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EMG Characteristics of the External Anal Sphincter Guarding Reflex and Effects of a Unilateral Ventral Root Avulsion Injury in Rhesus Macaques (*Macaca mulatta*)

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ABSTRACT

The external anal sphincter (EAS) is important for the maintenance of bowel continence and may be compromised by a variety of neuropathic conditions. However, large animal models for the study of EAS functions have been sparse. The EAS guarding reflex was examined by electromyography (EMG) in neurologically intact rhesus macaques (n=6) and at 4-6 weeks after a unilateral EAS denervation from an L6-S3 ventral root avulsion (VRA) injury (n=6). Baseline EAS EMG recordings were quiescent in all subjects, and evoked responses showed an initial large amplitude EMG activity, which gradually returned to baseline within 1-2 minutes. At 4-6 weeks post-operatively, the EAS guarding reflex showed a significantly reduced EMG response duration of 47±15 seconds and area under the curve (AUC) of 0.198±0.097 mV-s compared to the corresponding evoked EAS EMG duration of 102±19 seconds and AUC of 0.803±0.225 mV-s ($p<0.05$) in the control group. Detailed time and frequency domain analysis of the evoked EAS EMG responses for the first 40 seconds showed no difference between groups for the maximum amplitude but a significant decrease for the mean amplitude across the study period and an early AUC reduction for the first 10 seconds in the VRA injury group. Time-frequency analysis and power spectrum plots indicated decreased intensity and a narrower mid-range of frequencies in the VRA injury group. We conclude that the EAS guarding reflex in rhesus macaques shows characteristic EMG features in control subjects and signs of partial target denervation after a unilateral L6-S3 VRA injury.

Key words: Non-human primate; pelvic floor; electromyography; cauda equina injury; partial denervation

New and Noteworthy

The external anal sphincter (EAS) guarding reflex showed initial large amplitude peaks and a gradual return to a quiescent baseline after a rectal probe stimulus in rhesus macaques. At 4-6 weeks after a unilateral ventral root avulsion (VRA) injury, the EMG duration, mean amplitude, and area under curve measurements were decreased. Time frequency analysis and power spectrum plots indicated decreased intensity and a narrowed mid-range of frequencies in the VRA injury cohort.

INTRODUCTION

The pelvic floor diaphragm and external anal sphincter (EAS) provide anatomical support for the abdominal viscera, including the rectum, and an EAS constrictor function contributes with an important mechanism for continence to prevent accidental leakage of intestinal contents (Raizada and Mittal, 2008). The external anal sphincter (EAS) consists of a circular skeletal muscle, is normally in a constricted state, and receives somatic motor innervation by the pudendal nerve in higher primates (Sherrington, 1892; VanderHorst et al., 2000; Wunderlich and Swash, 1983). Bowel dysfunction with incontinence may result from a partial or complete EAS denervation, which may be caused by for instance, a thoracolumbar spine trauma with crush injury to the conus medullaris portion of the spinal cord, a cauda equina injury after compression, transection, or avulsion of lumbosacral nerve roots, or child-birth related injuries to the pudendal nerve (Park et al., 2016; Podnar, 2006; Rao, 2004). However, large animal models for the functional assessment of the EAS in the setting of lower motor neuron injuries have been sparse.

Previous electromyography (EMG) studies of the EAS in both humans and experimental models have shown the presence of tonic discharges at rest, and that the baseline rectal tone may be subject to modulation. For instance, the resting EAS tonic activity may be inhibited by distending the colon with an inflated balloon in cats (Bishop et al., 1956; Bishop, 1959; Dubrovsky, 1988). In contrast, augmented responses with increased EAS EMG activity may be induced in the cat by tactile stimulation of the circumanal region, compression of the abdomen, or the introduction of a rectal probe into the anus with activation of the guarding reflex response (Bishop, 1959). A similar augmentation of EAS EMG activity has been demonstrated in the rat following a rectal probe insertion (Holmes et al., 1998). Human subjects also show a reflex activation of the EAS muscle in response to inflation of the rectum (Denny-Brown and Robertson, 1935; Arhan et al., 1976; Vilensky et al., 2004). However, some caution is needed when directly comparing functional features of pelvic floor muscles between animal species, even between different primate species, as the physiological demands on the EAS and pelvic floor may vary greatly as a result of differences in e.g. posture, locomotion, and diet (Dubrovsky and Filipini, 1990; Fooden, 2000).

Macaque monkeys represent the most commonly used non-human primate model in biomedical research (Carlsson et al., 2004; Lankau et al., 2014), but functional studies of its pelvic floor and EAS have been in short supply. Therefore, a first goal for the present study was to characterize

EMG activity associated with the EAS guarding reflex in rhesus macaques. A second goal was to determine the effects of a unilateral avulsion injury of lumbosacral ventral roots in rhesus macaques on evoked EAS EMG activity as a potential model system for translational studies on partial denervation of pudendal nerve-innervated targets.

METHODS

Research subjects. A total of 10 female rhesus macaques *(Macaca mulatta)* were included in the study (Table 1). All subjects were adults, in good health, and without any history of neurological injury or impairments. The animals were divided into two study groups. One cohort of 6 subjects were neurologically intact and served as a control group. A total of 6 animals served as an experimental study group at 4-6 weeks after a unilateral lumbosacral ventral root avulsion (VRA) injury. Pre- and post-operative recordings from two of the animals contributed to both study groups. All animal procedures and study protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California at Davis and by the United States Department of Defense.

All subjects were housed and all experimental procedures performed at the California National Primate Research Center (CNPRC), a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal care was performed in compliance with the *Guide for the Care and Use of Laboratory Animals* provided by the Institute for Laboratory Animal Research (2011). The animals were housed indoor in stainless steel cages (Lab Product, Inc., Seaford, DE) and were exposed to a 12 hour light/dark cycle. Paired housing was attempted for all animals. The room temperature was maintained at 65-75°F, and the room humidity ranged between 30-70%. All subjects had free access to water and received commercial chow (High protein diet, Ralston Purina Co, St. Louis, MO) and fresh fruit supplements. The animals were fasted overnight before spine surgery and before EAS electromyography studies.

Surgical procedures. All spine surgery subjects (n=6) underwent an elective pre-operative and magnetic resonance imaging (MRI) study of the thoracolumbar spine under ketamine sedation (10 mg/kg IM) (Ohlsson et al., 2017). The imaging studies were performed to visualize the relationship between the lumbosacral spinal cord and the vertebral column. On the day of surgery, each subject was initially sedated using ketamine (10 mg/kg IM) and an endotracheal tube was placed. The spinous processes of the thoracolumbar spine were palpated and identified using surface anatomy landmarks. The identification of individual vertebral levels was

supported by a subsequent radiographic series in anteroposterior and lateral views. The subsequent spine surgery followed our established surgical procedures for lumbosacral ventral root dissections and injury (Ohlsson et al., 2013; Nieto et al., 2018). Each subject was next placed under a surgical plane of anesthesia provided by 1-2% isoflurane in O_2 via endotracheal tube and fentanyl (7-10 µg/kg/min IV). A skin incision was placed along the L1-L5 spinous processes and the fascia cut on the left side. The para-spinous muscles were dissected free on the left side to expose the dorsal surface of the lumbar spine, laminae, pedicles, and facet joints. A left-sided laminectomy from the caudal surface of the L1 vertebra to the rostral part of the L3 vertebra using rongeurs and a high-speed diamond-bit drill. The dura mater was opened and the lumbosacral dorsal roots were moved to the midline to expose the spinal cord and to visualize the ventral roots exiting from the ventral surface of the spinal cord. The L6-S3 ventral roots were identified based on their relationships to anatomical landmarks and characteristic caliber differences. The left L6-S3 ventral roots were next avulsed from the surface of the lumbosacral spinal cord by using a pair of fine forceps and gentle traction along the normal course for each root. The avulsed roots were deflected from the spinal cord, the dura mater closed using a continuous 6-0 Ethilon® suture, the paraspinous muscles and fascia closed in layers, and the skin closed using 4-0 Vicryl® sutures. All subjects recovered well after the procedures and received oxymorphine (0.15 mg/kg IM TID X 3 days) and ketoprofen (5 mg/kg IM daily X 5 days) for post-operative pain control as well as cefazolin 25 mg/kg IM BID X 5 days) intra-operatively and post-operatively as prophylactic treatment for infection.

EAS Electromyography. All subjects (n=12) were initially immobilized by an intramuscular injection of ketamine (approximately 10 mg/kg) followed by the placement of a peripheral intravenous access, and administration of ketamine by constant rate infusion (CRI) at approximately 12 mg/kg/hr. The CRI dose of ketamine was subsequently adjusted to maintain a light and stable plane of anesthesia and immobilization of each subject during the electrode placement and recording procedures.

