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Rat hind limb nociceptive withdrawal response to heat and mechanical stimuli depends on initial position of the paw but not stimulus location

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Rat hind limb nociceptive withdrawal response to heat and mechanical stimuli depends on initial
position of the paw but not stimulus location

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Sciences and Mathematics
James Madison University

by Giavanna Verdi

May 2017

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PUBLIC PRESENTATION

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III. Abstract

Mammals rapidly withdraw their hind limb in response to noxious stimulation, which is a protective movement known as the nociceptive withdrawal response (NWR). The NWR has been previously studied in spinalized, decerebrated and anesthetized non-human and human mammals; however, there is minimal information on the NWR in intact, unanesthetized non-human mammals.

The first specific aim was to identify the factors that determine the direction and magnitude of the NWR in intact, unanesthetized rats. Based on previous studies, we hypothesized that the location of stimulation and the initial position of the paw preceding the NWR will influence the direction and magnitude of the NWR. Rats were mechanically stimulated (“Von Frey” monofilament or 30-gauge needle) at five spots widely distributed over the plantar surface of the hind, left paw. In response to heat or mechanical stimulation to the plantar surface of the paw, rats withdraw and then replace the stimulated paw on the surface. The NWR was quantified as the vector between the initial and final positions of the stimulated paw. Unexpectedly, stimulus location did not significantly influence the direction of the response, falsifying our hypothesis. However, the initial position of paw was variable, suggesting an influence on the direction of response. Correlation between the initial position and the change in position rostral/caudally and lateral/medially revealed a significant and inverse effect on response direction. Thus, if the paw was initially rostral, it would move caudal after stimulation; if the paw was initially caudal, it would move rostral.

Second, after determining that the direction of the NWR depended on the initial position of the paw, we tested whether the rat used proprioceptive sensory feedback or corollary discharge to identify the position of the paw prior to stimulation. Based on previous studies, we

hypothesized that proprioceptive sensory feedback, rather than corollary discharge, would underlie the dependence on initial paw position. Rats were stimulated by heat with an infrared laser to a single region of the paw, which was placed on an independently movable glass plate. The plate was repositioned rostral-caudally (forward-backward) just before evoking the NWR to dissociate proprioceptive sensory feedback from corollary discharge. The NWR was unaffected by repositioning the paw prior to the evoking the NWR, consistent with proprioceptive sensory feedback being used by the rat to determine the direction and magnitude of the NWR.

Taken together, our results suggest the central nervous system in intact rats primarily uses proprioceptive information about limb posture, but not stimulus location, to determine the direction of the NWR movement. Thus, the NWR appears designed to both maintain posture, as well as protect the paw from injury. Since the NWR is the most widely used clinical test of reflexes, our results may enable improved understanding, diagnosis and treatment of neurological diseases and trauma.

IV. Introduction

Escape and withdrawal responses increase survival for animals in highly-predated and dangerous environments. Escape responses, in which the animal moves away from a threat, include rapid forward and backward movements (Zucker, 1972), turning (Eaton & Emberley, 1991; Domenici *et al.*, 2008) and random changes in direction (Firestone & Warren, 2010). As opposed to repositioning its entire body to escape a predator, some vertebrates and invertebrates withdraw only a part of their body away from the threat. For example, humans will only withdraw the skin area that was directly affected by cutaneous heat and pressure stimuli (Andersen *et al.*, 2001). Similarly, leeches (Lewis & Kristan Jr., 1998), *Caenorhabditis elegans* (Mohammadi *et al.*, 2013) and other common earthworms (Drewes, 1984) respond to the sense of touch by a bend of the segment away from the stimulus.

For both escape and withdrawal responses, the direction of movement may be critical to success. For some animals, the direction of the response is not surprisingly directly (Zucker, 1972; Kanou *et al.*, 2006) or partially away (Card & Dickinson, 2008) from the stimulus. Other animals tend to escape toward shelters or in random sets of directions to confuse predators (Domenici *et al.*, 2008). However, other animals exhibit unexpected strategies, such as moving toward the threat (Stankowich, 2009).

Previous studies have explored the factors that determine the direction of the escape and withdrawal response in non-mammals (Kanou *et al.*, 2006; Card & Dickinson, 2008; Domenici *et al.*, 2008; Domenici *et al.*, 2011a). First, the directionality of the stimulus influences the direction in which the animal responds, although the response may not be directly away (Kanou *et al.*, 2006). The initial posture of the animal has also been shown to modulate the response directionality, specifically in tarantulas (Domenici & Blake, 1993; McGuire *et al.*, 2007).

Another factor affecting the directionality of the withdrawal response is the type of stimulation delivered to the animal. Herring (*Clupea harengua*) larvae have been suggested to produce more responses that are directly away from visual stimuli, as opposed to tactile and sound stimuli (Blaxter & Batty, 1985; Yin & Blaxter, 1987; Batty, 1989). Modulation by the environment, including temperature and hypoxia, can also influence the direction in which the animal responds. In fish, rapid cooling (Preuss & Faber, 2003) and reduction of oxygen (Domenici *et al.*, 2007) have been shown to direct the response towards the stimulus. Lastly, the conditions of the surrounding environment, such as the presence of obstructions, modulate the tactics which an animal utilizes to move away from a stimulus (Eaton & Emberley, 1991; Domenici, 2010). For example, anurans avoid the direction in which a particular barrier was previously placed, in addition to keeping away from the obstacle when withdrawing from the stimulus (Ingle & Hoff, 1990).

Although mammals perform withdrawal responses upon recognition of a potential threat, the factors that determine the direction of the withdrawal response in mammals remain unclear. The overall research aim was to investigate two factors – stimulus location and posture – that may influence the direction of the nociceptive withdrawal response (NWR) in intact, unanesthetized rats.

While movement is regulated by diverse responses, the NWR may be the most crucial. As a protective response to avoid injury, the NWR is paramount for survival. Further, the spinal and supraspinal neural circuits that mediate the NWR may also underlie both posture (Ellrich, *et al.*, 2000) and locomotion (Duysens *et al.*, 2013). Clinically, the NWR is the most widely used test of reflexes (Sandrini *et al.*, 2005). Patients diagnosed with migraines and other primary headaches (Fields & Basbaum, 1978) and obesity (Pradalier *et al.*, 1980 & 1981) had decreased

levels of NWR, as opposed to the upregulation of NWR seen in patients diagnosed with fibromyalgia (Desmeules *et al.*, 2003; Banic *et al.*, 2004) and spinal cord injuries (Schwindt, 1981). A better understanding of the NWR in normal and disease states may lead to improved diagnosis and treatment of neurological diseases and trauma.

A. Nociceptive Withdrawal Response

The NWR is a rapid withdrawal movement of a specified part of the body in response to noxious (potentially tissue-damaging) stimuli, which may protect the body part that is being withdrawn (Sherrington, 1910; Andersen, 2007). The hind limb NWR incorporates coordinated rotation around the knee, hip and ankle, which results from the activation and inhibition of diverse muscles throughout the limb (Andersen, 2001; Baxendale & Ferrell, 1981; Kim *et al.*, 2007; Andersen, 2004). Puncture, extreme pressure, heat and cold, or exposure to chemical irritants are examples of cutaneous noxious stimulation (Bessou & Perl, 1969). The NWR has been demonstrated in varying animals, including rats (Cleland & Bauer, 2002; Chrzan, 2013; Seamon, 2015), rabbits (Harris & Clark, 2003), cats (Levinsson *et al.*, 1999) and humans (Peterson *et al.*, 2014).

a. Nociceptors

Nociceptors are specialized peripheral sensory receptors that transduce information about noxious stimuli of the skin of both vertebrates and invertebrates. The evolution of nociceptors may have begun with Cnidaria, which developed abilities to respond to noxious mechanical stimuli through unmyelinated axons, and continued to mammals, which acquired capabilities of detecting responses to noxious mechanical, heat, cold and chemical stimuli with the use of both myelinated and unmyelinated nociceptors (Smith & Lewin, 2009).

Nociceptors are activated by noxious changes in temperature ($>40^{\circ}\text{C}$), pressure to the skin and specific chemicals (e.g. capsaicin), as opposed to cutaneous receptors that respond to both non-noxious and noxious stimuli (Schepers & Ringkamp, 2009). Central nociception and pain arise only from nociceptors (Cleland & Gebhart, 1996).

