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Meijs, Suzan; Bjarkam, Carsten Reidies; Andreis, Felipe Rettore; Jensen, Winnie

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First Demonstration of Nociceptive and Non-Nociceptive Responses from Spinal Neurons in a Porcine Model

Suzan Meijs, PhD
Center for Neuroplasticity and Pain (CNAP)
Aalborg University
Aalborg, Denmark
smeijs@hst.aau.dk

Prof. Carsten Reidies Bjarkam, PhD, MD, Dr. Med.
department of neurosurgery
Aalborg University Hospital
Aalborg, Denmark
c.bjarkam@rn.dk

Felipe Rettore Andreis, PhD
Center for Neuroplasticity and Pain (CNAP)
Aalborg University
Aalborg, Denmark
fran@hst.aau.dk

Prof. Winnie Jensen, PhD
Department of Health, Science and Technology
Aalborg University
Aalborg, Denmark
wj@hst.aau.dk

***Abstract*— The spinal cord plays a key role in pain processing, but it remains unexplored in large animal models. We have developed a methodology to record from spinal neurons using three pigs. Here we aim to determine (1) at which rostro-caudal level ulnar nerve evoked responses can be recorded and (2) at which depth distinctly different responses can be recorded after noxious and non-noxious stimulation. Neural signals were evoked by ulnar nerve stimulation and recorded at different levels of the spinal cord in anesthetized pigs. Event-related potentials and peri-stimulus histograms showed that most activity was recorded at the C7 level, which diminished when the electrodes were moved towards C6 or C8. At 1 mm depth, spinal neurons responded primarily to noxious stimulation, which is typical for nociceptive specific neurons. While at 2 mm depth, neurons showed responses typical for wide dynamic range neurons by responding differently to noxious and non-noxious stimulation. Histological analysis showed that these signals may indeed have been recorded from lamina I/II and IV/V, respectively. This method opens new possibilities for studying pain and other spinal mechanisms in large animals and can be combined with peripheral and brain recordings to provide a more integrated picture of (chronic) pain mechanisms.**

Keywords—Spinal cord, PSTH, ERP, neural interfaces, pain

I. Introduction

Pain is defined as an "unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage [1]". Normally, pain serves to protect the organism again tissue damage. Pain can be amplified (hyperalgesia) for this reason, after injury or sunburn, for example. But pain can also be suppressed to protect the organism, in fight- or flight situations, for instance. Moreover, pain can become a pathology, where it no longer serves to protect the organism but becomes the only or primary complaint requiring medical intervention [2].

The spinal cord plays an essential role in the process of sensation and pain perception. Non-noxious signals arrive in the deeper laminae IV/V of the spinal cord and are sent via the dorsal columns to the brain, while noxious signals arrive both in deeper and more superficial lamina and are sent to the brain via the lateral spinothalamic tract. Sensory information is not just relayed, but also modulated at the spinal level. Two populations of neurons are responsible for pain processing at the spinal level: superficial nociceptive specific (NS) neurons and deep wide dynamic range (WDR) neurons. [3] For example, Pain can be increased via long-term potentiation of NS neurons in lamina I/II of the dorsal horn [4]. WDR neurons, on the other hand, receive inputs from both nociceptive and non-nociceptive primary afferents and are able to suppress nociceptive signaling and pain perception [5]. Moreover, pain can be amplified or suppressed by a descending drive from the brainstem [3].

Healthy pain amplification and suppression mechanisms can break down in pathology and there are indeed indications of impaired pain mechanisms in patients [6]. In particular, spinal long-term potentiation is held responsible for chronic pain symptoms hyperalgesia and allodynia [7], [8]. However, it remains difficult to pinpoint exactly which systems are impaired in patients and whether these were impaired already before the onset of chronic pain [3].

To study such mechanisms in more depth animal models are employed. Animal models of pain allow us to understand how the system functions in health, disease or induced disease models, recovery and how it responds to treatments of various kinds. In previous studies, we have recorded from peripheral nerves and the brain in large animal neuropathic models [9]–[11]. We are now expanding this by developing a method to study the spinal cord in the porcine model. To the best of our knowledge, we here present spinal signals recorded in a porcine model for the first time. The aim of the study was twofold (1) to identify at which level responses evoked by stimulation of primary afferents from the ulnar nerve could be recorded and (2) to determine at whether and at which depth (superficial and deep laminae) distinct nociceptive and non-nociceptive responses could be recorded.

