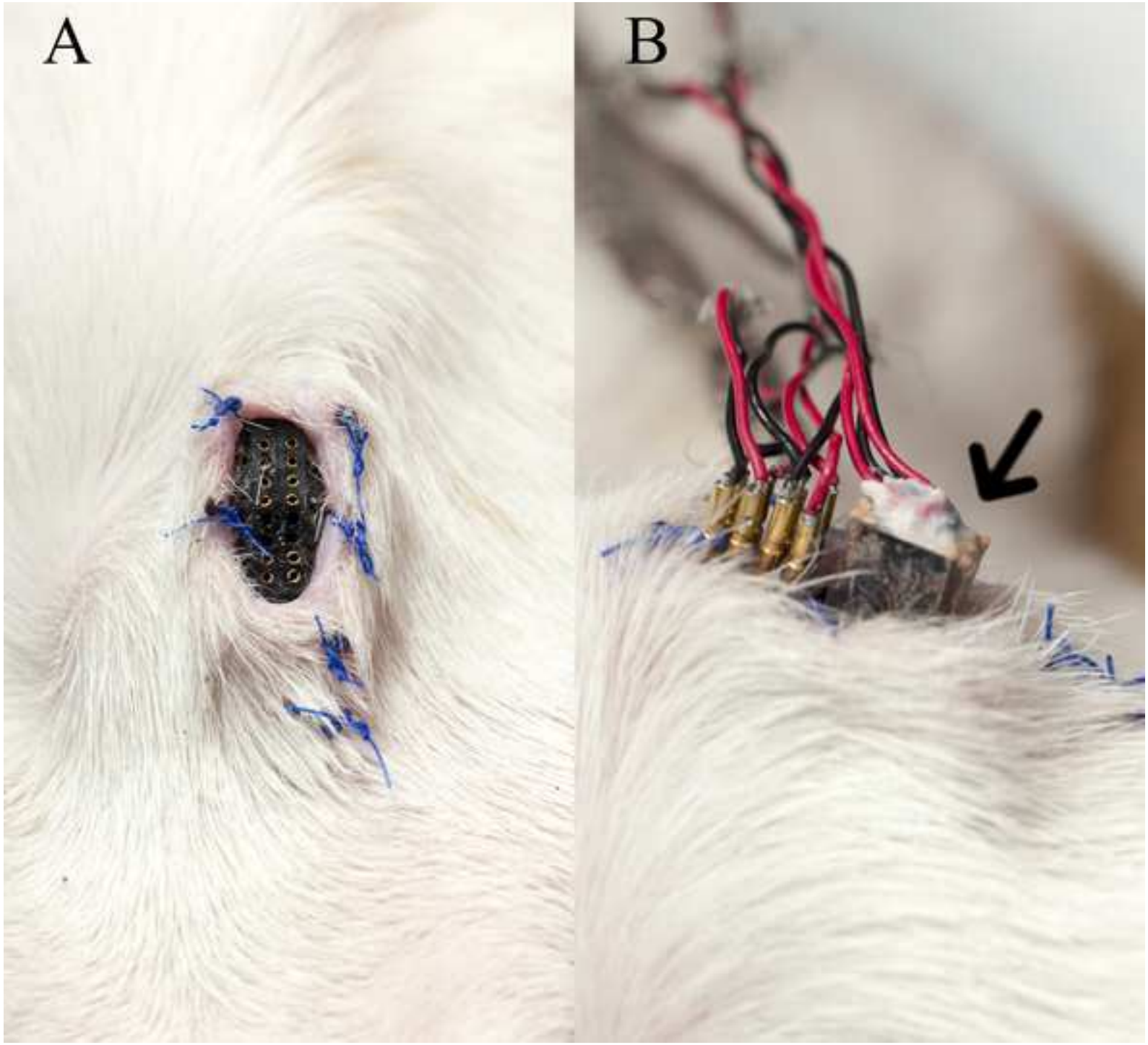

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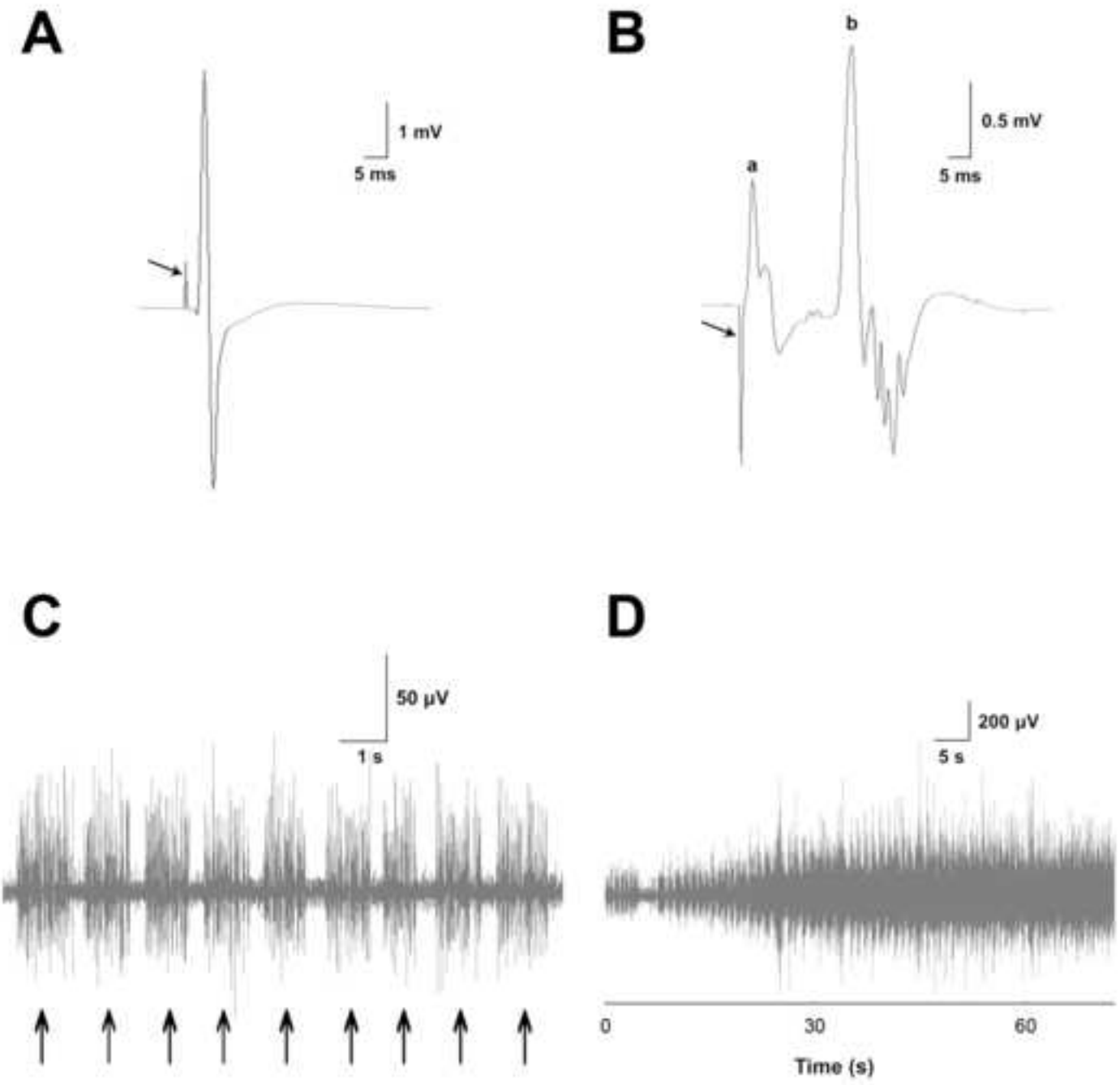

Table

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Stimulated nerve	Target muscle(s)	Ipsilateral vocal fold movement
RLN	PCA and/or TA-LCA	Yes
		No
SLN	TA-LCA	Yes
		No

Stimulus artifact	Causes
Absent (60-cycle noise present)	Discontinuity in the EMG input to preamplifier (e.g. lead, pin, cable);
Large	Cross-talk between stim and recording pins at the receptacle
Normal	Dislocation of EMG electrode
Absent	Discontinuity in stimulation circuit
Normal	1. RLN injury; 2. Cuff dislocation
Absent (60-cycle noise present)	Discontinuity in the EMG input to preamplifier (e.g. lead, pin, cable);
Large	Cross-talk between stim and recording pins at the receptacle
Normal	Dislocation of EMG electrode
Absent	Discontinuity in stimulation circuit
Normal	1. SLN or RLN injury; 2. Cuff dislocation