Paired 22 gauge, 2"/51 mm length, Large Hub Removable Needles, (Hamilton Company, Reno, NV) were used as bipolar electrodes and inserted into the left side of the EAS muscle. A separate ground electrode was placed into the gastrocnemius muscle of the left hind limb. The electrodes were next connected to a data acquisition system (MP150, Biopac Systems, Inc., Goleta, CA) equipped with the EMG amplifiers (Biopac Systems, Inc). An analog notch filter was used at 60 Hz to remove the powerline noise. In addition, the analog band pass filter was used between 10 and 500 Hz to remove the low-frequency and high-frequency noise. The high-pass

filtering was performed in attempts to remove movement artifacts, which may occur mostly as low-frequency components, whereas the low-pass filtering was performed to remove high frequency noise not originating from the EMG signal and to avoid aliasing (Gerdle et al., 1999; De Luca et al., 2010). Based on the Nyquist rate Sampling Theorem, postulating that data should be sampled with at least 2 points of the highest frequency, the sampling rate for EAS EMG activity was set at 1k Hz and digitized data were stored on a computer for analysis.

Baseline and evoked EAS EMG activity was recorded with each animal in prone position. For the evoked EAS EMG recordings, a lubricated glass probe, with a 10, 13, or 16 mm outer diameter, was inserted about 10-15 mm into the rectal opening to provide a gentle distension of the EAS. The probe was 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 was recorded and allowed to return to baseline levels before another attempt was made to activate this reflex response. At least three consecutive trials were performed and EAS EMG recordings collected. All subjects tolerated the procedure well and received a single dose of ketoprofen (5 mg/kg IM) upon recovery as discomfort prevention.

Data analysis. Time-frequency analysis of EAS EMG power was assessed using the zero-interval subtraction algorithm (Marchenko and Rogers, 2006), which uses a sliding zeroed segment to generate a series of fast Fourier transforms (FFTs) to calculate their difference spectra for adjacent time intervals. The zero-interval subtraction algorithm was chosen based on its speed and ability to more accurately calculate time-varying power at lower frequencies compared with parametric FFTs. Obtained time-frequency analysis were visualized in MATLAB® (The MathWorks, Inc., MA, USA) using the isocontour plots. A customized program was created and integrated by using Graphical User Interface (MATLAB®, The MathWorks, Inc., Natick, MA) in order to quickly analyze recordings and time-frequency analysis. The program had the ability to separate user-inputted time intervals from the whole data obtained by AcKnowledge (Biopac Systems, Inc.) to analyze. Also added to the program were the mean and maximum amplitude, and area under the curve (AUC) measurements. The signal was rectified for the amplitude measurements and AUC calculations. For the time-frequency analysis program, FFTs were performed using the inherent MATLAB functions to calculate the spectral density. Before the FFTs, however, the data were ran through digital notch filters at 180, 300, and 360 Hz to remove the harmonics of 60 Hz powerline noise.

Termination of experiments

All post-operative animals underwent intravascular perfusion using a paraformaldehyde solution at the end of the experimental studies, and the spine was harvested. Careful dissection of the spine allowed for the identification of avulsed ventral roots and validation of segmental level of injury and completeness of the VRA procedure.

Statistical analysis. Quantitative data are expressed as mean ± standard error. Time domain outcome measures included maximum and mean amplitude, and frequency domain outcome measures included peak, mean, and median frequencies. The peak frequency corresponded to the frequency of greatest intensity based on the power spectrum studies. For all parameters, a total of three measurements from consecutive evoked responses were averaged to create a mean value for each animal. One-way ANOVA was first applied to detect any statistical significance between samples, and the Tukey's multiple comparison tests were next used for comparisons among different time points by using Prism 4.0 (GraphPad Software, Inc, San Diego, CA). We regarded $p < 0.05$ to indicate a statistically significant difference between the experimental groups.

RESULTS

Electromyographic (EMG) recordings were obtained from the external anal sphincter (EAS) in neurologically intact rhesus macaques (n=6) and at 4-6 weeks after a unilateral L6-S3 VRA injury (n=6) (Table 1). Both baseline EMG recordings and responses following the activation of the EAS guarding reflex were studied in both groups. All subjects were female and the recordings were performed under a light sedating plane of ketamine anesthesia.

EAS EMG recordings from neurologically intact rhesus macaques

EMG recordings from the EAS in all neurologically intact subjects (n=6) showed a quiescent baseline at rest and an evoked response to the activation of the EAS guarding reflex (Figure 1). Specifically, a rectal probe with a diameter of 10 mm was inserted for 5 seconds to produce a brief and moderate distension of the EAS. Each rectal probe insertion resulted in an immediate and high amplitude EMG response. The probe removal resulted in a second high amplitude response followed by a gradual return of the evoked response to a quiescent baseline over 1-3 minutes. The amplitude reduction took place in a visually detectable and step-like pattern with a relatively shorter duration for each step of amplitude decline during the early portion of the evoked response and was followed by a low amplitude tail of longer duration before the end of the evoked response (Figure 1). Repeated probe stimulations resulted in highly reproducible responses (Figure 2).

To determine whether the magnitude of the evoked EAS EMG response may be influenced by the rectal probe size, comparative testing was performed in individual subjects using probes with an outside diameter of 10, 13, and 16 mm. The inner circumference of the rectal sphincter varied between subjects, and the use of larger sized probes was omitted in smaller subjects. Evoked EAS EMG activity was demonstrated in response to the insertion of rectal probes across a wide caliber range (Figure 2). The qualitative pattern of the evoked responses remained consistent between the different probe sizes, but the duration and/or AUC measurements may decline in response to a rectal distension stimulus provided by a larger probe, suggesting a non-linear relationship between the probe size and rectal distension-evoked EAS EMG response. As all subjects demonstrated evoked EAS EMG activity in response to the smallest probe used for testing, the 10 mm rectal probe size was used for all subsequent quantitative studies.

Effects of VRA Injury on EAS guarding reflex

To investigate the effects of a partial EAS denervation in rhesus macaques, EAS EMG recordings were compared between neurologically intact subjects (n=6) and at 4-6 weeks after a unilateral L6-S3 VRA injury (n=6) (Figure 3). A rectal probe was used to provide a gentle stretch of the EAS for 5 seconds and served as a stimulus to evoke the EAS guarding reflex.

At 4-6 weeks post-operatively, the EAS guarding reflex showed a significantly reduced EMG response duration of 47 ± 15 seconds and area under curve (AUC) of 0.198 ± 0.097 mV-s compared to the corresponding evoked EAS EMG duration of 102 ± 19 seconds and AUC of 0.803 ± 0.225 mV-s ($p<0.05$) in the control group (Figure 4). In both cohorts, most of the evoked EAS EMG activity took place during the early stages of the response periods. Therefore, a combination of time and frequency domain studies were next performed during the first 40 seconds of the evoked responses in all animals (Figure 5).

In the control group, the maximum EMG amplitude was 0.154 ± 0.016 mV at 0-5 seconds after the activation of the EAS guarding reflex, and the maximum amplitude had decreased significantly at 5-10 seconds ($P<0.05$) and at 10-20, 20-30, and 30-40 seconds ($P<0.001$) after the stimulus onset. However, there was no difference in the EAS EMG maximum amplitude between the control and VRA groups at any of the time points.

In the control group, the mean EMG amplitude was 0.023 ± 0.002 mV at 5 seconds after the EAS EMG activation, and it was significantly decreased at 10 seconds ($P<0.01$) and at 20, 30, and 40 seconds ($P<0.001$) after the stimulus onset. The mean EAS EMG amplitude was significantly reduced in the VRA group compared to controls at 5, 10, 20, 30, and 40 seconds after the EAS EMG activation ($P<0.01$).

In the control group, the area under the curve (AUC) measurement was 0.194 ± 0.040 mV-s at 10 seconds after the EAS EMG activation, and it was significantly reduced at 20, 30, and 40 seconds ($P<0.01$) after the reflex activation. The AUC measurement at 10 seconds was significantly reduced in the VRA group compared to the control group, but there was no difference between the groups at 20, 30, and 40 seconds after the EAS EMG activation.

In the control group, the peak, mean, and median frequencies were 130 ± 10 Hz, 180 ± 7 Hz, and 161 ± 8 Hz, respectively, at 0-5 seconds after the EAS EMG activation, and they remained

unchanged at 5-10, 10-20, 20-30, and 30-40 seconds after the stimulus onset. When comparing the corresponding frequency domain data between the control and post-operative groups, there were no differences for the peak and median frequencies between the two cohorts at any of the time points. The mean frequencies at 10-20 and 20-30 seconds after the stimulus onset were significantly reduced in the VRA group compared to controls.