Previous studies have demonstrated the presence of two types of nociceptors: myelinated A-delta (Leem *et al.*, 1993) and unmyelinated C-fiber nociceptors (Dubin, 2010). A-delta fiber nociceptors respond to either heat or pressure, while C-fiber nociceptors respond to both heat and pressure; thus the mechanical and heat stimulation will evoke the NWR in intact, unanesthetized rats. Although both A-delta and C-fiber nociceptors are present in the skin of the plantar surface of the rat (Fleischer *et al.*, 1983), A-delta afferents are more numerous than the C-fiber nociceptors (Leem *et al.*, 1993).

b. Spinal Mechanisms

The NWR can be evoked in animals with transection of the spinal cord (Eccles & Lundberg, 1959), demonstrating that the spinal cord is sufficient to mediate the NWR. Nociceptors' actions are polysynaptic, and the pathway through the spinal cord can be as short as two interneurons to excite motoneurons and three interneurons to inhibit motoneurons (Eccles & Lundberg, 1959). The spinal interneurons responsible for the NWR are located in lamina I, II and V of the dorsal horn (Schouenborg *et al.*, 1995), and include both multireceptive (MR) and nociceptive specific (NS) neurons (Morgan, 1998).

c. Long-Loop Reflexes

Supraspinal reflexes, in which sensory information is conveyed to the brain and then back down to the spinal cord, are known as long-loop reflexes (Macefield, 2009), and operate in parallel to spinal reflexes. Long-loop reflexes are distinguished by the presence of a long latency

in the muscle electromyogram (EMG) response; the long latency has been suggested to arise from the longer pathway of sensory information from the spinal cord to the brain and back to the spinal cord (Tracey *et al.*, 1980).

While little is known about long-loop NWRs, a previous study on the stretch reflex suggested the presence of a long-loop component. The characteristic long latency peak was observed in the EMGs of the upper limb of monkey and human subjects (Lee & Tatton, 1975).

Although there is limited evidence for the existence of a long-loop component of the NWR for normal human subjects, the EMG of the flexor reflex in the tibialis anterior muscle shows a second peak at a longer latency in response to electrical stimulation (Meinck *et al.*, 1985). Nevertheless, the long latency, second peak, responses could still be mediated by multi-synaptic spinal mechanisms (Tracy *et al.*, 1980).

d. Modulation

Spinal reflex pathways can be influenced by modulatory synaptic actions that do not evoke a new response, but rather inhibit or facilitate the existing reflex. Modulatory pathways can arise from spinal and supraspinal origins. The NWR is extensively modulated by descending supraspinal pathways, arising in part from the rostroventral medulla (RVM) of the brain, specifically the medial nucleus raphe magnus (Gebhart, 2004). Modulation acts as a protective mechanism, where the activation of descending facilitatory influences allows the protection of injured tissues from further damage (Gebhart, 2004). In addition to controlling motor output, descending motor pathways, such as corticospinal, vestibulospinal, reticulospinal and rubrospinal pathways, have been suggested to modulate the transmission of neuronal information from afferent fibers to the brain (Schwindt, 1981), and may influence the NWR.

Previous studies suggested that descending influences from the brain stem principally inhibited spinal nociceptive processing (Reynolds, 1969); however, recent studies have shown that descending modulation of nociceptive information can either be facilitatory or inhibitory. The sign of modulation can even vary with reflex intensity; there is inhibition of the NWR when the rat hind foot is electrically stimulated at high intensity, but facilitation of the NWR at low intensities. In contrast, mechanical stimulation at all intensities evoke both facilitation and inhibition of the NWR of the foot (Zhuo & Gebhart, 2002). The excitatory influences descend from the RVM and to the ventral/ventrolateral spinal cord, whereas the inhibitory influences were seen to descend to the spinal cord in the dorsolateral funiculi (Gebhart, 2004).

A recent study has suggested that modulation can also change the pattern of the NWR. Specifically, the spinalized rats exhibited a bias in the ventral direction, while the intact rats exhibited a bias in the dorsal direction when the NWR was evoked (Cleland & Bauer, 2002).

B. Dependence of the NWR on Stimulus Location

Early studies of non-human mammals demonstrated the independence of stimulus location on the NWR. In the hind limbs of spinalized cats and dogs, Sherrington (1910) observed that flexor muscles were activated and extensor muscles were inhibited when the sensory nerves were electrically stimulated at high intensity, presumably exciting nociceptors. Significantly, muscle contractions and relaxations involved with the withdrawal reflex were largely independent of which nerve was stimulated. Similarly, in spinal cord-injured humans, the NWR was also independent of stimulus location, as shown in experiments in which the location of electrical stimulation on the dorsal surface of the human foot did not influence the pattern of muscle activation in the NWR (Andersen *et al.*, 2004). The independence of stimulus location on the NWR leads to a minimal amount of choices for the animal to decide between,

thus the reduced time being spent on making a decision could result in less further damage to the animal (Schmidt & Lee, 1999).

In contrast, more recent studies suggest that the NWR depends on the locations of heat, electrical and mechanical stimulation in decerebrated, spinalized and anesthetized non-human mammals (Schouenborg & Kalliomaki, 1990; Schouenborg *et al.*, 1992; Cleland & Bauer, 2002; Harris & Clark, 2003). Using movement as the measure of response, the direction of the withdrawal movement response of spinalized rat tails depended on the location of localized heat stimulation (Cleland & Bauer, 2002), and the evoked withdrawal movement in spinalized and decerebrated rabbits depended on the location of electrical stimulation on the plantar surface of the paw (Harris & Clark, 2003). In both studies, the change in stimulus location resulted in a change in response, that directed the limb away from the stimulus. Using EMG as a measure of response, the EMG responses in the ankle muscles of anesthetized (Schouenborg & Kalliomaki, 1990) and spinalized (Schouenborg *et al.*, 1992) rats depended on the locations of mechanical and heat stimulation to the hind limb. The greatest EMG signals were observed in muscles that would withdraw the stimulated skin away from the noxious stimulus (Schouenborg & Kalliomaki, 1990; Schouenborg *et al.*, 1992). The dependence of stimulus location on the NWR causes the animal to move away from the stimulus, thus escaping from the threat with accuracy.

The NWR in intact, unanesthetized humans has also been shown to depend on stimulus location. In the lower limb, electrical stimulation at locations in the lateral region of the plantar surface of the human foot evoked eversion responses, while electrical stimulation at locations in the medial region evoked inversion responses; thus in both cases, the NWR caused the area of stimulated skin to move directly away from the stimulus (Andersen *et al.*, 1999 & 2001). In the upper limb, electrical stimulation of three different fingers of the human upper limb produced

different magnitudes and angles of force, resulting in the movement away from the stimulus (Peterson *et al.*, 2014).

A few previous studies suggest hybrid patterns, in which there was both a relative dependence and independence of stimulation location on the NWR (Andersen *et al.*, 2001; Cleland & Bauer, 2002). Spinalized rats showed withdrawal responses that were directed broadly away from the location of localized heat stimulation, which demonstrated that the direction of the NWR depends on stimulus location. However, the direction of the NWR remained confined to the ventral-lateral quadrant in support of independence of stimulus location (Cleland & Bauer, 2002).

In support of a second, but different, hybrid pattern, Andersen *et al.* (2001) demonstrated that the location of electrical stimulation on the foot's plantar surface influenced the pattern of the ankle, but not knee, muscle activity. Even at the highest stimulus intensity, EMG of the biceps femoris, a muscle responsible for the flexion of the knee, remained constant across multiple stimulus locations. In contrast, the EMG in the tibialis anterior, a muscle responsible for flexion of the ankle, was greatest when the stimulus was localized in the center of the foot (Andersen *et al.*, 2001).

Since previous studies showed dependence of stimulus location in decerebrated, spinalized and anesthetized human and non-human mammals, Chrzan (2013) and Seamon (2015) hypothesized a similar dependence for intact, unanesthetized mammals. In their studies, conducted in our laboratory, rats were stimulated with heat at multiple locations along the plantar surface of the hind paw, resulting in brisk withdrawal and replacement movements of the stimulated limb. The direction of the NWR was measured by the difference in the foot's location before and after completion of the resulting NWR. Unexpectedly, the location of stimulation

along the plantar surface of the rats' hind paw did not significantly impact the direction of the NWR. More specifically, the change in position in the rostral/caudal direction for rostral and caudal stimulus locations, as well as the change in position in the lateral/medial direction for lateral and medial stimulus locations, did not significantly depend on where the stimulus was applied on the plantar surface. Similarly, the change in foot angle after the NWR for intact, unanesthetized rats did not significantly depend on the location of heat stimulation (Chrzan, 2013; Seamon, 2015). However, since these studies were done using *heat* stimuli; the dependence on the location of *mechanical* stimulation remained unknown for intact, non-human mammals.

