EVALUATION OF CALCIUM-MEDIATED PERSISTENT INWARD CURRENTS IN SPINAL MOTOR NEURONS OF AWAKE BEHAVING RODENTS

by

Julius Vellutato

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As members of the Master's Committee, we certify that we have read the thesis prepared by: Julius Vellutato
titled: Evaluation of Calcium-Mediated Persistent Inward Currents in Spinal Motor Neurons of Awake Behaving Rodents

and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree.

Andrew Fuglevand — Date: May 11, 2022

Daniela Zarnescu — Date: May 11, 2022

James V Proffitt — Date: May 11, 2022

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.

Andrew Fuglevand — Date: May 11, 2022
Thesis Committee Chair
Department of Physiology

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
EMG	Electromyography
ITC	Intrathecal Catheter
PIC	Persistent Inward Currents
MU	Motor Units
MN	Spinal Motor Neurons
LTCC	L-Type Calcium Channel
5-HT	Serotonin
ALS	Amyotrophic Lateral Sclerosis
IACUC	Institutional Animal Care and Use Committee
CaV1.2	Alpha 1C Subunit L-Type Calcium Channel
CaV1.3	Alpha 1D Subunit L-Type Calcium Channel

ABSTRACT

In order to test the natural synaptic input of motor neurons, we developed and tested a protocol for training rats to perform a voluntary motor behavior during which electromyography (EMG) recordings could be collected during natural synaptic input. Four rats were successfully trained to perform voluntary plantar flexion in conjunction with EMG recordings and another set of four naïve rats were used to gain insight into our surgical method of intrathecal catheter (ITC) implantation. All four naïve rats survived surgery with little to no complications, which prompted us to attempt implantation of two of the trained animals to no avail. To further evaluate this ITC method, verification methods including Lidocaine injections and laminectomy dissections were performed. Results were often confounding but provided useful knowledge about further use of this technique, which requires additional investigation and a larger sample size to make a definitive conclusion. Regardless, preliminary EMG data obtained with a separate cohort of rats, which were generously implanted by surgical specialist Kerry Gilbraith of the University of Arizona Department of Pharmacology, suggests that pharmacological blockade of persistent inward currents (PICs) with nifedipine results in a decreased firing rate of individual motor units (MUs) and compensatory MU recruitment to achieve the same target force. Again, further verification data is needed to rule out confounding factors associated with this data.

INTRODUCTION

The most basic neurophysiology schema is that sensory neurons receive a stimulus that is processed by central neurons and conveyed to motor neurons through synapses. Due to the simple nature of this arrangement, knowledge about the processing of a stimulus can be interpreted by measuring the output, or the behavior displayed by a model organism. Though this schematic may satisfy a circuit-based neural model, it fails to recognize the importance of intrinsic (ie. non-synaptic) processes that may drive neural activity.

Spinal motor neurons (MNs) have elaborate dendrites that receive up to 50,000 synaptic contacts[1]. As such, these last order neurons in the central nervous system (CNS) are hubs of convergence that integrate supraspinal, spinal, and peripheral inputs. Spiking output of MNs is conveyed to the periphery by axons that ramify to contact hundreds of muscle fibers in a target muscle. Because of the potency of the synapse at the neuromuscular junction, each presynaptic action potential in a MN axon provokes an action potential in every postsynaptic muscle fiber. This causes all the muscle fibers innervated by a MN to contract together as a group. Consequently, the MN and its innervated muscle fibers constitute a functional entity- the motor unit (MU). From a practical standpoint, because spiking of MNs is broadcast and amplified in an accessible tissue, it is relatively straightforward to record activities of MNs with electrodes placed in muscle in awake subjects, including humans.

Partly because of their accessibility, MNs have long been used as model neurons to understand general principles of synaptic integration. Pioneering work of Granit, Kernell, Schwindt & Crill[2-5] suggested that spike frequency output of MNs is roughly proportional to the net excitatory synaptic input received. Indeed, once brought to spiking threshold, the relationship between injected depolarizing current (as a proxy for synaptic input) and MN firing rate is remarkably linear over most of the range of inputs[2-5]. Consequently, theoretical ideas and computational models related to information processing in the brain oftentimes rest on the idea that above-threshold neuron output is a more-or-less a linear function of synaptic input.

In the late 1970s, an intrinsic, long-lasting depolarizing current was discovered in MNs[6], which later became known as persistent inward currents (PICs)[7]. PICs appear to be caused by activation of voltage-gated channels that increase conductance primarily to calcium ions, enabling an inward flow of cations to depolarize the membrane[8-11]. While other forms of PIC exist (e.g., sodium or non-specific cation), calcium-mediated PICs appear to be the dominant form of PICs in MNs[10]. In fact, PICs in MNs are largely abolished in the presence of the drug nifedipine[10] and other dihydropyridine class antagonists[12], which are all known L-type calcium channel (LTCC) blockers. Therefore, we focus our attention on Ca^{2+} PICs, and hereafter refer to them simply as PICs.

The LTCCs underlying PICs inactivate slowly (on the order of seconds) and thereby can provide long-lasting depolarization (sometimes referred to as a plateau potential) in MNs. For PICs to be activated, two conditions must be met: 1) the membrane needs to be depolarized by about 10-15 mV (through the action of extrinsic synaptic inputs)[13-14] and 2) the channels must be enabled by an intracellular biochemical cascade instigated by the binding of monoamine neuromodulators[8,15-16] to metabotropic receptors[11,17]. Axons projecting from brainstem nuclei to the spinal cord provide the primary source of these widely broadcast neuromodulators, such as serotonin (5-HT) and noradrenaline[18-19]. Indeed, the reason why early investigators did not observe nonlinearities in motor neuron firing associated with PICs is likely due to their use of anesthetics that impaired activity of neurons responsible for releasing neuromodulators[20].

Finally, to extinguish PICs once engaged, active synaptic *inhibition* is thought to be required[15,21-22].

In general, the depolarizing current provided by PICs can be thought of as providing an added boost to that delivered synaptically. This boost can be substantial; some investigators have suggested that the current provided by PICs can be 3-6 times larger than that provided by synaptic drive alone[13-14, 23-24]. Moreover, PICs have been implicated in self-sustained activity of MNs whereby MN activity continues even after the cessation of excitatory synaptic drive[15,21,25-26]. As such, PICs have been proposed to facilitate prolonged activity in MNs needed for maintenance of posture[27-28], to boost excitability of MNs during locomotion[27], and to provide the *primary* source of depolarizing current to MNs during normal motor behaviors[24,29]. Furthermore, impaired regulation of PICs may underlie hyperexcitability associated with spasticity following spinal cord injury[30-32] and stroke[33] and may promote calcium-mediated excitotoxicity in amyotrophic lateral sclerosis (ALS)[34-35]. Furthermore, PICs are thought to play a role in a variety of functions throughout the nervous system, including formation of place fields in the hippocampus[36] production of sustained pacemaker activity in dopaminergic cells in the substantia nigra[37], mediation of pain sensitization in dorsal horn neurons of the spinal cord[38], fear learning in the anterior cingulate cortex[39], and short-term working memory in prefrontal cortex[40].