Time-frequency analysis and assessments of evoked EAS EMG power were performed to determine which different frequencies were present at different time points during the first 40 seconds of the evoked EAS EMG responses in both the control and experimental groups (Figure 6). The resultant power spectrum plots were next compared to the corresponding evoked EAS EMG recordings for the same time period in each subject. The power spectrum analysis showed that frequencies ranged from below 100 Hz to over 350 Hz during the recordings with the broadest range of frequencies present during the first 5-10 seconds. In all subjects, the highest intensity of spectral analysis was evident during the first 5 or 10 seconds of the recordings, corresponding to the insertion and removal of the rectal probe and the associated high amplitude EAS EMG responses. Compared to the control group, the subjects of the VRA injury series showed frequencies that were more centered in the mid-range of the spectrum and an overall decreased intensity of their spectral analysis.

DISCUSSION

The EAS guarding reflex was studied in neurologically intact rhesus macaques and after a unilateral L6-S3 VRA injury. A characteristic and robust time and frequency domain response to the insertion and removal of a rectal probe was demonstrated. Residual EMG activity ipsilaterally to the VRA injury provide support to the notion of the EAS being innervated by the bilateral pudendal nerves. A marked reduction of the evoked EMG activity after a unilateral VRA injury is consistent with a partial denervation of the EAS response.

Baseline and evoked EAS EMG activity

Continuous contractile activity at rest has been demonstrated in the EAS muscle in many species. In humans, resting EAS EMG activity is present during awake and sleep states (Kawakami, 1954; Podnar and Vodusek, 2000; Podnar et al., 2000, 2002). Tonic and persistent discharges have also been demonstrated during EMG recordings from the EAS in decerebrate cats (Bishop, 1959; Bishop et al., 1956). In rats, spontaneous EMG activity was detected in the EAS for only a subset of neurologically intact subjects, which had recovered from a combination of ketamine and xylazine anesthesia used for EMG electrode placement, with the baseline muscular tone of the sphincter being sufficient to keep the anal orifice closed (Holmes et al., 1998). In the present study, all rhesus macaques were lightly sedated by ketamine, and no spontaneous EAS EMG activity was present during baseline recordings in any of the subjects. The utility of EAS EMG recordings for the identification and mechanistic studies of spinal cord injury-induced EAS hyperreflexia was first demonstrated in the rat model (Holmes et al., 1998, 2005). The present studies introduce a motor neuron injury model in the non-human primate and provide additional support for the notion that EAS function may be evaluated in experimental models using evoked EMG responses. Prior studies have also demonstrated that other stimuli, including a sensory stimulus to the perianal area may evoke the guarding reflex Dubrovsky and Filipini, 1990). Care was taken not to include other triggers of EAS EMG activation outside of the rectal probe in the present studies.

It is possible that the use of anesthesia may influence EAS EMG recordings in non-human primates. Previous studies have demonstrated that the use of anesthesia choice, depth, and delivery method may influence physiological studies, including EMG recordings in experimental models. For instance, a variety of anesthetic agents have been evaluated in studies of pelvic

functions in rats and shown to suppress both bladder contractions and EMG activity detected in the external urethral sphincter (Matsuura and Downie, 2000; Cannon and Damaser, 2001; Chang and Havton, 2008). The ano-anal reflex function is similarly affected by several different anesthetic agents, including ketamine/xylazine, urethane, and chloral hydrate, as demonstrated in studies of EAS hyperreflexia in spinally transected rats (Holmes et al., 1998). For the present study, we used a ketamine CRI protocol to immobilize and provide a stable plane of light sedation of each subject for the EAS EMG studies. Ketamine is well tolerated by rhesus macaques and is the most commonly used anesthetic in nonhuman primates for brief clinical procedures and physiologic studies (Steelman et al., 1991; Ghoniem et al., 1996; Lee et al., 2010; Christe et al., 2013). Although baseline EAS EMG activity was not detectable in the present study, evoked EMG activity was readily evoked in all subjects.

The magnitude of evoked responses by skeletal muscles may also depend on the degree of rectal distension. The output can in experimental situations be estimated in the form of generated force or EMG activity. A comprehensive review on the relationship between sarcomere length and relative muscle force in several different muscles and across multiple species has suggested that many muscles normally operate over a relatively narrow sarcomere length and that the operating range may different between muscles carrying out different functions (Burkholder and Lieber, 2001). The normal operational length for a muscle may also be different from the length at which it generates the most force, the optimal length, as has been suggested by studies of length-tension relationships of the EAS in cats (Krier et al., 1989). In combined in vitro and in vivo studies in the rabbit, it was demonstrated the insertion of a rectal probe of increasing size resulted in increased anal canal pressure and increased sarcomere length, suggesting that the operational length for the EAS sarcomeres is significantly shorter than its optimal length (Rajasekaran et al., 2008). In subsequent studies on length-tension relationships for the EAS in humans, it was similarly demonstrated that anal canal stress increased with increasing rectal probe size, whereas EAS EMG activity did not change (Mittal et al., 2011). However, as a robust activation of the guarding reflex was obtained in all subjects using a 10 mm in diameter probe, quantitative EMG studies were performed based on the responses to this probe size. Interestingly, the present study showed two amplitude peaks representing probe insertion and removal, suggesting that distension and relaxation may both serve as a stimulus for increased EAS EMG activity. A similar reflex response with two peaks of activation has previously been demonstrated during EMG studies in human subjects with evoked EAS muscle contractions noted during both rectal inflation and deflation (Shafik, 1997).

However, an alternative possibility for the observed variation in EAS EMG response to probes of different size is that the EAS guarding reflex is under increased inhibition under conditions of greater rectal distension.

Effects of a unilateral VRA injury on EAS EMG activity

In the present study, a unilateral avulsion injury of lumbosacral ventral roots resulted in a markedly decreased, but not absent, ipsilateral EMG activation of the EAS guarding reflex. The residual EMG responses may be explained by an incomplete injury to the ipsilateral motor axons innervating the EAS or by the presence of bilateral pudendal nerve innervation of the EAS. Although both the control and experimental groups were within the adult age range, the injured cohort showed a significantly higher body weight and number of live births. However, there was no difference in BCS between the groups, suggesting that there was no difference in body fitness between the groups. The number of prior conceptions were not different between the groups. A possible contribution by these demographic differences cannot be ruled out and efforts for future studies will aim at randomizing subjects based on multiple aspects of subject demographics.

Rhesus macaques show seven lumbar vertebrae and corresponding spinal cord segments, but about 20% of rhesus macaques demonstrate a set of supernumerary ribs attached to the L1 vertebra (Ohlsson et al., 2017), and motoneurons contributing to the pudendal nerve typically reside in the lower lumbar and upper sacral spinal cord (Akita et al., 1995; VanderHorst et al., 2000). For instance, EAS contractions took place in response to stimulation of the cut ends of the L7, S1, and S2 ventral roots in rhesus macaques (Sherrington, 1892), and stimulation of the EAS-innervating branch of the pudendal nerve resulted in evoked response in primarily the L7-S2 ventral roots and a rare response in the L6 ventral root of rhesus macaques (Rockswold et al., 1980). These functional mapping reports have been supported by subsequent anatomical studies. The somata of retrogradely labeled motoneurons innervating the pudendal nerve in male and female macaque monkeys were detected in the ventral horn of the L7 and S1 segments with rare labeled cells encountered in the S2 segment (Roppolo et al., 1985; Ueyama et al., 1985). It would therefore be expected that the unilateral L6-S3 VRA injury performed in the present study will sever the axons of all EAS-innervating motoneurons, and that the residual EAS EMG activity on the ipsilateral side of the injury is provided by motoneurons on the contralateral side of the spinal cord and with peripheral axons crossing the midline in the EAS

muscle. However, a component of sprouting with spread of motor axons to the denervated EAS areas cannot be excluded at 4-6 weeks after the ipsilateral VRA injury in the present studies.

In order for a complete unilateral denervation of the EAS to take place, it is critical that the correct ventral roots were identified during surgery. In order to identify the correct lumbosacral ventral roots intro-operatively, despite normal anatomical variation between subjects with regards to the relationship between the lumbar spine and the lumbosacral spinal cord segments, several measures were taken. Pre-operatively, all subjects underwent radiographic and MRI imaging of the spine to identify the L6-S3 spinal cord segments and their relationship to the spine (Ohlsson et al., 2017). Intra-operatively, characteristic caliber differences between the L6-S3 ventral roots were also taken into consideration in determining segmental levels (Ohlsson et al., 2013; Nieto et al., 2018). At the end of the experiments, the spine was harvested and anatomical studies validated the anatomical levels and completeness of injury.