Name of Material/ Equipment	Company
20 G x 1" Gauge hypodermic needle	BD
23 G x 1" Gauge hypodermic needle	BD
25 G x 1" Gauge hypodermic needle	BD
3-0 absorbable sutures, COATED VICRYL	Ethicon
3-0 monofilament, nonabsorbable sutures, Prolene	Ethicon
4-0 monofilament, nonabsorbable sutures, Prolene	Ethicon
6-0 monofilament, nonabsorbable taper needle suture, Prolene	Ethicon
7-0 monofilament, nonabsorbable sutures, Prolene	Ethicon
Adhesive silicone solvent-Hexamethydisiloxane 98%	ACROS
Bone cement	Zimmer
Buprenorphine (Buprenex, ampules of 1ml)	Reckitt Benckiser Healthcare (UK) Ltd
CCD video camera attached to the endoscope	Sony
Cefpodoxime (Simplicef 100mg tablets)	Zoetis
Data acquisition device , PowerLab 16/35	ADInstruments, Inc
Deep-brain stimulation (DBS) electrodes	Abbott
Digital oscilloscope	Tektronix
Implantable pulse generator (IPG), Infinity	Abbott
Knitted polyester graft	Meadox Medical Inc
Medical Grade Polyethylene Micro Tubing	Amazon.com
Metal female pin	Allied Electronics & Automation
Metal male pin	CDM electronics
Prefabricated coiled leads	Medical innovations Inc.
Silastic Laboratory Tubing	Cole-Parmer
Silastic Medical Adhesive Silicone	Dow corning
Stainless steel monofilament wire	The Harris Products Group
Sterile Disposable Biopsy Punch (4mm)	Sklar Instruments
Strip connector	CDM electronics
Teflon-coated multi-filament stainless steel wire	Medwire
Tiletamine and Zolazepam combination, Telazol - 5mL	Zoetis
Tissue-compatible antiseptic solution, Nolvasan - 1 Gal.	Zoetis
Zero-degree rigid endoscope	Karl Storz

Catalog Number	Comments/Description
305175	
305145	
305125	
J219H	
8684G	
8871H	
8805	
M8735	
code 194790100	for dilution of modical adhesive silicone
1102-16	20g powder 10ml liquid
12496-0757-1	
MCC500MD	
5228	
5761-E	
6172ANS	
DPO71304SX	
6660ANS	
92220	20mm in diameter
BB31695-PE/13-10	OD 0.156", ID 0.094"
220-S02-100	
220-p02-1	
2415569	OD 0.125", ID 0.062"
Type A, 2 oz	
type 316	0.008" (coated), 0.005" (bare)
96-1146	
2.6 x 11.6 x 101.5 mm	single row, round, through hole
Part 316, ss7/44T	
004866	
540561	
8712AA	

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3. *Please provide at least 6 keywords or phrases.*

   Done.

4. *Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Teflon, Telazole, Prolene, Karl Storz, Germany, Sony Corp., Tokyo, 319 Japan, PowerLab 16/35 device (ADInstruments, Inc., Colorado Springs, Colorado), etc.*

   Done.

5. *Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 60-63, 65-72, 80-84, 265-266, 271-274, 276-279, 300-303, 318-320, 449-450, 452-457, 467-471, 473-475, 477-479, 482-485.*

These sections identified and the entire manuscript as well have been completely rewritten. New information has been introduced throughout the manuscript and previously published information omitted.

6. *Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"*

   Done.

7. *Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."*

   The protocol has been rewritten as required.

8. *The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.*

   Done.

9. *The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.*

   We have limited the actions to 4 sentences per step as required.

10. *Please ensure you answer the "how" question, i.e., how is the step performed?*

    Done.

11. *1. Please use complete sentences in imperative tense.*

    Done.

12. *2.1.4: Please make substeps if describing action. We cannot have paragraphs of text in the protocol section.*

    Done. Refer to step 1.4 in the revised manuscript.