Despite the significance of PICs, and the large number of studies carried out to characterize the cellular mechanisms underlying PICs in reduced animal preparations, little is known about the actual physiological conditions during which PICs are activated in MNs. On one hand, it has been suggested that only during ‘fight or flight’ conditions might there be sufficient monoamine input to activate PICs and thereby augment MU activity in situations of high stress or arousal[41]. On the other hand, it has been argued that PICs may provide the majority of the current to drive normal recruitment and rate coding in MNs[29].

The reason why the physiological role of PICs has not been established is that it is not possible to identify the relative extents of synaptic versus PIC inputs to MNs in awake behaving animals. To address this challenge, methods have been developed that are thought to reveal the presence of PICs in human subjects. One widely used method involves measuring the differences in recruitment and derecruitment thresholds in pairs of MUs during triangular isometric contractions[42-44]. Another involves characterizing the duration and extent of involuntary contractions associated with muscle stimulation or vibration[45-48]. A third approach compares MU firing during normal voluntary contractions to that in which PICs are putatively disabled by activation of an inhibitory reflex[49]. All these approaches have provided valuable insights into the control of MUs and possible role of PICs. Nevertheless, they are all indirect, and they rest on several assumptions. Furthermore, mechanisms other than PICs can account for some of the responses attributed to PICs[50].

Therefore, the overall goal of my project was to directly evaluate the role that PICs play during normal voluntary muscle contraction. We did this by recording MU activity in plantar flexor muscles of rats during voluntary contraction in the presence and absence of nifedipine injected into the intrathecal space surrounding the lumbar spinal cord. If PICs normally play an important role in amplifying MU firing rates, then disabling PICs by nifedipine should lead to reduced firing rates. Consequently, impaired rate coding should lead to compensatory MU recruitment mediated by synaptic drive as the rat attempts to hold a designated target force. We also plan to perform the converse experiment by augmenting PIC activation by injection of 5-HT into the intrathecal space. In this case, higher discharge rates and lower levels of recruitment for the same target force are anticipated. Collectively, these causal manipulations should provide direct insight into the physiological role played by PICs during normal muscle activity.

METHODS AND MATERIALS

Animals. All procedures were performed in accordance with the policies and recommendations of the International Association for the Study of Pain, the National Institutes of Health guidelines for the handling and use of laboratory animals, and by the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona. Eight male Sprague Dawley rats ranging in weight of 400-500 grams were used as participants in this study. Four of these were naïve to our training protocol whereas the other four were trained for the duration of the study. All four naïve rats and two trained rats underwent intrathecal catheter (ITC) implantation whereas the other two trained rats were used for baseline data. Rats were initially housed together 2 per cage and were maintained in a climate-controlled room on a 12-hour light/dark cycle with access to water ad libitum. Food was restricted to approximate the least amount each rat needed prior to training every day. Rats were trained 3-5 days a week.

Behavioral Training. Rats learned to perform voluntary isometric plantar flexion over several weeks of progressively more intense training. Animals were placed inside of a KentScientific nose cone animal holder with a rectangular hole cut out in the bottom of the tube **(Fig 1A)** through which the left hindlimb could be extended and secured to a small pedal with a strip of Velcro material. This pedal was connected to a FT03 Force-Displacement Transducer (Grass Instruments) that measured the force exerted on the pedal during plantar flexion. The front of the tube was closed with an opaque nose cone with a small opening at the front to enable access to liquid reward. In addition, a hole was cut through the top part of the cone **(Fig 1B)** to allow for access of an implanted catheter. The position of the nose cone could be adjusted to increase or decrease the length of the tube. The rear of the tube was closed off with a flat plastic disc. A slot in the disc allowed for the rat's tail to remain outside of the tube.

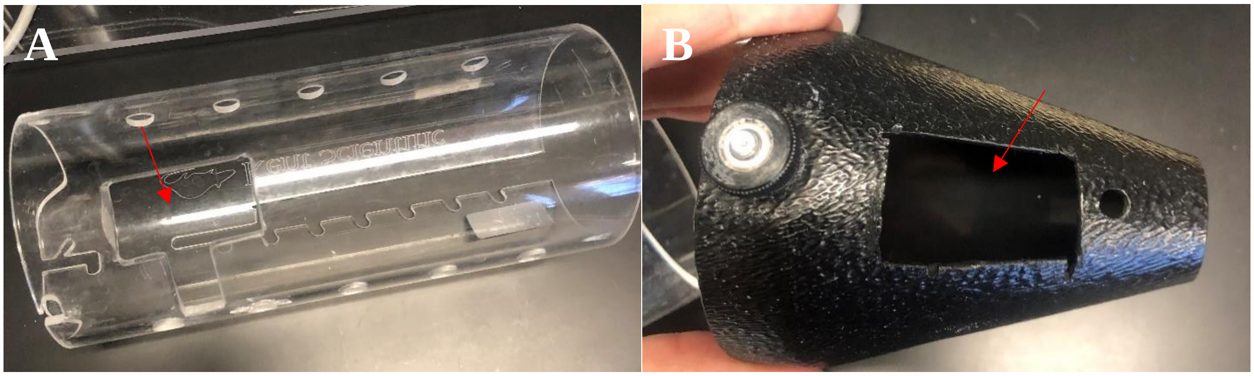

Figure 1. Modifications to Rat-Sized Restraint tubes from KentScientific. **A)** An opening was made in the bottom of the restraint tube that was large enough for a rat to comfortably extend its left leg through. **B)** An opening in the nose cone that allows access to an implanted catheter, which would be covered for training purposes. Note: each modification is indicated by a red arrow.

Force signals were recorded with a computerized data acquisition system (CED Power 1401). Custom scripts were used to determine whether the exerted force was within a specified range. The initial range was 5-25 grams but was wide (usually 5-25 grams) but could be adjusted to better coincide with the strength of each rat and to target different force levels. If the rat successfully produced force within the target range, the script triggered a peristaltic pump (LungerPump) to deliver liquid chocolate Ensure as a reward through a modified syringe held in place at the opening of the nose cone. In addition to the liquid reward, rats were notified that the force was in the target range by activation of a buzzer tone.

Training advanced in stages based on the comfort level of each animal. Animals were introduced to new conditions every two days. Stages were prolonged if individual animals showed greater than normal difficulty accepting the new conditions and required more adjustment.

During the initial phases of training, rats were given access to the chocolate Ensure reward ad libitum to reduce their stress via positive distraction. The first stage of training simply involved letting the rat sit in the tube (with the nose cone placed at its most distal position) to acclimatize rats to confinement in the tube. Animals turned around often in this stage to explore both ends of the tube and sometimes showed initial disregard of the Ensure reward. Over the next few days, the internal dimensions of the tube were shortened until the rat could no longer turn around. Usually by this stage, the Ensure reward was enthusiastically received. Nevertheless, food restriction was used to help motivate rats to seek the Ensure reward. The rats were weighed before each training session to make sure their weight did not fall below 85% of their designated age-based weight.

