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Methodology for Quantifying Excitability of Identified Projection Neurons in the Dorsal Horn of the Spinal Cord, Specifically to Study Spinal Cord Stimulation Paradigms

Trevor M. Smith[1], Dongchul Lee[2], Kerry Bradley[2] and Stephen B. McMahon[1]

[1] King's College London, Wolfson CARD (London, United Kingdom)

[2] Nevro Corporation, (Redwood City, California, United States)

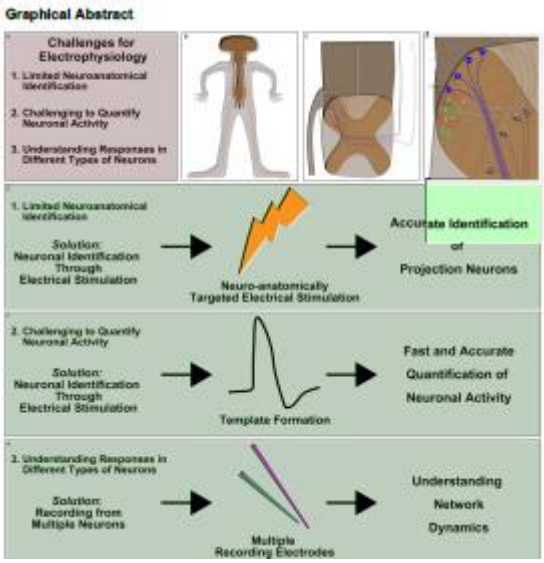
For correspondence:

Prof. Stephen B. McMahon[3] = Stephen.mcmahon@kcl.ac.uk

Highlights

- Neuroscience, particularly electrophysiology, struggles from being able to stringently identify the type of neuron being recorded from, especially with *in vivo* preparations.
- Furthermore, recording from the exact same neuron over time is challenging due to the dense neuroanatomy of the dorsal horn in the spinal cord.
- Here, we present refinements and combinations of established techniques to identify whether a dorsal horn neuron projects to the brain and to ensure that the same neuron is recorded from over 3-hours.
- Additionally, this preparation introduces a Matlab algorithm to generate a well-characterised template from controlled antidromic C2 stimulation that allows accurate and fast quantification of neuronal excitability in response to peripheral stimulation.

Graphical Abstract



Abstract

Background

Using *in* and *ex vivo* preparations, electrophysiological methods help understand the excitability of biological tissue, particularly neurons, by providing microsecond temporal resolution. However, for *in vivo* recordings, in the context of extracellular recordings, it is often unclear precisely which type of neuron the tip of the electrode is recording from. This is particularly true in the densely-populated central nervous system, such as the spinal cord dorsal horn at both superficial and deep levels.

New Method

Here, we present a detailed protocol for the identification of superficial dorsal horn spinal cord neurons that receive peripheral input and project to the brain, using multiple surgical laminectomies and the careful placement of electrodes. Once a superficial projection unit was found, quantification to electrical peripheral stimulation was performed using a Matlab algorithm to form a template of projection neuron response to controlled C2 stimulation and accurately match this to the responses from peripheral stimulation.

Results

These superficial spinal projection neurons are normally activated by noxious peripheral stimuli, so we adopted a well-characterised wind-up protocol to obtain a neuronal excitability profile. Once achieved, this protocol allows for testing specific interventions, either pharmacological or neuromodulatory (e.g., spinal cord stimulation) to see how these affect the neuron's excitability. This preparation is robust and allows the accurate tracking of a projection neuron for over 3-hours.

Comparison with Existing Method(s)

Currently, most existing methods record from dorsal horn neurons that are often profiled based on their excitability to different peripherally-applied sensory modalities. While this is well-established, it fails to discriminate between interneurons and projection neurons, which is important as these two populations signal via distinctly different neuronal networks. Using the approach detailed here will result in studies with improved mechanistic understanding of the signal integration and processing that occurs in the superficial dorsal horn.

Conclusions

The refinements detailed in this protocol allow for more comprehensive studies to be carried out that will help understand spinal plasticity, in addition to many considerations for isolating the relevant neuronal population when performing *in vivo* electrophysiology.

Keywords:
Electrophysiology, *in vivo*, Extracellular, Neuron, Spinal Cord, Projection Neuron, Matlab, Template Matching, Spike sorting.

1. Introduction

1.1 Peripheral Nervous System in the Spinal Cord

The human somatosensory nervous system is responsible for minimising tissue damage to external stimuli by conveying the sensation of pain from the periphery to highly complex neuronal networks in the central nervous system (CNS) (Basbaum et al., 2009; Peirs et al., 2015). However, a myriad of factors such as injury, genetic abnormalities, or neuronal plasticity can result in this warning system maladapting. This regularly results in chronic pain, which is common across society and cost an estimated $635 million in 2010 (National2011, n.d.), but remains poorly treated due to incomplete efficacy and dose-limiting effects of pharmacological compounds (Gilron et al., 2013). Understanding the nervous system's activation, integration and response to peripheral stimuli will facilitate the development of analgesics to provide specific and robust therapeutic options. While substantial progress has been made, the mechanistic role of specific subtypes of neurons has not

been thoroughly characterized, particularly using *in vivo* experiments. There is remarkable heterogeneity amongst neurons, which include interneurons (INs) and projection neurons (PNs), and understanding the firing properties of these different types will improve our understanding and hopefully assist the development of better treatment therapies.

Within the spinal cord is the superficial dorsal horn, which consists of neuroanatomically distinct lamina in the cat (Rexed, 1952), the rat (Molander and Grant, 1986) and humans (Schoenen, 1982). The dorsal horn is neuronally dense with an incredible heterogeneity of neurons, resulting in 15 different inhibitory INs and 15 different excitatory INs, in addition to PNs (Häring et al., 2018). In particular, Lamina I, the most superficial, is comprised of 95% INs and 5% PNs (Spike et al., 2003), which results in ~400 PNs at the L4 segment (Polgár et al., 2010). Amongst PN types, the neurokin-1 receptor positive PNs are critical in pain processing, as knock-out of neurons expressing this receptor using a Saporin toxin conjugate prevents the development of chronic inflammatory pain (Mantyh et al., 1997; Nichols et al., 1999). However, developing a pharmacological treatment to specifically target these neurons has been a significant challenge and has not yet been accomplished (Hill, 2000). However, whether chronic pain treatments, either pharmacological or neuromodulatory, are acting on these superficial dorsal horn PNs has not been robustly characterized and requires the ability to accurately record from these PNs.

