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TITLE: Use of GDNF-Releasing Nanofiber Nerve Guide Conduits for the Repair of Conus Medullaris/Cauda Equina Injury in the Nonhuman Primate

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<b>14. ABSTRACT</b> The present study evaluated a surgical approach for repair of conus medullaris/cauda equina injury in rhesus macaques using a biodegradable bridging graft that releasing the trophic factor, GDNF. All subjects have been entered into the study. All subjects have also undergone pre-surgical testing, including magnetic resonance imaging, pain behavioral testing, urodynamic recordings, electromyography of external anal sphincter, and locomotor testing. All subjects have also undergone surgery with 5 subjects in each group undergoing either 1) unilateral L6-S3 ventral root avulsion (VRA) injury, 2) L6-S3 VRA injury followed by replantation into the spinal cord using a peripheral nerve bridging graft, 3) L6-S3 VRA injury followed by root replantation into the spinal cord using a GDNF-releasing nerve guide channel as bridging graft, or 4) L6-S3 VRA injury followed by root replantation into the spinal cord using an empty (control) nerve guide channel as bridging graft. The surgery was tolerated well and subjects have undergone additional, longitudinal functional assessments using locomotor training, urodynamic recordings, electromyography of the pelvic floor, and pain behavioral testing. At 18 months after the surgery, a final set of imaging and functional assessments were obtained, following by termination of the study and harvesting of spinal cord and nerve root tissues for anatomical studies. Collected materials and data are presently undergoing additional analysis and are being prepared for manuscript submissions. To summarize, subjects with either peripheral nerve bridging grafts or GDNF-releasing nerve guide channels have shown that axonal regeneration by myelinated fibers are capable of entering the graft and reinnervate the periphery.					
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## INTRODUCTION

Cauda equina and conus medullaris forms of spinal cord injury commonly results in paralysis, sensory deficits, pain, and autonomic impairments, including bladder, bowel, and sexual dysfunction (Hoang and Havton, 2006; Havton and Carlstedt, 2009; Dobkin and Havton, 2012). The present translational study investigated the feasibility of a new neural repair strategy in rhesus macaques using a GDNF-releasing nerve guidance channel as a bridging graft between the spinal cord and ventral roots that had been separated from the surface of the spinal cord by a traumatic avulsion injury. The experimental studies aimed to repair avulsed lumbosacral ventral roots by reconnecting them to the spinal cord via a bridging nerve guidance channel. For control purposes, the investigations also included the use of a guidance channel without trophic factor release and a peripheral nerve graft to bridge the tissue gap. A comprehensive set of behavioral, electro-diagnostic, imaging, and morphological study protocols were developed to provide comprehensive and detailed outcome measures. The research protocols and data collected from these translational research studies will also be available to the general research community for non-human primate studies and serve as a guide for the planning of future clinical studies on neural repair after cauda equina/conus medullaris injuries. The present report summarizes major accomplishments, including descriptions of surgical models and methodology for new outcome assessments, over the course of this 3-year (plus 1-year no-cost extension) collaborative and multi-investigator project.

## BODY

Our studies have been going well and have made good progress. At the beginning of the project period, Dr. Havton had just moved from UCLA to UC Irvine, where he had accepted a position as Professor. At the end of the project period, Dr. Havton was recruited back to UCLA, where is now serving as Professor in the Departments of Neurology and Neurobiology in the David Geffen School of Medicine at UCLA. In this collaborative partnership project, all three of the participating PIs (Leif Havton at UC Irvine/UCLA, Ahmet Höke at Johns Hopkins University, and Kari Christe at the California National Primate Research Center (CNPRC, at UC Davis) have interacted frequently and on a regular basis. In particular, Dr. Havton has visited the CNPRC about 1-2 times per month throughout the study period, especially during times of the development and implementation of surgical procedures and physiologic data collection. At those visits, Dr. Havton and Dr. Christe meet in person to review progress and any issues related to the study subjects. Dr. Havton also has met with Dr. Höke in person, at least 2-3 times per year and at those visits also reviewed and discussed progress. In addition, the PIs and members of the research team have communicated extensively by email and phone to promote project progress and for any needed experimental trouble shooting.

This project included the development of new research tools, including the surgical model of lumbosacral ventral root injury and repair in rhesus macaques (*Macaca mulatta*) using peripheral nerve grafts and glial derived neurotrophic factor (GDNF)-releasing nerve guidance channels as bridging grafts. The studies also developed protocols for new outcome measures, urodynamic recordings, electromyography (EMG) of the guarding reflex of the external anal sphincter, and spine imaging methods using a combination of radiographs and magnetic resonance imaging (MRI), which were developed for pre-surgical planning of conus

medullaris/cauda equina procedures in rhesus macaques. Below is a methodological description of step-by-step approaches for these new tools.

### **3.1 Lumbosacral ventral root avulsion injury and repair using peripheral nerve and biodegradable nerve guidance channels as bridging grafts.**

The follow section describes the methods and protocols for laminectomy, ventral root identification and avulsion injury as well as ventral root repair using either a peripheral nerve graft or a nerve guidance channel strategy. It also includes the new imaging strategy using radiographs and MRI studies to determine the surgical anatomy of the lumbosacral spinal cord and cauda equina.

1. A pre-operative magnetic resonance imaging (MRI) study of the thoracolumbar spine is performed under ketamine sedation (approximately 10 mg/kg IM) to identify the conus medullaris and determine its anatomical relationship to the vertebral column. This imaging study is useful as there is extensive inter-individual variation with regards to the location of the caudal end of the spinal cord and its relationship to the vertebral column. This imaging study is preferentially performed at least a few days pre-operatively to allow research subjects to recover from the ketamine anesthesia before the scheduled spinal surgery and to provide the research team with ample time for image evaluation and surgical planning.
2. On day of surgery, each subject is sedated (approximately ketamine 10 mg/kg IM) and an intravenous catheter is placed. An X-ray series of the spine is obtained to visualize the thoracolumbar spine anatomy. This is an important step to determine the potential presence of an anatomical variant, approximately 20% of subjects have a set of supernumerary ribs, which are attached to the L1 vertebra when present (Ohlsson et al., 2017). Consideration of a normal vs variant spine anatomy is critical for the identification and correct nomenclature of the nerve roots during subsequent surgical procedures. Next, the animal is intubated and then placed in prone position on the surgical table, 1-2% isoflurane (Abbott, North Illinois, IL) in O<sub>2</sub> is administered via the endotracheal tube to achieve a surgical plane of anesthesia. The back is shaved and prepped for surgery using three applications of Betadine® scrub solution alternating with sterile wipes saturated with 70% isopropyl alcohol.
3. A skin incision is performed over the L1-L5 spinous processes, and the fascia is cut on the left side, adjacent to the lumbar spinous processes. The left para-spinous muscles are next dissected free from the dorsal surface of the lumbar spine, and the laminae, pedicles, and facet joints are exposed. The use of a high-speed diamond-bit drill and rongeurs allow for a left-sided lumbar laminectomy extending from the caudal aspect of the L1 vertebra to the rostral aspect of the L3 vertebra.
4. The dura mater is opened using a pair of micro-scissors and a dural scalpel equipped with a size 15 blade. The lumbosacral dorsal roots are gently moved from the dorso-lateral side to the dorsal side of the spinal cord in order to visualize the lumbosacral ventral roots exiting from the ventral surface of the spinal cord. The L6-S3 ventral roots are identified based on anatomical landmarks and their characteristic caliber differences. In rhesus macaques, the L6 and L7 nerve roots are normally of large and similar caliber,

whereas the S1 ventral root is considerably smaller and is rostral to even smaller S2 and S3 ventral roots.

5. The L6-S3 ventral roots are avulsed and separated from the surface of the spinal cord by using gentle traction along the normal course of each ventral root. A pair of fine forceps is used to apply traction to avulse the unilateral L6-S3 ventral roots.
6. Individual avulsed ventral roots can then be surgically repaired using one of two different strategies, when a potential tissue gap needs to be bridged between the spinal cord and the avulsed roots. For our study, a peripheral nerve graft or the use of a bioengineered nerve guidance channel (NGC) can be used (see details for either approach in items #7 and #8 below).
7. For use of a bridging peripheral nerve graft, an inter-costal nerve graft from a lower thoracic segmental level is suitable, as the caliber of such peripheral nerves is similar to the caliber of the lower lumbar ventral roots in rhesus macaques. For this purpose, an incision is made over the lateral portion of the T11 rib and careful dissection of the neurovascular bundle allows identification of the intercostal nerve. An approximately 2 cm long intercostal nerve segment is removed and kept moist in saline until being grafted,
8. For use of a bridging biodegradable NGC segment, the NGCs were fabricated and comprised of electrospun polycaprolactone (PCL) tubes with an outer diameter of 1.00, 1.25, or 1.50 mm and an inner layer of aligned PCL nanofibers for topographical guidance and a PVA-collagen gel for controlled glial cell derived neurotrophic factor (GDNF) release (300ng/tube). For control studies, NGCs without GDNF release are also used as a bridging graft.
9. For bridging the gap between the spinal cord and avulsed lumbosacral roots, the avulsed end of ventral root is trimmed with a microscissor to obtain an even cut end and sutured to one end of the peripheral nerve graft using 10-0 Prolene® suture. When an NGC is used as bridging graft the end of each avulsed ventral root is trimmed and inserted approximately 1-2 mm into one end of the NGC and held in place by a stitch using a 10-0 Prolene® suture. The opposite end of either the peripheral nerve graft or NGC is surgically attached to the spinal cord as detailed below (item #10).
10. Using a #11 scalpel blade, a longitudinal incision (3-4 mm long and 1-2 mm deep) is made into the ipsilateral lateral funiculus of each lumbosacral spinal cord segment to receive a replanted ventral root (see Note 8). The proximal end of either the peripheral nerve graft or the NGC is surgically placed about 1 mm into the incision and secured to the arachnoid using a 10-0 Prolene® suture.
11. Closing of the dura is performed using a continuous 6-0 Ethilon® suture. Next, the paraspinous muscles and skin are closed in individual layers. The skin closure is performed using 4-0 intradermal resorbable Vicryl® sutures.
12. Post-operatively, animals recover from the surgical procedure and receive oxymorphone (0.15 mg/kg IM TID X 3 days) and ketoprofen 2-5 mg/kg IM SID x 3 days) for post-operative pain control.