The next phase of the training involved rats having their left hind paw touched and extended from the plastic tube by the experimenter. This aspect of training typically required the most time-usually from three days to over a week for rats to adjust to this manipulation. Once the foot could be comfortably extended from the tube, the next phase involved securing the paw to the instrumented pedal with a Velcro strap.

Rats were next trained to plantar flex the ankle to exert force on the pedal within the assigned target range. This phase also could involve several sessions. Initially, while secured in the tube and with their paw fixed to the pedal, rats would inadvertently push against the pedal with sufficient force to trigger delivery of Ensure reward and activation of the buzzer. Over time, rats learned that such an action was reliably associated with delivery of the reward and would then have to hold the force in the target range for long durations to obtain continuous reward. To elicit repeated trials of plantar flexion, the duration of time during which reward was delivered was curtailed after a defined period of time (initially 60 s). The animal was then required to relax to allow the force to drop below the minimal threshold of the target range. Once this relaxation occurred and force was re-exerted within the target range, positive reinforcement returned. Unloading the foot was also a challenging maneuver for the rats to learn. Once learned, the duration of reward window was shortened to increase the number of plantar flexion-relaxation cycles.

Electromyography. When rats were successfully trained, we initiated intramuscular electromyographic (EMG) recordings from the lateral gastrocnemius muscle, a plantar flexion at the ankle joint, to measure the activity of single motor units reflecting the spiking output of spinal motor neurons.

We used 75 µm diameter bipolar hooked wire electrodes made using two strands of insulated stainless-steel wire (California Fine Wire Company) threaded through a 27G hypodermic needle. One end of each wire was folded so that they made small "hooks" at the tip of the needle. The electrodes were then sterilized and stored for recordings. A separate surface electrode was placed on the tail that served as the ground.

Prior to recording, the left hindlimb of the rat was shaved to make insertion of the electrode easier. This was typically done a day or two before the recording to minimize stress on recording days. Electrodes were removed from their sterilization pouches and inserted medially into the gastrocnemius muscle using the hypodermic needle. When the needle was removed, the hooked ends of the wires remained lodged in the muscle. The electrodes were connected to a differential amplifier (gain=1000, bandpass 300-3000 Hz, Grass Instruments model RPS 107) and the EMG signal led to the data acquisition system (CED). Noise levels were small with this electrode configuration and the insertion appeared to produce minimal discomfort in the animals.

If needed, slight adjustments of the intramuscular electrodes were made by gently tugging on the leads to improve motor unit detection.

Catheter Implantation and Drug Delivery. Once rats had reached a stage in their training where they were voluntarily pushing the pedal, they underwent intrathecal catheter (ITC) implantation. These catheters were prepared from 0.28 mm I.D. x 0.64 O.D. micro medical tubing (Scientific Commodities Inc.) that were measured to rat length to ensure they extended down the spinal cord to the first vertebra of the lumbar region. They were then sterilized, pre-loaded with ~0.01 mL of 1,000 USP units/mL heparin saline (Sagent Pharmaceuticals) and sealed via cautery prior to implantation.

At the start of surgery, rats were injected with a 5 mg/kg dosage of 50 mg/mL Carprofen (Zoetis- Rimadyl) dosage and subsequently anesthetized with 4% Isoflurane. The surgical site was then prepared by shaving the nape of the neck and back of the head and disinfecting the skin with 3 cyclic scrubs of 70% ethanol and betadine solution. The rat was then positioned into a stereotaxic frame with the head bent at a nearly 90-degree angle to facilitate catheter insertion. Isoflurane was adjusted to an appropriate level (~2-3%) throughout surgery depending on responsiveness to a toe pinch. A heating pad placed beneath the animal helped support core temperature (not monitored) during the surgery.

A midline incision was made from the back of the skull to the C1 vertebra into the skin and muscle to expose the atlanto-occipital membrane. A retractor was then set in place to expand the surgical site and a #11 blade was used to carefully incise the membrane and expose the intrathecal space. The ITC was then advanced slowly to the lumbar region. During insertion, any twitches indicating potential spinal cord damage by the catheter were noted. A loose knot in the

ITC situated outside of the entry point served as an anchor, around which the muscle incision area was sutured back in place using 5-0 absorbable suture (Veterinary Sutures). The skin was then closed using surgical staples with 4-4.5 cm of the ITC remaining outside the body to be accessible for drug injection. The rat then received an 8-10 mg/kg dose of 100 mg/ml Gentamicin Sulfate (VetOne), a 1 mg/kg dosage of Buprenorphine-SR (ZooPharm), and 3 mls of physiological saline (ICU Medical, Inc). Rats were then placed on a warming pad for recovery. The rats required about one week of recovery time during which we injected 2 additional doses of 5 mg/kg Carprofen (Zoetis- Rimadyl) and monitored for motor impairment, weight loss, and chromodacryorrhea (red tears).

A group of four naïve 400 g male rats were first implanted to determine whether the ITC was performed appropriately before attempting the surgery in trained animals. To verify correct catheter placement in these rats, we injected 0.02-0.05 mL of 2% Lidocaine (VetOne) to induce temporary paralysis. If the catheter was situated correctly, then such an injection should cause a brief period of selective hindlimb paralysis. Each time an injection was performed, the end of the ITC was cut and then resealed via cautery. As such, it progressively became shorter as more injections took place.

Two trained 500 g male rats were then used for surgery. Unfortunately, both animals displayed paralysis immediately after the surgery and were euthanized. These two animals were then used for laminectomy dissections (discussed below) to help identify the cause of paralysis. Nifedipine injections via ITC were obtained and some of those data are described here. Those surgeries were performed by surgical specialist Kerry Gilbraith at the University of Arizona Department of Pharmacology.

Dissections. Rats that exhibited significant paralysis after surgery were immediately euthanized and frozen for later dissection. This consisted of making a midline incision over the spine from the nape of the neck to the L1 vertebra using a #10 blade. Skin was then peeled back by making horizontal cuts at these two landmarks and muscle was cleared away from the vertebra as thoroughly as possible. The spinous processes and dorsal laminae of the vertebrae were removed using rongeurs to cut through the laminae bilaterally starting at the lumbar vertebrae and moving rostrally until the implanted ITC was exposed. In one case, both deionized water and TMD-6 Tissue Marking Dye (Triangle Biomedical Sciences) diluted 100x with saline were injected into the ITC to mark the spinal site at which the catheter ended.

Data Analysis. The recorded EMG data were analyzed using CED Spike2 Data Acquisition and Analysis Program (Spike2). Fifteen-30 minutes of data were typically recorded and included the unprocessed EMG, the force exerted by the rat on the pedal, time of reward delivery, and indications of when drugs were injected. Segments approximately 10-30 seconds in duration that contained minimal noise and unambiguous firing of motor units were used for further analysis. Action potentials of individual motor units were identified based on waveform amplitude and shape. Instantaneous and average firing rates were then computed in both the control and experimental conditions.