1.2 Recording Neuronal Activity

Over past decades, to examine neuronal excitability, electrophysiology has been commonly used. There are many different variations, but extracellular recording protocols were first used to record from single neurons (Hubel, 1957) and this was followed by single-unit recordings in the superficial dorsal horn (Wall, 1965). More recently, there have been dramatic improvements in the temporal and spatial resolution of extracellular electrophysiology (Buzsáki, 2004; Harris et al., 2016).

While there are excellent protocols for performing *in vivo* electrophysiology of dorsal horn neurons, these do not differentiate between either PNs or the many different types of INs in both the rat (Svendsen et al., 1999; Urch and Dickenson, 2003) and mouse (Cuellar et al., 2004). There have been many informative *in vivo* electrophysiological studies to examine the effect of therapies such as neuromodulation on the excitability of dorsal horn neurons (Yakhnitsa et al., 1999; Shechter et al., 2013), but, again, these have not differentiated between INs and PNs. There are published

methodologies for a rat preparation to identify superficial PNs using antidromic stimulation (McMahon and Wall, 1983), though they haven't been widely adopted. This technique is reliable for PNs as INs are unable to follow 100 Hz reliably (Lipski, 1981; McMahon and Wall, 1983). However, by combining the nuances of these techniques, experiments can be far more informative and neurons can be identified by their functional neuroanatomy.

In addition, technological improvements offer incredible opportunity for more robust methodologies. For extracellular electrophysiology, spike sorting (Medrano et al., 2016) has improved significantly using software algorithms (Barnett et al., 2016) and this helps to track multiple units from a single recording position over time, especially in response to various stimuli. Therefore, this study introduces Matlab code to robustly quantify neuronal activity from neurons that receive input from the periphery and project from the spinal cord to the brain. Additionally, this preparation allows for the examination of different types of therapeutic intervention; in this case, a specific type of neuromodulation for pain: spinal cord stimulation (SCS) (Verrills et al., 2016). However, the principles of this extracellular recording and identification technique can be used both with different equipment and for neurons in other neuroanatomical structures.

1.3 Understanding Current Therapies

Neuromodulation offers many advantages over traditional pharmacological interventions, since neuromodulatory therapy can be far more specific through careful placement of the electrodes and a vast range of stimulation protocols to differentially affect neuronal activity (Verrills et al., 2016; Shamji et al., 2017). In this methodology, SCS was focussed on, as this is a widely-used therapy to treat pain, with two different protocols depending on the frequency: low-frequency and high-frequency (Chakravarthy et al., 2017). Currently, it is not clear whether these treatments act to affect the excitability of superficial dorsal horn neurons. Improvements in the understanding of this should help to offer dramatically improved therapies for individuals that suffer from pain.

2. Materials and Methods

The methodology presented here offers refinements to well-established techniques to help improve the quality of the information obtained from *in vivo* extracellular dorsal horn electrophysiology. The workflow is summarised below (Fig. 1.A.) and each step has a subsection offering details. Additionally, while this methodology uses specific equipment, most of it is interchangeable for similarly functioning hardware.

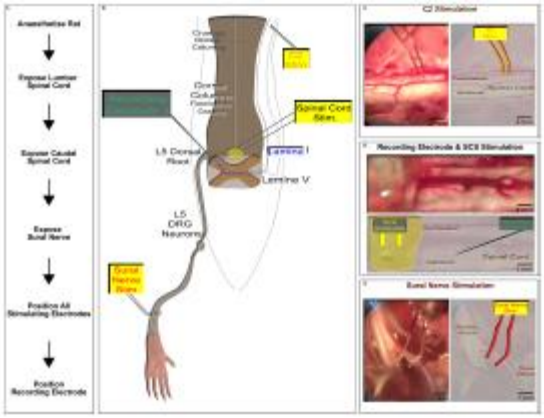


Figure 1. Detailed workflow showing the steps for targeted identification of a dorsal horn projection neuron, a schematic of the neuron and electrode positions, and photos of the relevant surgical exposures. (A) Experimental workflow, shown sequentially, to allow for testing the relevant stimulation paradigms. (B) Schematic showing the positions of the electrodes in both the peripheral nervous system and central nervous system. (C) Photo showing the position of the stimulating electrodes at C2, in the side opposite to the recording site, for antidromic activation of the projection neurons, along with labels placed over the photograph. (D) Photo showing the recording electrode and the position of stimulating electrodes for spinal cord stimulation with agar used as a medium for the cerebral spinal fluid to test various paradigms of neuromodulation, along with labels placed over the photograph. (E) Photo showing the position of the peripheral electrodes on the Sural nerve, for stimulation to test the wind-up test, along with labels placed over the photograph. Scale bars = 2 mm (C) ; 1 mm (D & E).

2.1 Animals

All animal procedures conformed to the United Kingdom Home Office Animals (Scientific Procedures) Act 1986, in addition to institutional guidelines at King's College London. For all experiments, adult male and female Wistar rats (175-250 g; Envigo Laboratories, U.K.) were used. For the data presented below, animals treated with Complete Freund's Adjuvant (CFA) were used. 5-8 days prior to recording, CFA (100 µl ; intra-plantar ; F-5881, 10 ml ; Sigma-Aldrich, U.K.) was applied sub-dermally to the hindpaw to induce inflammation (Larson et al., 1986) that produces increased pain (Ren and Dubner, 1999) and increases the excitability of peripheral nerves (Djouhri et al., 2006).

2.2 Anaesthesia

To anaesthetise rats, urethane was chosen as this is a stable and long-lasting anaesthetic for *in vivo* electrophysiology (Hildebrandt et al., 2017) and previous studies have used ether for dorsal

horn recordings (McMahon and Wall, 1983). For delivery, the route of administration of anaesthetics is an important consideration (Turner et al., n.d.) and in this case, intra-peritoneal delivery was chosen to facilitate quick absorption. Rats were carefully placed in a transparent triangular plastic bag (KCMCICE50, Kitchen Craft, U.K.), which had the end cut off so that the animal could breath while being safely restrained (Stewart and Schroeder, 2017), with minimal stress. Once calm and restrained, rats were administered intraperitoneal Urethane (1.5 g kg^{-1}; i.p. ; U2500, Sigma-Aldrich, U.K.). After administration, rats were allowed to freely roam around a box with environmental enrichment, while the anaesthetic took effect, which ranged from 10-30 minutes based upon the absorption of the anaesthetic. Once anaesthesia was observable, rats were tested for deep anaesthesia using an absence of response to the cornea reflex and areflexia to noxious pinch. If surgical level anaesthesia was not accomplished, then an additional top-up of Urethane (0.5 g kg^{-1}; i.p.) was delivered. Upon surgical anaesthesia, fur was shaved off at three locations: 1) the area just beneath the head, around the neck at the cervical level of the spinal cord; 2) the area around the lumbar enlargement; and 3) the area around the kneecap, to allow exposure of the Sural nerve.