II. Methods

A. Surgery

Three female Danish landrace pigs weighing 36-58 kg were used for this pilot study. All experiments were approved by the Danish Veterinary and Food Administration under the Ministry of Food, Agriculture and Fisheries of Denmark (protocol number 2020-15-0201-00514).

The animals were tranquilized with an adjusted zoletil blend without ketamine (5 ml zoletil - tiletamine 25 mg/ml and

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zolazepam 25 mg/ml-, 6.25 ml xylazine (20 mg/ml), and 2.5 ml butorphanol (10 mg/ml)), intubated and maintained during surgery with sevoflurane (1.0 %) combined with propofol (10 mg/ml) and fentanyl (50 µg/ml). During the recordings, sevoflurane was turned off to prevent any depression of the neural signals. Two percutaneous wires were temporarily implanted in the forearm in the area of the ulnar nerve to evoke spinal responses. Movement responses were used to verify the placement of these electrodes.

To access the spinal cord, the first and second thoracal (T1 and T2, respectively) spinal process was identified. A 20 cm incision was made at the midline until a few centimeters beyond the T2 spinal process. Back muscles were split at the midline, and the spinal processes were cleared from muscle tissue. The fourth cervical (C4) until the T1 spinal processes were removed. The dura was carefully incised at the midline, and warmed saline was dripped on the spinal cord to keep it moist. A custom-made 3D-printed spinal micromanipulator holder was placed on the T2 spinal process. A 4-contact needle array was fabricated from four epoxy-insulated tungsten needles (AM Systems, Sequim, Washington, USA). These were inserted approximately 0.5-1 mm medial to the dorsal root entry zones at different rostral-caudal levels. Evoked responses driven by ulnar nerve stimulation were expected at C7-T1 [12].

B. Stimulation and Recording

Peripheral stimulation was set at non-noxious and noxious levels, which were defined as two times the motor threshold and ten times the motor threshold, respectively [13]. Each recording set consisted of 100 non-noxious and 100 noxious stimuli at a frequency of 1 Hz. Stimulation was performed every 10 minutes using a programmable stimulator (STG4008, Multichannel Systems, Reutlingen, Germany). The motor threshold was found by increasing the stimulation amplitude from 50 µA in steps of 200 µA. Once a motor response was detected (by palpation), the amplitude was decreased in steps of 50 µA until the response disappeared and increased again with 50 µA until a response was perceived; this was considered the threshold. An asymmetric rectangular charge-balanced biphasic pulse was used, with the secondary phase having an amplitude of 10% of the primary phase and an inter-pulse interval of 10 ms.

Spinal signals were recorded using a system from Tucker-Davis Technologies (TDT, Alachua, FL, USA), including a pre-amplifier (model SI-8), a processor (model RZ2), and a workstation (model WS8). Spinal signals were sampled at 25 kHz, as these were single-neuron responses. Evoked responses were visualized online to confirm the placement and functionality of the electrodes. Spinal signals crossing a manually set threshold were fed into an audio speaker to determine whether stimulation evoked spinal responses.

C. Data processing

Data was processed for visualizing event-related potentials (ERP) by bandpass filtering between 5 and 300 Hz with a 6^{th}-order Butterworth filter. A notch filter was applied between 47 and 53 Hz using a 10^{th}-order Butterworth filter. Harmonics were filtered using a 20^{th}-order Butterworth filter applied ±1 Hz. Non-noxious and noxious epochs were averaged individually.

Latencies of the spinal signals were extracted from the averaged ERPs. The spinal ERP could be positive or negative depending on the depth of the electrode; therefore, both the minimum and maximum and their latencies were derived for spinal ERPs. Due to fluctuations in the background signals, a window of 50 ms was set for detecting spinal minima and maxima. In the dorsal horn, a depolarization is expected, while in the ventral horn, a hyperpolarization is expected [14]. Since the electrode can be either in the dorsal horn or in the ventral horn, either the minimum or maximum is used as the response latency depending on the ERP shape.

To construct peri-stimulus histograms (PSTH), spinal signals were band pass filtered from 152 Hz to 10 kHz using a second-order Butterworth filter. Line noise was filtered up to the 9^{th} harmonic using a first-order Butterworth filter applied ±1 Hz. All filters were applied in a back-and-forth manner, doubling the effective filter order. Spikes were detected in the filtered data using a threshold of 2.5 times the root means square (RMS) of the noise floor. Spikes were counted from 50 ms before until 450 ms after stimulation onset. PSTHs were created using a 1 ms bin width.