A bilateral pattern of EAS innervation has also been proposed in prior studies of large mammals. Early physiologic studies in rhesus macaques showed bilateral EAS muscle contractions in response to unilateral stimulation of lumbosacral nerves (Sherrington, 1892). In the cat, EMG activity was similarly detected on both sides of the EAS following unilateral stimulation of the pudendal nerve (Bishop, 1959). Anatomical studies of the EAS muscle has provided additional support for the notion that each pudendal nerve contributes with innervation to both sides of the sphincter. Specifically, histological analysis of the EAS showed denervation atrophy of skeletal muscle fibers and a mixed pattern of normal and degenerated intramuscular nerve fibers on both sides of the sphincter after a unilateral transection injury of the pudendal nerve in macaque monkeys (Wunderlich and Swash, 1983).

Comparisons between the control and experimental groups showed a significant reduction in select time domain parameters, including overall duration and AUC measurements, after a unilateral VRA injury. In contrast, frequency domain parameters, including peak, mean, and median frequencies remained stable in the experimental group. However, time-frequency analysis of evoked EAS EMG activity in all subjects showed both similarities and differences between the control and experimental groups. All subjects showed a differential pattern of signal intensity. Specifically, there was a wide range of frequencies and a high intensity of firing associated with the rectal probe insertion and removal as was supported by power spectrum plots. Later phases of the evoked EAS EMG recovery showed a more restricted and mid-range of frequencies to appear most prevalent. Although the frequency domain analysis showed no

change in peak, median and mean frequencies after the unilateral VRA injury, the frequency variability was reduced as demonstrated also by the power spectrum plots. Increased EMG synchronization after VRA injury may be a consequence of reduced neuromuscular jitter (Stålberg, 2012). The present time-frequency analysis support the notion that this approach may be a useful tool to identify major frequency bands and their relationship to various stimuli and physiological response patterns, as has been suggested in prior studies on, for instance, pelvic-to-pudendal nerve and pudendo-to-pudendal nerve reflexes in rats (Chang et al., 2004) and electrocorticogram recordings in a reversible cortical inactivation model for assessments of bladder hyperreflexia in cats (Pikov and McCreery, 2009). Although the unilateral VRA injury did not result in a compromise in continence, the detected VRA-induced changes in both time and frequency domain parameters suggest identification of physiologic signatures that may serve as diagnostic biomarkers to identify partial denervation of the EAS.

Conclusions

We conclude that the evoked EAS guarding reflex may be recorded under a light and stable plane of ketamine anesthesia and shows a characteristic EMG activation pattern in adult rhesus macaques. A unilateral avulsion injury of the L6-S3 ventral roots resulted in a markedly reduced EAS guarding reflex on the ipsilateral side at 4-6 weeks after the injury. The presence of residual EAS EMG activity on the ipsilateral side of the injury was consistent with EAS innervation by the bilateral pudendal nerves. Our findings support the use of a unilateral lumbosacral VRA injury as a model system for partial denervation studies of pudendal nerve-innervated target tissues.

Author contributions

All authors contributed significantly to the manuscript. Designed studies: HHC and LAH. Performed experiments: JHN, KLC and LAH. Analyzed and interpreted data: HHC, UL, TV, VP, and LAH. Prepared figures and manuscript: HHC and LAH. Edited and revised manuscript: HHC, UL, TV, VP, JHN, KLC, and LAH. All authors approved the final version of the manuscript.

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Competing interest

None of the authors have any competing interests with this study.

FIGURE LEGENDS

Figure 1. Representative tracing of evoked EAS EMG activity. Following the insertion and removal of a rectal probe (10 mm outer diameter), EAS EMG activity was evoked. Two peaks of large amplitude EMG activity were present at the beginning of the tracing and corresponded to the probe stimulus and removal. The amplitude of the evoked response subsequently followed a step-like decrease over time until a quiescent baseline was re-established. Each step was identified visually by a sharp shift to a lower amplitude plane. A-K indicate the different components and phases of a typical EAS EMG response to the probe stimulus.

Figure 2. A. Evoked EAS EMG activity using rectal probes of different sizes. Rectal probes with an outside diameter of 10, 13, and 16 mm were used to evoke EAS EMG responses. Probe size may influence response duration and AUC measurements. B. Representative example of three consecutive evoked responses in same subject using a 10 mm probe size. Note similar duration and AUC measurements for all three responses.

Figure 3. Evoked EAS EMG activity in neurologically intact rhesus macaques (n=6) and at 4-6 weeks after a unilateral L6-S3 VRA injury on the ipsilateral side (n=6). A rectal probe was used to activate the EAS guarding reflex. Note a markedly decreased response duration and mean amplitude for the VRA series.

Figure 4. Comparison of EAS EMG response duration and area under curve (AUC) measurements between neurologically intact rhesus macaques (n=6) and in subjects after an L6-S3 unilateral VRA injury (n=6). At 4-6 weeks post-operatively, the EAS guarding reflex showed a significantly reduced EMG response duration of 47 ± 15 seconds and area under curve (AUC) of 0.198 ± 0.097 mV-s compared to the corresponding evoked EAS EMG duration of 102 ± 19 seconds and AUC of 0.803 ± 0.225 mV-s ($p<0.05$) in the control group. The guarding reflex was activated by a 10 mm in diameter rectal probe. The AUC was calculated for the full duration of the evoked response. * indicates $p<0.05$.

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Figure 5. Time and frequency domain analysis of evoked EAS EMG activity in intact rhesus macaques and in subjects at 4-6 weeks after an L6-S3 unilateral VRA injury. Data were collected for the time periods of 0-5, 5-10, 10-20, 20-30, and 30-40 seconds after stimulus onset using a rectal probe to activate the EAS guarding reflex. The maximum and mean amplitudes and AUC measurements were most prominent at the onset of the recordings and decreased significantly over time in both control and experimental groups. In contrast, the peak, mean, and median frequencies remained without change across the studied time periods for each group. The symbols *, **, and *** indicate $p<0.05$, $p<0.01$, and $p<0.001$, respectively, for statistical comparisons between indicated time point and first time point for control subjects. The + symbol indicate a significant difference between control and experimental groups at indicated time point.

Figure 6. Time-frequency analysis and power spectrum plots for evoked EAS EMG activity in two representative rhesus macaques of the control series and two representative subjects of the VRA post-operative cohort. Each power spectrum plot is displayed with its corresponding tracing of an evoked EAS EMG recording. The highest intensity of signals and broadest range of frequencies were present during the early portion of each tracing, corresponding to the time of probe stimulation and probe removal. Note that the subjects of the VRA series show decreased intensity of responses and a narrower range of frequencies compared to the control subjects.

REFERENCES

Akita K, Sakamoto H, Sato T. Muscles of the pelvic outlet in the rhesus monkey (Macaca mulatta) with special reference to nerve supply. *Anat Rec* 241: 273-283, 1995.

Arhan P, Faverdin C, Persoz B, Devroede G, Dubois F, Dornic C, Pellerin D. Relationship between viscoelastic properties of the rectum and anal pressure in man. *J Appl Physiol* 41: 677-682, 1976.

Bishop B. Reflex activity of external anal sphincter of cat. *J Neurophysiol* 22: 679-692, 1959.

Bishop B, Garry RC, Roberts TD, Todd JK. Control of the external sphincter of the anus in the cat. *J Physiol* 134: 229-240, 1956.

Burkholder TJ, Lieber RL. Sarcomere length operating range of vertebrate muscles during movement. *J Exp Biol* 204: 1529-1536, 2001.

Cannon TW, Damaser MS. Effects of anesthesia on cystometry and leak point pressure of the female rat. *Life Sci* 69: 1193-1202, 2001.

Carlsson HE, Schapiro SJ, Farah I, Hau J. Use of primates in research: a global overview. *Am J Primatol* 63: 225-237, 2004.

Chang HY, Havton LA. Differential effects of urethane and isoflurane on external urethral sphincter electromyography and cystometry in rats. *Am J Physiol Renal Physiol* 295: F1248-F1253, 2008.

Chang HY, Peng CW, Chen JJ, Cheng CL, de Groat WC. The time-frequency analysis of the pudenda-to-pudendal nerve and pelvic-to-pudendal nerve reflexes in anesthetized intact rats. *J Med Biol Eng* 24: 17-21, 2004.