2.3 Physiological Monitoring

Appropriate physiological monitoring is essential during multi-hour *in vivo* experiments (Tremoleda et al., 2012), and there are clear guides for experiments requiring artificial ventilation (Grimaud and Murthy, 2018). In this instance, core temperature, respiratory rate/carbon dioxide (CO_2) expiration, blood pressure, and heart rate were all monitored through specialised equipment. Additionally, a respiratory pump was used to maintain respiration after the delivery of the neuro-muscular blocker. For temperature, rats had a rectal temperature probe inserted and through a feedback system, were kept warm using a heating blanket adjusted to maintain their core temperature around 35 °C (Harvard Apparatus; U.K.). For respiration, an incision was made along the exposed skin and the tracheal muscles were bluntly dissected to expose the trachea, before a tracheotomy and the insertion of a y-shaped cannula (73-2834, Biochrom, U.S.A.), with a respiratory pump (Model 7025, Ugo Basile, Italy) and required tubing ready for later use. The outflow was also connected to an end-tidal CO~2~ monitoring machine (Capstar-100, CWE, U.S.A.), which had a BNC cable out to a 1401 Power Mk II (Version 3, Cambridge Electronic Design, Cambridge, U.K.), which was controlled using Spike2 software (Version 8, Cambridge Electronic Design, Cambridge, U.K.) for continuous monitoring during the experiment. The external jugular vein was cannulated (2.5F, O.D. 0.75 mm, Portex, U.K.) to allow for administration of 0.9% physiological saline (10 ml vial, GKT-IL, GKT Immunoregulation Laboratory, Cambridge, U.K.), warmed to 37 °C using a water bath every hour throughout the experiment to maintain hydration. For monitoring of blood pressure, the carotid artery was also cannulated using tubing (P00/100/200, I.D. 0.58 mm, O.D. 0.96 mm, Portex, U.K.) filled

with hepinarised (20 units / ml ; 10429693, Fischer-Scientific, U.K.) saline and connected to a blood pressure transducer (NL108T2, Digitimer, U.K.) which was connected to a Neurolog unit (NL108A, Digitimer, U.K.) and also wired into the 1401 and continuously visible on Spike2. The blood pressure remained between 80 - 120 mmHg throughout the experiment. Finally, an EKG neurology unit (Digitimer, U.K.) was used with two stainless steel electrodes placed intra-muscularly on the left and right forelimbs to continuously monitor the heart rate.

To improve stability during the recordings, the rat was clamped in multiple places. The head was held in location using a nose clamp and ear bars on a stereotaxic frame (Model 1430, Kopf Instruments, CA, U.S.A.). At the hips, bars were positioned to improve stability and the tail was clamped to provide axial tension.

2.4 Lumbar Spinal Cord Exposure

To gain access to the relevant part of the dorsal horn (in this preparation's case, the lumbar region), a laminectomy was made starting with an incision along the spinous processes of the T12-L2 vertebrae. Then, a custom-made, approximately 10-centimetre (cm) long by 5 cm wide oval metal loop was affixed to the skin to create a pool around the area of the lumbar laminectomy. After removal of the muscle and other connective tissue over the vertebrae, a custom-made spinal cord clamp was attached in the caudal-rostral direction around the lumbar enlargement to minimise movement. Then, the vertebrae were carefully chipped away using bone rongeurs (16015-17, Fine Science Tools, Germany). This lumbar laminectomy was then covered in blue paper tissue and submerged in warmed saline, until the placement of the electrodes, later in the preparation.

2.5 Cervical Spinal Cord Exposure

After completion of the lumbar laminectomy, the cervical laminectomy was performed by using a scalpel blade to make an incision between the C1-C6 vertebrae. The incision naturally created a parting of muscles that allowed this area to be filled with solution to create a pool. The spinal cord was exposed, the dura was left intact, and covered in blue tissue and warmed saline.

2.6 Sural Nerve Exposure

Using a scalpel blade, an incision was made along the knee region to isolate the triceps surae muscles, that lie over the sciatic nerve. Then, a custom-made, approximately 5-centimetre (cm) long by 4 cm wide oval metal loop was used to tie the skin to and create a pool. The muscles were bluntly

dissected and the sciatic nerve and branch of the sural nerve was exposed. The sural nerve is comprised of ~75% nociceptors, making it ideal to examine the central responses to noxious input (Schmalbruch, 1986; Swett et al., 1991). Then, the nervous tissue was covered in blue tissue that had been dipped in warmed 0.9% physiological saline. Dental glue (Xantropren XL; Kent Express, U.K.) was applied around the blue tissue to create a pool, which was then filled with warmed mineral oil (Product 31911, Alfa Aesar, MA, U.S.A.).

2.7 Positioning of Stimulating Electrodes

After the completion of all of the surgeries, stimulating electrodes were placed in three positions (Fig. 1. B): the sural nerve, the C2 region of the spinal cord, and a vertebral segment rostral to the recording area in the lumbar region of the spinal cord (the latter position was used to examine the effect of neuromodulatory paradigms on the dorsal horn projection neurons). The sural nerve stimulating electrodes consisted of two platinum wires (0.25 mm diameter, PT540707, Advent Research Materials, U.K.), spaced about 1 mm apart. These were placed underneath the Sural nerve while not touching any of other tissue and surrounded with warm mineral oil.

For the C2 stimulating electrodes, the blue tissue was removed and all excess saline was absorbed. Then, the dura was carefully lifted and cut. After absorbing excess CSF and any other fluids, a few drops of Lidocaine (2% w/v; Braun, U.K.) were applied to the spinal cord and fine metal watchmaker forceps were used to crush only the dorsal columns at C4 (McMahon and Wall, 1983). The Lidocaine was then absorbed using a cotton bud and mineral oil was applied to the pool. The dorsal columns were crushed to help improve the signal to noise by removing a confounding and completely different neuronal population. Bipolar stimulating tungsten electrodes (20-um exposed tip; A-M Instruments, U.K.) were inserted superficially into the contralateral side of the spinal cord using a Kopf micro manipulator to a depth of ~200 μm. This is the best neuroanatomical location for activating these projection neurons using both functional electrophysiological (McMahon and Wall, 1983) and histological (Polgár et al., 2010; Todd, 2010) studies. Then, the tissue was submerged in mineral oil that had been warmed to 35 °C in the water bath.