D. Histology

The spinal cord of one euthanized pig was removed and drop-fixed in formaldehyde to investigate the spinal neuroanatomy at the cervical level. The specimens were stored in a refrigerator for more than 14 days, after which they were rinsed and paraffin embedded. The samples were sliced into approximately 3 mm pieces, resulting in two sections of each dorsal root entry zone. The samples were sectioned into 10 µm slices and stained with hematoxylin & eosin (H&E) and the Nissl stain (Toluidine blue). On the resulting sections, the depth to the dorsal horn (lamina I), substantia gelatinosa (lamina II, where noxious signals terminate), lamina III-VI (where non-noxious and noxious signals terminate) and to the ventral horn was measured.

III. Results

Signals were recorded on all channels and no epochs were rejected due to noise contamination or artifacts.

A. Spinal event-related potentials

Spinal signals were strongest at the C7 dorsal root entry zone and decreased in amplitude when moving towards the C6 or C8 dorsal root entry zone (Fig. 1). When the electrodes were placed superficially, at the level of the dorsal horn, a depolarization was observed, while a hyperpolarization was seen at the level of the ventral horn. Table I shows that the first spinal evoked potential with either polarity arrived within 10 ms after the stimulus.

B. Spinal PSTH

Most spiking activity was observed when electrodes were placed in the caudal area of the C7 dorsal root entry zone. Responses decreased when the electrodes were placed at the C8 dorsal root entry zone. The largest response at C8-level was recorded with the most rostral electrode, while no responses were recorded with the most caudal electrode.

TABLE I. Latencies of the spinal first negative and positive peaks for each pig. Latencies were averaged over all trials and all channels. Either a negative or a positive peak was detected based on the ERP shape.

	First negative spinal peak (ms)		**First spinal positive peak (ms)**	
Pig	***noxious***	***non-noxious***	***noxious***	***non-noxious***
1 (58 kg)	8.1 ± 0.6	8.1 ± 0.5	8.0 ± 0.5	7.9 ± 0.8
2 (56 kg)	9.3 ± 0.4	9.2 ± 0.3	8.0 ± 0.3	7.9 ± 0.2
3 (38 kg)	6.9	7.1 ± 0.3	7.9 ± 0.4	8.4 ± 0.6

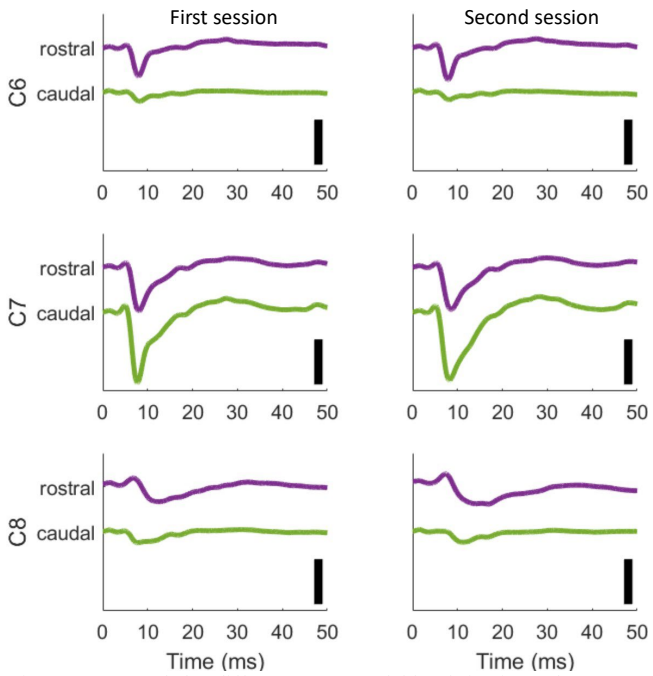

Fig. 1. ERP recorded at different rostro-caudal levels in pig 1. The ERP at the level of the C7 dorsal root entry zone has the largest amplitude. The recording sessions were repeated at each location at a 10 min interval to show stability of the responses. Traces of the same color are recorded with the same electrode. Scale bar: 200 µV.