13. *Lines 151-153, 234-237, 307-311: Please number all the steps or convert to a note.*

    Any non-actionable information has been either retained as a note or omitted throughout the protocol.

14. *3: Will the live animal be available during the day of filming?*

    Yes, an animal will be available for filming but it will be a cadaver.

15. *3: Any age or sex-specific bias? Do you check the depth of the anesthesia? Please include throughout how do you perform each step.*

    The animal model is a 1 to 2-year old, 20-25 kg canine of either sex. We check the depth of anesthesia. We have provided a detailed description for each step.

16. *There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*

    They have been highlighted in the manuscript.

17. *Please expand the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.*

    Done.

18. *Please include a one-liner title for each figure legend.*

    Done.

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    Done. Copyright permission has been obtained in a form of letter and will be uploaded at the time of resubmission.

20. *Figure 4: Please convert sec to s. Please include a single space between the number and unit e.g. 1 mV.*

    Done.

21. *As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:*
    a) Critical steps within the protocol.
    b) Any modifications and troubleshooting of the technique
    c) Any limitations of the technique
    d) The significance with respect to existing methods
    e) Any future applications of the technique

    We have completely rewritten this section as a 6-paragraph discussion and included the type of information that is appropriate for this journal.

22. *Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in a .xlsx file. Please sort the table in the alphabetical order.*

    Done.

Reviewer #1:

1. *Manuscript Summary:*
   Overall, the electrode assembly presented in this manuscript appears to be an excellent approach for the manufacture of electrodes for chronic (long term) stimulation of nerves and recording of EMG potentials from the awake animal. However, it is very difficult to understand the procedures as written, and revised or additional figures would enhance the quality of the manuscript.

   Although I present detailed questions and comments below, the best solution for modifying this proposal is to generate drawings of the steps made for the creation and assembly of the stimulation and recording electrodes. The photographs of the finished products add very little to the clarity of the procedures to be followed as described in the text.

   We appreciate the difficulty this reviewer had in following our detailed description of process in manufacturing and utilization of the implant with limited visual aid. However, this is a journal focusing on the presentation of steps in the protocol using video format. All the important steps will be videotaped for the final publication. The authors are unaware whether additional review will take place after the video phase has been completed.

2. *Major concerns:*
   Abstract
   Here and elsewhere in the text you refer to the EMG recording only in the context of nerve stimulation. However, these electrodes could also be used in the absence of nerve stimulation, and it would add to the significance of these techniques to mention that these electrodes could be used in a much wider context of recording EMGs in the awake, behaving animal. You add this at the end of your discussion, but you could mention it here as well.

This information has been added to the abstract and embellished in the discussion. Refer to lines 45-46, and 569-570

3. *Line 32. Here you mention the SLN, but I think it should be made clear that you are referring to the internal branch of the SLN and not the external branch. In the discussion you could mention that the techniques could be used for stimulation of the cricothyroid muscle by applying a nerve cuff to the ESLN.*

   Change has been made.

4. *Introduction*
   Line 63. Here and elsewhere it would be good to mention that the RLN stimulation would activate the interarytenoid muscles along with the "TA-LCA complex" for glottal closure.

   Change has been made.

5. *Lines 117-118. You mention the coiling device. It would be good to describe this device in more detail or else provide a reference to a description of the device if it has been published elsewhere. You should also mention in more detail that the coil allows for movement of the stimulation wires in a way that reduces the chance that bending of the wires would eventually lead to breakage. You do mention this somewhat later in the discussion, but adding it here as well would be good.*

   This is a “how-to-do-it” journal and the protocol has to comprise only actionable steps with minimal non-actionable notes. The video will show the coiling device and the actual coiling process of a lead. There is no previous publication describing the device. The importance of coiling leads for chronic study in awake animals is thoroughly detailed in the discussion section (Lines 546-550).

6. *Line 126. You say the "…needle is pierced through the tubing…" You should say 'wall of the tubing' to make it clear that the needle is not pushed into the tubing from the end of the tube.*

   Change has been made.

7. *Line 127. You say "The 10mm end"; this was not previously specified. Also, it is not clear what you mean by "backfilled into the tip". By adding drawings it would greatly ease the description of these procedures.*

   Change has been made. These steps will be videotaped.