Finally, for the testing of neuromodulation effects on PNs by spinal cord stimulation (SCS), 4% agar in 0.9% saline was prepared on a magnetic hot plate stirrer that was set to ~90°C. SCS electrodes consisted of a bipolar pair of TEFLON-insulated silver electrodes (electrodes separated ~1.12 mm; each electrode 0.5 mm diameter; 99.99% silver wire insulated with TEFLON; Gi2479; Advent

Research Materials Ltd.). Using a coarse micromanipulator, these electrodes were lowered to ~0.5 mm off of the cord. Then, the blue tissue and saline was removed. The dura was cut between T11 - L5 and the excess CSF was absorbed. Then, using the previously prepared agar, a faux cerebrospinal fluid layer was formed by surrounding the electrode tips in rapidly cooling 4% liquid agar, made with dilute 0.9% saline. Once the agar had hardened, the whole pool was filled with warm mineral oil.

To establish the required testing amplitude of the SCS, the motor threshold, which is the stimulation amplitude required to produce activation of motor fibres visible from muscle twitch, was determined. This was performed using the stimulator on a 2 Hz cycle and slowly increasing the amplitude by 40 μA steps until muscle twitch was observed. The lowest current amplitude required for muscle twitch was recorded as the motor threshold (MT).

2.8 Paralysis of the Rat

To ensure stability of the recording electrode and the other electrodes in the preparation, once the MT had been determined, the animal was paralysed using a non-depolarising neuromuscular blocker (Itoh et al., 2004). In this case, Gallamine was administered intravenously in 1 ml volumes at a dosing volume of 5 mg / ml dosing volume to produce a final dose of ~ 25 mg / Kg. Once Gallamine had been administered, the respiratory pump was immediately connected to both the ‘in’ and ‘out’ flows of the Y-shaped tracheal cannula and switched on.

2.9 Positioning of Recording Electrodes

For the recording electrode setup, Neurolog units were used, starting with the NL100 differential headstage, which was connected to a Kopf micromanipulator with fine and coarse adjustment (1460, Kopf Instruments, CA, U.S.A.) The A input was connected to a Carbostar microelectrode (Carbostar-1, Kations Scientific, Hungary) which was lowered onto the spinal cord, while the B input was connected to a stainless steel pin inserted into the muscle on the contralateral side, which was not recorded from. To explore tip size, the Carbostar’s were photographed using a light microscope. Additionally, tungsten electrodes with 2.0 MΩ impedances (Microelectrodes, Cambridge, U.K.) were photographed and tested.

The recorded signal was digitised at 10000 Hz and then processed using the following hardware: the NL100 differential amplifier was connected to a pre-amplifier (NL 104, Digitimer, U.K.) with a gain of 1000. This was connected to filters (NL125, Digitimer, U.K.) set to bandpass frequencies of

500-5000 Hz, which then connected to an AC/DC amplifier (NL106, Digitimer, U.K.) with a gain of 1. Mains noise, present at 50 Hz in the U.K., was removed using a Humbug (Quest Scientific, Vancouver, Canada). After this, the signal was converted from analog to digital using a Power 1401 (Ver. 3, Cambridge Electronic Design, Cambridge, U.K.) and recorded using Spike2 software (Ver 8, Cambridge Electronic Design, Cambridge, U.K.). All data was recorded using Spike2 and was exported to Matlab.

2.10 Identification of a Projection Neuron

When recording with a single electrode, the micromanipulator approached at an angle of about 30° parallel to the rostrocaudal axis of the spine, to achieve the smoothest entry into the spinal cord. The recording electrode was positioned on the white matter, ~1 mm off of the centre of the cord. Then, the electrode was slowly advanced at a rate of ~5 µm to a maximal depth of ~500 µm from the top of the cord, which included white matter. The depth of lamina I, which is the target and the location of PNs implicated in chronic pain, varies with the distance from the centre of the spinal cord (Seagrove et al., 2004), due to the varying thickness of white matter. For this reason, the depths, from the most dorsal part of the spinal cord, vary dramatically based on the medial-lateral position of the recording electrode.

Once the recording electrode was in position, C2 stimulation (3 monophasic pulses at 100 Hz, 200 µs, ~ 500 μA; every 0.5 Hz) was initiated and depths were constantly monitored to ensure that the superficial lamina was still being targeted (Fig. 2. B-C). Then, the C2 stimulation protocol was activated and the electrode was slowly advanced until a signal with a constant latency was evoked by the C2 stimulation. Once a signal was located, the electrode was repositioned to maximise the signal's amplitude. Also, previous experiments confirmed that these units are activated specifically by their axons running primarily through the dorsolateral funiculus (dorsal column stimulation at C2 did not activate these neurons (McMahon and Wall, 1983)).

Three C2 stimulations, at 100 Hz, were delivered to determine the type of unit, as INs are unable to follow consecutive C2 stimulation at 100 Hz (Fig. 2.D), while a PN doesn't have any synaptic delay and does successfully follow the 100 Hz stimulation (Fig. 2.E) (Lipski, 1981). Additionally, across experiments, PNs always followed consecutive stimuli and followed all three stimulations > 50% of the time. Finally, only units demonstrating an initial signal-to-noise ratio (SNR) of at least double the background activity were used, and the electrode was repositioned until this was achieved. An

additional check that can be run is a collision test, where neurons are activated anti- and ortho-dromically to show that collision results in an absence of firing and this has been doing using a similar preparation (Keller et al., 2007). In this preparation, often multiple tracks would have to be made before finding a suitable unit.