C. Dependence of the NWR on Initial Position

Posture has also been shown to influence the NWR of decerebrated and spinalized subjects. Baxendale and Ferrell (1981) and Kim *et al.* (2007) showed that the NWR depends on the joint angle of the knee and hip, respectively. In decerebrated cats, the non-noxiously evoked flexion withdrawal and crossed extensor reflexes were altered by the changes in knee joint angle, in which extension of the knee increased the activity of the flexor muscles of the lower limb (Baxendale & Ferrell, 1981). Similarly, in spinally-injured human subjects, increase in hip extension resulted in larger NWR in the hip (Kim *et al.*, 2007).

Previous studies have also tested the dependence of the NWR on initial position in intact subjects. While applying electrical stimulation to the fingers of intact humans, the direction of the withdrawal response was altered based on the initial position of the upper limb; from flexion to extension of the limb, the NWR was in the posterior-lateral direction, and then shifted to the posterior direction, in which the limb moved further away from the electrical stimulus (Peterson *et al.*, 2014). In intact, unanesthetized rats, the direction of the NWR depended on the initial

position of the hind limb prior to movement. For example, when the rat spontaneously placed the paw rostrally, or more forward, the paw moved caudal, or more backward, in response stimulation. This inverse relationship was also seen in reverse; when the paw was initially caudal, the paw would move rostral in response to stimulation. A similar pattern was observed when the rat placed its paw within the lateral-medial direction (Chrzan, 2013; Seamon, 2015).

D. Proprioceptive Sensory Feedback vs. Corollary Discharge

The mechanism of how the initial position determines the direction of the NWR, as well as what source of information the CNS (central nervous system) utilizes for this determination, remains unknown. The CNS may use two sources of information to determine the position of the limb before stimulation: proprioceptive sensory feedback from the limb and central corollary discharge. Proprioceptive sensory feedback provides information about the muscle length, force and joint angles in the limb (Onushko et al., 2013). Corollary discharge is an internal copy of a motor command that resides within the CNS (Crapse & Sommer, 2008); in this case, the command that moved the foot to its initial position.

Proprioceptive information can arise from the sensory receptors in skin, joints, tendons and muscle spindles (Hasan, 1992). Kim *et al.* (2007) proposed that hip primary spindle receptors increased the NWR in the human lower limb, due to observations of larger torque responses in the ankles when the *hip* joint was extended, compared to when the hip joint was flexed. In contrast, in decerebrated cats, flexed and extended positions of the knee joint determined the intensity of the NWR, suggesting that *knee* joint afferent activity was responsible for this observed pattern. Further, injection of a local anesthetic into the knee joint to block joint receptors abolished modulation of the NWR when a change in the knee joint angle occurred, thus supporting the role of knee joint receptors (Baxendale & Ferrell, 1981).

An alternative source of information about the location of the foot is corollary discharge. Although there is no evidence suggesting that corollary discharge modulates the NWR, previous studies have shown that corollary discharge regulates other reflexes. For example, rapid eye movements, or saccades, have been suggested to be controlled by corollary discharge mechanisms within the CNS (Zaretsky & Rowell, 1979). Similarly, in studies with fish, Sperry (1950) suggested that corollary discharge compensated for the retinal displacement of a visual image caused by voluntary movement. In the context of the modulation of the NWR, corollary discharge could allow the animal to store the information of where the paw is initially located, and this information could be used to direct the paw after a stimulus has been delivered (Crapse & Sommer, 2008).

E. Specific Aims

The overall research goal was to investigate the factors and mechanism that determine the direction and magnitude of the NWR in intact, unanesthetized rats. The direction of the NWR has been previously studied in spinalized, decerebrated and anesthetized non-human and human mammals; however, there is conflicting evidence on the dependence of stimulus location, minimal information about the underlying mechanisms and few reports on the NWR in intact, unanesthetized non-human mammals.

The direction of the NWR for intact, unanesthetized rats was quantified by measuring the change in location of a rat's hind paw before and after noxious heat or mechanical stimulation at multiple locations along the plantar surface of the foot.

The first specific aim of the project was to determine whether the location of *mechanical* stimulation influenced the direction of the NWR. Noxious stimuli, with a "Von Frey" nylon monofilament and a 30-gauge needle, were given at multiple locations along the plantar surface

of the hind paw to determine if the location of mechanical stimulation affected the final position of the rat's paw. Based on previous research using *heat* stimulation in a similar experiment (Chrzan, 2013; Seamon, 2015), we hypothesized that the initial position of the paw, rather than the location of mechanical stimuli, would alter the NWR.

The second specific aim of the project was to determine the source of limb position information used by the CNS to determine the direction of the NWR; either proprioception or corollary discharge. The experiment was designed to dissociate between proprioceptive sensory feedback and corollary discharge by changing the position of the paw immediately before evoking the NWR. Based on limited previous studies (Baxendale & Ferrell, 1981; Kim *et al.*, 2007), we hypothesized that proprioceptive sensory feedback, rather than corollary discharge, would determine the direction of the NWR after heat stimulation was applied.

V. Methods

A. Animal Care

Male Sprague Dawley rats (n=23; 426 mean \pm 54 S.D. grams; 27 \pm 12 weeks; Harlan, Indianapolis) were placed in separate or individual cages with continuous access to water and food, as well as exposure to 12 hours of light and 12 hours of darkness (7am-7pm). Inspections of the room occurred daily in order to verify temperatures were between 68-79°F, humidity levels were between 30-70%, monitor cleanliness, and to check food and water availability. The animal care facility and protocol were approved by the James Madison University Institutional Animal Care and Use Committee.

B. Rat Preparation

For both sets of experiments, rats were restrained in an acrylic box and lightly anesthetized with exposure to a mixture of 2-5% isoflurane in 100% oxygen. Anesthesia was necessary to shave the hind end of the rat and to mark the bottom of the paw with three to five stimulus locations and an approximate center of mass (COM) location for tracking using a black permanent marker. Points, approximately 1 mm in diameter, were arranged rostral-caudally or along both rostral-caudal and lateral-medial axes (Figures 1A and 2A). The sites of stimulation were marked to improve absorption of the heat stimulus and to restrict the stimulus to only blackened areas of the paw, as unmarked areas absorbed heat at a much slower rate. The center of mass location was used to determine the distance and magnitude of movement of the response. Before beginning the experiment, there was a 45-minute recovery period in order to reduce any possible influence on rats' behavior from the anesthesia (Hagbarth, 1952).

