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

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14. ABSTRACT The present study is evaluating 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 underwent additional, longitudinal functional assessments using locomotor testing, 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 are obtained, following by termination of the study and harvesting of spinal cord and nerve root tissues for anatomical studies. A subset of subjects are still undergoing evaluations. Preliminary studies of subjects undergoing VRA injury and root repair show that the intercostal nerve is well innervated by regenerating axons. In a first subject undergoing post-operative morphological evaluation of GDNF-releasing nerve guide channels, it is shown that regenerating fibers are also capable of entering the graft.					
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INTRODUCTION

Cauda equina and conus medullaris forms of spinal cord injury result in paralysis, sensory impairment, and autonomic dysfunction (Hoang and Havton, 2006; Havton and Carlstedt, 2009). The present translational research study investigates the effects of neural repair in the rhesus macaques using a GDNF-releasing nerve guidance channel as a bridging graft. The studies aim to repair avulsed lumbosacral ventral roots using a bridging strategy. For comparison, the studies will also include the use of a guidance channel without GDNF release and a peripheral nerve graft collected from an intercostal nerve to bridge the tissue gap. A comprehensive set of electrodiagnostic, imaging, pain and locomotor behavioral and morphological investigations will provide detailed data about the outcome of the intervention. We are hopeful that this translational research study may guide the planning of future clinical studies on neural repair after cauda equina/conus medullaris injuries. The present report will summarize the major accomplishments within the third year of this 3-year project.

BODY

Our studies are going well and have made good progress . At the beginning of the reporting period, Dr. Havton moved from UCLA to UC Irvine, where he had accepted a position as Professor and Vice Chair for Research in the Department of Anesthesiology & Perioperative Care. Dr. Havton also joined, as a member, the Reeve-Irvine Research Center for Spinal Cord Injury Research. In this collaborative partnership project, all three of the participating PIs (Leif Havton at University of California, Irvine, Ahmet Höke at Johns Hopkins University, and Kari Christe at the California National Primate Research Center (CNPRC) at UC Davis) interact frequently and on a regular basis. In particular, Dr. Havton routinely travels to the California National Primate Research Center and has been present for all surgeries, EMGs, and urodynamic studies. Dr. Havton interacts and communicates regularly in person or by phone and email with the participating PIs (Dr. Kari Christe at the CNPRC at UC Davis and Dr. Ahmet Höke at Johns Hopkins University).

At the onset of the project, research subjects in the form of adult female rhesus monkeys were selected for pre-surgical testing and were enrolled in these studies. The selection and screening process was extensive, as behavioral components were very important for the success of these studies. Not all subjects that are initially assessed were able to pass the behavioral criteria needed for successful participation in our study. During the first year of the project, we developed an algorithm to ensure selection of suitable subjects for these studies. While the pre-surgical selection, screening, and testing of our subjects was extensive and time consuming, we were successful in developing a method for optimal subject selection, trained staff to perform the behavioral screening and evaluation, as well as successfully implementing our procedures for animal enrollment and testing. These algorithms for subject identification, selection and evaluation were also implemented during years 2 and 3 of the project.

Prior to the start of surgical procedures, each enrolled rhesus macaque underwent extensive screening and training. Behavioral records and profiles were reviewed to select subjects that are likely to cooperate with training. Next, each animal was introduced to transfers from cage to a carrying cage

using a chute. This was followed by the introduction of the treadmill environment, which is enclosed by a plexi-glass cage. Animals were trained to walk on the treadmill belt at various speeds, and the desired behavior was encouraged using various food rewards. The training requires multiple sessions to be successful. Extensive variation between subjects was recognized with regards to how many sessions that were needed for a subject to be a reliable and consistent treadmill walker. Extra time for training was at times needed and provided to accommodate for this individual variation. However, when a subject required additional sessions for treadmill training, there was subsequently a delayed start of the other behavioral training sessions, e.g. chair training for sensory threshold and pain screening.

Successful treadmill training was a requirement for subsequent chair training, which was needed in order to perform sensory testing using von Frey hairs and an Electro-von-Frey device to obtain baseline sensory thresholds pre-operatively. In addition, the chair training was needed for the application of paint markers over the hip, knee, ankle, distal metatarsal bone, and distal portion of the fifth toe. The paint markers were filmed while the subjects performed treadmill walking. These digital recordings allow for subsequent detailed kinematics analysis.