RESULTS

Our methods of shaping behavior have proved useful to elicit voluntary, targeted force production in hindlimb muscles of awake rats. When initially introduced to the enclosed setting, rats typically had no interest in drinking the Ensure and would attempt to turn around in the tube **(Figure 2)** in an attempt to explore their space. Eventually, rats would discover the liquid reward and would spend more time licking at the spout. Then, over a set of sessions, we would gently manipulate the paw until the rat was comfortable with the experimenter extending the leg through the opening of the tube. Next, the rat's paw was strapped to the force pedal for extended periods of time during which reward was continuously available. Lastly, the rat needed to learn to push on the pedal within a set force range to trigger delivery of reward **(Figure 3)**. As animals became more adept at this, they would continuously exert force in the target range. To increase the number of task trials, after a set period (initially 60 s), the reward was curtailed, and the rat needed to relax force below the target level to instigate another trial. This reward period was then decreased over sessions (to about 20 s) to further increase the number of target force trials.

In tandem with training data from pilot work, it took an average (± SD, n=21) of 21.3 ± 6.5 training sessions until the paw could be fixed to the pedal and 27.0 ± 8.2 training sessions until rats were able to produce their first instance of spontaneous plantar flexion **(Table 1)**. However, four rats were unable to successfully learn to relax their hindlimb before reapplying force. On average (± SD, n=17), it took 34.2 ± 8.1 training sessions to accomplish this feat. Of all 21 rats, five of them never underwent surgery, ten were euthanized immediately following surgeries, three were euthanized due to complications days to weeks after surgery, and three survived with no complications.

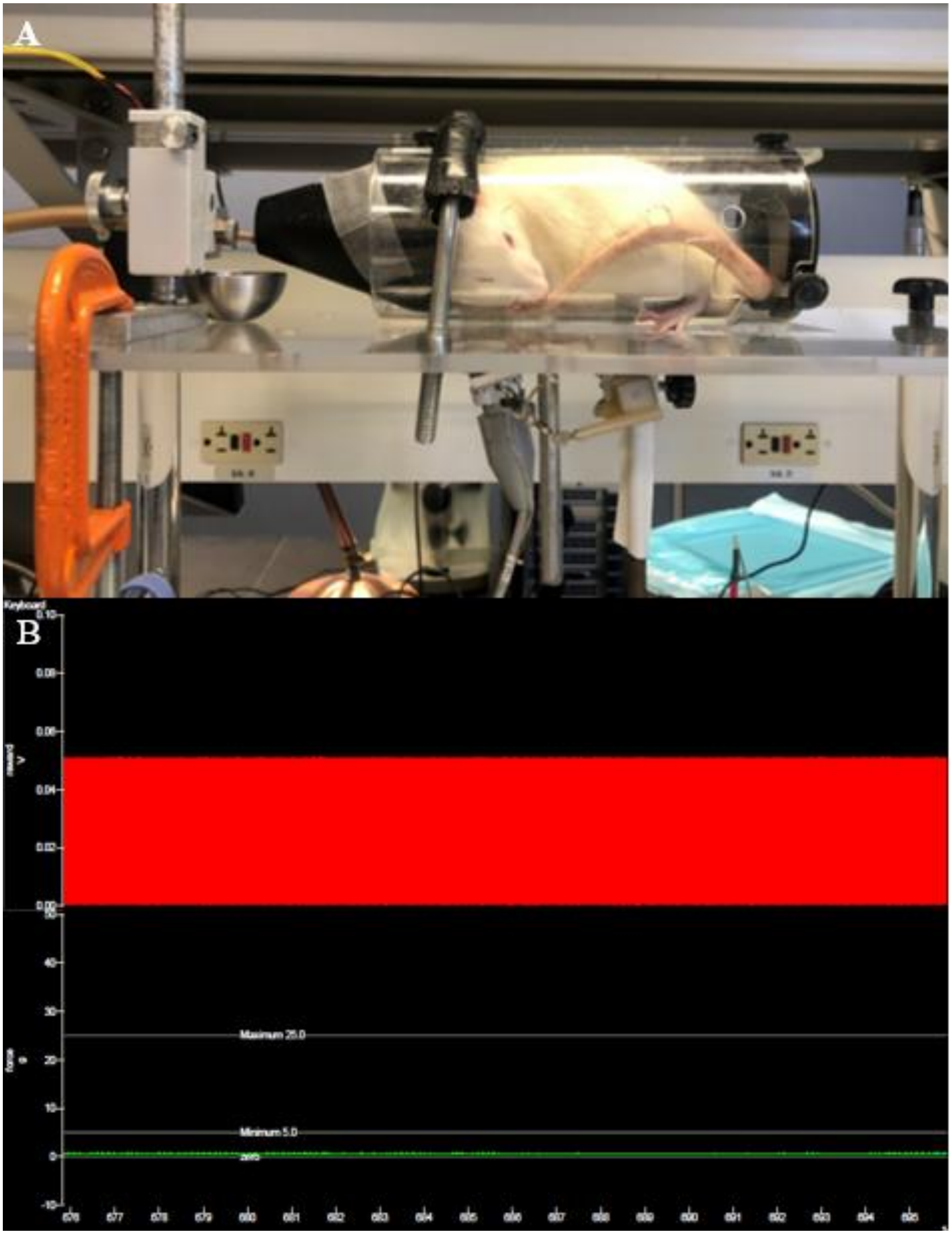

Figure 2. Early Training Observations. **A)** A naïve rat on his third day of training showing no interest in the chocolate Ensure reward, who was turning around in the tube. **B)** Continuous reward delivery (red trace) used to entice rats to face forward in the tube. Because the paw was not attached to the pedal at this stage, no force is recorded (green trace).