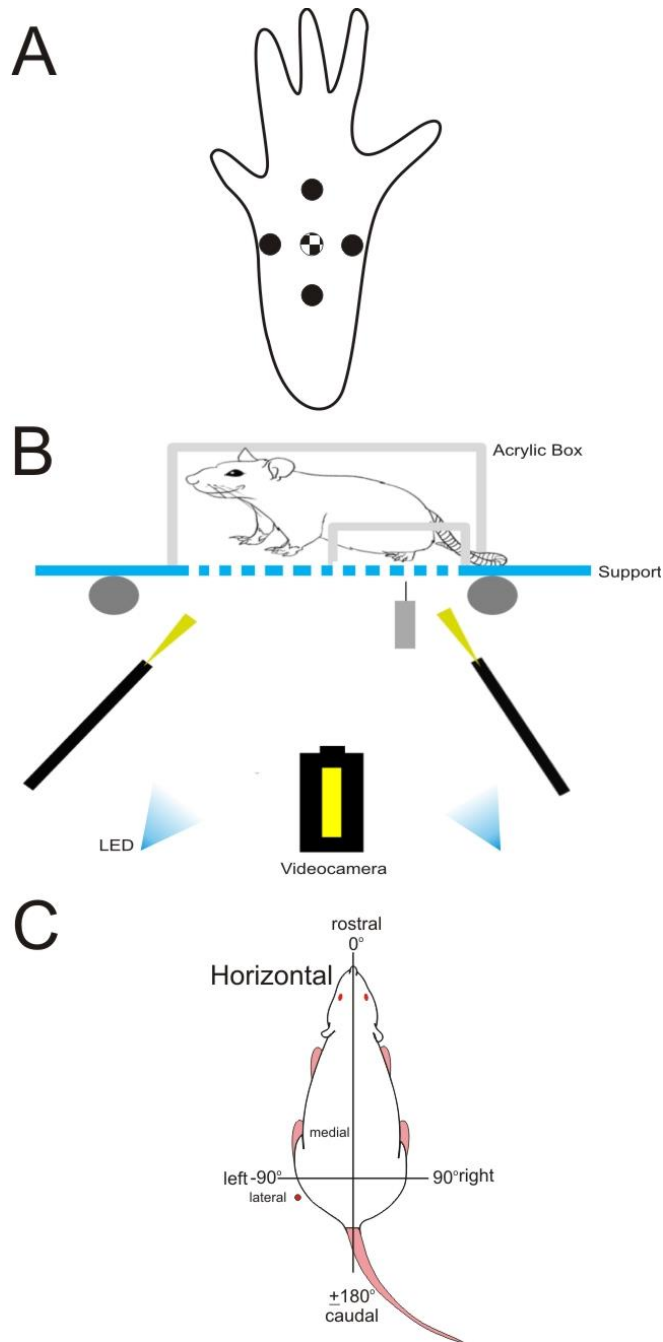


Figure 1. Methods – Mechanical Stimuli Experiments. **A** Localized mechanical stimuli were delivered to five locations (circles) along two dimensions on the plantar surface of the foot: rostral-caudal (3 locations) and lateral-medial (3 locations). The center (checkerboard circle) plantar location, which represents the center-of-mass (COM) of the foot and was stimulated as part of both rostral-caudal and lateral-medial dimensions, was also utilized to quantify the movement of the foot after a stimulus was delivered. **B** The cartoon shows the experimental design. Intact rats were placed on a mesh platform, a fenestrated acrylic box was placed around the rat, and a conventional video camera (60 fps) was positioned underneath. Mechanical stimuli (Von Frey and needle) were delivered to the plantar surface of the foot through the wire mesh. Illumination of the foot was provided by two LEDs from underneath the platform. **C** Measurements are reported in the Cartesian coordinate system for the frontal plane of the rat. The urinary orifice was used as the origin, with the axes as shown in the figure.

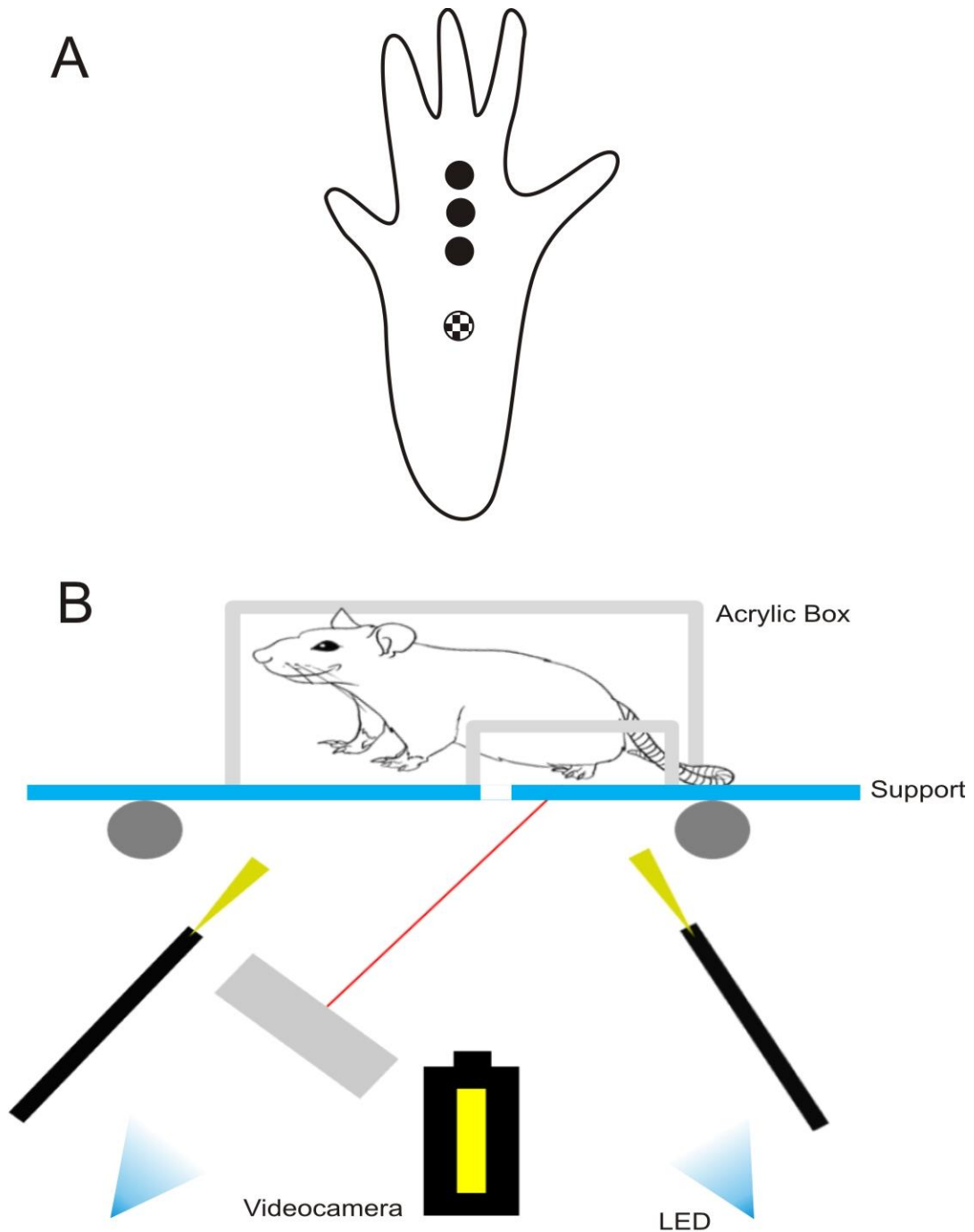


Figure 2. Methods – Moving Plate Experiments. **A** Localized mechanical stimuli were delivered to three closely spaced locations (circles) along the rostral-caudal dimension on the plantar surface of the foot. The checkerboard location, which represents the COM of the foot, was utilized to quantify the movement of the foot after a stimulus was delivered. **B** The cartoon shows the experimental design. Intact rats were placed on a glass platform, a fenestrated acrylic box was placed around the rat, and a conventional video camera (60 fps) was positioned underneath. The hind, left paw was placed on a separate glass plate, in order to independently move the paw along the rostral-caudal dimension. The heat stimulus (980 nm infrared laser) was delivered to the plantar surface of the foot at each of the three black targets through the glass plate. Illumination of the foot was provided by an LED from underneath the platform.

C. Experimental Setup and Stimulation

Rats were placed on either a mesh (Specific Aim 1; mechanical stimuli) or a glass (Specific Aim 2; heat stimuli) platform (2' X 1'), both of which were supported by two horizontal steel bars. Rats were loosely restrained on top of the mesh or glass surface by a fenestrated acrylic box to limit voluntary repositioning during and between trials. There were two cutouts in the acrylic box: one on the left side to allow uninhibited movement of the hind, left paw, and one smaller cutout in the back to allow natural extension of the tail. The platforms were raised 51 cm from the table to allow the video camera, light emitting diode (LED) lights and laser to fit underneath the rat (Figures 1B). A light source underneath the mesh and acrylic platform was needed in order to capture the NWR and to easily identify the video frame for which the heat stimulation began. One high-power, low heat, LED (SugarCube; Vergennes, VT) light illuminated the platforms from underneath, and for heat stimulation, the LED light was triggered to turn on at the same time as the delivery of the stimulus (Figure 2B).

In the first set of experiments, two methods of mechanical stimulation were used to determine their effect on the direction of the NWR, a “Von Frey” nylon monofilament (Le Bars *et al.*, 2001) and a 30-gauge needle (Anand *et al.*, 1998). The Von Frey monofilaments deliver a constant force that is proportional to the thickness of the nylon monofilament. The needle stimulus was delivered through the mesh with constant and minimal pressure to minimize tissue damage, but to also induce a response, reddening with repeated stimulation on occasion.

In the second set of experiments, a 980 nm infrared laser diode (v. Frey, 1897; Le Bars *et al.*, 2001; BWTEK; Wilmington, DE), focused by a condenser lens, was positioned underneath the glass plate to deliver a 1 mm diameter heat stimulus (Figure 2B). The infrared laser light was positioned directly over the specified blackened stimulus location, and the delivery of the

stimulus was activated by a foot switch. Infrared lasers are known to quickly increase the temperature of the stimulated skin area; however, this method of stimulation appears to better match the natural aversive stimuli than electrical stimulation in evoking a NWR (Morch *et al.*, 2007). The heat stimulus was delivered at intensities between 4-4.5 watts to evoke the NWR. To obtain latencies between 1-3 seconds, the intensity of the heat stimulus varied during each trial. For both experiments, the three to five locations along the plantar surface were stimulated in a random order, in which randomizing the stimulus order counter-balanced the effect that the order of stimulus location could have on the response (Nielsen & Arendt-Nielsen, 1998).