### **3.2 Urodynamic studies using cystometrogram and urethral pressure recordings**

The following section describes the methods for urodynamic studies in rhesus macaques, as these functional assessments are relevant after injury and repair of the conus medullaris/cauda equina portions of the spinal cord.

1. For urodynamic studies, each subject is first sedated (approximately ketamine 10 mg/kg IM), an intravenous catheter is placed, and ketamine anesthesia is administered by constant rate infusion (CRI, 10-12 mg/kg/hour IV). The ketamine CRI administration allows for stable physiological studies without significant fluctuation of evoked reflexes. Next, an endotracheal tube is placed for airway protection.
2. The perineal area is prepared for an aseptic transurethral bladder catheterization using three alternating applications of a 10% povidone-iodine solution and 70% isopropyl alcohol.
3. A 7-Fr triple lumen transurethral bladder catheter is placed. This choice of catheter allows for separate and concurrent recordings of bladder and urethra pressures as well as infusion of saline into the bladder as a stimulus to trigger micturition reflexes. The cystometry port and the urethral pressure profile (UPP) port at the distal end of the catheter are placed within the bladder and the urethral canal, respectively. The urethral length has initially been measured using a Foley catheter with a balloon at the tip to estimate positioning of the triple lumen catheter for each animal.
4. The proximal ends of the cystometry port and the UPP port are connected to separate TSD 104A pressure transducers (Biopac), which are connected to an MP150 Data Acquisition System (Biopac). The Data Acquisition System is connected to a personal computer equipped with the *AcqKnowledge* software (Biopac).
5. For urodynamic studies, the bladder is emptied using a 60 ml syringe attached to the fill port of the triple lumen bladder catheter.
6. To evoke a micturition reflex, the bladder is manually infused with saline using 60 ml pre-loaded syringes. The bladder is partially filled from a baseline pressure of 0-5 cm H<sub>2</sub>O to a pressure of 20-25 cm H<sub>2</sub>O.
7. After the partial filling of the bladder, a reflex micturition response is evoked after a delay of typically 10-40 seconds. The reflex response consists of the initiation of a bladder contraction and voiding.
8. Cystometrogram (CMG) and urethral pressure recordings are viewed collected on a personal computer, and evoked the micturition reflexes next undergo quantitative analyses. Determination of maximum and threshold pressures, voiding efficiency, and post-voiding residual volumes are determined. An elevation of the urethral pressure during the micturition reflex response is also a useful biomarker for onset of voiding and urine flow.
9. At the end of the urodynamic data collection, the transurethral bladder catheter is removed and the animal allowed to recover.

### **3.2 Electromyography (EMG) recordings of the external anal sphincter guarding reflex**

The following section describes the procedures for EMG recordings of the external anal sphincter (EAS) guarding reflex. This is a relevant outcome measure for injuries to the

lumbosacral spinal cord and cauda equina, as the EAS is innervated by the pudendal nerve, which originates primarily from the L7 and S1 spinal cord segments in rhesus macaques.

1. For EAS EMG studies, each subject is sedated with ketamine (approximately 10 mg/kg IM), and an intravenous catheter is placed to administer ketamine by constant rate infusion (CRI, 10-12 mg/kg/hour IV). The ketamine CRI administration allows for stable physiological studies without significant fluctuation of evoked reflexes. Ketamine is also a suitable anesthetic agent, as it preserves neuromuscular reflexes well, especially a light sedating plane of anesthesia.
2. The perineal area is cleaned using three alternating applications of a 10% povidone-iodine solution and 70% isopropyl alcohol.
3. Paired 22 gauge, 2"/51 mm length, Large Hub Removable Needles, (Hamilton Company, Reno, NV) are used as bipolar electrodes and inserted into the left and right sides of the EAS muscle. A separate ground electrode is placed into the gastrocnemius muscle of the left hind limb. The electrodes are next connected to a data acquisition system (MP150, Biopac Systems, Inc., Goleta, CA) equipped with the EMG amplifiers (Biopac Systems, Inc.). Recorded EAS EMG activity are digitized at 1k Hz and stored on a computer for analysis.
4. Baseline and evoked EAS EMG activity was recorded. For the evoked EAS EMG recordings, a lubricated glass probe, with a 10 mm outer diameter, is inserted about 10-15 mm into the rectal opening to provide a gentle distension of the EAS. The probe is held in place for 5 seconds and next removed to allow for the EAS to relax. EAS EMG activity evoked by the brief insertion and removal of the glass probe is recorded and allowed to return to baseline levels before another attempt is made to activate this reflex response. At least three consecutive trials are performed and EAS EMG recordings collected to assure of consistent responses and allow for averaging responses during data analysis. The procedure is overall well tolerated and subjects receive a single dose of ketoprofen (2- 5 mg/kg IM) upon recovery as discomfort prevention.
5. The EAS EMG recording are next analyzed with regards to the duration of evoked responses, maximum and mean amplitudes, area under curve, as well as frequency domain parameters using the AcKnowledge software product (Biopac Systems, Inc) and the MATLAB® signal processing tool kit (MathWorks, Inc., Natick, MA).

At the onset of the project, a total of 20 research subjects, adult female rhesus macaques, were selected for pre-study testing and entered into the these studies. The selection and screening process was extensive, as behavioral components were very important for the success of these long-term studies. Not all subjects that are initially screened were able to pass the behavioral criteria needed for being included in the studies. During the first year of the project, an algorithm was developed to ensure selection of suitable research subjects for these studies. While the pre-surgical selection, screening, and testing of our subjects was extensive, detailed, and needed dedicated time and effort, we were successful in developing a method for optimal selection of animals, trained staff to perform the behavioral screening and evaluation, as well as in implementing our procedures for subject behavioral testing and project



enrollment. These algorithms for subject identification, selection and evaluation were implemented and consistent for the duration of the project.

Prior to the start of experimental procedures and research data collection, each enrolled subject was extensively screened and trained. Behavioral records and profiles for each considered rhesus macaque were reviewed to select subjects that are most likely to cooperate with training. Next, each animal was trained in transfers from its home cage to a carrying cage using a chute. Next, each subject was introduced to the treadmill environment, which is enclosed by a plexi-glass cage. As the planned surgeries, which include a multi-level lumbosacral ventral root avulsion injury, are expected to result in ipsilateral leg weakness, the treadmill training will provide an added benefit of encouraging use of the weak leg and limit potential muscular contractures. The subjects were trained to perform treadmill walking on the moving belt at various speeds, and the desired locomotor behavior was supported using food rewards. The training required multiple sessions for the learned behavior to be successful. Extensive variation between subjects was present with regards to the number of sessions required to become and reliable and consistent treadmill walker. Extra training sessions was needed for some subjects at times to accommodate for and overcome this individual variation between animals. However, such extra training sessions resulted in a delayed start of surgical procedures for some subjects or other types of training, including chair training for somato-sensory threshold testing and pain screening.

Successful treadmill training was a requirement for the next step in subject evaluation, i.e. chair training, which was needed sensory testing in awake subject using von Frey hairs to obtain baseline and pre-surgical sensory threshold data. The chair training was also needed at the onset of the study for the application of paint markers over joints at the hip, knee, ankle, distal metatarsal bone, and distal portion of the fifth toe. The paint markers were applied before filming subjects while undergoing treadmill testing. These digital recordings with paint markers in place allowed for subsequent videos and kinematics analysis. Later during the studies, a new marking system was introduced and consisted of the application of tattoos using dye of different colors over the joints. The tattoos represented a significant improvement as they were more consistent between recording sessions to identify the joint movements for kinematic analysis and also represented a permanent marker system.

Pre-operative electromyography (EMG) recordings from the external anal sphincter muscle were obtained pre-operatively as baseline records. The external anal sphincter muscle was chosen as it is normally innervated by primarily the L7 and S1 spinal cord segments and nerve roots. As a result, the sphincter is directly affected by the unilateral L6-S3 ventral root avulsion injury (VRA) in our experimental model. As a result of the VRA injury in the present study, the external anal sphincter muscle was partially denervated, allowing only innervation from the contralateral side of the spinal cord to remain. We developed the evoked external anal sphincter EMG recordings in the non-human primate during this project, and it represents an example of innovation and a new customized outcome measure. The analysis of the control data were expanded and included customized development of computer program applications to make possible both time domain and frequency domain data to be interpreted. These EMG studies also allow for longitudinal studies and subjects were tested at multiple time points during the course of the studies.