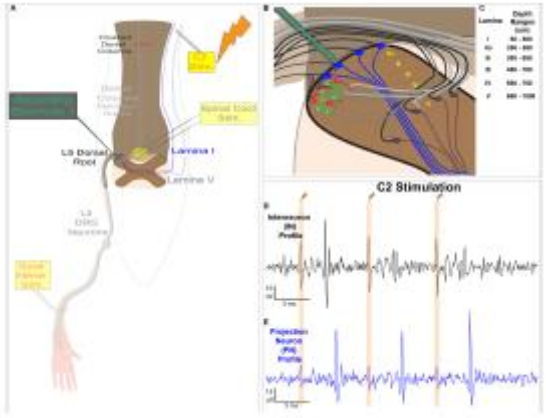


Figure 2. Importance of using antidromic stimulation to identify and isolate dorsal horn projection neurons. (A) Schematic showing the relevant electrodes for this part of the preparation, with C2 stimulation (orange lightning bolt) to antidromically activate the projection neuron located in the superficial dorsal horn being recorded from using recording electrode 1. (B) Enlarged schematic of the superficial dorsal horn and the neurons that act upon superficial dorsal horn projection neurons. Green neurons are excitatory interneurons (e) and red neurons indicate inhibitory interneurons (i). (C) Quantification of the electrode depths taken perpendicularly from the white matter on the most dorsal part of the spinal cord. (D) A representative trace of a dorsal horn interneuron that is unable to follow the C2 antidromic stimulation at 100 Hz. (E) A representative trace of a dorsal horn projection neuron that is able to follow C2 antidromic stimulation at 100 Hz.

2.11 Peripheral Threshold Determination

To test the excitability of a particular neuron, a robust and reproducible activation protocol is required. While physiological stimulation helps to identify the type of neuron being recorded from, responses can be inconsistent and require more time (Urch and Dickenson, 2003), whereas electrical stimulation is more stable and commonly used (Mendell, 1966; Urch and Dickenson, 2003; Cuellar et al., 2004). Therefore, in this preparation, after noting the neuron's response to various physiological stimulations applied to the hindpaw, electrical stimulation was used to determine an excitability profile. Other preparations electrically stimulate the periphery by inserting pin electrodes into the paw and activating much larger areas of the periphery using larger current amplitudes (Urch and Dickenson, 2003; Guan and Raja, 2010; Shechter et al., 2013). Instead, to use less electrical

current by delivering to a more specific location ((Pubols, 1990), we isolated the sural nerve and stimulated peripheral nerve fibres directly. Due to the distance of the Sural nerve to the PN (~14 cm), the latency of the PN's activity could reveal which peripheral nerve fibre type was activated by the Sural nerve stimulation (Fig. 3.A). For this preparation, these windows consisted of Aα/β between 0 – 20 ms; Aδ between 20 – 70 ms ; C between 70 – 300 ms ; and a post-discharge window between 300 – 1000 ms, which are similar to other studies (Urch and Dickenson, 2003) (Fib. 3.B).

The peripheral stimulation protocol (biphasic pulses at 0.5 Hz, 50 µs) determined thresholds by increasing stimulation amplitude by ~ 20 μA at every repeated stimulation. Biphasic stimulation was used as this is less tissue damaging than monophasic (Merrill et al., 2005). When units were active in successive traces at latencies for the windows for each of the Aα/β-, Aδ-, or C- fibre types, then the higher value was recorded as the threshold. In the example below, the thresholds were 20 μA for no nerve fibre activation (Fig. 3.C), 140 μA for Aα/β-fibres (Fig. 3. D), 160 μA for Aδ-fibres (Fig. 3. E), and 420 μA for C- fibres (Fig. 3. F).

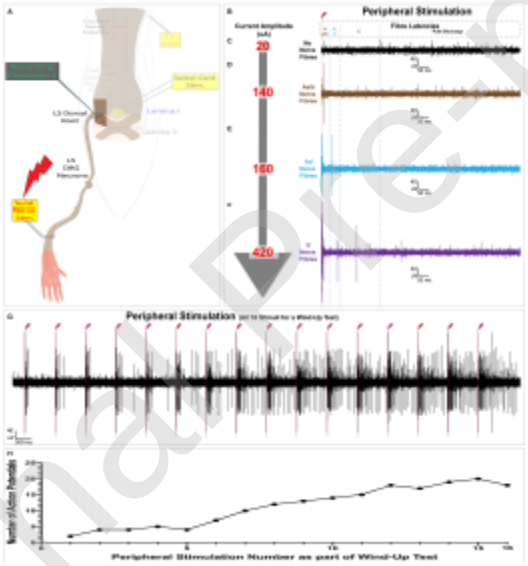


Figure 3. Importance of determining peripheral thresholds for activation of dorsal horn projection neurons for wind-up tests. (A) Schematic showing the relevant part of the preparation, with peripheral stimulation of the Sural nerve to orthodromically activate the projection neuron located in the superficial dorsal horn. (B) Schematic indicating the latency at which the different peripheral nerve fibre types are activated. (C) Example trace when the peripheral stimulation is sub-threshold for all fibre types. (D) Example trace when the peripheral stimulation is supra-threshold for Aα/β-fibres. (E) Example trace when the peripheral stimulation is supra-threshold for Aδ-fibres. (F) Example trace when the peripheral stimulation is supra-threshold for C-fibres. (G) Example of 16 sequential peripheral stimulations at 2 x C-fibre threshold to determine whether wind-up is present, with quantification of this neuronal activity (H).

2.12 Peripheral Wind-Up Test

Neurons are able to display remarkable plasticity in response to repetitive stimuli. This is a well-known phenomenon, termed 'wind-up,' for spinal cord neurons that fire increasingly to the same strength stimulus, if there is a short-enough inter-stimulus gap (Mendell and Wall, 1965; Mendell, 1966). While many preparations determine the electrical threshold for C-fibre stimulation and multiply this by 3, in this preparation, we demonstrate that a multiplication of 2x C-fibre threshold and the conventionally used 16 stimulation pulses (Urch and Dickenson, 2003; Cuellar et al., 2004) is sufficient to observe wind-up (Fig. 3. G). The wind-up is particularly visible when quantified (Fig. 3. H), with the last few stimuli showing multiples more activity than the first few stimuli. Traditionally, the quantification of activity in response to a wind-up protocol is relatively laborious and also, challenging in situations where there is a low SNR, or electrode movement that might result in a different unit being recorded from.