Electromyography (EMG) recordings of the external anal sphincter were obtained pre-operatively as baseline records. The external anal sphincter muscle was chosen as it is innervated by primarily the L7 and S1 spinal cord segments and therefore directly affected by the unilateral lumbosacral ventral root avulsion injury in our experimental model. As a result, the external anal sphincter muscle is partially denervated and only innervation from the contralateral side of the spinal cord remains. The development of external anal sphincter EMG recordings in the non-human primate was developed by us during this project and reflects innovation and a new outcome measure. The data from control subjects are presently in the late stages of preparation for a research manuscript. The analysis of the control data were expanded over the past year by development of customized computer programs to allow for also quantitative analyses of firing frequency as a component of the EMG studies. As a result, our data interpretation has strengthened and markedly improved conclusions made in our pending research manuscript. We have also collected external anal sphincter EMG recordings from all of the post-operative subjects at multiple time points. These data are presently undergoing detailed analysis.

In addition, We have developed a method for urodynamic recordings in the non-human primate. The continued development and refinement of the urodynamic procedures primarily took place during year 2 of the project. This was an achievement as well, as no previously established methods for urodynamic studies in non-human primates are present in the literature. We developed a method for performing cystometrogram recordings, urethra pressure recordings, and external urethral sphincter EMG recordings under ketamine anesthesia. In addition, we developed a method for obtaining concurrent external abdominal wall EMG recordings during the urodynamic procedures. The abdominal wall EMG recordings associated with bladder filling allow us to monitor for visceral pain development. These combined bladder pressure and EMG studies are novel and serve as additional clinically relevant outcome measure. Similar to our EMG studies of the external anal sphincter, the urodynamic studies are reportable as scientific findings. Currently, we are analyzing the data from the pre-surgical series and are preparing the data for an initial control study on micturition reflexes in rhesus macaques. In

addition, post-operative urodynamic data are also collected and analyzed for the purpose of subsequent publications on micturition reflexes after ventral root injury and repair in our non-human primate model for studies of cauda equina injury and repair. The post-operative studies will include urodynamic recordings obtained throughout the experimental series, which will be completed in March, 2014.

Magnetic resonance imaging (MRI) has been performed of the lower spine to visualize the lumbosacral spinal cord and associated nerve roots. This approach has proven to be a very valuable component of the studies, as pre-operative imaging allows for detailed identification of lumbosacral roots and surgical planning. The post-operative imaging has shown to be valuable as well as an MRI signature is suggested with regards to ventral root size, signal intensity, and contrast enhancement patterns after ventral root injury and repair.

All 20 subjects, adult female rhesus monkeys, were selected, screened, and successfully enrolled for full pre-surgical testing, including MRI, treadmill training, chair testing, and pain screening using a von Frey testing approach. The majority of these subjects underwent the surgical spine procedure with ventral root avulsion injury and repair in year 2 of the studies. Year three has primarily included ongoing behavioral, physiological, and imaging data collection and analyses.

A no-cost extension request for this project has also been 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 behavioral studies and undergo somatosensory testing. This delayed the date for the first surgical procedures. However, the subsequent studies have progressed well but additional time was needed to complete the ongoing studies. During the period of no-cost extension, anatomical studies will also be performed in the light and electron microscope to evaluate for successful axonal regeneration.

KEY RESEARCH ACCOMPLISHMENTS

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

Development of a method for obtaining interpretable quantitative EMG recordings from the external anal sphincter pre- and post-operatively (Figures 1-4). Development of comprehensive urodynamic methods, which allow for screening for visceral pain in addition to obtaining functional micturition data.

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 MRI, collection of urodynamic recordings, EMG recordings of the external anal sphincter, and sensory threshold testing using manual von Frey hair and Electro-von-Frey approaches.

Demonstration of utility of MRI studies for assessing time-dependent changes in longitudinal studies after ventral root injury and repair.

Demonstration that surgical use of both peripheral nerve grafts and of GDNF-releasing nerve guidance conduits are feasible and well tolerated as bridging grafts between the spinal cord and avulsed ventral roots in rhesus macaques (Figure 5).