To better assess autonomic dysfunction that may result from the lumbosacral ventral root injury model in the present project, we have also developed a comprehensive method for urodynamic recordings in rhesus macaques. The development and refinement of the urodynamic procedures took place primarily during the second year of the project. This was also a protocol achievement that improved our functional assessments of the subjects. We developed a method for concurrent collection of evoked cystometrogram recordings and urethral pressure recordings in subjects sedated by ketamine anesthesia. In addition, we developed a method for the screening of visceral pain by collecting concurrent external abdominal wall EMG recordings during the urodynamic procedures. These combined bladder pressure and EMG studies are clinically relevant and also represent protocol development and refinement. The urodynamic studies have throughout the project undergone methodological development with data being collected in a longitudinal study design, and the collected data are presently still undergoing analyses.

Both radiography and magnetic resonance imaging (MRI) of the thoracolumbar spine were performed of the lower spine to visualize the vertebral column and the lumbosacral spinal cord and associated nerve roots. These studies were instrumental for surgical planning, as the location of the caudal portion of the spinal cord may vary extensively between subjects. An anatomical variant, represented by subjects with a set of supernumerary ribs, has also been identified in about 20% of subjects, highlighting the need for multiple bony and soft tissue landmarks to be taken into consideration for surgical planning of surgical procedures targeting the cauda equina and conus medullaris. The post-operative imaging has also been useful in developing an MRI signature for root injuries and repair.

Following pre-study screening, a total of 20 subjects, adult female rhesus monkeys, were enrolled for full pre-surgical testing, including MRI, treadmill training, chair testing, and pain screening using a von Frey testing approach. All subjects underwent the surgical spine procedure with ventral root avulsion injury and repair in years 2 and 3 of the studies. Years 3 and 4 has included ongoing behavioral, physiological, and imaging data collection and analyses. Anatomical material collected at the end of the study has been preserved and is still being processed for ongoing light and electron microscopic investigations. The extensive morphological material continues to be subject for extensive studies and analyses on axonal regeneration and repair after the VRA injury and repair procedures using different bridging strategies.

A no-cost extension request for this project was submitted and approved. The rationale for the request were related to the expansion of scope for the performed studies at the time of funding. In addition to the original research plan, we incorporated pain behavioral studies that had been suggested by the Scientific Review Committee and also recommended by the Scientific Program Officer for the Spinal Cord Injury Research Program. As a result of this addition, all animals needed to be trained for extended periods of time before surgical procedures could take place. However, the subsequent surgeries and longitudinal imaging, behavioral, and physiological studies have progressed well but additional time was needed for their completion. During the period of no-cost extension, anatomical materials have been collected and morphological studies of an extensive tissue material are ongoing and progressing well.

## KEY RESEARCH ACCOMPLISHMENTS

Development of an algorithm for selection of rhesus macaques based on behavioral and treadmill locomotor criteria.

Development of a new outcome method for obtaining interpretable quantitative EMG recordings from the external anal sphincter pre- and post-operatively (**Figures 1-4**). Development of comprehensive and new urodynamic outcome methods, which allow for screening for visceral pain in addition to obtaining functional micturition data in both pre- and post-operative subjects and ability to demonstrate injury and repair effects (**Figure 5**).

Collection of comprehensive pre-surgical data, including treadmill locomotor studies with an automated digital recording system, Imaging of the lumbosacral spinal cord and lower extremity muscles using a combination of radiography and MRI, collection of comprehensive evoked urodynamic recordings, evoked EMG recordings of the external anal sphincter, and behavioral and sensory threshold testing, including manual von Frey hair and Electro-von-Frey approaches.

Demonstration of utility of MRI studies for pre-operative assessments for surgical planning as well as for monitoring assessing time-dependent changes in longitudinal studies after ventral root injury and repair (**Figures 6, 8**).

Demonstration that surgical use of peripheral nerve grafts, harvested as intercostal nerve segments, can be used to bridge tissue gaps between the spinal cord and avulsed lumbosacral nerve roots in rhesus macaques in long-term studies over 18 months (**Figures 7-11**).

Demonstration that axonal regeneration is feasible and successful by myelinated and non-myelinated axons and can be promoted and directed by GDNF-releasing nerve guidance conduits to bridge tissue gaps after conus medullaris/cauda equina injuries in long-term studies (**Figures 12-20**). The nerve guidance channels are also well tolerated and without any detected adverse effects by the spinal cord and nerve root tissues. In addition, no signs of tissue rejection of nerve guidance channels by host tissues were detected.

## REPORTABLE OUTCOMES

Extensive sets of reportable data have been collected and undergoing analysis and preparations for comprehensive manuscripts for peer-reviewed journals. Outcomes that are currently being analyzed include pelvic floor EMG recordings, pain behavioral monitoring, urodynamic studies, locomotor behavior, and combined radiographic and MRI studies as well as morphological outcome measures after the collection of nerve root and spinal cord tissues. Multiple manuscripts have been published or are in various stages of submission to peer-reviewed journals (please see list below). These manuscripts include presentations of surgical, imaging and physiological approaches, including studies of evoked activation of external anal sphincter guarding reflex EMG activity in rhesus macaques in pre- and post-operative studies. Detailed analyses of signal processes have allowed for both amplitude and frequency based

quantitative studies. We have also demonstrated feasibility of performing comprehensive urodynamic studies in pre- and post-operative non-human primates, and that shows that such functional outcome measures of micturition reflexes are suitable for studies of spinal cord injury and repair in non-human primates. Here, evoked bladder contractions and voiding are determined using both cystometrogram recordings and urethra pressure recordings, and subsequent calculations for voiding efficiency is performed. We are also preparing manuscripts that demonstrate successful axonal regeneration by motor axons into peripheral nerve grafts and GDNF-releasing nerve guidance channels for long-term studies in non-human primates.

### **Meeting Abstracts**

We have presented and submitted abstracts related to funded studies at the following national scientific meetings:

Lee U, Chang HH, **Christe KL**, Havton LA. Evoked electromyographic activity in the external anal sphincter muscle of non-human primates differs from corresponding patterns in humans. Annual Meeting of the Society for Neuroscience, 2011, 41.

Havton LA, Ohlsson M, Nieto JH, **Christe KL**. Retrograde degeneration of motoneurons and sprouting of spared sensory fibers into denervated ventral root segments in a non-human primate model for cauda equina injury. Annual Meeting of the Society for Neuroscience, 2011, 41.

Lee U, Chang HH, **Christe KL**, Havton LA. Evoked electromyographic activity in the human external anal sphincter muscle of non-human primates differs from corresponding patterns in humans. Annual Winter Meeting of the Society of Urodynamics and Female Urology, Feb. 28 - March 3, 2012, New Orleans, LA.

Lee U, Chang HH, **Christe KL**, Havton LA. Evoked electromyographic activity in the external anal sphincter muscle of non-human primates differs from corresponding patterns in humans. American Urological Association (AUA) Annual Meeting, May 19-23, 2012.

Havton LA, Nieto JH, Ohlsson M, Chang HH, Mao HQ, Höke A, **Christe KL**. Use of GDNF-releasing nanofiber nerve guide conduits for repair of conus medullaris/cauda equina injury in the non-human primate. Military Health System Research Symposium, August 13-16, 2012, Fort Lauderdale, FL.

Ohlsson M, Nieto JH, **Christe KL**, Havton LA. Effects of lumbosacral ventral root avulsion and replantation in a non-human primate model for cauda equina injury. Annual Meeting for the Society of Neuroscience, New Orleans, CA, 2012, 42.

Lee U, Chang HH, **Christe KL**, Havton LA. Evoked voiding contractions and corresponding urethral sphincter electromyography in non-human primates differ from rodents. American Urological Association Annual Meeting, San Diego, CA, May 4-8, 2013.

Ohlsson M, Nieto JH, Villablanca P, **Christe KL**, Havton LA. Perioperative imaging studies and intra-operative EMG monitoring as guide for surgical repair of avulsed ventral roots using intercostal nerve grafts in rhesus macaques. Annual Meeting of the Society for Neuroscience, 2013, 43.

Biscola NP, Nieto JH, Martin R, Ohlsson M, **Christe KL**, Mao HQ, Hoke A, Havton LA. Repair of a lumbosacral ventral root avulsion injury using GDNF-releasing nerve guidance channels to bridge tissue gaps between the spinal cord and avulsed ventral roots in rhesus macaques. Annual Meeting for the Society of Neuroscience, Washington, DC, 2017, 47 (Submitted)

## **Publications/Manuscripts**

The following papers have been published, submitted, or are in late preparatory states for submission to peer-reviewed journals:

**Christe, KL**, Lee U, Lemoy MJ, Havton, LA. Generalized Seizure Activity in Association with Ketamine Anesthesia and Urodynamic Studies in an Adult Rhesus Monkey (*Macaca mulatta*), *Comparative Medicine*, October 2013, Vol 63, No 5; 1-3.