2.13 Peripheral Wind-Up Test Quantification using Matlab Code

This extracellular electrophysiological recording experimental design uses the C2 stimulation to identification of the unit of interest and with this protocol, templates can be formed in response to the 1st, 2nd and 3rd C2 stimulations (Fig. 2. F). The beginning and ending of the antidromic stimulation of the unit measured from the microelectrode, for each of the three consecutive stimuli (S1→ Response1 [R1], S2 → R2, S3 → R3), were manually selected from digitized recordings using a customized graphical software interface developed in Matlab (Mathworks, MA, USA). Ensemble averages, from more than 10 repeated trials, generated three templates (T1, T2, T3), one for each response to the consecutive stimuli. The latencies, from C2 stimulation, were examined to ensure that there was consistency over all three traces. If traces showed slight jitter in their times, only visually-consistent responses were used. Additionally, the three responses, R1, R2, and R3, often varied slightly in latency, with the 2nd stimulation having a shorter latency than the first, a well-characterised phenomena (Waxman and Swadlow, 1977). Once the templates, T1, T2 and T3, were formed, the peripherally-driven wind-up traces were scanned for these templates, with a similarity algorithm (~70% similarity) providing quantified activity for predefined time windows: Aα/β (0 - 20 ms); Aδ (20 - 70 ms); C (70 - 300 ms) and a post-discharge (300 - 999 ms). Total activity was quantified by summating Aδ-, C-, and post-discharge values. Aα/β values were not included as sometimes the stimulation artefact was so large that it obsured the recordings in this 0-30 ms window. These quantified values were then available and could be copied to other software, such as Microsoft Excel or Graphpad Prism.

2.14 Recording from Two Projection Neurons

During the optimisation of this preparation, questions arose about how other PNs were responding to these protocols. Therefore, the recording hardware was mirrored and another electrode was inserted into the cord, but rather than at a 30° angle, it was perpendicular to the cord, to ensure that there was enough space for both recording electrodes. The perpendicular electrode was inserted first, as this caused the largest dimpling of the tissue, and once a PN was found and isolated, the second recording electrode was inserted and another PN was found. With these electrodes, there are a huge number of permutations available, as these could be in the same neuroanatomical location in the cord, or in adjacent segments, or different depths. Also, for these experiments, C2 stimulation was delivered using 2 pulses at 100 Hz, with a frequency of 0.5 Hz. Once the PNs are found, it is slightly harder to ensure that they both respond to Sural nerve stimulation. This technique would allow a detailed mapping of the functional neuroanatomical terminations of sensory neurons onto projections neurons at a specific vertebral spinal segment.

2.15 Use of Therapeutic Intervention

This technique describes the ability to test neuromodulatory paradigms on the excitability of dorsal horn PNs. In particular, SCS relies upon electrodes that sit epidurally, just above the superficial dorsal horn. Therefore, this preparation allows for testing of SCS that relies upon either of low- and high-frequency current delivery. Furthermore, these electrodes can be placed at multiple neuroanatomical positions. In this preparation, the electrodes were placed for application of high-frequency SCS, which is directly above the segments that they seek to affect. Additionally, this protocol allows the ‘tracking’ of a PN over time, with the C2 latency being known; thus, almost any therapy, whether neuromodulatory or pharmacological, could be tested in this setup. We then re-tested the C2 stimulation protocol and retracted our recording electrode ~20 µm from our confirmed dorsal horn PN, reducing the SNR, to account for the swelling of the cord over time.

3. Results

3.1 Preparation

The methodology presented here aims to improve current *in vivo* electrophysiological extracellular recordings from single neurons in the dorsal horn of the spinal cord. An advantage of this preparation is the stability, with a PN being tracked for 3-hours and continuing to wind-up over 16 peripheral stimuli (Fig. 4). Most importantly, the C2 antidromic stimulation allows for verification of the same

unit being recorded from throughout the entire preparation: 0-mins (Fig. 4. A), 45-mins (Fig. 4. E), 90-mins (Fig. 4. I), 135-mins (Fig. 4. M), and 180-mins (Fig. 4. Q). Additionally, the SNR remains at ~7, or higher, over all of these timepoints.

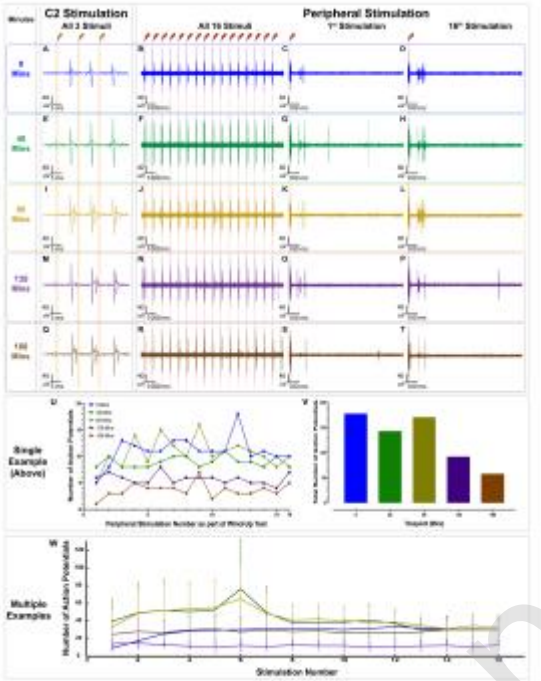


Figure 4. Data showing the stability of this recording methodology over 3s hours, with C2 stimulations remaining constant and the response to peripheral stimulation also remaining. Testing was performed every 45-minutes with C2 and peripheral responses matched for 0-mins (A-B), 45-mins (C-D), 90-mins (E-F), 135-mins (G-H), and 180-mins (I-K). (U) The number of action potentials to each of the 16 stimuli was quantified using our unique template matching algorithm and plotted on a single graph. (V) The total number of action potentials was also calculated and plotted for each timepoint. (W) From multiple experiments, the number of action potentials to each of the 16 stimuli was quantified using our unique template matching algorithm and plotted on a single graph with the mean and standard error of the mean (n = 4 rats at all time points, except 135-minutes, where n = 3 rats).

By establishing the consistency of the C2 stimulation, templates for each time point can be generated to ensure that the data can rapidly be analysed using a custom-made Matlab script, which quantifies the activity of this particular unit in response to the wind-up protocol (16 peripheral stimulations ; 2x C-fibre threshold ; 1 Hz frequency). Typically, quantification requires setting numerous, variable parameters to form a template and then attempts to quantify the activity. In cases where the SNR lowers over time, this can result in challenging data analysis. The technique presented here allows for a fixed set of parameters to analyse every dataset.

In this particular dataset, the unit continues to wind-up, although it is minimal, demonstrated by the activity of the last stimulation being more than the average activity to the first stimulation at each time-point: 0-mins (Fig. 4. B-D), 45-mins (Fig. 4. F-H), 90-mins (Fig. 4. J-L), 135-mins (Fig. 4. N-P), and 180-mins (Fig. 4. R-T). Quantification of the activity to each stimuli does reveal the wind-up phenomena at each time point (Fig. 4. U), although the total activity over the 16-stimuli does decrease over the duration of the preparation (Fig. 4. V) by 19.66 % at 45-mins, 3.93% at 90-mins, 48.31% at 135-mins, and 67.42% at 180-mins. Across multiple experiments (n = 4 rats), the total activity over the 16-stimuli was variable and increased by 29.27 ± 17.48% at 45-mins, 84.72 ± 87.21% at 90-mins, before decreasing by 10.92 ± 55.02% at 135-mins, and then increasing again by 17.79 ± 66.33% at 180-mins. The decrease at 135-minutes was affected by the loss of a unit due to variance in the C2 latency time resulting in the recording of another unit.