Christe KL, Lee UJ, Lemoy MJ, Havton LA. Generalized seizure activity in an adult rhesus macaque (Macaca mulatta) during ketamine anesthesia and urodynamic studies. *Comp Med* 63: 445-447, 2013.

Clingerman KJ, Summers L. Validation of a body condition scoring system in rhesus macaques (Macaca mulatta): inter- and intrarater variability. *J Am Assoc Lab Anim Sci* 51: 31-36, 2012.

De Luca CJ, Gilmore LD, Kuznetsov M, Roy SH. Filtering the surface EG signal: Movement artifact and baseline noise contamination. J Biomech 43: 1573-1579, 2010.

Denny-Brown D, Robertson EG. An investigation of the nervous control of defecation. *Brain* 58: 256-307, 1935.

Dubrovsky B. Effects of rectal distension on the sphincter ani externus and levator ani muscles in cats. *Am J Physiol* 254: G100-G106, 1988.

Dubrovsky B, Filipini D. Neurobiological aspects of the pelvic floor muscles involved in defecation. *Neurosci Neurobehav Rev* 14: 157-168, 1990.

Fooden J. Systematic review of the rhesus macaque, *Macaca mulatta* (Zimmermann, 1780). Field Zool 96: 1-180, 2000.

Gerdle B, Karlsson S, Day S, Djupsjöbacka M. Acquisition, processing and analysis of the surface electromyogram. In: Modern techniques in neuroscience research, edited by Windhorst U, Johansson H. Berlin, Heidelberg, Germany: Springer-Verlag, Chapter 26, pages 705-755, 1999.

Ghoniem GM, Shoukry MS, Monga M. Effects of anesthesia on urodynamic studies in the primate model. *J Urol* 156: 233-236, 1996.

Holmes GM, Rogers RC, Bresnahan JC, Beattie MS. External anal sphincter hyperreflexia following spinal transection in the rat. *J Neurotrauma* 15: 451-457, 1998.

Holmes GM, Van Meter MJ, Beattie MS, Bresnahan JC. Serotonergic fiber sprouting to external anal sphincter motoneurons after spinal cord contusion. *Exp Neurol* 193: 29-42, 2005.

Institute for Laboratory Animal Research. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press, 2011.

Kawakami M. Electro-myographic investigation on the human external sphincter muscle of anus. *Jpn J Physiol* 4: 196-204, 1954.

Krier J, Meyer RA, Percy WH. Length-tension relationship of striated muscle of cat external anal sphincter. *Am J Physiol* 256: G773-G778, 1989.

Lankau EW, Turner PV, Mullan RJ, Galland GG. Use of nonhuman primates in research in North America. *J Am Assoc Lab Anim Sci* 53: 278-282, 2014.

Lee VK, Flynt KS, Haag LM, Taylor DK. Comparison of the effects of ketamine, ketamine-medetomidine, and ketamine-midazolam on physiologic parameters and anesthesia-induced stress in rhesus (Macaca mulatta) and cynomolgus (Macaca fascicularis) macaques. *J Am Assoc Lab Anim Sci* 49: 57-63, 2010.

Marchenko V, Rogers RF. Time-frequency coherence analysis of phrenic and hypoglossal activity in the decerebrate rat during eupnea, hyperpnea, and gasping. *Am J Physiol Regul Integr Comp Physiol* 291: R1430-R1442, 2006.

Matsuura S, Downie JW. Effect of anesthetics on reflex micturition in the chronic cannula-implanted rat. *Neurourol Urodyn* 19: 87-99, 2000.

Mittal RK, Sheean G, Padda BS, Lieber R, Raj Rajasekaran M. The external anal sphincter operates at short sarcomere length in humans. *Neurogastroenterol Motil* 23: 643-e258, 2011.

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. *Meth Neurotrauma Res*, Editors: Risling M, Danielsson S, 2018 (In Press).

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

Park SE, Elliott S, Noonan VK, Thorogood NP, Fallah N, Aludino A, Dvorak MF. Impact of bladder, bowel and sexual dysfunction on health status of people with thoracolumbar spinal cord injuries living in the community. *J Spinal Cord Med* 31: 1-12, 2016.

Pikov V, McCreery DB. Spinal hyperexcitability and bladder hyperreflexia during reversible frontal cortical inactivation induced by low-frequency electrical stimulation in the cat. *J Neurotrauma* 26: 109-119, 2009.

Podnar S. Bowel dysfunction in patients with cauda equina lesions. *Eur J Neurol* 13: 1112-1117, 2006.

Podnar S, Mrkaić M, Vodusek DB. Standardization of anal sphincter electromyography: quantification of continuous activity during relaxation. *Neurourol Urodyn* 21: 540-545, 2002.

Podnar S, Vodusek DB. Standardization of anal sphincter electromyography: uniformity of the muscle. *Muscle Nerve* 23: 122-125, 2000.

Podnar S, Vodusek DB, Stålberg E. Standardization of anal sphincter electromyography: normative data. *Clin Neurophysiol* 111: 2200-2207, 2000.

Rajasekaran MR, Jiang Y, Bhargava V, Littlefield R, Lee A, Lieber RL, Mittal RK. Length-tension relationship of the external anal sphincter muscle: implications for the anal canal function. *Am J Physiol Gastrointest Liver Physiol* 295: G367-G373, 2008.

Raizada V, Mittal RK. Pelvic floor anatomy and applied physiology. *Gastroenterol Clin North Am* 37: 493-509, 2008.

Rao SS. Pathophysiology of adult fecal incontinence. Gastroenterology 126: S14-22, 2004.

Rockswold GL, Bradley WE, Chou SN. Innervation of external urethral and external anal sphincters in higher primates. *J Comp Neurol* 193: 521-528, 1980.

Roppolo JR, Nadelhaft I, de Groat WC. The organization of pudendal motoneurons and primary afferent projections in the spinal cord of the rhesus monkey revealed by horseradish peroxidase. *J Comp Neurol* 234: 475-488, 1985.

Shafik A. Deflation reflex: description and clinical significance. *Anat Rec* 249: 405-408, 1997.

Sherrington CS. Notes on the Arrangement of some Motor Fibres in the Lumbo- Sacral Plexus. *J Physiol* 13: 621-772, 1892.

Stålberg EV. Quantifying normal and abnormal needle electromyography. In: Plenary I: How do we measure up? Quantitation in EDX and clinical practice, edited by Robinson LR, Cornblath DR, Stålberg EV. Rochester, MN: American Association of Neuromuscular & Electrodiagnostic Medicine, 2012.

Steelman R, Seale NS, Bellinger L, Harris M, Wagner M, Williams F. Conscious sedation and analgesia with rectal ketamine in the Macata fuscata monkey. *Anesth Prog* 38: 50-56, 1991.

Ueyama T, Mizuno N, Takahashi O, Nomura S, Arakawa H, Matsushima R. Central distribution of efferent and afferent components of the pudendal nerve in macaque monkeys. *J Comp Neurol* 232: 548-556, 1985.

Vanderhorst VG, Terasawa E, Ralston HJ, Holstege G. Monosynaptic projections from the nucleus retroambiguus to motoneurons supplying the abdominal wall, axial, hindlimb, and pelvic floor muscles in the female rhesus monkey. *J Comp Neurol* 424: 233-250, 2000.

Vilensky JA, Bell DR, Gilman S. "On the physiology of micturition" by Denny-Brown and Robertson: a classic paper revisited. *Urology* 64: 182-186, 2004.

Wunderlich M, Swash M. The overlapping innervation of the two sides of the external anal sphincter by the pudendal nerves. J Neurol Sci 59: 97-109, 1983.