Chang HH, Havton LA. A ventral root avulsion injury model for neurogenic underactive bladder studies. *Experimental Neurology*, 2016; 285: 190-196.

Ohlsson M, Nieto JH, **Christe KL**, Villablanca JP, Havton LA. Radiographic and magnetic resonance imaging identification of thoracolumbar spine variants with implications for the positioning of the conus medullaris in rhesus macaques. *Anatomical Record* 2017, 300: 300-308.

Nieto JH, Chang HH, Ohlsson M, Lee U, Villablanca JP, **Christe KL**, Havton LA. Surgical replantation of avulsed lumbosacral ventral roots and urodynamic studies in a rhesus macaque (*Macaca mulatta*) model of Cauda Equina/Conus Medullaris Injury and Repair. *Methods Molecular Biology* 2017 (In Press)

Chang HH, Lee U, Vu T, Pikov V, **Christe KL**, Havton LA. EMG activity of the external anal sphincter guarding reflex shows different time and frequency domain signatures in rhesus macaques (*Macaca mulatta*). 2017 (Submitted)

Lee C, Nieto JH, Ohlsson M, Christe KL, Havton LA, Cannesson M. Changes in pulse oximeter perfusion index and laser doppler flowmetry are closely correlated during spinal surgery: an in vivo study. (In Preparation).

Havton, LA, Nieto JH, Ohlsson M, Do R, Rana S, Chang HH, Villablanca P, **Christe KL**, Hoke A. Replantation of avulsed lumbosacral ventral roots using intercostal nerve grafts promotes motoraxonal regeneration and root reinnervation in the non-human primate. (In Preparation).

Biscola NP, Nieto JH, Martin R, Amin A, Ohlsson M, **Christe KL**, Mao HQ, Hoke A, Havton LA. Repair of a lumbosacral ventral root avulsion injury using GDNF-releasing nerve guidance channels to bridge tissue gaps between the spinal cord and avulsed ventral roots in rhesus macaques (In Preparation)

## **CONCLUSION**

Our studies have made significant progress and been successful in the overall goal, demonstrating surgical feasibility and pre-clinical safety of using GDNF-releasing nerve

guidance channels in long-term studies as an alternative to peripheral nerve grafts to promote long-term axonal regeneration after surgical repair of conus medullaris and cauda equina injuries. Several functional and imaging outcome measures and approaches have been developed along the course of these studies, and these technical and methodological advancements will also serve as valuable tools to other investigators performing translational studies in large mammals, including non-human primates, as well as to the general research community. The collected data will guide discussions and provide critical pre-clinical feasibility and safety data to be considered for possible clinical translation of the use of trophic factor-releasing nerve guidance channels for select populations of spinal cord injured patients.

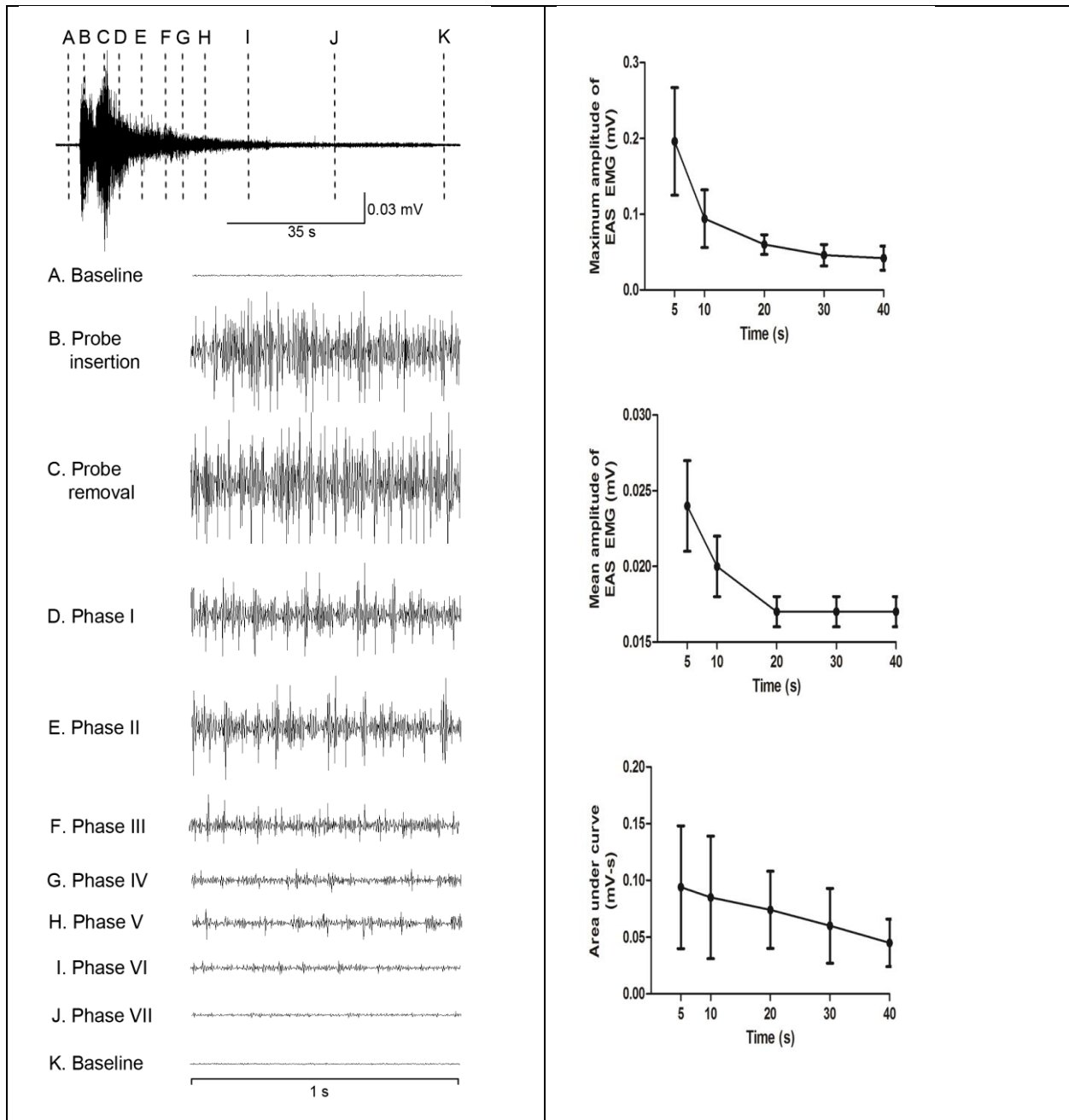
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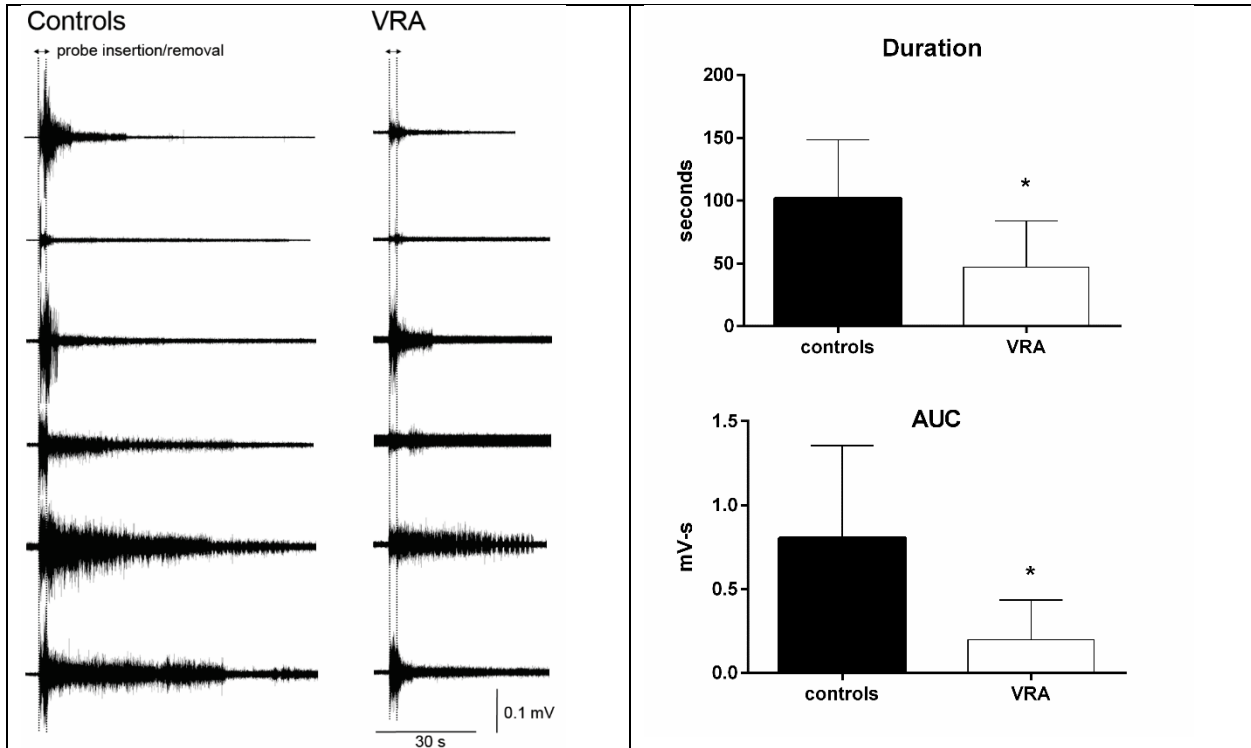
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APPENDICES/SUPPORTIVE DATA