3.2 Template matching and similarity Computation

As previously mentioned, one of the strengths of the methodology presented here is in the quantification of peripheral activity using the template formed from C2 stimulation. Here, the 'similarity' was defined by a point-by-point comparison between the template and candidate response. To generate a binary decision (e.g., accept or reject the response), a 'similarity threshold' (ST, ranging from 0-1) was created. To optimize the ST, different ST values were applied to recorded data and the success of single-unit detection was compared to visual judgement by human expert. To optimize the discriminability of the template-matching technique, we first computed the similarity of all three templates to orthodromic single-unit firings of the target neuron in response to repetitive activation of afferent hind limb nerves. Similarity value had peaks for each AP. Depending on the selection of templates (T1, T2, T3), similarity values for expert-identified APs were slightly different. Similarity (Y axis) of AP to T2 & T3 was compared to the similarity of AP toward T1 (X axis). Most points were located below unity line, indicating that the AP were more similar to T1 than other templates. With extracellular recordings, APs can have varying morphologies (e.g., because of noise from neighboring neurons), but with this template matching algorithm, the unit of interest could not only be identified but isolated from the neighbouring units. This can be viewed in Fig. 4.F, where a smaller unit has fired a few times, but this activity was not quantified. This is a major advantage of any extracellular electrophysiological technique that simply uses thresholding to quantify neuronal activity. Furthermore, thresholding does not reveal whether the electrode has moved over the duration of the preparation. We did observe recordings where there was drift and the C2 latencies changed or disappeared, revealing interneurons with similar peripheral response profiles.

3.2. Electrodes

To optimise the recording and the chances of success, different types of recording electrodes were tested for their efficiency at optimising recordings from superficial dorsal horn PNs. Magnified photographs and sample traces are shown for Tungsten microelectrodes with 2.0 MΩ impedance (Fig. 5. A,C.), and carbon-fibre microelectrodes with 1.0 MΩ impedance (Budai and Molnár, 2001) (Fig. 5. B, D.). In multiple preparations, the same PN was found and stimulated using C2 using both Tungsten (Fig. 5. E) and Carbostar (Fig. 5. F) to allow for direct comparison of SNRs and properties. A comparison of the SNRs of these electrodes is also shown (Fig. 5. J.) and revealed that there no differences between these different types of electrodes, although the Carbostar did show less variability. One difference observed was the ease with which the electrode entered the cord, with the thinner profile Carbostars entering the tissue more easily, with less dimpling and carbon offering stable long term neuronal recordings (Guitchounts et al., 2013).

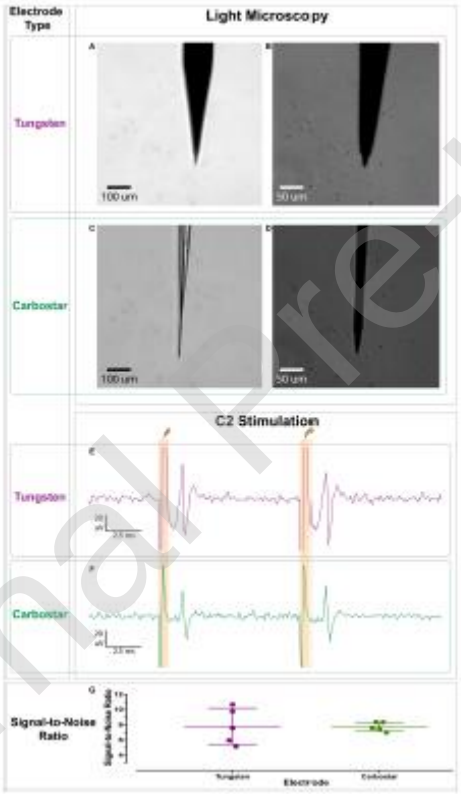


Figure 5. Comparison of different electrodes and impedances for recording superficial dorsal horn projection neurons. Light-microscope images of Tungsten microelectrode with 2.0 MΩ impedance (A,C), and carbon fibre microelectrode with 1.0 MΩ impedance (B, D). (E,F) Sample recordings showing the amplitude of the unit while using tungsten (E) and Carbostar (F) microelectrodes. (G) Quantification from multiple parts of the same trace showing the SNR for each microelectrode.

3.3 Recording from two projection neurons

This technique offers a robust way to examine the excitability of superficial dorsal horn PNs, however, examining responses in multiple neurons will be essential to fully understand nervous system plasticity. While imaging has offered fantastic insights into the activity of larger populations, the trade-off is that Calcium imaging has decreased temporal resolution of seconds, not microseconds, (Ran et al., 2016) and two-photon imaging is only able to record up to depths of about 20 μm (Fenrich et al., 2012). Other electrophysiological preparations, such as *ex vivo*, paired recordings have offered incredible insights into how neuronal excitability is regulated (Magnuson et al., 1987; Hachisuka et al., 2016). *In vivo* recordings are far more technically challenging, but the neuron is recorded from in the natural environment with structures largely intact. Therefore, performing this technique while recording from two neurons, particularly if two PNs in different neuro-anatomical areas (i.e. Lamina I and V), could reveal informative datasets (Fig. 6. A-B.).

After duplicating all the required hardware, the biggest challenge was the positioning of the microelectrodes above the spinal cord. For this reason, one recording electrode entered at the normal 30 ° and the other came in at 90 °, which caused more dimpling and was therefore always inserted first. However, this technique, after searching and getting the electrodes to the required depths in the dorsal horn (Fig. 6. B-C.), can isolate two PNs that are activated by C2 stimulation on recording electrode one (Fig. 6. D.) and recording electrode two (Fig. 6. E.). These were overlaid (using an oscilloscope) to validate that the stimulation artefacts were the same and that the latencies of the units were different (Fig. 6. F). Next, delivering the appropriate peripheral stimulation was challenging, as it was not easy to find two neurons that respond to sural nerve stimulation with the exact same dermatomes. Although mechanical stimulation, such as pinching, can activate both neurons (data not shown), the usefulness of this data will be impacted by how robustly they can be activated in the periphery using electrical stimulation. In this preparation, both units did respond to electrical peripheral stimulation, as shown by raw traces recording electrode one (Fig. 6. G.) and recording electrode two (Fig. 6. H.). However, as indicated by the green arrow, recording electrode one only responded with A-fibre latency, while recording electrode two had other fibre-latencies present as well as indicated by the purple arrows.