Preliminary studies have demonstrated that regenerating axons may enter the GDNF-releasing nerve guidance conduits as evidenced by light microscopy of plastic embedded sections and by immunohistochemistry for the detection of axons (Figure 5).

REPORTABLE OUTCOMES

Most of the reportable data outcomes are currently being analyzed; they include locomotor behavior, EMG recordings, pain behavioral monitoring, and MRI studies as well as morphological outcome measures after the collection of nerve root and spinal cord tissues. However, do not forget many of our collected pre-surgical data were novel and reflect innovation and new knowledge. For instance, our baseline anal sphincter EMG and comprehensive urodynamic recordings are examples of new and original findings.

We are currently in the stages of preparing a manuscript describing evoked activation of external anal sphincter EMG activity in rhesus macaques and provide support and rationale for these studies to serve as quantifiable outcome measures in primate spinal cord injury and repair models. Detailed analyses of signal processes here have allowed for both amplitude and frequency based quantitative studies.

We are also preparing a manuscript that demonstrates feasibility of performing comprehensive urodynamic studies in non-human primates, and 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.

During year 3, we presented at two national scientific meetings:

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.

During year 3, we published one paper with a number of other papers in preparation.

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.

CONCLUSION

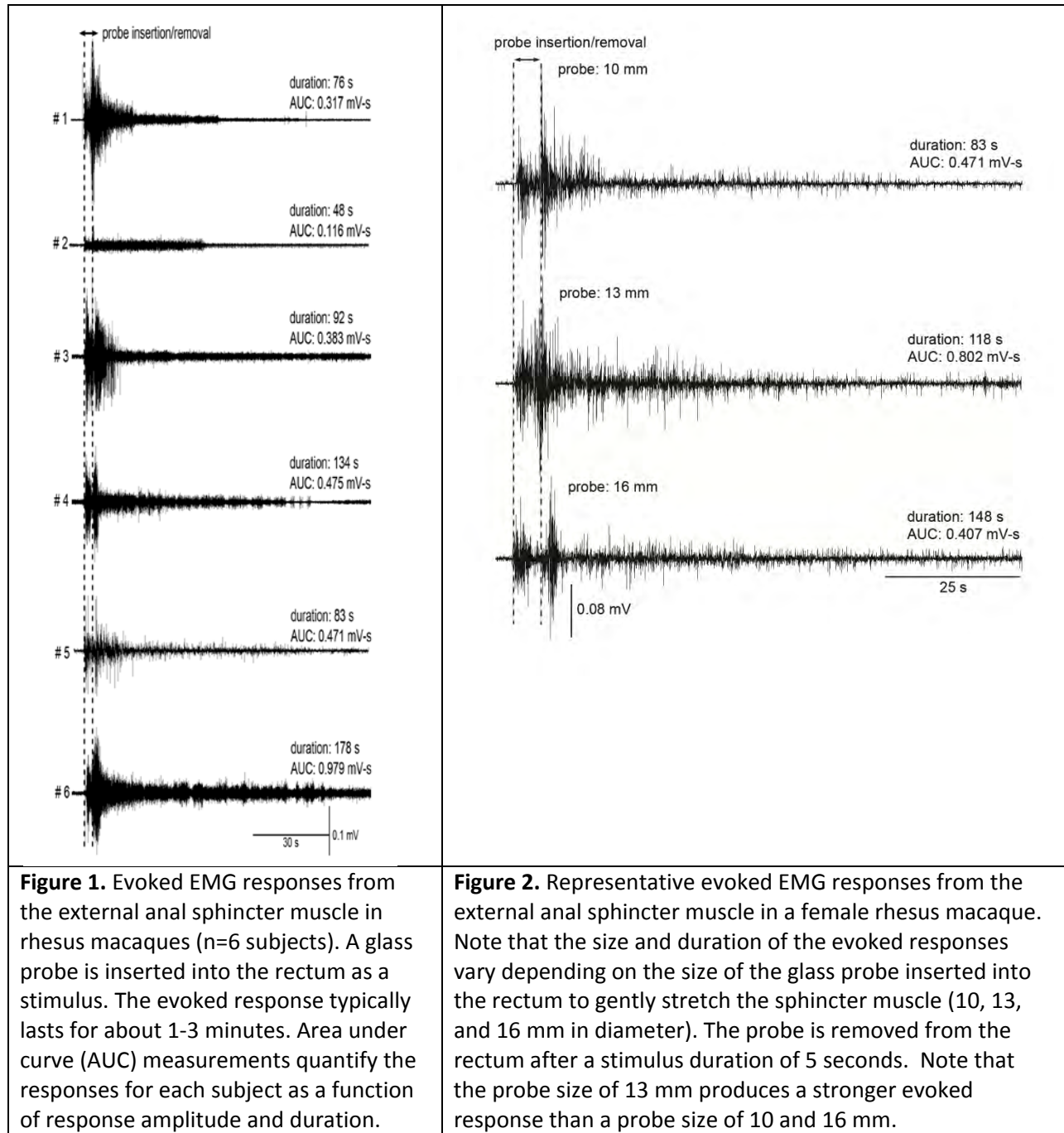
Our studies have made significant progress and the data collected is still being analyzed for more information. We developed an algorithm for pre-surgical testing of nonhuman primates, including locomotor treadmill studies, pain behavioral assessments, urodynamic recordings, MRI studies, and anal sphincter EMG studies. We demonstrated feasibility of a lumbosacral ventral root avulsion procedure with animals recovering well after the surgical spine and spinal cord procedure. We also demonstrated the feasibility of using peripheral nerve grafts and GDNF-releasing nerve guide conduits to bridge tissue gaps between the spinal cord and avulsed ventral roots. All surgeries for the studies (20 subjects) were completed in year 2. During year 3, we continued our ongoing collection of comprehensive functional and imaging data for the planned study time of 18 months for each subject. The data collection will be completed by March, 2014, and ongoing data analysis of collected and incoming behavioral, physiological, imaging, and anatomical data will be prepared for manuscripts including both control physiologic recordings in the Spring, 2014. Manuscripts that include new data collected from both control subjects and post-operative data sets are presently being prepared for manuscripts, which are aimed for submission to peer-reviewed journals, also in 2014.