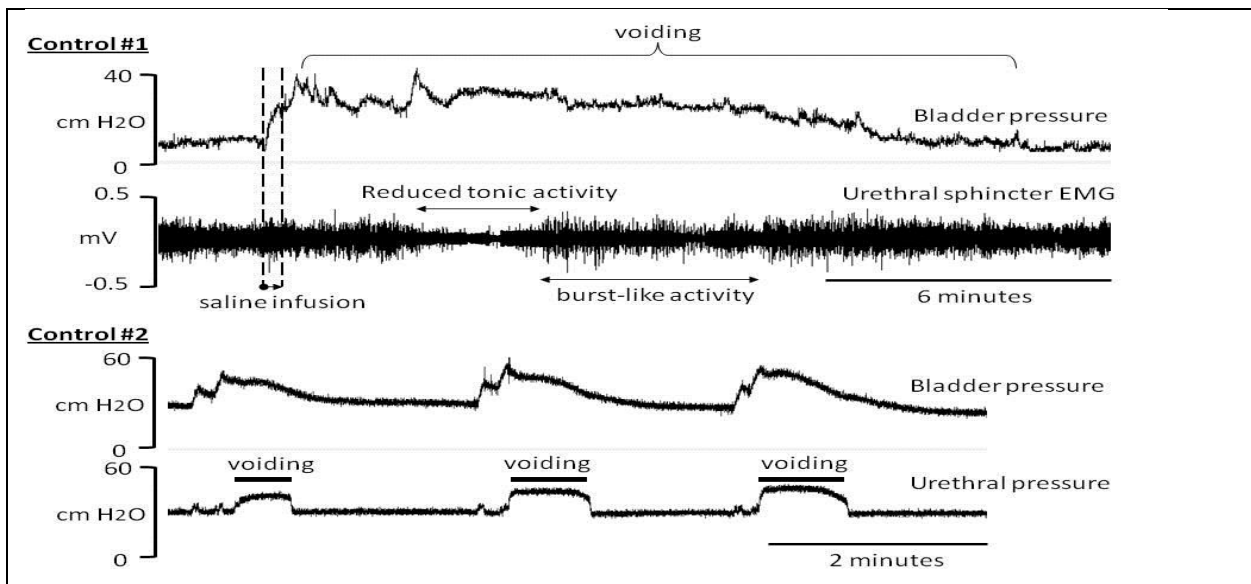
**Figure 1.** Evoked EMG guarding reflex responses from the external anal sphincter muscle following rectal insertion and removal of a glass probe (10 mm diameter). Stimulus duration was 5 seconds. A-K indicate different time points of stimulus and evoked responses. Note step wise decrease in EMG amplitude until return of quiescent baseline.

**Figure 2.** Quantitative studies of evoked guarding reflex EMG responses from the external anal sphincter muscle. The responses are presented as maximum and mean amplitude as well as area under curve measurements over the first 40 seconds after stimulus. Note gradual decrease of all three outcome measures over time.

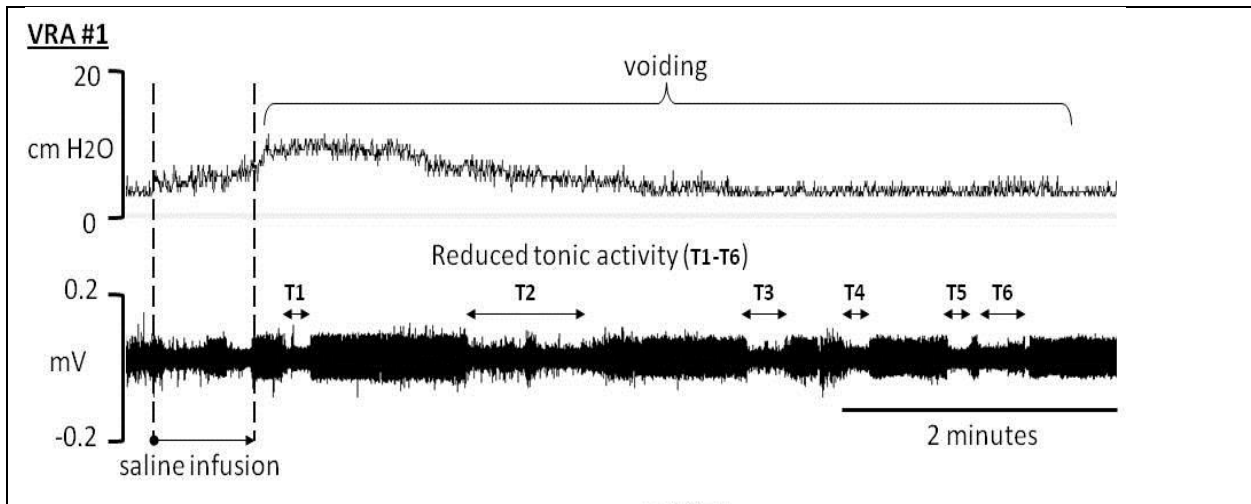


**Figure 3.** EMG recordings from the external anal sphincter (EAS) in control rhesus macaques (n=6) and at 4-6 weeks after a unilateral ventral root avulsion (VRA) injury (n=6). Note overall decreased evoked EAS guarding reflex responses in the VRA cohort compared to control subjects.

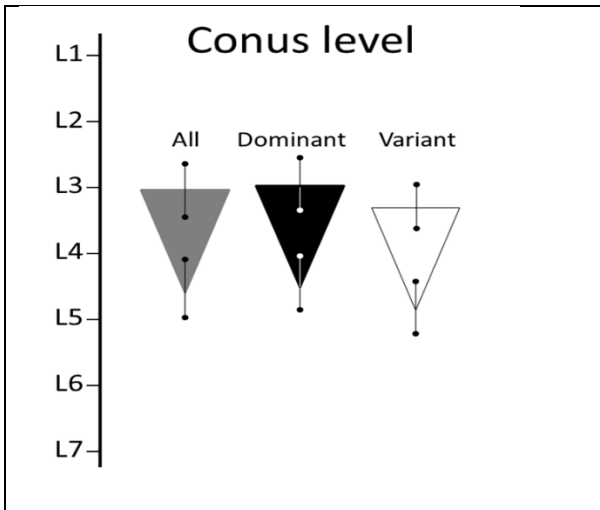
**Figure 4.** At 4-6 weeks after a unilateral L6-S3 VRA injury (n=6), significantly reduced duration and area under the curve measurements of evoked EAS guarding reflex responses are demonstrated compared to corresponding recordings in control subjects (n=6).