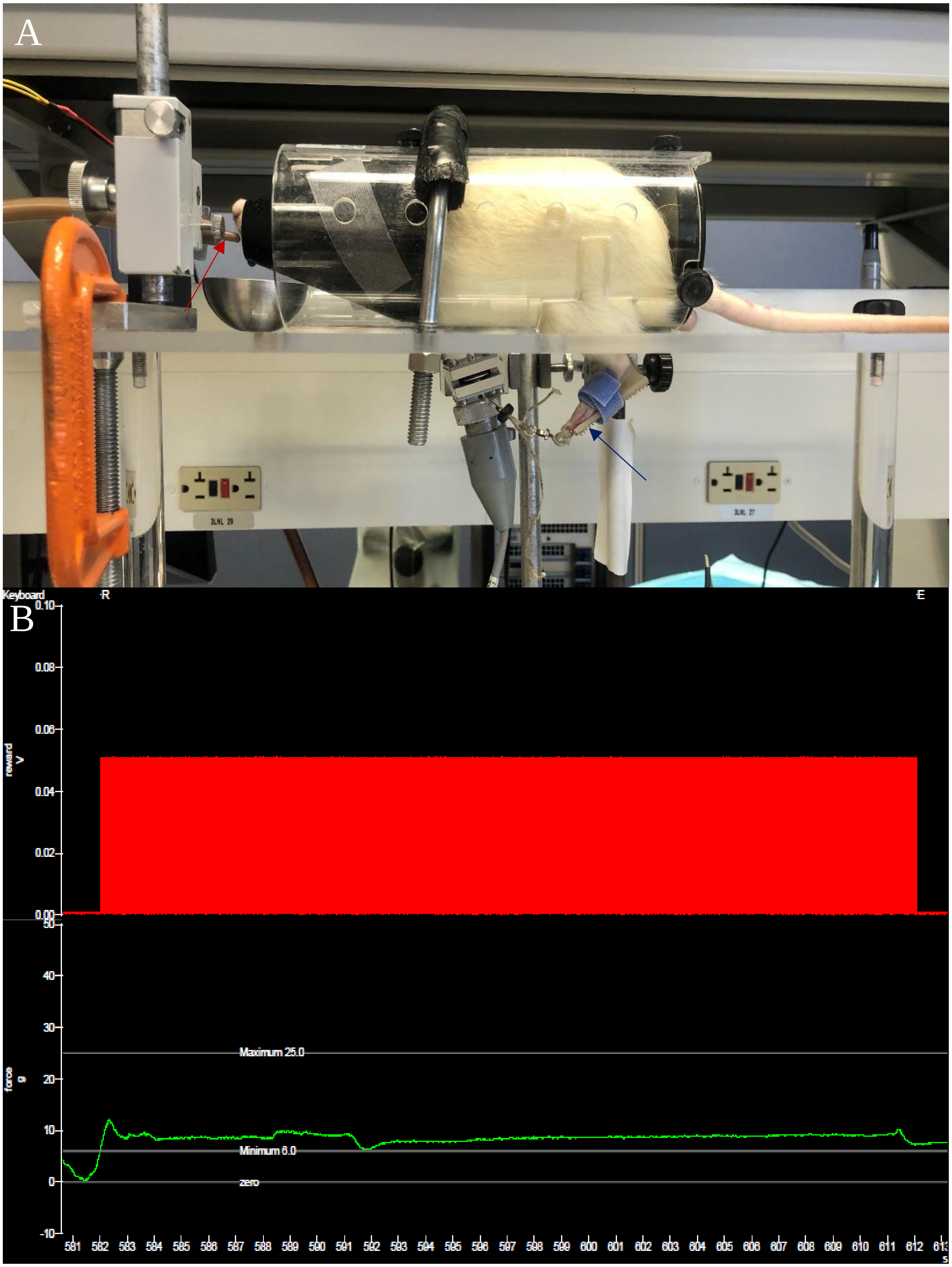

Figure 3. Late-Stage Training. **A)** A trained rat with the paw fixed to the force pedal (indicated by blue arrow) and exerting force for liquid reward (indicated by red arrow). **B)** Rat exerted force (green trace) above threshold (6 grams) which then triggered delivery of reward (red trace). After 20 seconds, the reward was halted, requiring the rat to reduce the force below the threshold (not shown) to initiate another trial.

Rat	Training sessions until paw could be fixed to pedal	Training sessions until first successful plantar flexion	Training sessions until reliable relaxation	Survived Surgery?
G	11	27	39	N*
Y	15	19	19	Y
Pu	15	17	23	Y
O	23	24	-	N*
Bl	23	30	-	Y**
Pi	24	27	-	Y**
Uno	20	33	35	N*
Dos	23	34	36	Y
Tres	20	33	-	N*
Four	30	38	40	N*
Five	30	36	38	N*
Six	30	36	38	N*
Seven	36	38	43	N*
Eight	29	39	50	Y**
C	18	24	35	-
E	18	23	37	-
F	18	23	35	-
1α	16	16	37	-
2α	17	17	25	-
3α	16	16	30	N*
4α	16	16	22	N*
Average Training Sessions:	21.3 ± 6.5	27.0 ± 8.2	34.2 ± 8.1	-

Table 1. A table showing the number of training sessions required for the rats to reach milestones in their training. Data expressed as average ± SD. Dashes indicate that the rat did not reach a particular milestone. One asterisk indicates euthanasia immediately following surgery. Two asterisks indicate euthanasia due to worsening paralysis, self-mutilation, or removal of catheter after surgery.

Once rats were trained, we then initiated recording of EMG while the rats performed the task. Rats were relatively unperturbed by the insertion of the electrodes and performed the task per usual with the electrodes in place. From the EMG recording, we are able to identify the timing of action potentials generated by different motor units in the vicinity of the electrode based on their distinct waveforms. For example, **Fig. 4A** shows a brief sample of EMG (lower trace) in which the action potentials associated with five different motor units (shown in different colors, upper trace) could be distinguished. From such processing, we could characterize the instantaneous firing rates of individual motor units during the task **(Fig 4B)**.

Once rats were trained to adequately perform the targeted force task, they were implanted with ITCs that extended to the lumbar spinal cord where the motor neurons supplying the gastrocnemius reside. To practice this procedure, we first implanted four naïve rats, all of which survived the surgery with little to no complications. However, rats were prone to pulling them out which eventually occurred in three of the four practice rats. We were able to perform Lidocaine injections on two of these rats **(Supplemental Videos)**. The first of these was performed 11 days post-implantation **(Supplemental Video 1)**, in which we noted successful temporary paralysis of both hindlimbs and no disturbance of the forelimbs. The second of these was injected 5 weeks post-implantation **(Supplemental Video 2)** with slightly less success. While we noted temporary paralysis of both hindlimbs, paralysis of the forelimbs soon followed.