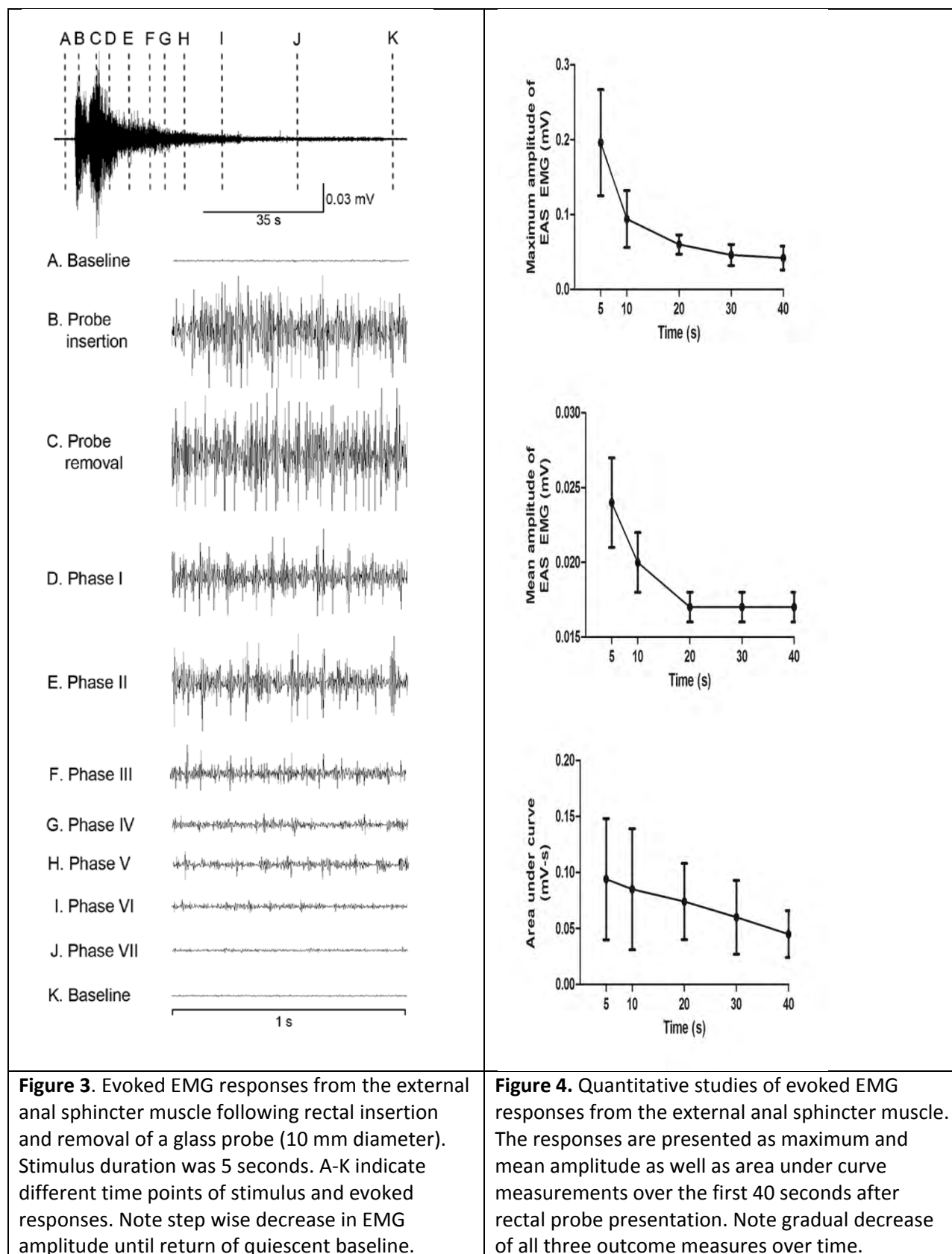
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SUPPORTIVE DATA