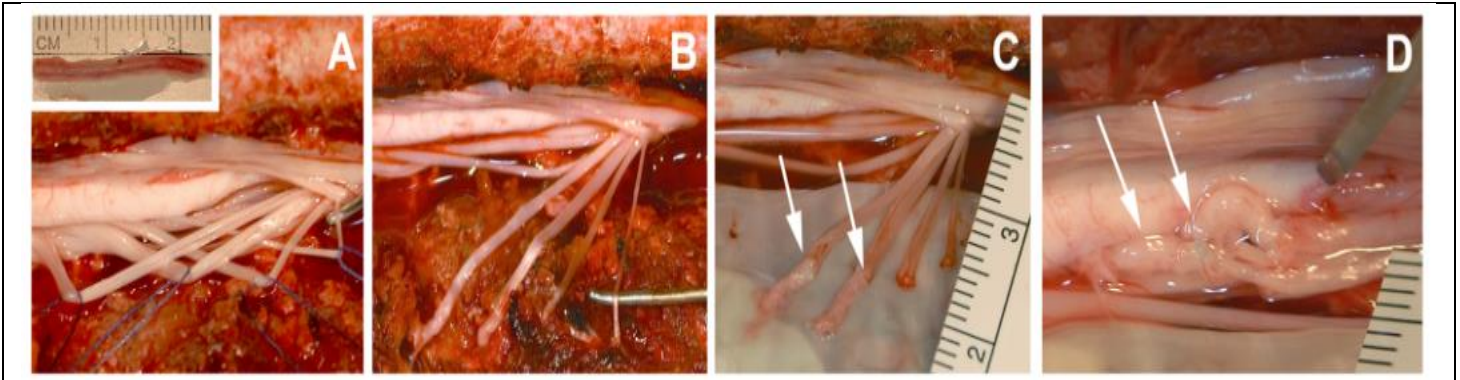




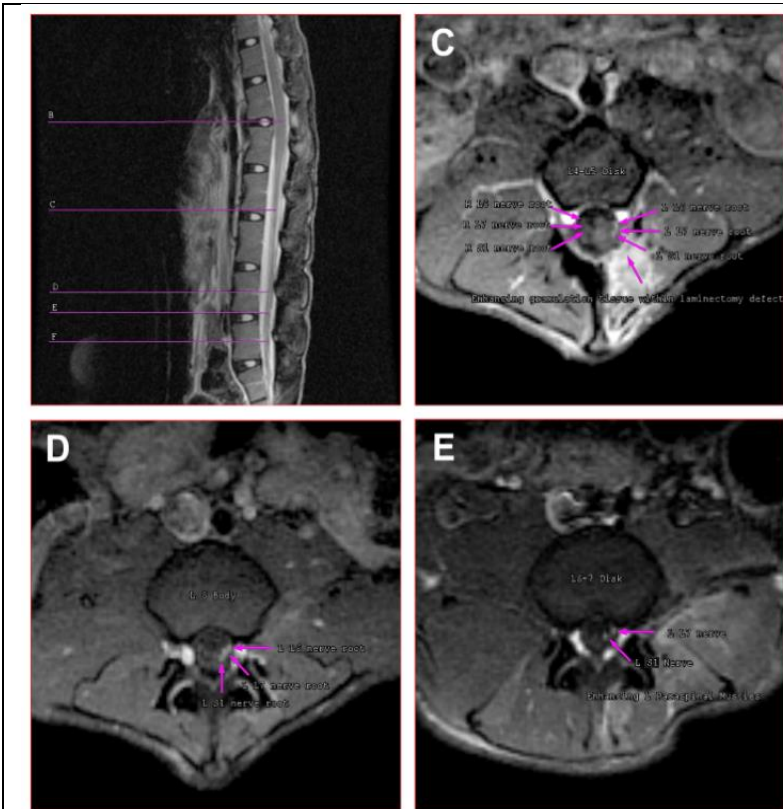
**Figure 5.** Comprehensive urodynamic recordings were performed in control and post-operative rhesus macaques. For cystometrogram recordings, a triple lumen bladder catheter was placed and attached to two pressure sensors for bladder and urethral pressures. Saline was infused into the bladder and triggered an evoked bladder contraction and voiding. Concurrent urethral pressures were recorded and increased pressure was associated with voiding. Needle electrodes were placed into the external urethral sphincter for electromyography (EMG). There was reduced tonic activity identified by EMG in the external urethral sphincter during voiding (upper image). Note that the postoperative recordings at 6 weeks post-op (lower image) suggested a reduced maximum bladder pressure being generated, interpreted as a likely effect of denervation of peripheral pelvic ganglia from unilateral lumbosacral ventral root avulsion injury and in the absence of any root repair.



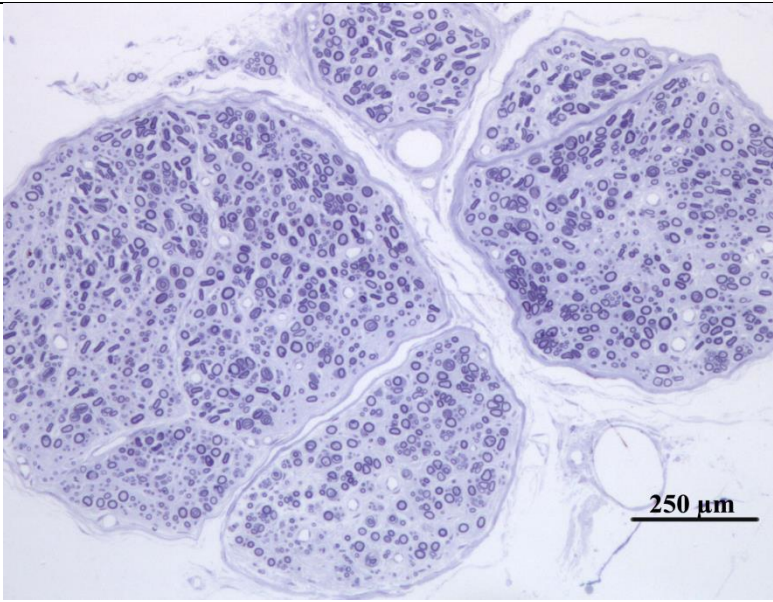
**Figure 6.** A combination of radiographic studies and magnetic resonance imaging (MRI) of the spine was performed pre-operatively in all rhesus macaques to determine the location of the conus medullaris for planning of surgical root procedures. In a total sample of 44 rhesus macaques, we detected about 20% of subjects showing a set of supernumerary ribs. We determined, that based on the relationship between the conus medullaris and the vertebral column, this anatomical variant should be considered as showing an L1 vertebra with supernumerary ribs rather than demonstrating a T13 vertebrae (Ohlsson et al., 2017). The image shows the anatomical relationship between the top and the tip of the conus medullaris of the spinal cord for all, dominant, and variant anatomical subjects and the vertebral column.



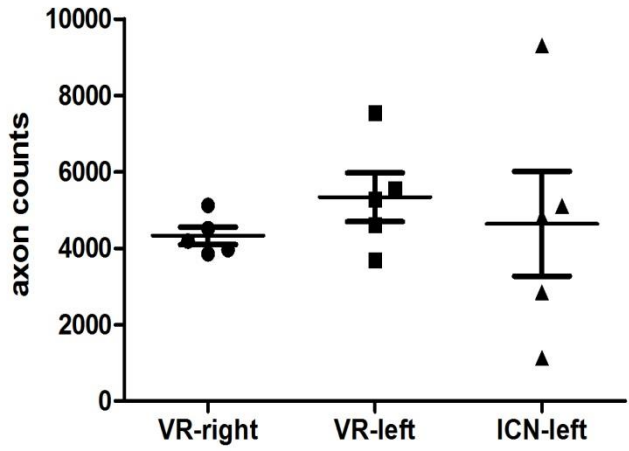
**Figure 7.** Intra-operative images of lumbar VRA injury and replantation using an intercostal nerve graft to bridge between avulsed roots and the spinal cord. **A:** Four consecutive ventral roots of the L6, L7, S1, and S2 segments are identified **A (inset):** Harvested T11 intercostal nerve graft. **B:** The L6, L7, S1, and S2 ventral roots (left-to-right) are avulsed from the surface of the spinal cord. **C:** An approximately 8 mm long intercostal graft segment was attached to the avulsed end of the L6 and L7 ventral roots using microsurgical techniques and 10-0 Prolene® sutures. **D:** The free end of the intercostal nerve graft segments are implanted into the lateral funiculus of the spinal cord and the implanted graft is attached to the leptomeninges using a 10-0 Prolene suture.



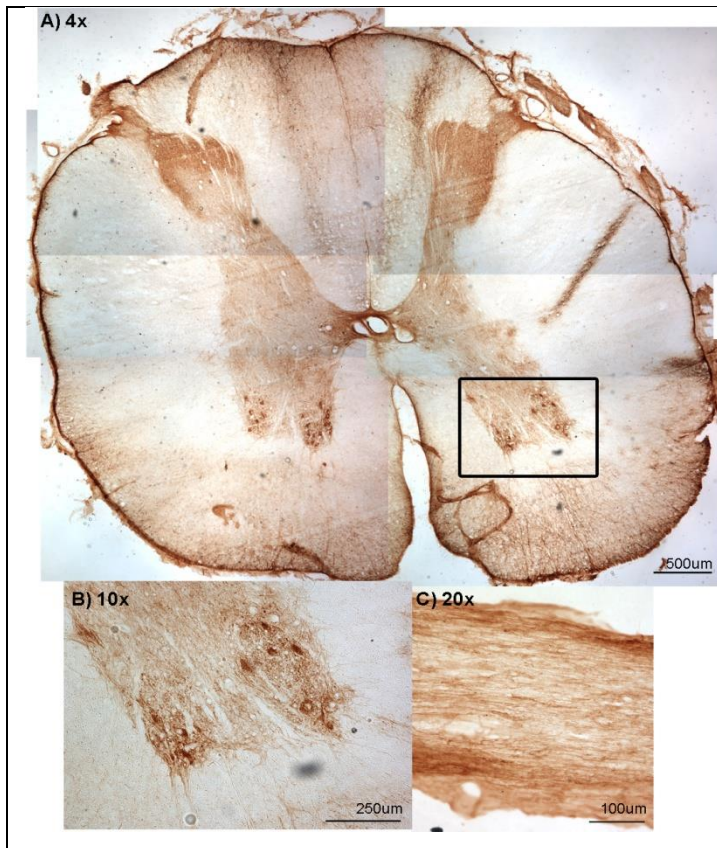
**Figure 8.** MR imaging of the lumbar spine at 6 weeks after L6, L7, and S1 VRA injury and L6+L7 ventral root repair using a peripheral nerve bridging graft between the spinal cord and the avulsed ventral root. **Upper left:** T2 sagittal scout image. Images at the indicated **C, D, E** planes of the sagittal scout image were obtained using axial T1 post-contrast and fat saturation protocols. **Upper right:** At the level of the L4-L5 disk space, some granulation tissue is present in the central thecal sac, whereas individual lumbo-sacral nerve roots lie laterally as shown. **Lower left:** At mid-L6 vertebra level, there is excellent delineation of avulsed and surgically repaired left L6 and L7 ventral roots and the avulsed S1 ventral root, which all strongly enhance, suggestive of blood-nerve barrier disruption compared to the non-enhancing, contralateral intact roots. **Lower right:** At L6-L7 disk space level, the L6 ventral root has exited (and is no longer seen), whereas the traversing left L7 and remaining left S1 ventral roots are readily identified.