8. *Line 136. The "V" shape straddling the nerve is unclear. A better description or drawing would be much better.*

We have provided a new subfigure (Figure 2G) of the “V” shape for orientation. These steps will also be videotaped.

9. *Line 139. The S-shaped slit issue is unclear. Drawing?*

   It will be videotaped.

10. *Line 162. What is the implantable pulse generator (IPG)? Please describe.*

    A brief description has been provided. See lines 171-174.

11. *Line 163. Deep brain stimulation electrode. What is this and why is it implanted into a submuscular pocket? Why is it not implanted into the brain? What is a submuscular pocket?*

    This section has been rewritten for clarification. A brief description of the DBS electrode in the context of PCA muscle stimulation has been provided.

12. *Line 178-179. I have trouble understanding the mesh and how it is used to anchor the electrode lead to the thyroid cartilage. A drawing could clarify this.*

    It will be videotaped.

13. *Lines 180-182. A drawing showing an illustratiom of this configuration would be a big help. I don't think I could do this without a better description or else a drawing.*

    It will be videotaped.

14. *Line 217. What is the "aorta patch"? More explanation is necessary.*

    It is described as “knitted polyester graft” now. The receptacle skirt made from this material is shown in Figure 2. Refer to section 5.6.

15. *Line 245. Please reword "…to distance recording pins from…"*

    It has been moved to a note to provide a better explanation. It has been reworded as “… to separate recording pins from stimulation pins.” (lines 248-249).

16. *Line 246. What does this mean: "pinched into a hole"*

    A detail description of the insertion and locking of a pin in its hole with a modified hemostat has been provided, line 301-304: “One jaw of the hemostat has an end slit leading to a counter-sink hole. The lead wire can be positioned through the slit into the hole and the counter-sink placed against the head of the pin. The second jaw is placed on the opposite side of the receptacle. Squeezing the hemostat presses the pin into its respective receptacle hole.”

17. *Line 276. Again, the DBS electrode is a mystery to me. Is this electrode used to stimulate the PCA muscle or just record the EMG from the PCA?*

    The DBS electrode interfaces with the IPG and is used strictly to stimulate the PCA muscle. A separate EMG electrode is attached to the DBS electrode to record EMG potentials.

18. *Line 288. Again the "implantable pulse generator" is unclear.*

    It has been explained, see lines 171-174

19. *Line 332. Something is missing here: "during give"*

    This section has been moved, rewritten and typo corrected.

20. *Line 375-6. It would be good to mention that the cricothyroid muscle is not paralyzed; hence some laryngeal function would remain intact.*

    In general, the terms of bilateral vocal fold paralysis (BVFP) or bilateral laryngeal paralysis, indicate only injury to the RLNs. A combined injury of both RLNs and both SLNs is much less common and is usually referred to as a combined injury. Under this assumption, it may be unnecessary to specify that the CT muscle is not paralyzed.

Reviewer #2:

1. *Manuscript Summary:*
   This effort has been previously published in the article titled "An Implantable system for in vivo chronic electromyographic study in the larynx", Muscle & Nerve, 2017. The text and figures are essentially the same. If this is allowed, then I think it would be useful to see these techniques in video form, and the project should be presented in jove.

   This manuscript has been completely rewritten. New information has been introduced throughout the paper and a large portion of previously published information omitted. This paper focuses on how to manufacture and implant this system in video format, in contrast to our previous publication, which focused only on a description of the technology. A video will provide a useful supplement in step-by-step fashion for readers who want to reproduce this technology in their own research fields.

2. *Major Concerns:*
   The authors claim that there are no EMG systems available that enable periodic EMG recordings in a non-invasive manner, but there are published methods for implantable connection ports and several commercial options for wireless EMG telemetry systems that should be recognized.

We have retracted the statement that there are no EMG systems available for periodic EMG recordings in a non-invasive manner. In addition, previous studies using implantable neuromuscular stimulation/recording technology are now cited. Some of these citations use wireless transmission.

3. *The manuscript heavily cites the authors' prior work. However in many locations there are more appropriate sources that should be cited instead of, or at least in addition to, the authors work.*

   We have identified pertinent literature of other authors and added these papers to our reference list.