When a good response was audible, it was assumed the right rostro-caudal location was identified. The electrodes were then gradually moved down 0.5 mm at a time attempting to record different noxious and non-noxious responses at different depths. Fig. 2 shows that at a depth of 1.0 mm, neurons primarily responded to noxious stimulation at a latency of 7 ms. The response lasted for approximately 20 ms. Neurons responded at the same latency indifferently to both noxious and non-noxious stimulation with a response duration of approximately 10 ms. At 2.0 mm depth, the response to non-noxious stimulation was comparable to that seen at 1.5 mm depth, while with noxious stimulation the response duration was stronger and longer.

C. *Spinal neuroanatomy*

The depth of the dorsal boundary of the dorsal horn, the substantia gelatinosa (lamina II), lamina III-VI and the ventral horn increased with more rostral sections (Table II). The

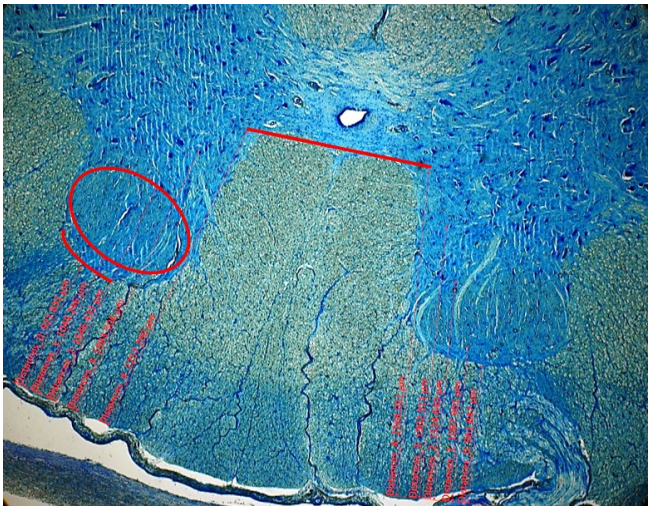

Fig. 3 The dorsal and ventral horn and substatia gelatinosa were clearly distinguishable on Nissl-stained sections. This section is at the C7 level, where the ventral horn is relatively large compared to the dorsal horn.

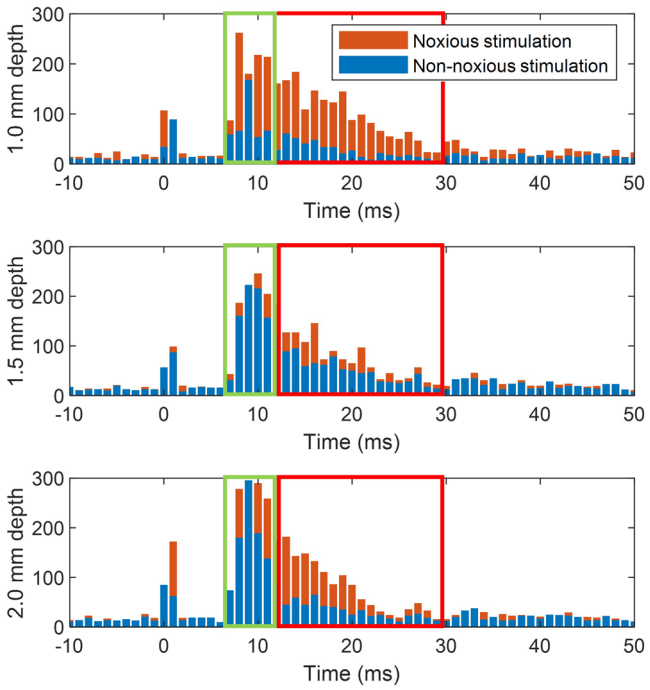

Fig. 2. PSTH showing different response profiles at different depths of the spinal dorsal horn in pig 2. The green and red box indicate the suspected latency for A-beta (non-noxious) and A-delta (noxious)fiber activation.

ventral horn was visibly larger in rostral sections compared to the more caudal sections (see Fig. 3). Greater variability was also observed in the more rostral sections, indicating a difference between two sections taken at different levels of the same dorsal root entry zone.

IV. Discussion

To our knowledge, it is the first time that spinal signals were recorded in a porcine model. We were able to control the positioning and depth of the spinal electrodes, resulting in the recording of distinct response patterns after non-noxious and noxious stimulation. The pig is considered a more translational model, as it is physiologically closer to the human than the rodent, especially regarding spinal neuroanatomy [15]. These features are particularly relevant for the study of pain and spinal cord stimulation for the treatment of chronic pain.