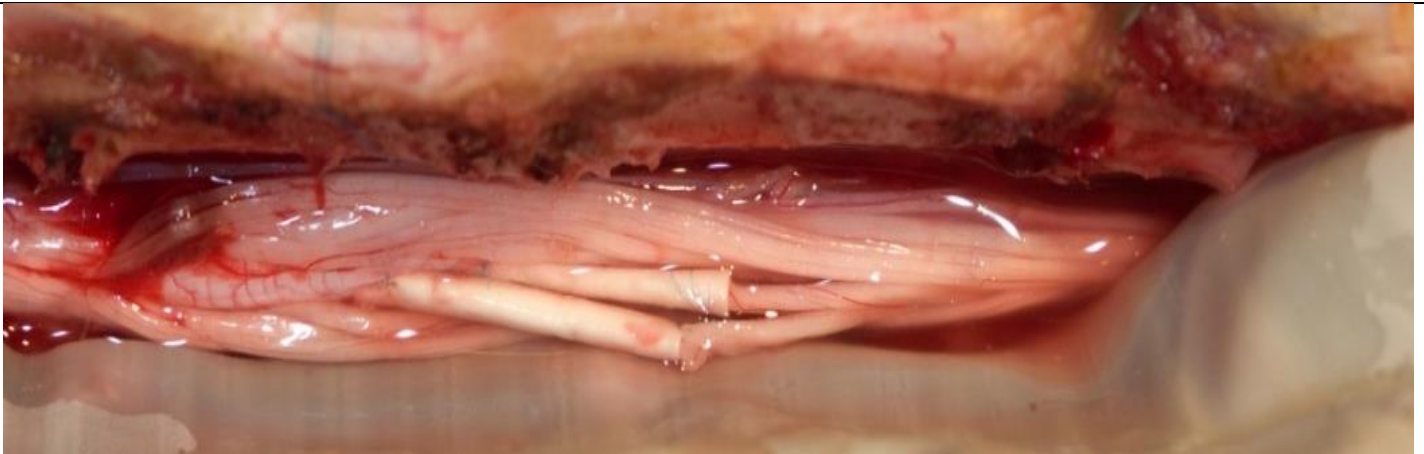
**Figure 9.** Light microscopic studies of plastic embedded and toluidine blue stained intercostal nerve graft, which was used to bridge between the conus medullaris and the avulsed L7 ventral root after a unilateral L6-S2 ventral root avulsion injury in a rhesus monkey. These studies were performed at 18 months after the root repair. Note the large number of regenerating axons in nerve graft and fibers showing myelination. Stereological studies show a total of 2,606 small and large myelinated nerve fibers in graft. The studies demonstrate the feasibility of using a mixed peripheral nerve as a bridging graft to overcome a tissue gap caused by a proximal ventral root injury for long-term studies in the non-human primate.



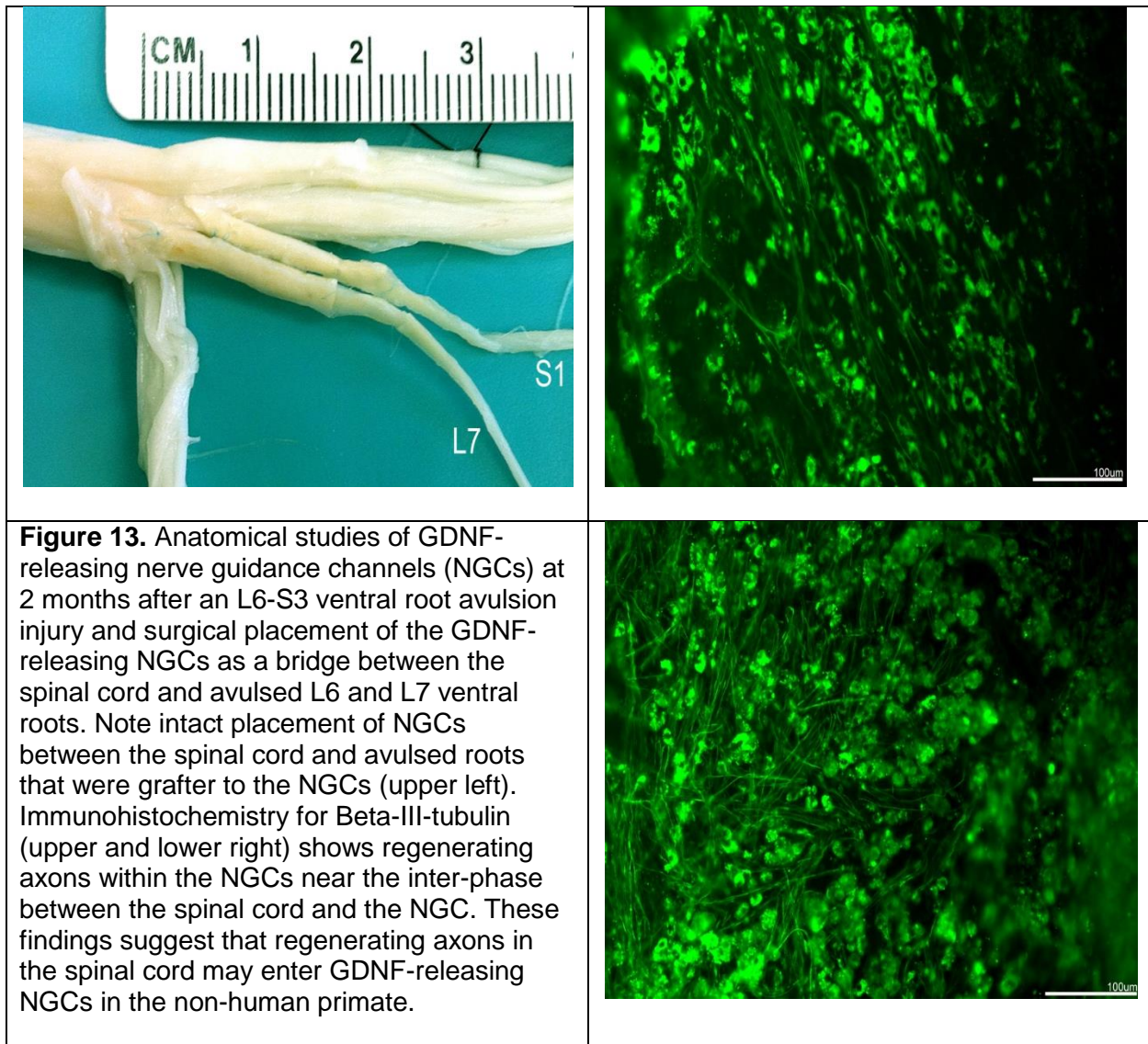
**Figure 10.** Quantitative studies on axon counts in the L7 right ventral root (VR-right), the L7 left ventral root (VR-left) and the intercostal nerve (ICN) segment, which was used as a bridging graft between the spinal cord and avulsed L7 ventral root (ICN-left). Note that both the control side (right) and the experimental side (left) show similar mean numbers of myelinated axons in the L7 ventral root and in the ICN graft. The findings suggest that axonal regeneration takes place on the experimental side, and that it is an important contributor to the repopulation of the grafted intercostal nerve segment and avulsed ventral root.

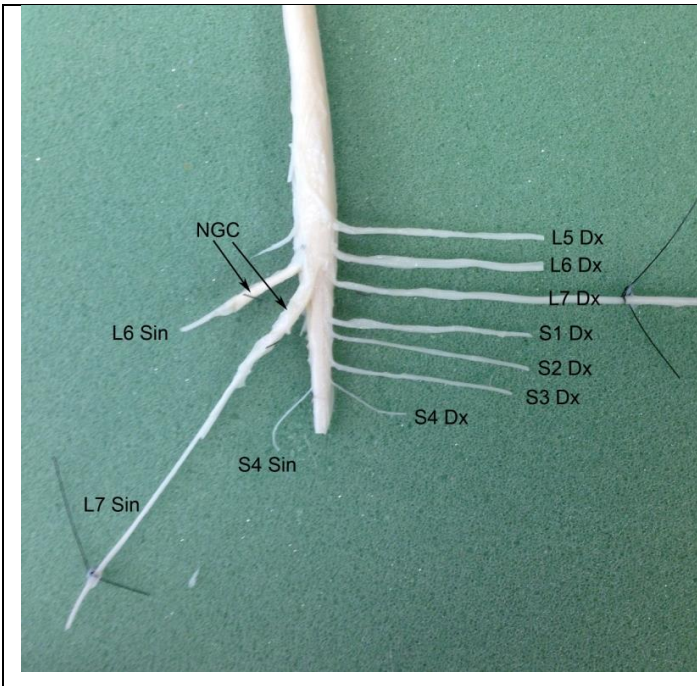


**Figure 11. A.)** Photomontage of lumbar spinal cord section from rhesus macaque at 18 months after an L6-S3 VRA injury and surgical repair of the L6 and L7 ventral roots using an intercostal nerve bridge between the spinal cord and the avulsed roots. The section had been reacted with a primary antibody for choline acetyl transferase (ChAT) and a diaminobenzidine (DAB) protocol was used to achieve a light stable reaction product. **B.)** Detail of ventral horn showing ChAT-immunoreactive motor neurons. **C.)** Longitudinal section of the repaired L6 ventral root shows that numerous axonal profiles are immunoreactive for ChAT. The latter finding suggests that cholinergic spinal cord neurons have regenerated axons into the peripheral nerve graft and subsequently also extended into the avulsed and repaired L6 ventral root. In this subject, quantitative studies of plastic embedded and toluidine blue stained sections showed a total of 3,977 myelinated axons in the L6 ventral root at 18 months after avulsion injury and repair using a nerve graft.