D. Movement Recording

Conventional video cameras (Sony HDR-CX130; 60 fps @ 1080p) were placed beneath the platform (mesh or glass) and directly facing the ventral surface of the rat. The focus, exposure and zoom were manually adjusted prior to each experiment. Movement was recorded in pixels on each video and later converted to millimeters. To perform this calibration, one frame was recorded with a ruler placed on the glass plate and mesh plate in approximately the same location as where the rat's hind limb would have been.

E. Experiment Protocol – The Effect of Mechanical Stimulation Location on the NWR

The first specific aim was to determine whether stimulus location or initial location of the paw influenced the direction of the NWR in intact, unanesthetized rats using mechanical stimuli. Rats were placed on the mesh to become adjusted to the new environment for 10 minutes. Once oriented in the proper direction, the fenestrated acrylic box was placed over the rat to restrict movement. For each rat, (n=17) each of five locations were stimulated, and each individual location along the plantar surface was stimulated three to four times in a random order

(15-20 trials per rat). Before the trials could begin using the Von Frey monofilament, thresholds had to be completed to determine which Von Frey monofilament to use.

Once the trials were ready to begin, a Von Frey monofilament or 30-gauge needle was applied perpendicularly to the plantar surface at a specific stimulus location, which was determined through a random ordering of stimulus locations. The conventional video camera underneath the mesh platform began recording approximately 30 seconds before the mechanical stimulus was delivered, and the camera was stopped once the rat's paw returned to the mesh platform and was positioned completely flat (Figure 4). After each trial, there was a 6-minute waiting period to minimize the influence of repeated stimulation on the NWR.

F. Experimental Protocol – The Determination of Information Used by the CNS

After determining that stimulus location did not have an effect, the second specific aim was to determine whether proprioceptive sensory feedback and corollary discharge information influenced the direction of the NWR in intact, unanesthetized rats using heat stimuli. For each rat, (n=6) each of three locations were stimulated, and each individual location along the plantar surface was stimulated five to six times in a random order (15-20 trials per rat). In the second set of experiments, three closely spaced distinct stimulus locations were used to minimize tissue damage. Experiments on an individual rat were conducted over a 7-day period, at all times of the day.

Rats were placed onto an acrylic plate to adjust to the new environment for 10 minutes. Once the rat was oriented in the proper direction for recording and stimulation, the acrylic box was placed over the rat. The left, hind limb was placed on the separate glass plate, and no other paws were on this plate during trials (Figure 2B). For trials where the rats' paw was repositioned, the camera started recording before the paw was moved either 10 mm forward or

backward. A heat stimulus was applied along the rostral-caudal direction on the plantar surface about 4 seconds after movement, and the camera stopped recording once the paw reached the glass surface. After each trial, a 4-minute interval was initiated before the next trial, as other studies have utilized randomized intervals between 10 and 30 seconds in between heat stimulated trials (Morch *et al.*, 2007).

Two types of trials were conducted in order to distinguish between proprioception and corollary discharge. In half of the trials, the paw was stimulated after it was positioned by the rat. In the other half of the trials, the paw was repositioned manually by the experimenter for approximately 5 seconds before stimulation. Repositioning directions were approximately 10 mm and usually based on the initial posture of the paw along the rostral-caudal direction. When the paw appeared to be positioned more forward, the experimenter would reposition the paw in the backward direction, and the same was true when the paw appeared to be positioned more backward.

G. Movement Tracking

Video files were converted to a series of “jpeg” image files using Video Mastering Works 5 (TMPGEnc) in order to be compatible with ProAnalyst software. The onset of illumination from the LED lights was used to determine the frame number when the heat stimulus application began. Videos were cut approximately 50 frames before the NWR, and for heat-stimulated trials with repositioning, videos were cut several frames before the movement. For each trial, images were analyzed using ProAnalyst (Xcitex, Cambridge, MA) in order to quantify the initial and final positions of the paw. Prior to analysis, a Cartesian coordinate system was produced for each rat by setting the origin at the urinary orifice, the positive X axis at the medial and the positive Y axis at the center of the rat in the rostral direction (Figure 1C). The withdrawal

response consisted of a rapid removal of the rat's paw from the surface, followed by quick replacement of the paw on the surface in a new location. The changes in initial and final positions of the NWR were analyzed by recording the rostral-caudal (X) and lateral-medial (Y) coordinates of the heel, COM and directly beneath the middle toe prior to and following the movement.

Coordinates from ProAnalyst were recorded onto an Excel spreadsheet, where the direction and magnitude of the response vector were calculated. Analysis and graphing were conducted using SigmaPlot (Systat Software, San Jose, CA) and custom programs in Matlab (Mathworks, Natick, MA) developed by Dr. Corey Cleland. Significance, indicated as α , was set to a p-value of 0.05, and error bars were standard errors of the mean. Parametric and non-parametric statistics were selected based on the distribution conformity. Directional data were analyzed using circular statistics. Some data were presented using boxplots, in which the line in the center of the box represented the median, the upper and lower boundaries of the box indicated the 75th and 25th percentiles, the whiskers demonstrated the 90th and 5th percentiles.

VI. Results

A. The Effect of Mechanical Stimulation Location on the NWR

The NWR resulting from mechanical stimulation applied to the plantar surface of the paw of intact, unanesthetized rats was characterized by rapid removal and replacement of the paw on the mesh platform (Figure 3). Typically, the withdrawal and replacement of the paw occurred within 50 milliseconds.

In order to represent the two-dimensional nature of the movement in the frontal plane of the rat, the responses are shown as vectors (Figure 4A), for both Von Frey (n=178 trials) and needle (n=101 trials) stimulation. The origins of the vectors represent the initial position of the rat's paw prior to the stimulation, and the arrow ends of the vectors represent the paw's final position following replacement on the mesh platform. Overall, the rat moved its paw in all possible directions (Figure 4B). The frequency histogram of the response directions is clearly bimodal and suggests a preference for movement in the caudal-lateral direction for needle stimulation and in the rostral-medial direction for Von Frey stimulation. Statistically, the direction of the responses significantly differed between the methods of mechanical stimulation used (Figure 4F, $p=0.001$, circular Watson-Williams F-test). The frequency histograms of the response magnitudes (Figure 4C) suggest that there is a larger change in the position of the paw with needle stimulation (median=10.4 mm) than with Von Frey stimulation (median=6.4 mm). Statistically, the magnitude of the responses significantly differed between the methods of mechanical stimulation used (Figure 4E, $p=0.002$, Mann-Whitney test). Strikingly, there appears to be a relationship between the direction and magnitude of the NWR; responses in the caudal-lateral and rostral-medial directions were the largest in magnitude (Figures 4B and 4D).

A Delivery of stimulus



B Response to stimulus

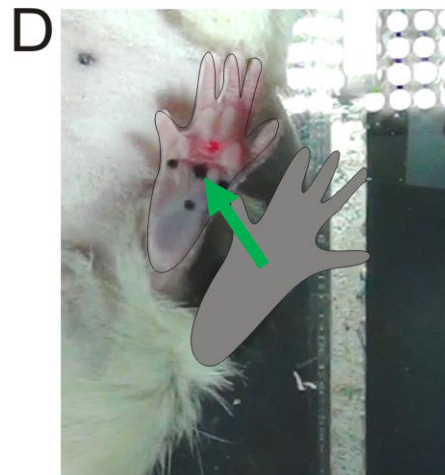
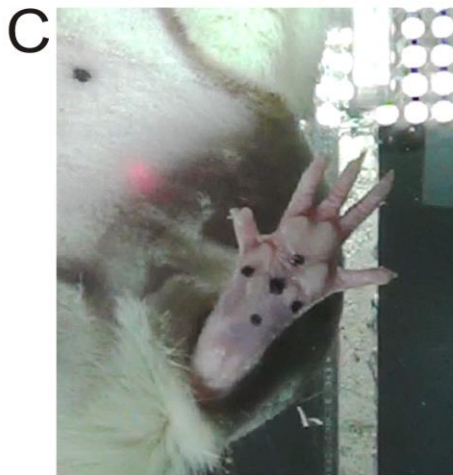
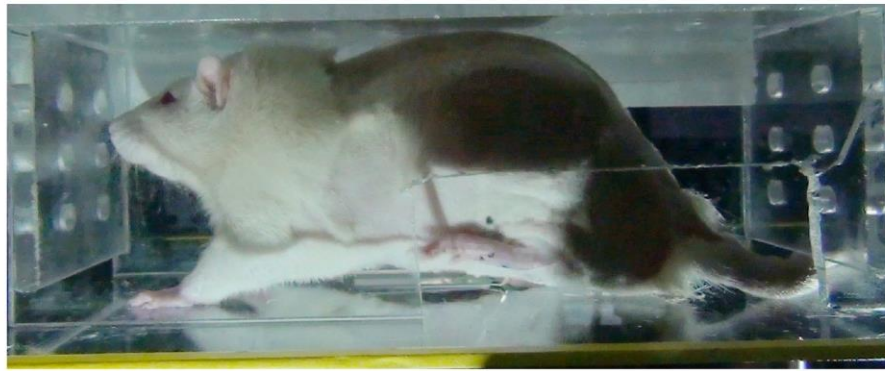