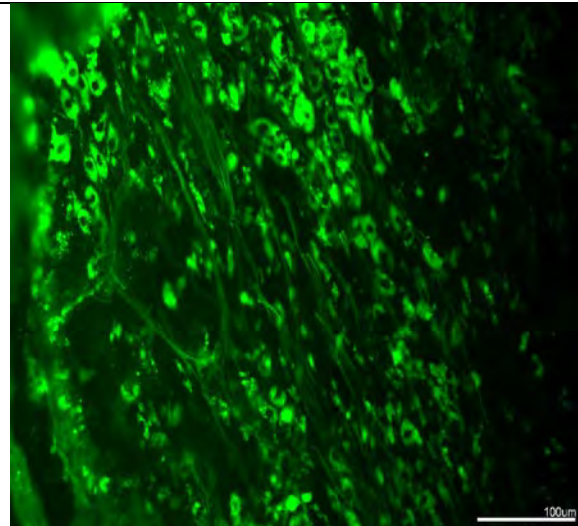
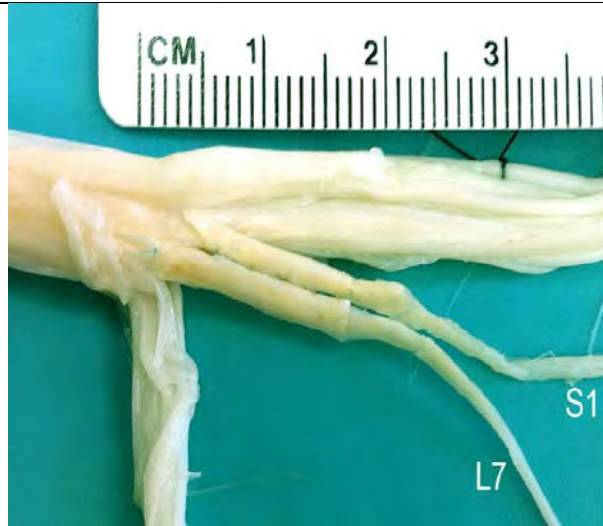


Figure 5. Anatomical studies of GDNF-releasing nerve guidance channels (NGCs) at 2 months after an L6-S3 ventral root avulsion injury and surgical placement of the GDNF-releasing NGCs as a bridge between the spinal cord and avulsed L6 and L7 ventral roots. Note intact placement of NGCs between the spinal cord and avulsed roots that were grafted to the NGCs (upper left). Immunohistochemistry for Beta-III-tubulin (upper and lower right) shows regenerating axons within the NGCs near the inter-phase between the spinal cord and the NGC. These studies suggest that regenerating axons in the spinal cord may enter GDNF-releasing NGCs in the non-human primate.