4. *There is inadequate cross-referencing between the products listed in appendix and those mentioned in the text. Are the items listed in the appendix in the order of appearance in the text? There seems to be a lack of organization. Also, please provide details about the implanted stimulator (IPG). Please spell-out manufacturer names, and do not list amazon.com as a manufacturer unless they actually fabricated the medical tubing listed.*

   It is a requirement of this journal that all materials appear in generic terms in the text. The materials are further delineated commercially in the table of materials. The table is organized alphabetically according to the name of the material. The details of the IPG and DBS leads have been provided. In most cases, the source of material is the manufacturer. However, the distributer and catalog number are provided when the manufacturer is unknown. The major concern is that the reader can acquire these materials without difficulty.

5. *A large portion of the Methods description is dedicated to the fabrication of the Skin Interface Receptacle (section 2.5). However, the authors should acknowledge that this piece could be easily and inexpensively printed in biocompatible polymer (like MED610 by Stratasys). It would greatly simplify the entire process and provide a way of shielding/encasing the parts that are toxic to tissue, obviating the need for the synthetic aorta patch.*

   This is an excellent suggestion and is now presented in the discussion section as an alternative strategy to the current approach (lines 512-514).

6. *Please describe the pre-operative anesthetic regimen, or was the I.V. line placed in a non-sedated animal? Were heating pads used? Was supplemental oxygen provided during nerve stimulation and EMG recordings.*

   The paper has incorporated these suggestions. Specifically, the preoperative anesthesia is administered by intravenous injection of Telazol (2-4 mg/kg) in both aseptic surgery and nerve stimulation-EMG recording sessions. Anesthesia is maintained by isoflurane during surgery and by IV administration of Telazol during the sessions. A heating pad is used in both procedures. Supplemental oxygen is generally not required during sessions but is available.

7. *For sections 3.10 and 5.6, what are the post-operative analgesics and antibiotics?*

   Information has been added to the text and the table of materials.

8. *Please provide stimulation parameter details in section 4.4, 4.5. and 6. Include pulse train frequency, pulse width, amplitude, shape, etc.*

   This information has been added to the protocol (steps 8.4, 8.5 and 10)

9. *The Discussion dedicates much of the text to ways of avoiding infection, but the implant was reported to have been infection-free. How were these multiple risks identified if infection didn't occur?*

   The discussion has undergone a major revision and expansion with a reduction in the text describing infection issues, since this information was presented in our previous paper. This protocol represents a refinement in the implant technology that is evolved in our lab over many years and animal models, including rat, cat, canine, and monkey.

10. *Minor Concerns:*
    If the midline incision heals for 10-14 days or longer after the initial surgery before being re-opened to perform the second surgery (Section 5.1), didn't it require more than simply removing the sutures to open the neck? (Section 5.3). In my experience with dog neck incisions of this nature, the necks are healed adequately for suture removal (without the incision opening) in about 10 days.

    We revised the text to indicate that a blunt dissection is required.

11. *Are the dummy pins held within a single inserted piece? If so, please show example.*

    No, the pins are inserted individually into female pins and do remain in place. However, one could use a plug to protect the receptacle if desired.

12. *Correct instances where silicon should be silicone.*

    Done.

13. *Correct instances where simulation should be stimulation*

    Done.

14. *Correct the instance where operation should be operating*

    Done.

15. *Correct the sentence that states "assembly format will allow facility in the pinhole"*

It has been revised to “This double-row assembly format will provide convenience in the assignment of pinholes for left side versus right side electrodes”.

16. *Correct the sentence that states "complex during give a good index"*

    Done.

17. *Some product sources/models are listed in the main text and in the appendix, whereas others (like the synthetic aorta patch) are only described in the appendix. Please list all material sources or state their appendix location at the start of the Methods.*

    Each material mentioned in the text is now listed in the table of materials.

18. *What size biopsy punch was used?*

    It is 4mm in diameter. Information has been added in the text and the table of materials.

19. *Based on the implanted hardware photo, the skin did not appear clipped of fur. Wouldn't this help avoid infection (in addition to the daily cleaning mentioned)?*

    We only shaved around the receptacle when the hair interfered with making connections. It presented no risk of infection.

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