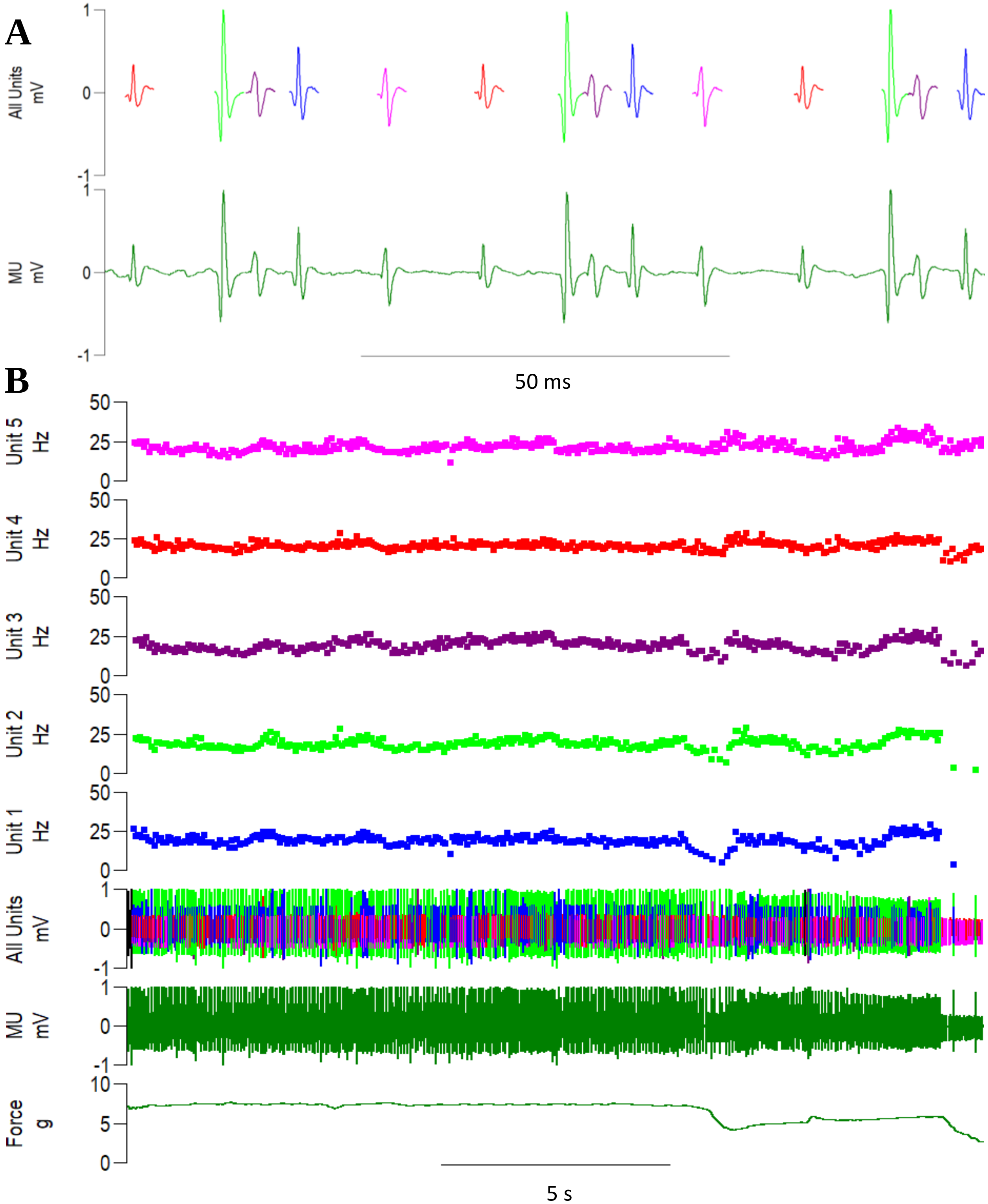

Figure 4. Example EMG recording data analysis. **A)** ~0.1 s of EMG data (lower trace) and discriminated spike waveforms of five different MUs (different colors, upper trace). **B)** ~20 seconds of a 15-minute EMG recording showing the instantaneous firing rates of the five motor units identified in **A**). Bottom two traces: Pedal force and intramuscular EMG data. Third trace: Discrimination of five different MUs. Top five panels: Instantaneous firing rates of the five MUs after discrimination.

These two rats were eventually euthanized and underwent laminectomy dissections. The first rat eventually partially pulled the catheter out 2-3 cm **(Fig 5A)**. Upon dissection, the ITC was not found to be laying on top of the spinal cord, as it should. The laminae of the remainder of the vertebrae up to the point of ITC insertion were then removed **(Fig 5B)**, and TMD-6 Tissue Marking Dye was injected into the catheter. The ITC was observed to travel to the right lateral side of the spinal cord. The dye pooled near the T1 region but was also inside the spinal cord near the C2-C3 region. When the spinal cord was retracted laterally, the ITC was seen to penetrate a shallow segment of the spinal cord ~2 cm near the upper thoracic region. The second rat experienced some right forelimb weakness immediately after surgery and forelimb paralysis with the Lidocaine injections. This suggested a potential tear in the ITC. After dissection, a slight crimp in the catheter was notice that could have allowed leakage of Lidocaine into the thoracic and cervical regions **(Fig 5C)**.

Dissection was also performed on the two trained rats that were euthanized after surgery because of paralysis. In one, a slight twitch was noted in the left forepaw during implantation. Upon dissection, the catheter was to have made a complete U-turn into the left wall of the spinal canal around the T6 and T7 vertebrae **(Fig 5D)**. In the other rat, the entire body spasmed upon advancement of the ITC during surgery. Dissection revealed that this was due to longitudinal penetration of most of the spinal cord. Cross-section of the spinal cord was needed to see that the end of the catheter had ended up near the L1 vertebra **(Fig 5E)**.