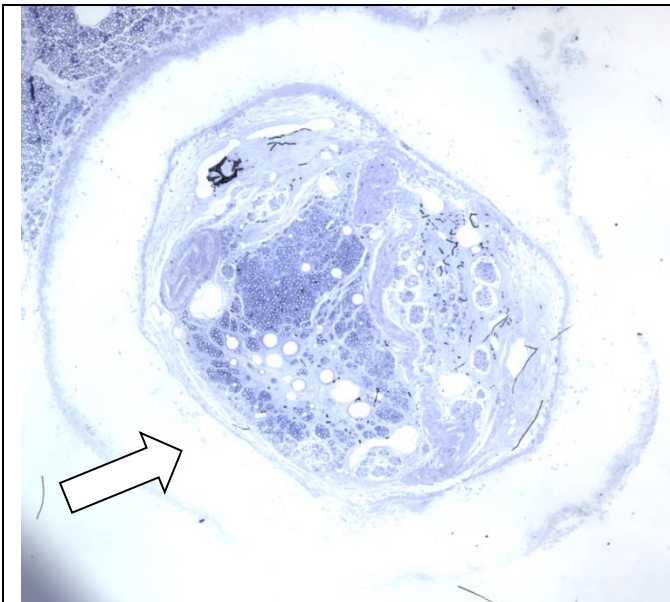


**Figure 12.** In adult rhesus macaques, a lumbar laminectomy and dura opening was followed by a unilateral avulsion injury of the L6-S3 ventral roots. Next, GDNF-releasing NGCs were surgically placed as a bridge between the spinal cord and the avulsed L6 and the L7 ventral roots. The proximal end of each NGCs was placed into the lateral funiculus of the L6 or L7 segments. The avulsed tip of L6 and L7 ventral roots was placed into the lumen of the NGC and secured with a fine suture. Next, the dura was closed and the paraspinal muscles, fascia, and skin closed in layers. All subjects were allowed to recover and studied for 2 months (n=1) or 18 months (n=4). The procedures were tolerated well and the NGCs were identified and in original surgical position at the end of the study periods (see Figures 12-14).

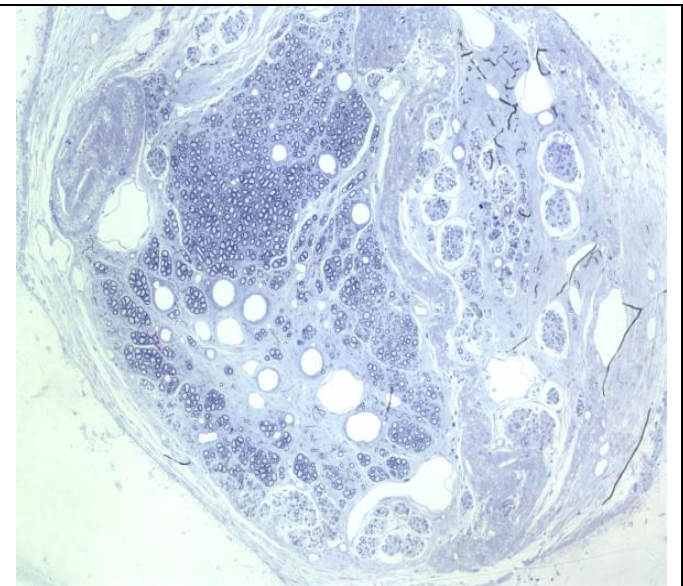




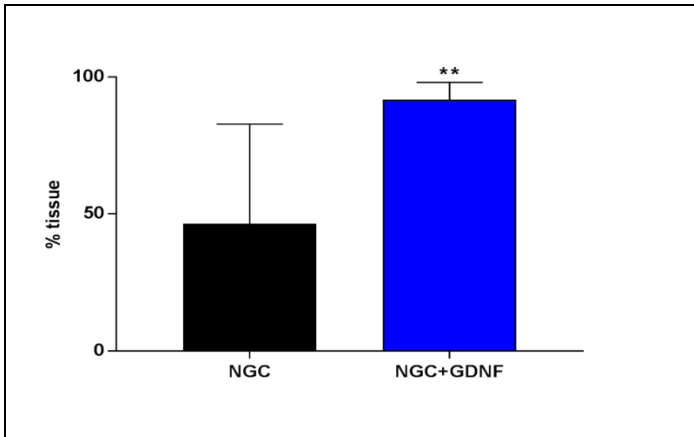
**Figure 14.** Lower part of the spinal cord from rhesus macaque at 18 months after L6-S3 ventral root avulsion injury and repair of the L6 and L7 ventral roots using a GDNF-releasing nerve guidance channel (NGC) as a bridge between the spinal cord and avulsed ventral roots. Note the left sided (sin) well-preserved NGCs that are in place and attached to the appropriate L6 and L7 spinal cord segments. Note absence of S1-S3 ventral roots on the left side, as these roots were avulsed at the time of the original injury. The bilateral S4 ventral roots are present, however. The left (Sin) and right (Dx) L7 ventral roots were identified and attached with a small suture at the time of tissue dissection for identification purposes. The findings provide anatomical support for feasibility for using NGCs as a bridge between the spinal cord and avulsed ventral roots in nonhuman primates for long-term studies on axonal regeneration.



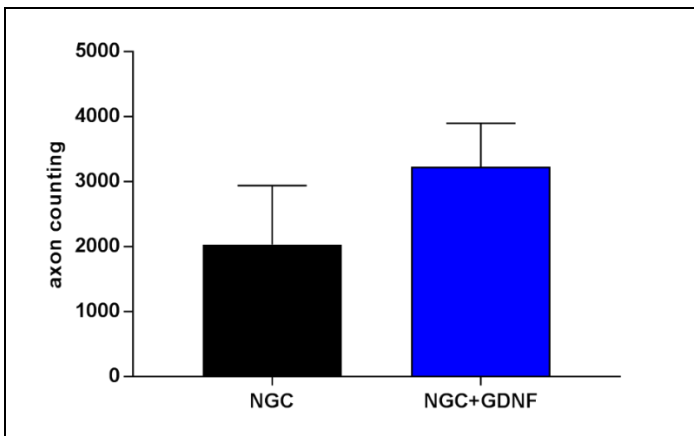
**Figure 15.** Light micrographs of a GDNF-releasing NGC at 18 months after a unilateral L6-S3 VRA injury and surgical placement of the NGCs as a bridging graft between the spinal cord and the avulsed L6 ventral root in an adult female rhesus macaque. Note the well preserved integrity of NGC wall (arrow) and complete filling of the GDNF-releasing NGC lumen by connective tissue, blood vessels, and myelinated axons.



**Figure 16.** A larger magnification presentation of the contents of GDNF-releasing NGC in Figure 14. Note the presence of several hundred myelinated axons in various sized bundles within the NGC lumen as well as the presence of many vascular structures. The findings show feasibility for trophic factor-releasing NGCs to attract and support regenerating axons in non-human primates in long-term studies.



**Figure 17.** At 18 months post-operatively, GDNF-releasing NGCs (n=8) showed a significantly larger area of their cross-sectional surface occupied more by tissues compared to the corresponding lumens of control NGCs, which did not release any trophic factors. The GDNF-releasing NGCs showed  $91.7\% \pm 12.8\%$  (SD) of their lumen occupied by tissue, whereas  $46.5\% \pm 36.3\%$  (SD) of the control NGC lumens (n=8) were filled with tissues. The tissues within both GDNF-releasing NGCs and control NGCs included blood vessels, connective tissues, Schwann cells, and myelinated axons.



**Figure 18.** At 18 months post-operatively, GDNF-releasing NGCs (n=8) showed an average of  $3,230 \pm 699$  myelinated axons, whereas control NGCs (n=8) showed  $2,033 \pm 906$  myelinated axons. There was no difference in axon numbers between the two groups. We conclude that both GDNF-releasing NGCs and control NGCs are capable of supporting regenerative growth by myelinated axons. Although all GDNF-releasing NGCs showed marked growth of regenerating axons, there was also a large variation in number of myelinated axons between individual NGCs.



**Figure 19.** Electron microscopic (EM) image of contents of control NGC at 18 months post-operatively. The NGC shows myelinated and non-myelinated axons, Schwann cell nuclei and extracellular collagen fibers.



**Figure 20.** EM image of contents of GDNF-releasing NGC at 18 months post-operatively. The NGC shows both myelinated and non-myelinated axons, Schwann cells, and extracellular collagen fiber collections.