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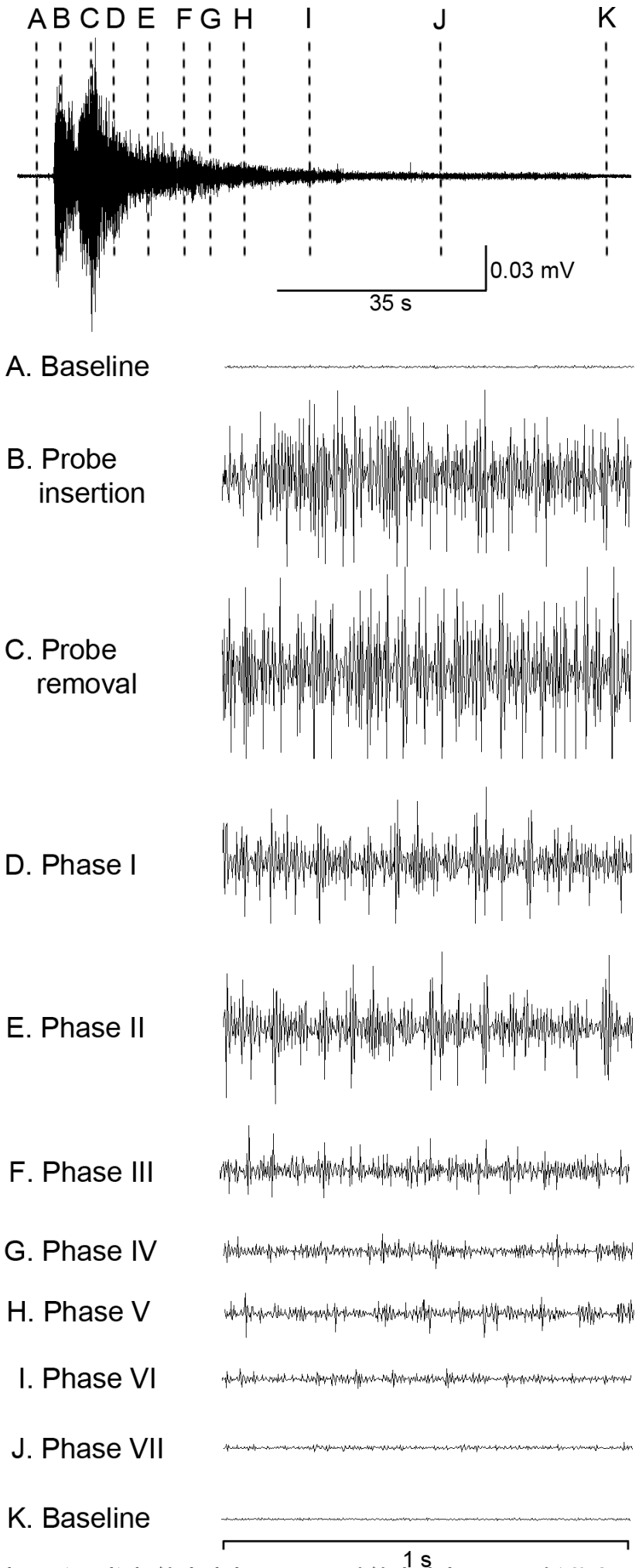