In line with Greenspon [14], we observed depolarizations at the level of the dorsal horn (1-2 mm depth) and positive deflections at deeper levels of the dorsal horn (>2 mm). The depolarizations after non-noxious stimuli occurred earlier in the smaller pig than in larger pigs, which is likely due to a larger distance between the stimulation wires and the spinal cord. Slight variation was seen in ERP latencies after noxious stimulation. Differences between pigs of approximately the same size are likely due to variability in the location of the stimulation wires, as the ulnar nerve has a relatively long straight path along the lower forelimb along which the wires can be placed [12].

The strongest evoked activity was recorded at the C7 level, which decreased when the electrodes were moved in the

TABLE II. Average depths from the surface to various regions in the spinal cord.

Depth from surface to	**C6 (mm)**	**C7 (mm)**	**C8 (mm)**
Lamina I	1.07 ± 0.07	0.87 ± 0.09	0.72 ± 0.03
Lamina II	1.26 ± 0.07	1.02 ± 0.07	0.83 ± 0.02
Lamina III	1.93 ± 0.16	1.58 ± 0.03	1.31 ± 0.05
Ventral Horn	2.8 ± 0.26	2.09 ± 0.2	1.85 ± 0.09

caudal direction. In the smaller pigs, this resulted in small responses, as the electrodes could only be placed in the C6 and the rostral area of the C7 dorsal root entry zone. Challenges placing the spinal micro-manipulator holder were anticipated, which was therefore printed in different sizes. However, the manipulator could not be moved caudal enough to access the C8 dorsal root entry zone. The design of the micromanipulator holder will be optimized for future experiments in smaller pigs, which is one of the advantages of a 3D printed design.

The recordings from the dorsal horn at different depths showed clearly distinct response patterns, in line with [16]. At 1 mm depth, a large response to noxious stimulation is observed, while non-noxious stimulation does not result in a clear response. At this depth, at the caudal C7 level (see Table II), we are likely recording from nociceptive specific neurons in lamina II. At 1.5 mm, there is little difference between noxious and non-noxious responses. This may correspond to the mechanoreceptors of nucleus proprius. At 2.0 mm depth, a stronger and prolonged response pattern is recorded after noxious compared to non-noxious stimulation. Wide dynamic range neurons, located in layer V of the dorsal horn could be responsible for this.

The spinal neuroanatomy of the pig at the cervical level is not well-described. However, the locations of the dorsal and ventral horns are similar to those described for the lumbar and sacral levels. The C8 level is most comparable to the rostral end of the lumbar enlargement (L2), while the C6 and C7 levels are more comparable to the central part of the lumbar enlargement (L3-L6) [15].

One limitation of this study is that we did not record which primary afferent fibers were activated by stimulation. It is generally accepted that 10x motor threshold activates nociceptive Aδ and C-fibers [13], however, this was not verified in the present work. Our data indicates that some Aδ-fibers were activated at 2x motor threshold, as some activity in the superficial dorsal horn was evoked (see Fig. 2), which was not the case in rodents [13]. In order to investigate pain mechanisms in the future, it would be beneficial to establish at which intensities fiber populations are activated, because activity-dependent plasticity requires C-fiber activity [3]. In a future study, we should therefore combine spinal recordings with a methodology like velocity-selective recording [9].

To further understand the chronic pain pathology and to understand the function and malfunction of different pain mechanisms, it is necessary to understand neuronal function at different levels of the nervous system. It has long been known that peripheral and spinal mechanisms influence the brain [17] and that brain processes influence the spinal nociceptive pathways [3]. Even in pain conditions that are considered purely central, like post-stroke pain, a peripheral treatment may bring about pain relief [18]. It is therefore probable that peripheral, spinal and cortical systems are all affected by the chronic pain pathology. The methodology presented in this paper may therefore also be combined with cortical recordings to understand the interplay between these mechanisms better [10], [11].

V. Conclusion

Spinal signals have been recorded in the porcine model for the first time. Largest activity was recorded from the caudal area of the C7 dorsal root entry zone. Different activation profiles were recorded after non-noxious and noxious stimulation at different depths in the dorsal horn. The depths and the activation profiles indicate that signals originate from NS and WDR neurons.

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