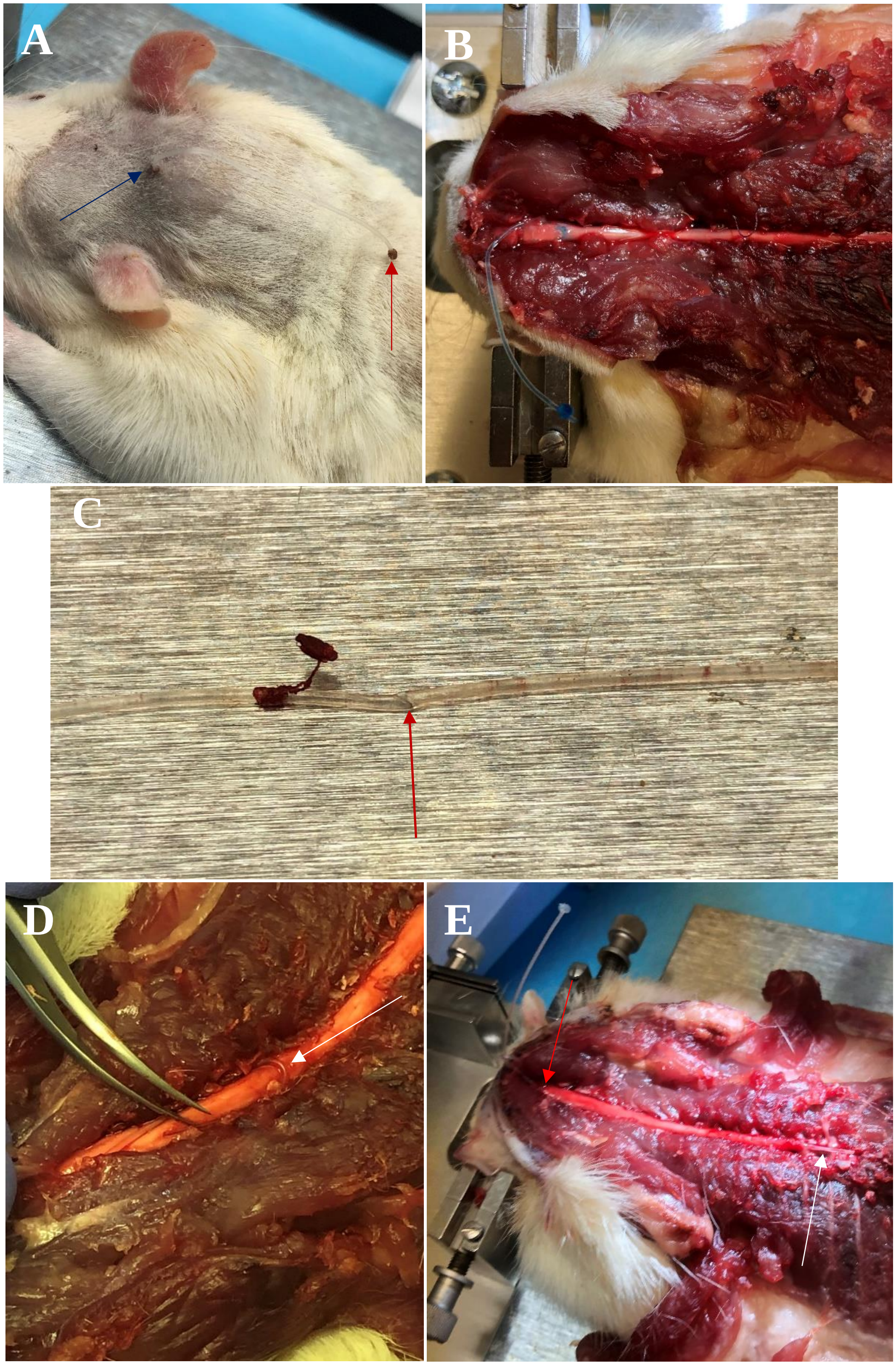
A
B
C
D
E

Figure 5. Laminectomy Dissections for Verification of ITC Placement. **A)** Pre-dissection of a rat displaying that ITC was pulled out by ~2-3 cm. Note: The red arrow displays the initial scab that was pulled out with the ITC from its initial more rostral location (blue arrow). **B)** Dissection of the rat displayed in **(A)**, revealing that the catheter was inserted to the right lateral side of the spinal cord. Note: this particular ITC is blue due to injection of TMD-6 Tissue Marking Dye to reveal that fluid was accumulating inside the cervical region of the spinal cord. **C)** Catheter that was pulled out accidentally during dissection, displaying a scab that was at the surface interface. Immediately caudal to this site is a crimp (marked by the red arrow) that may have occurred during surgery. **D-E)** Dissected spinal cords of rats who displayed significant paralysis post-surgery. **(D)** displays that the ITC made a U-turn (marked by the white arrow) between the T6 and T7 vertebrae whereas the ITC in **(E)** penetrated the spinal cord at the site of insertion (marked by the red arrow) entirely all the way down to the L1 vertebra (marked by the white arrow).

Though none of the surgeries performed on trained animals were successful, preliminary data on trained, implanted rats were obtained in a previous pilot experiment. Intramuscular EMG signals were used to identify and discriminate the spike timing of different MU potentials. In the pre-drug period **(Fig. 6A)**, MU 1 (blue) discharged steadily with an average rate of ~ 20 imp/s. A second MU (red) discharged sporadically but was not tonically active. Following a 10 µg nifedipine injection **(Fig. 6C)**, and while the exerted force was practically the same (~5 gm), the activity of MU 1 was diminished, with an average firing rate of ~12 imp/s. At the same time, MU 2 (red) was recruited and discharged tonically. **Figures 6B and 6D** show the overlaid action potential waveforms of the two motor units, indicating the same motor units were detected before and after drug delivery. These results imply that voltage-gated calcium channels associated with PICs likely provide a significant component of the depolarizing current underlying spiking activity in spinal motor neurons. Because the process of bringing motor neurons to recruitment threshold are thought not to involve PICs, previously quiescent motor units (i.e. unit 2, Figure 6) can be activated to compensate for the loss of force production normally mediated by rate coding but impaired with PIC blockade.

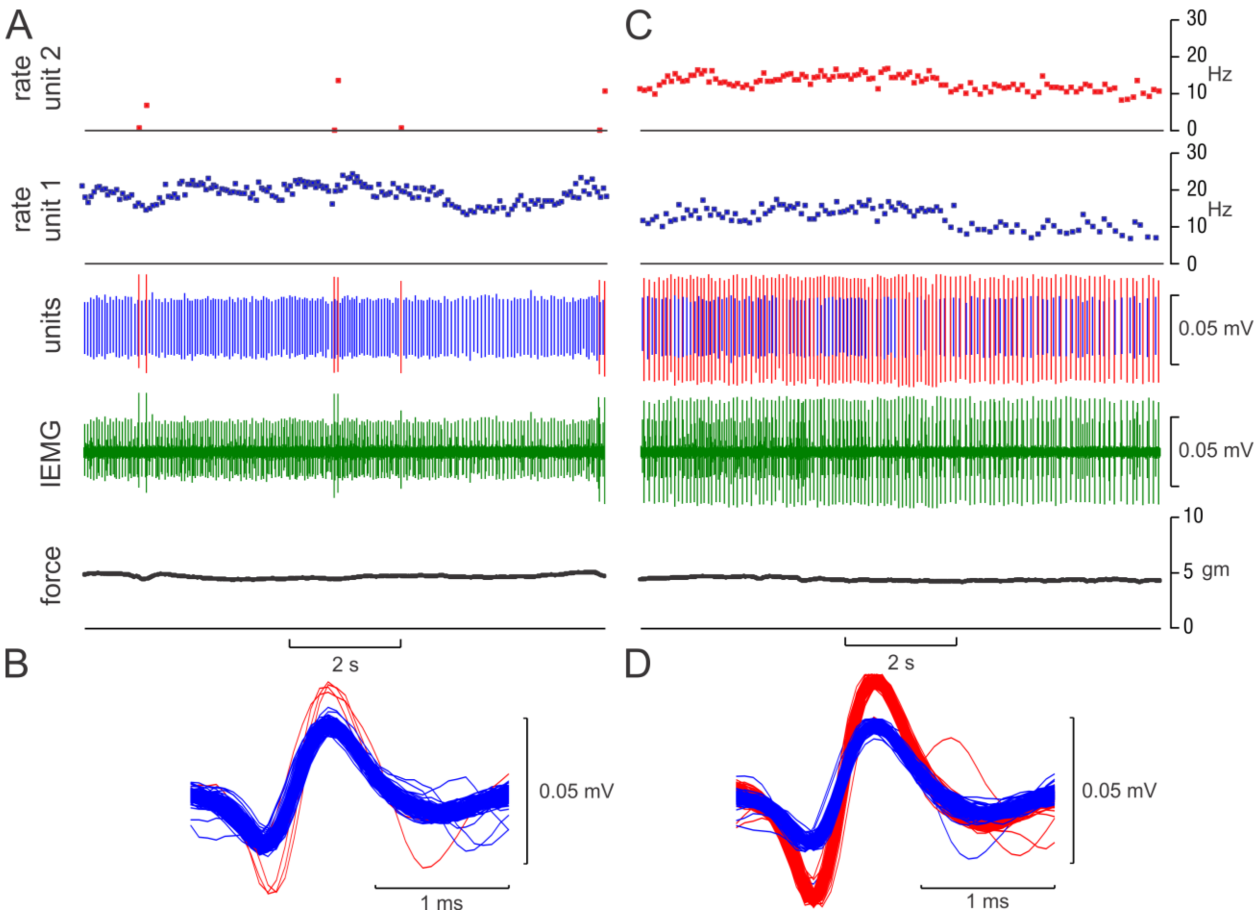

Figure 6. Effects of nifedipine on motor unit discharge during a voluntary force holding task. A) During the pre-injection period, one MU (blue) discharged tonically at an average rate of ~ 20 imp/s. A second MU (red) discharged only sporadically. B) superimposed MU potentials of discharges shown in panel A. C) Following nifedipine injection (10 µg), blue MU firing rate dropped to about 12 imp/s while red MU now fired tonically. D) superimposed MU potentials of discharges shown in panel B.