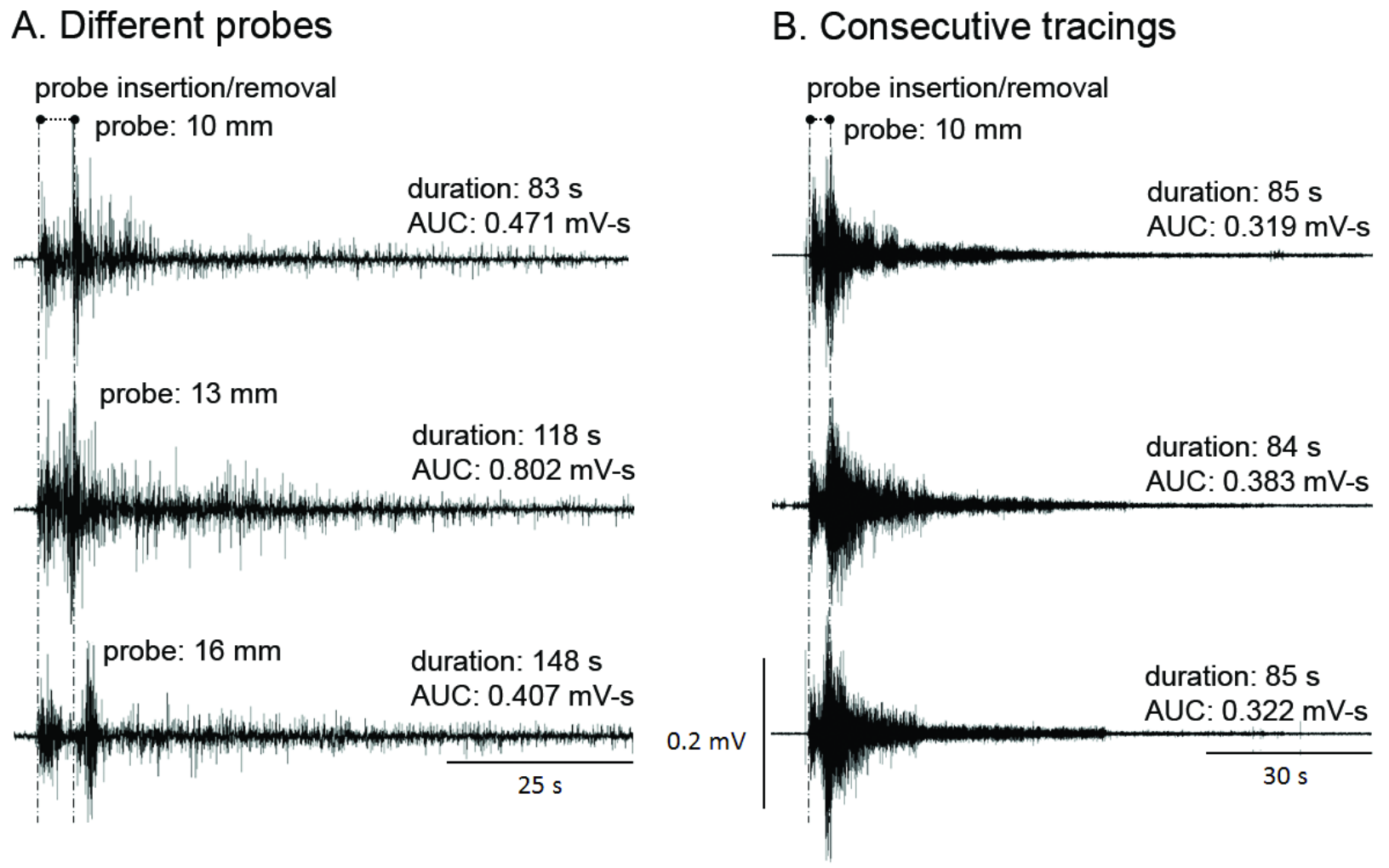

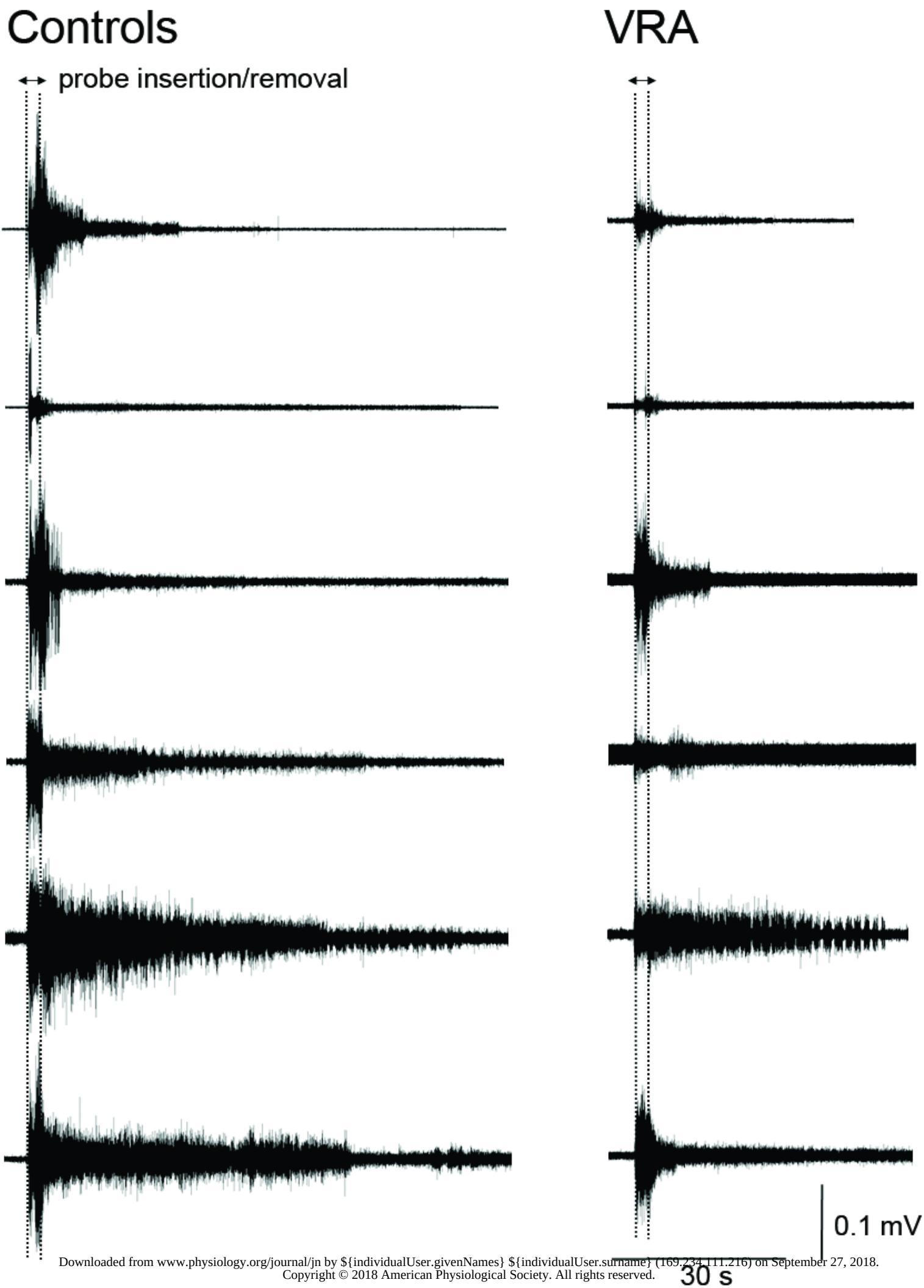

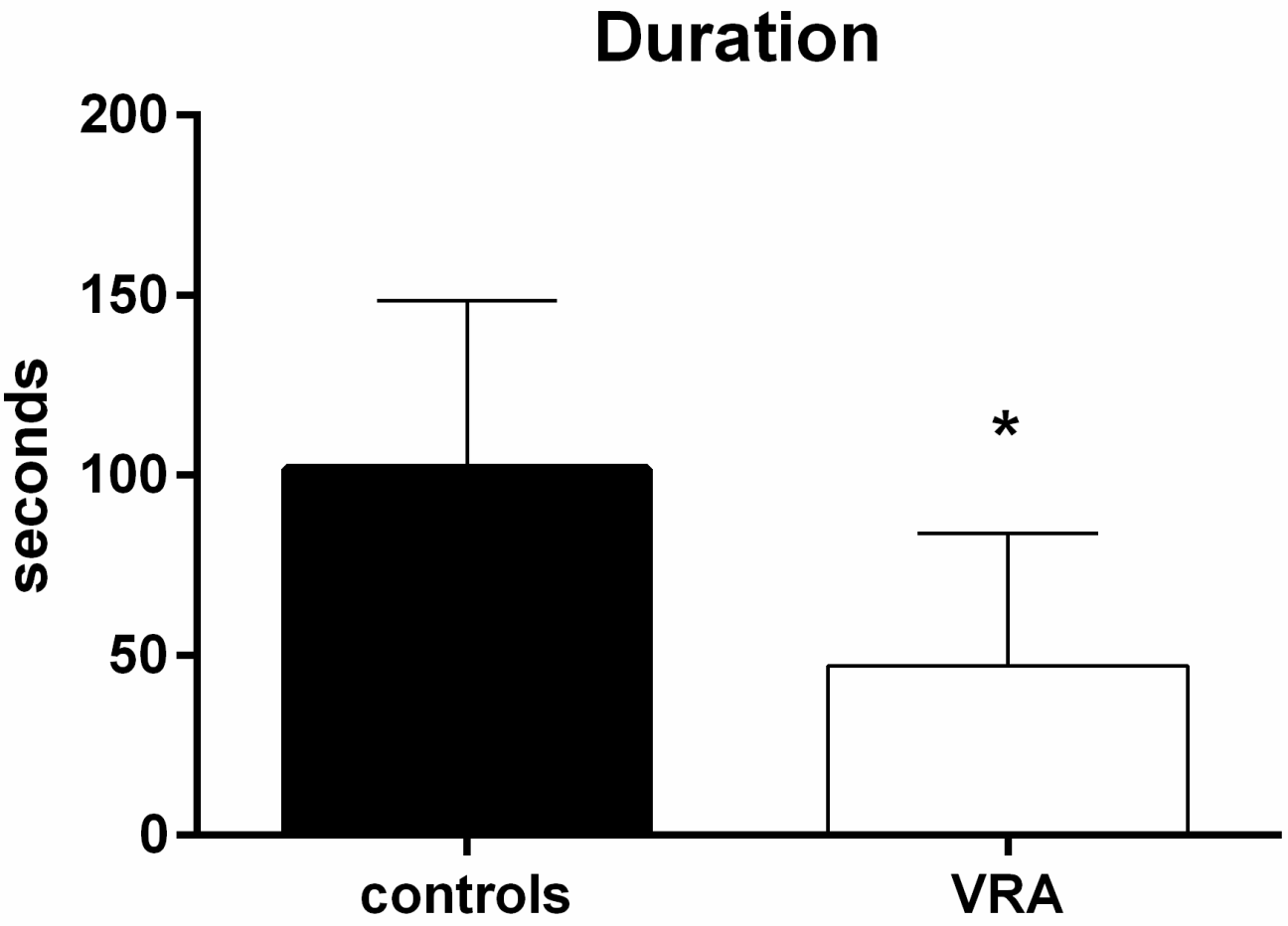

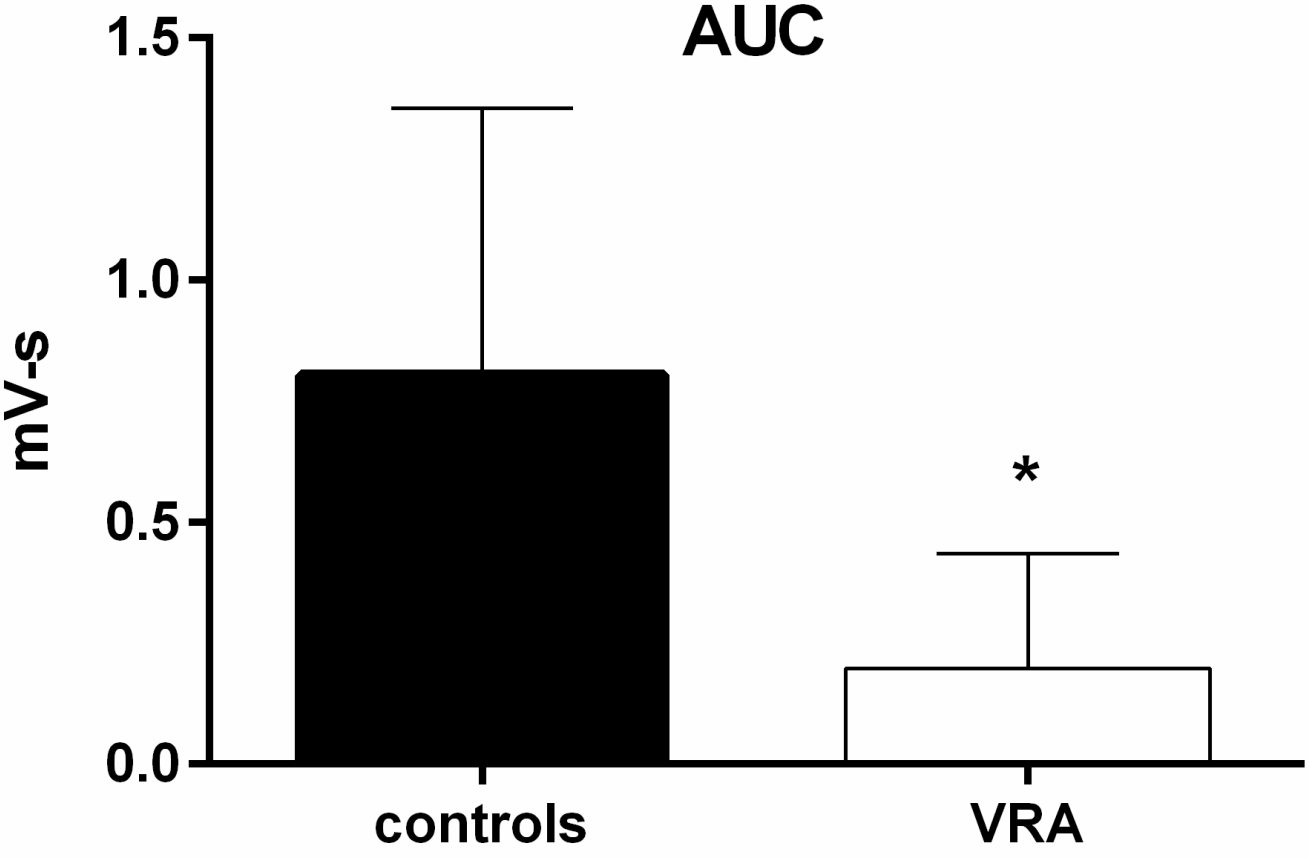

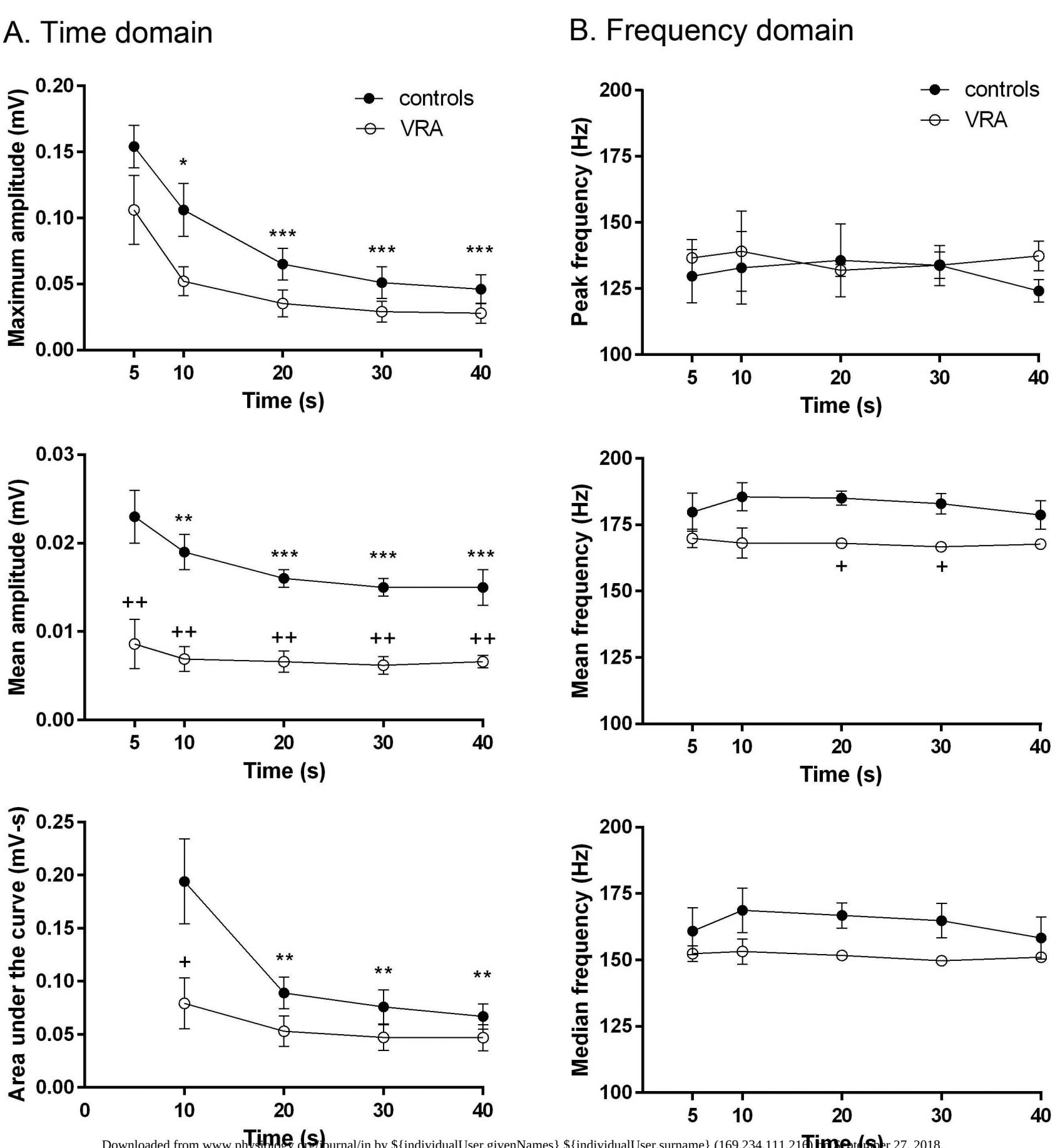

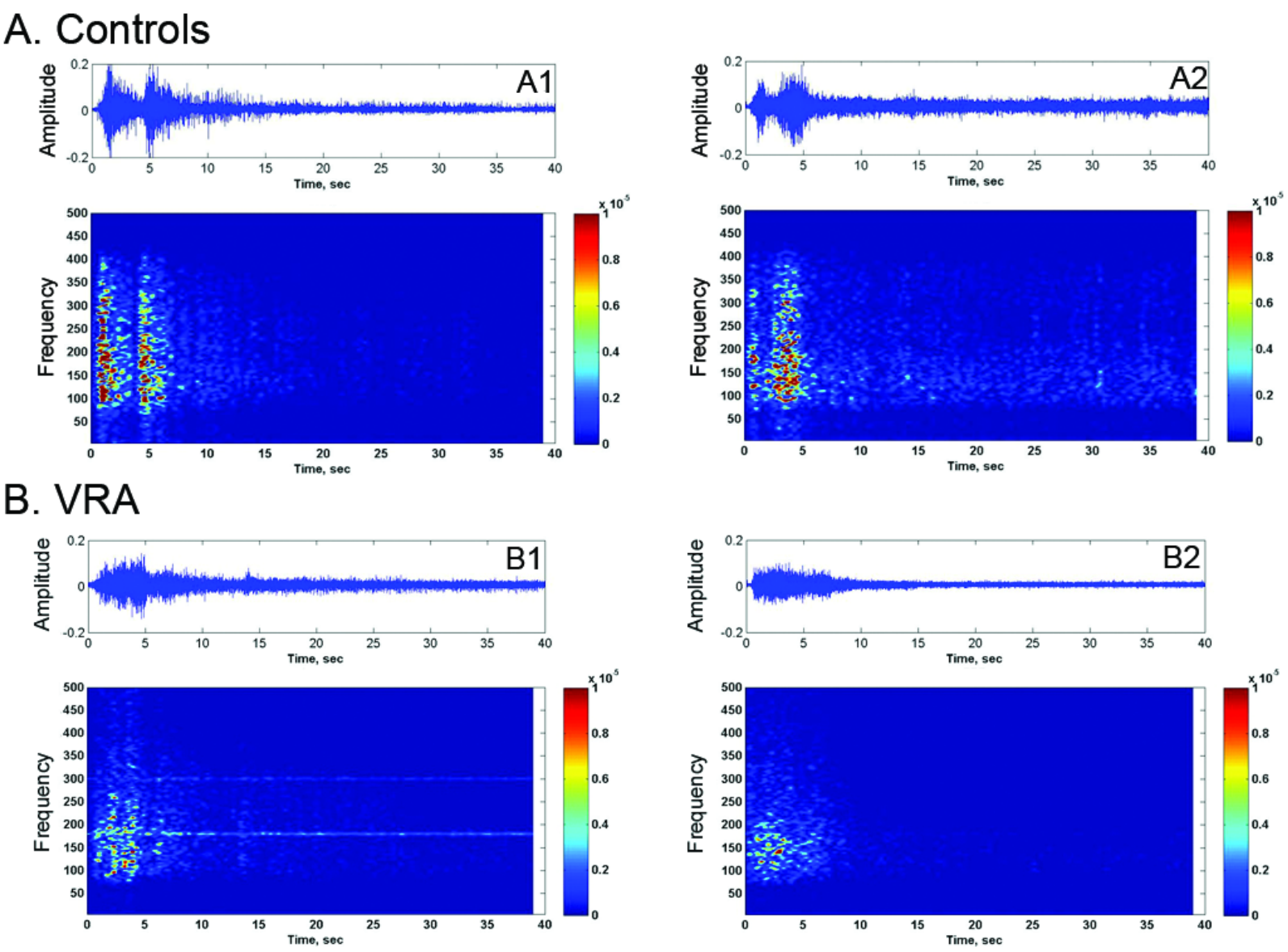

Table 1. Demographic information for study subjects; VRA = ventral root avulsion; BCS = body composition scoring (Clingerman and Summers, 2012); P values indicate statistically significant difference between groups; ns = not statistically significant.

Condition	Subjects	Gender	Anesthesia	Age (years)	Weight (kg)	BCS	Conceptions	Live births
Control	N=6	Female	Ketamine	6.4 ± 0.5	9.6 ± 0.7	3.2 ± 0.1	3.3 ± 0.6	3.2 ± 0.5
VRA	N=6	Female	Ketamine	7.7 ± 0.5	7.4 ± 0.5	2.9 ± 0.2	2.1 ± 1.0	1.3 ± 0.7
				ns	P<0.05	ns	ns	P<0.05