Figure 3. NWR movement of the hind limb. Video recording from the rat's left side illustrates the NWR evoked by stimulation (heat) of the plantar surface of the hind, left paw. Typically, the withdrawal and replacement of the paw occurred within 50 milliseconds. Video from below the animal shows the initial position of the hind limb before the stimulus (**A, C**) and following the stimulus-induced movement (**B, D**). The initial position prior to the stimulus is represented by the shaded region. The illustration shows the initial and final positions of the paw viewed from below the rat. The green arrow represents the movement vector describing the NWR.

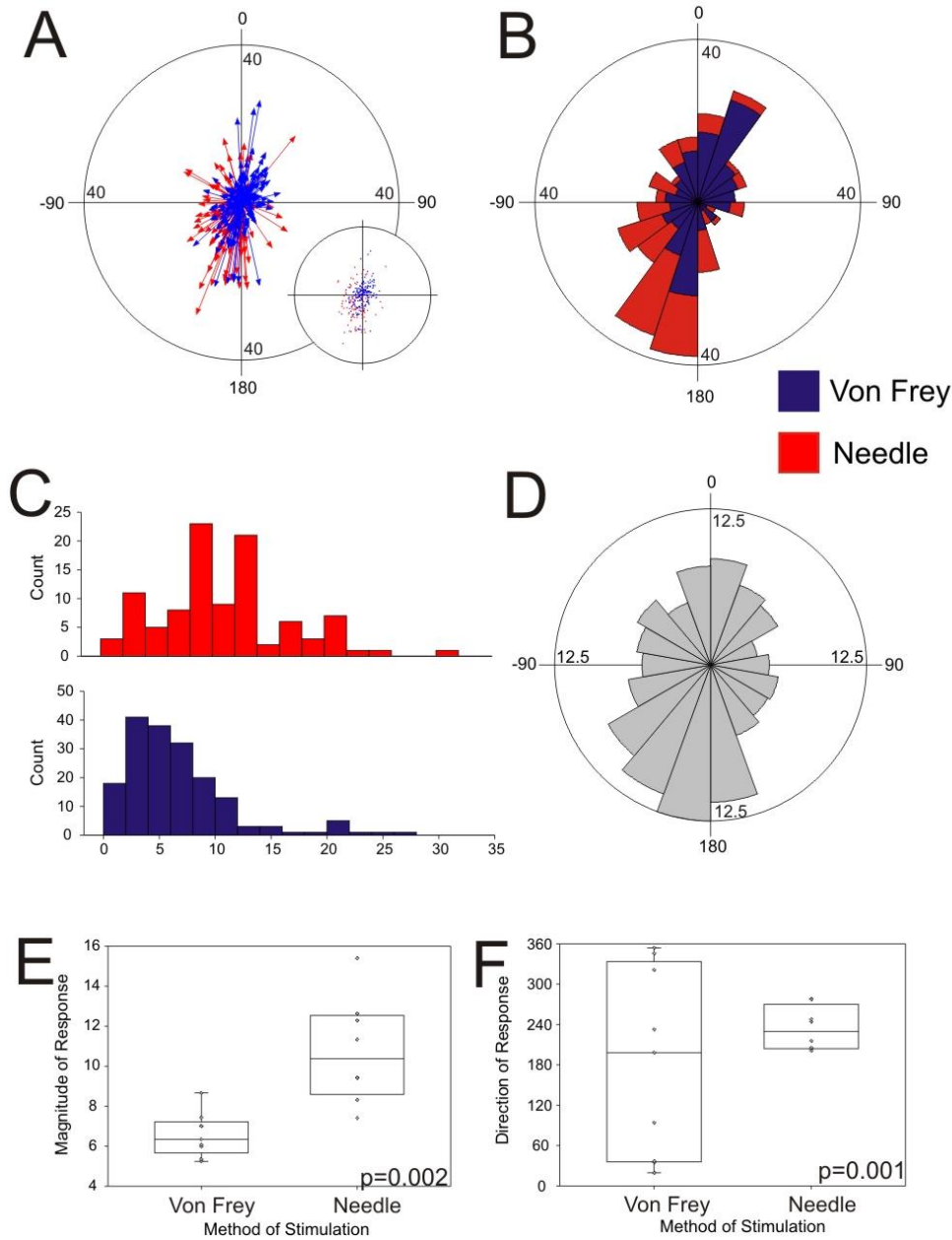


Figure 4. Nociceptive withdrawal responses for Von Frey and needle stimulation. **A** Vectors show the directions and magnitudes of the changes from initial to final positions of the paw, separately for Von Frey (blue, $n=178$ trials, $n=9$ rats) and needle (red, $n=101$ trials, $n=8$ rats) stimulation. The scatter plot better depicts the short vectors. **B** The polar frequency histogram visualizes the distribution of the direction of response vectors separately for Von Frey and needle mechanical stimulation delivered to the plantar surface (bin width = 18° ; frequency represented by the radius of the wedge). The mean directions of responses, averaged over 2-5 replicates within each animal, were significantly different for Von Frey (median= 197.6°) and needle (median= 229.8°) stimulation (**F**, $p=0.001$, $n=9$, circular Watson-Williams F-test). **C** The frequency histograms represent the distributions of the magnitudes of responses for the Von Frey and needle mechanical stimulations. The distances of responses, averaged over 2-5 replicates within each animal, were significantly different for Von Frey (median= 6.4) and needle (median= 10.4) stimulation (**E**, $p=0.002$, Mann-Whitney test). **D** The distance of the response, shown as a function of response direction, is largest for movement downwards and to the left (caudal-lateral), which matches the downward-left peak in directions shown in **B**.

The first question asked in this study was whether or not the stimulus location on the plantar surface of the paw determined the direction and magnitude of the NWR in intact, unanesthetized rats. Stimulation at each of all five locations produced responses with diverse directions and magnitudes (Figure 5), suggesting no difference between the five stimulus locations.