DISCUSSION

The goal of this project was to record motor unit activity from a hindlimb muscle while awake rats performed a voluntary force-target task in the presence and absence of drug meant to prevent PIC activation in lumbar motor neurons. We were able to train these rats to perform the task in as little as 6 weeks. Furthermore, we were successful in recording motor unit activity during this task. These methods are critical on their own for testing motor unit firing patterns in awake, behaving mammals, but can be coupled with an additional strategic manipulation to evaluate mechanisms of spiking activity in motor neurons.

The idea that PICs may have an important, non-synaptic influence on motor neuron discharge was originally articulated in the 1970s[6-7]. Furthermore, the role of L-type calcium channels (LTCCs) and monoamines in this phenomenon have been thoroughly characterized in isolated preparations[8-11, 13-16]. In addition, an extensive list of dihydropyridine class LTCC antagonists have been developed and characterized[10,12], which aided in the development of the experimental design for this project. That is, we could use a well-established (although fickle) surgical technique of rodent ITC implantation to manipulate MNs in tandem with EMG recording of motor unit activity and characterize the effects that PIC blockade or activation has on MU firing rate and recruitment patterns.

In practice, however, the surgery represented a significant hurdle that can set back progress after substantial effort was put toward training these animals. While 67% of the animal that underwent surgery in this project survived (compared to ~50% in pilot work), few were suitable for the study. Some exhibited forelimb neuropathic injuries and others pulled out the catheter.

There are certainly some steps we can take to improve this ITC surgery. One would be to purchase a standard stereotaxic frame for rodents. The custom-built frame used in this project presented difficulties in anesthesia setup and required an inordinate amount of time to appropriately secure animals in the frame (particularly larger animals). Furthermore, this frame made it a challenge to achieve a 90-degree flexion of the head as is usually prescribed for this surgery. An additional improvement might be to lubricate the ITC to enable it to slide through the intrathecal space easier. Regardless of next steps, it seems vital to continue to perform verification dissections to check for any unnoticed neuropathic injuries. Indeed, such injuries could obscure the physiological effects of PICs that we are attempting to observe. It also highlights the importance of increasing the sample size, since the results presented here demonstrate the possibility for many unique complications associated with these surgeries.

Other methods might also be useful in manipulating the LTCCs. One alternative involves implantation of an osmotic mini pump, such as the one developed by Tan et al[51], with a specialized catheter inserted through a laminectomized vertebra. This would allow for scheduled release of drugs into the intrathecal space and may be less of an irritation to the rat. It would allow usage as long as the catheter stays patent.

Another method would be to use genetic modifications to knock out appropriate LTCC subunits in specific MNs caudal to the L1 vertebra. This approach too poses significant challenges. Firstly, genetic engineering of the laboratory rat has lagged behind such advances in the mouse[52]. With the exception of the CRISPR/Cas gene editing system, many of the popular genetic tools like Cre-LoxP editing systems are ineffective on rats or larger organisms. Secondly, the issue with CRISPR/Cas is that injection of this system anywhere into the intrathecal space would not be exclusive to the specific set of MNs caudal to the L1 vertebra. This would require a more complex design to achieve cell specificity, in which microRNA-dependent expression of anti-CRISPR proteins in off-target cells may would need to be explored[53]. Thirdly, and perhaps most importantly, successful usage of any genetic construct would preclude the acquisition of baseline data in the same motor units. It can then be seen why the pharmacological approach offers a better chance to make conclusions about the role of PICs in the context of our current experimental design.

Despite these difficulties, it was possible in some cases to record motor unit activity when LTCC blocker had been injected into the intrathecal space surrounding the lumbar spinal cord **(Figure 6)**. For example, one motor unit clearly showed a decreased firing rate after injection of nifedipine while a second unit was activated. This suggests compensatory recruitment mediated by increased synaptic drive to attain the same target force in the face of diminished firing rate capacity caused by blockade of PICs. It should be noted that PIC activation threshold is thought to occur at about the same membrane potential as spiking threshold[14]. As such, raising membrane potential to spiking threshold depends primarily on synaptic input and not on the production of PICs. Therefore, the ability to recruit motor neurons should not be substantially impaired when PICs are blocked with nifedipine.

If PIC blockade and amplification are successful with our current pharmacological approach, the next step would then be to delineate a more specific mechanism with further pharmacological analysis using more selective LTCC antagonists and 5-HT receptor agonists. The main limitation with nifedipine and other dihydropyridine antagonists is that they are relatively non-specific in this blockade. There are four LTCC subtypes that are named according to their main alpha subunit and two of them are highly expressed in the central nervous system: CaV1.2 and CaV1.3[54]. Though these are two similar channels, they have been shown to have different locations throughout the neuron[54] and display distinct electrophysiological and pharmacological properties[55-58]. Similarly, they have been shown to play different roles throughout the CNS, including in peripheral pain processing in dorsal horn neurons[59], rhythmic pacemaking in the substantia nigra[37], and long term potentiation (LTP) in the hippocampus[60]. Thus, it would benefit future research to characterize the contributions of these two subtypes. Again, since there are no known pharmacological blockers that are specific for either subtype, a more feasible option would be a knockdown at the transcript level with antisense nucleic acid constructs such as those used by Radwani et al[59].

A similar limitation with delineating a specific mechanism with our approach is that 5-HT can activate any 5-HT receptor in a non-specific manner. Indeed, there are many studies indicating that extrinsic 5-HT mediates PICs[8,11,15-19], but characterization of the specific 5-HT receptor subtypes involved in this mechanism can help understand its implications in pathology. There are 14 different 5-HT receptor subtypes, with 13 of them being metabotropic and 5 of those being crucial when discussing pathologies like ALS and Parkinson's Disease[61-65]. It is thought that these receptors are important for their inhibitory role on glutamate release, in which serotonergic denervation leads to dysregulated glutamate-induced excitotoxicity[61]. Though it is not practical to make conclusions about pathogenesis using our current experimental design, differential activation of each of these specific 5-HT receptors as well as other monoamine-mediated metabotropic receptors could provide some insight into PICs of pathological or physiological origins.

These last two ideas emphasize the potential for the work presented here. I have shown early preliminary data indicating reduction of motor neuron firing rate and compensatory MU recruitment with PIC blockade. Improvement in surgery, larger sample sizes, Lidocaine induced hindlimb paralysis as verification of catheter placement, and follow-up dissections are needed to help ensure the utility of this approach. With such improvements and verifications, there are many new directions one could take using the setup described here.

SUPPLEMENTAL VIDEOS

Supplemental Video 1: https://arizona.box.com/s/93slxywtdzfe0dygoklrec8286brvrls

8 minutes post 0.5 mL ITC Lidocaine injection, 11 days post ITC implantation of Rat 217.

Supplemental Video 2: https://arizona.box.com/s/k7jyr0rseenk21km3xu0l22u5p8emdn6

5 minutes post 0.4 mL ITC Lidocaine injection, 5 weeks post ITC implantation of Rat 210.

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