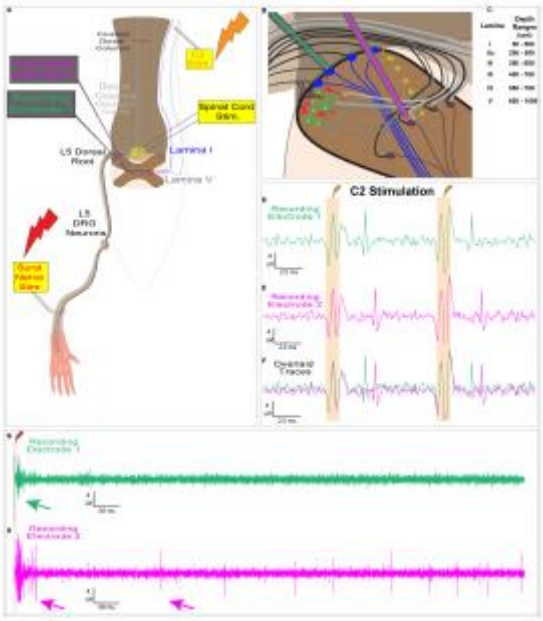


Figure 6. Results from isolation and recording of two dorsal horn projection neurons. (A) Schematic showing the position of the electrodes during this methodology. (B) Schematic showing positions of the recording electrodes in the dorsal horn during this particular recording. Green neurons are excitatory interneurons (e) and red neurons indicate inhibitory interneurons (i). (C) Depths permitted to isolate neurons in specific laminae. (D-F) Data trace showing antidromic activation, from C2 stimulation, of the two projection neurons in recording electrode one (D), recording electrode two (E) and both traces overlaid (F). (G-H) Data trace showing peripheral stimulation, in this case, electrical stimulation, of both neurons in recording electrode 1 (G) and 2 (H).

Additionally, at any point, this technique could also be used to confirm whether a neuron projects and if it doesn't, then knowing an IN is being recorded from could also be useful information. Some of the INs had responses to peripheral stimulation that matched the PNs (data not shown).

4. Discussion

In order to offer better treatment for chronic diseases, especially illnesses involving maladaptive plasticity of the nervous system, such as pain, there needs to be better understanding of the precise mechanisms involved in the development of these illnesses. Therefore, techniques are required to have with greater resolution with accurate tracking of the neuronal signals of interest, so that more power is provided from the results of each study. This methodology offers the following improvements for examining the excitability of dorsal horn neurons using single-unit recordings:

1. Functional neuroanatomical identification of the neuron being recorded form by confirming whether it projects to the brain.
2. Accurate tracking of the neuron of interest, so that over time, the unit can be validated to be the same.

3. An *in vivo* methodology to record from two neurons in either different or the same lamina the dorsal horn, either INs or PNs, to study the effects of their activity over time in response to a treatment.

While this paper offers a precise type of electrophysiological recording, these principles are applicable across all extracellular recordings using either single-electrode or multiple-electrodes.

The accurate identification of the neuron being recorded from is critically important to be able to put results in context. The methods for PNs in the superficial dorsal horn presented here could be even further enhanced using a collision test, which uses concurrent ortho- and antidromic activation of the unit to observe cancellation when activity is expected (Lipski, 1981). For processing peripheral inputs, these PNs are important as knock-out studies have demonstrated (Mantyh et al., 1997; Nichols et al., 1999) for local spinal circuits, as well as for PN projection targets, which consist of multiple brain regions, with ~95% reaching the lateral parabrachial nucleus, ~33% reaching the periaqueductal gray, ~25% reaching the nucleus tract solitarus, and ~5% reaching the thalamus (Todd, 2010). Therefore, understanding the responses of these neurons in normal and disease states offers a way to improve treatment.

Additionally, the use of the peripherally-driven wind-up protocol is widespread and wind-up in spinal neurons has been well characterised (Herrero et al., 2000). Using this protocol, in the example presented here, there was a decrease in activity compared to 0-mins at 135- and 180-mins. This could well be a physiological adaptation of this particular neuron, as the dorsal horn neurons are highly plastic to allow for processing of sensory inputs, especially noxious information (Basbaum et al., 2009; Todd, 2010; West et al., 2015; Peirs and Seal, 2016). Additionally, with this technique, neurons can be physiologically identified using a standardised set of physiological stimuli (Harper and Lawson, 1985), as the primary afferents and their terminals are fully intact. Typically, PNs are classified into nociceptive, non-nociceptive, or unresponsive (McMahon and Wall, 1983; Craig and Kniffki, 1985). However, the responses to physiological stimuli are typically more variable than to electric and improvements are being made to automate the application of nociceptive mechanical stimuli (Dale et al., 2019).

Many *in vivo* electrophysiologists are now using electrodes with multiple contacts to record from more than one neuron during a protocol, with a single probe containing 384 recording channels (Jun et al., 2017) or being inserted into multiple lamina within the spinal cord (Greenspon et al., 2018). However, most of these arrays are not-flexible and if a large unit is lost, it is exceptionally hard to re-adjust the whole array. Additionally, accurate identification of the units being recorded from are more important than ever and the principles of experimental design here should prove useful.

Regarding the loss of a unit due to movement, while the two-electrode protocol also suffers with this, with each electrode on its own micromanipulator, the precise position of microelectrodes can be adjusted to optimise the SNR or adjust the position in the middle of a preparation. The antidromic stimulation from C2 and the unit's latency allow confirmation that the unit is the same. Additionally, spike-sorting algorithms for multi-electrode arrays are improving rapidly, but don't seem to have yet embraced the use of electrical stimulation for unit identification and template formation (Pachitariu et al., 2016; Steinmetz et al., 2018).

5. Conclusion

Using repetitive antidromic stimulation at the C2 spinal vertebrae level of the spinal cord, superficial dorsal horn PNs were activated and stably recorded for up to 3-hours, which allowed the formation of templates of the unit's response. With these templates, accurate quantification of peripherally-evoked activity from hind limb nerve stimulation was easily and robustly performed. We found our template-matching technique was a rapid, reliable, semi-automated technique for consistently identifying single unit firing in this experimental preparation for SCS.

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