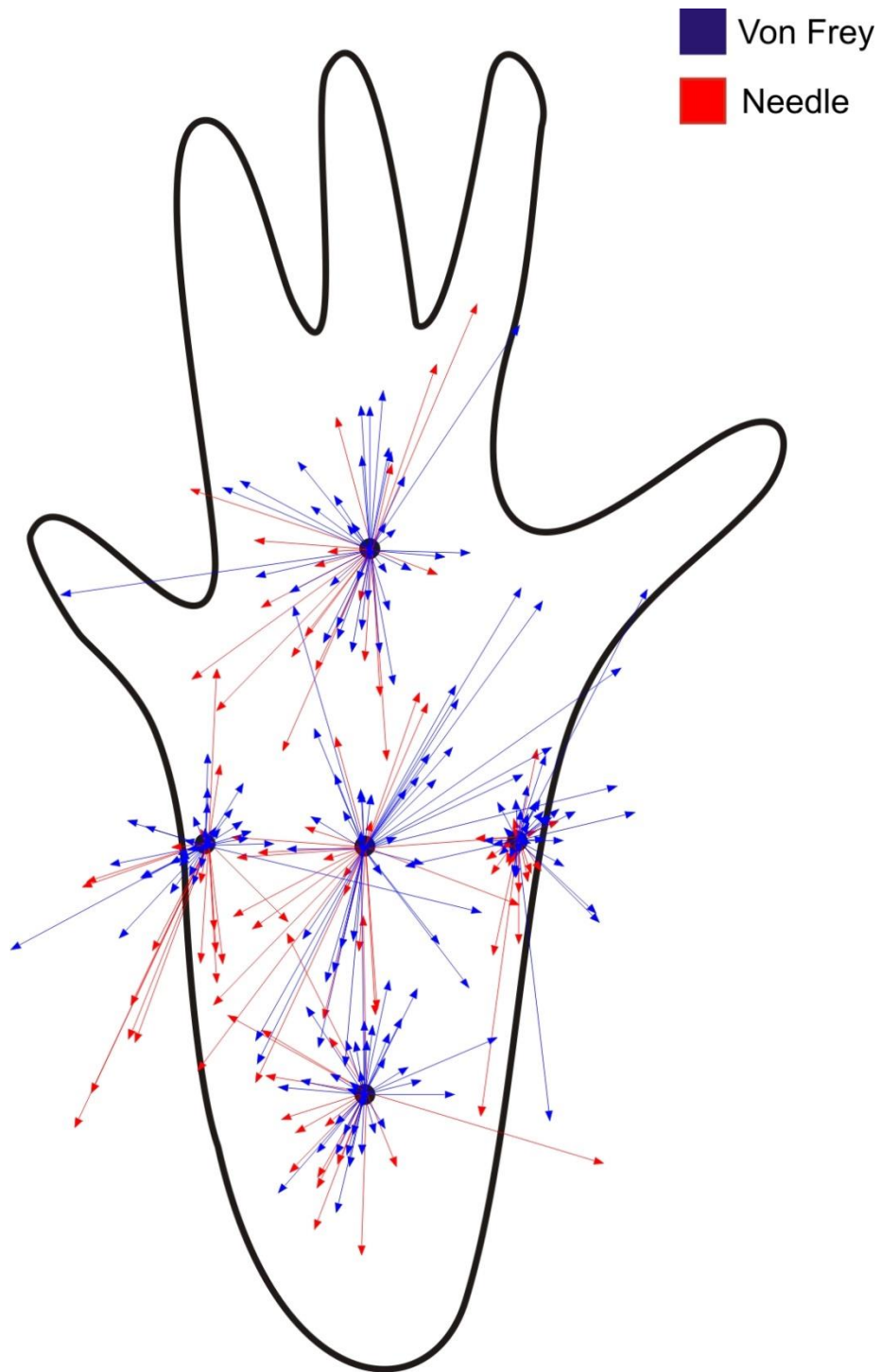
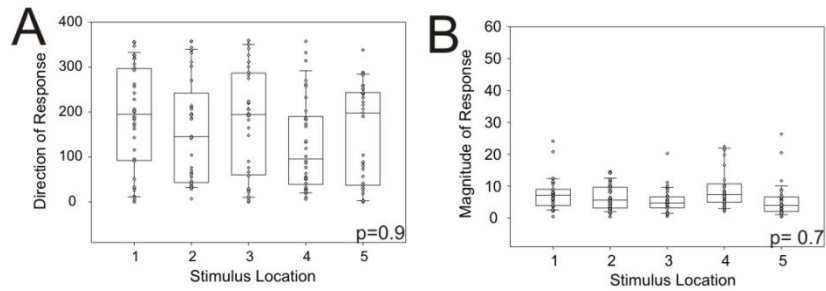


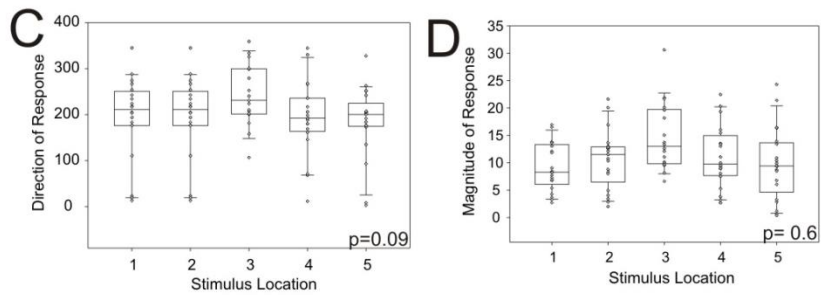
Figure 5. Nociceptive withdrawal responses for individual stimulus locations. Response vectors grouped by location according to each of the 5 plantar rostral-caudal and lateral-medial stimulus locations and grouped by color for Von Frey (n=178 trials) and needle (n=101 trials) stimulation. Vectors, not to scale with the foot, show the varying directions and magnitudes of the changes in foot stimulus location from initial to final positions.

In order to determine if the stimulus location had a significant effect on the direction and magnitude of the response, the responses were averaged over 2-5 replicates at each stimulus location within each animal and graphed separately for Von Frey and needle stimulation. For Von Frey, the stimulus locations did not significantly affect the direction (Figure 6A, $p=0.9$, circular Mardia-Watson Wheeler Test) or the magnitude (Figure 6B, $p=0.7$, ANOVA on Ranks) of the response. Similarly, the direction (Figure 6C, $p=0.09$, circular Mardia-Watson Wheeler Test) and the magnitude (Figure 6D, $p=0.6$, ANOVA on Ranks) of the NWR did not depend on the location of needle stimulation. Combining the results to increase the statistical power still showed no significant effect on the stimulus location on the direction (Figure 6E, $p=0.5$, circular Mardia-Watson Wheeler Test) and magnitude (Figure 6F, $p=0.6$, ANOVA on Ranks) of the NWR when the data was combined for Von Frey and needle stimulation. Together, these results falsified the hypothesis that stimulus location influenced the direction or magnitude of the NWR.

Von Frey



Needle



Combined

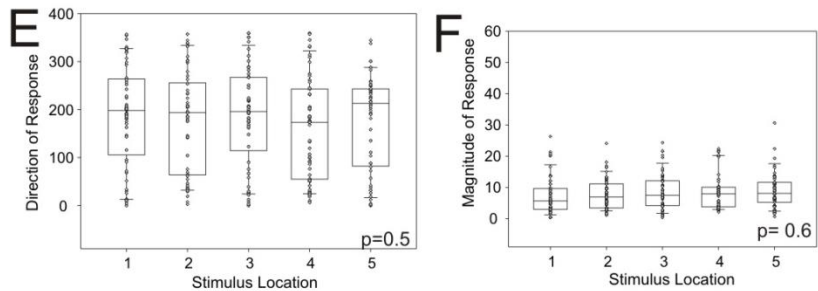


Figure 6. The effect of stimulus location on the magnitude and direction of the NWR for Von Frey, needle and combined stimulation. Direction and magnitude ($n=279$ trials, $n=17$ rats) from each trial are represented as both boxplots and individual data points. For Von Frey, stimulus location did not significantly affect direction (**A**, $p=0.9$, circular Mardia-Watson Wheeler Test) or magnitude (**B**, $p=0.7$, ANOVA on Ranks) of the NWR. Similarly, the direction (**C**, $p=0.09$, circular Mardia-Watson Wheeler Test) and the magnitude (**D**, $p=0.6$, ANOVA on Ranks) of the NWR did not depend on the location of needle stimulation. When the data is combined for Von Frey and needle stimulation, there continues to be no significant dependence of stimulus location on direction (**E**, $p=0.5$, circular Mardia-Watson Wheeler Test) and magnitude (**F**, $p=0.6$, ANOVA on Ranks) of the NWR.

It is possible that the differences in the initial position of the rat's paw could have obscured a relationship between the stimulus location and direction and magnitude of the NWR. To test this possibility, multiple regressions were used to “subtract” the effects of initial position from the effects of stimulus location. However, for comparison between movement in both rostral-caudal and lateral-medial directions, there was still no dependence on stimulus location.

Since stimulus location did not significantly affect the direction nor the magnitude of the response, other variables may determine the diverse directions and magnitudes of the NWR. We observed that the rat positioned its stimulated paw (hind, left) in various positions between trials (Figure 7), suggesting the hypothesis that the initial position of the paw might influence the magnitude and direction of the NWR.

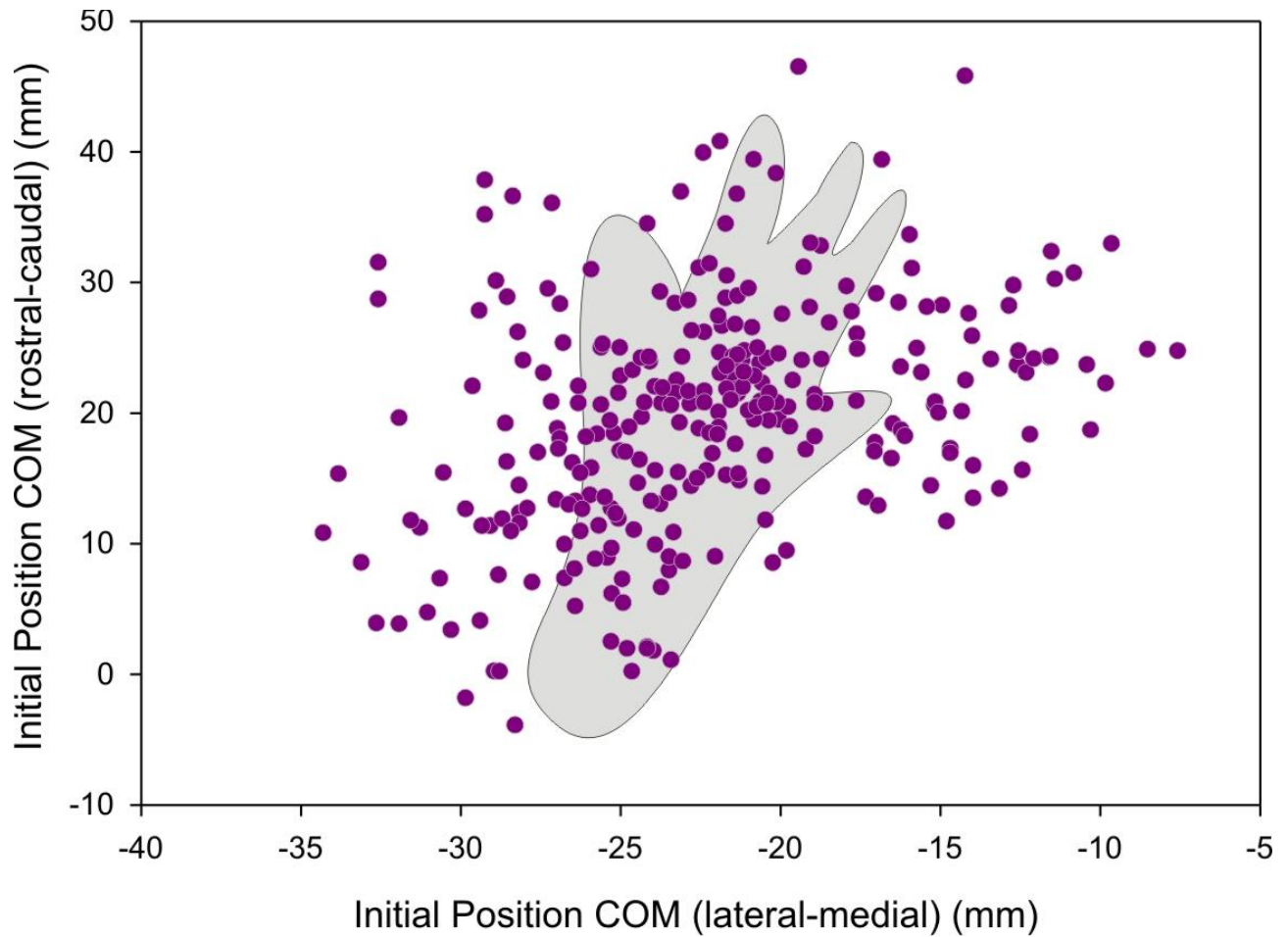


Figure 7. Initial positions of the paw prior to mechanical stimulation. The initial positions of the COM of the paw are shown in the lateral-medial and rostral-caudal planes, and the variability reflects distribution of initial paw positions before stimulation.

From analysis of the change in direction between the initial and final positions of the rat's paw, there appears to be an effect of initial position on the direction of the NWR, following mechanical stimulation applied to the plantar surface. The changes in directions for each individual trial are represented by vectors (Figure 8A, n=279 trials); similarly, the origins of the vectors represent the initial position of the rat's paw, and the arrow ends of the vectors represent the paw's final position. To minimize the amount of overlap, the vectors' lengths were scaled down, which makes the vectors appear smaller in size. The changes in directions for each individual trial are clearly represented by vectors (Figure 8B), where each vector represents an average of the vector tails that fall within a designated 5mm X 5mm box on the graph. The directions of the vectors suggest a pattern similar to Chrzan (2013) and Seamon (2015); when the rat spontaneously placed the paw rostrally, or more forward, the paw moved caudal, or more backward, in response stimulation. This inverse relationship was also seen in reverse; when the paw was initially caudal, the paw would move rostral in response to stimulation. A similar pattern was observed when the rat placed its paw within the lateral-medial direction.

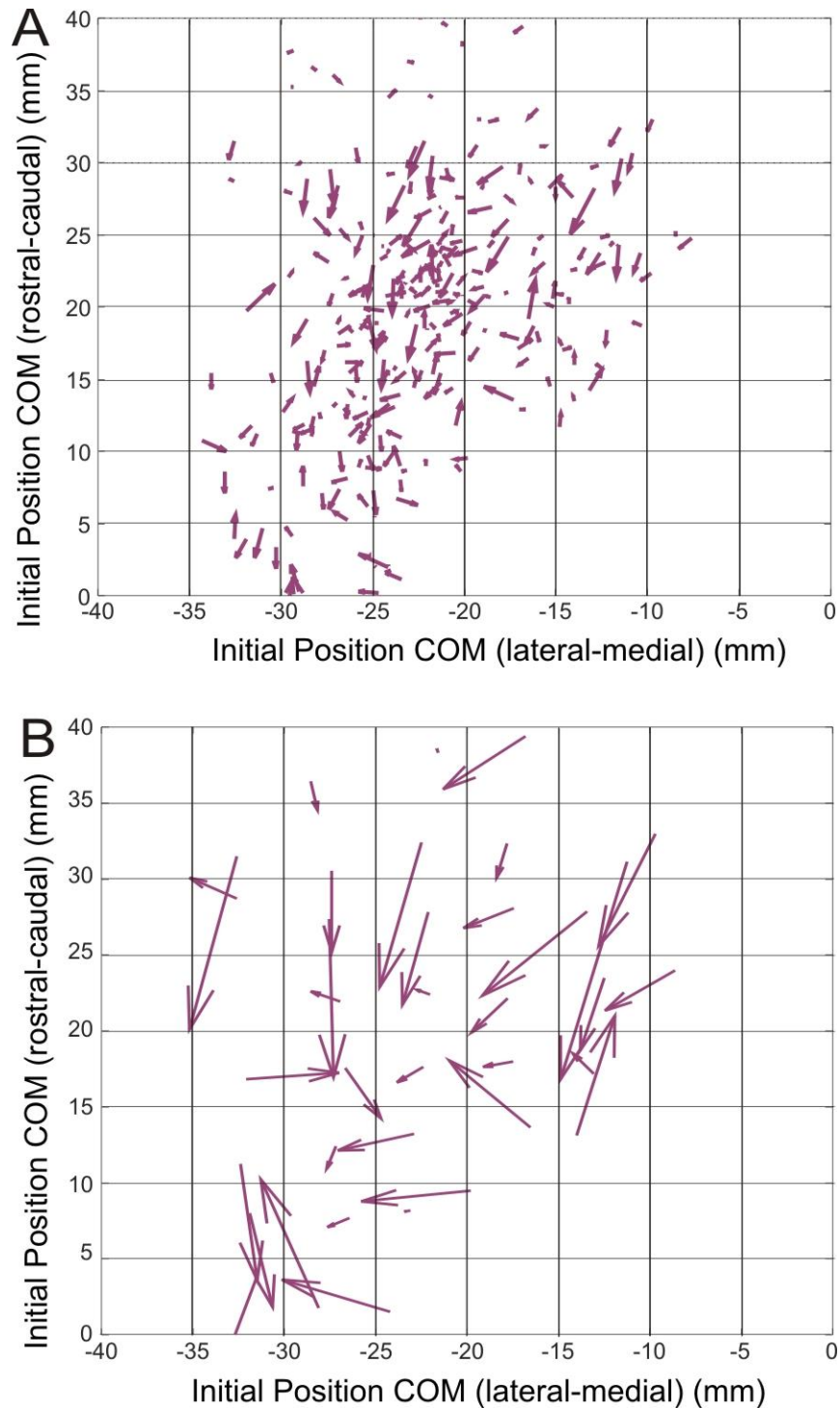


Figure 8. Spatial distribution of response vectors after mechanical stimulation. Vectors show the directions, represented by the orientation, and magnitudes, represented by the length, of the changes from initial to final COM positions of the paw. The origins of the vectors represent the initial COM positions, while the arrow ends of the vectors represent the final COM positions. **A** Each vector represents an individual trial, for both Von Frey and needle ($n=279$ trials) stimulation. To minimize the amount of overlap, the vectors' lengths were scaled down. **B** Each vector represents an average of the vector tails that fall within each 5mm X 5mm box on the graph.

To determine if the initial position of the paw significantly affected the withdrawal response, regression and correlation were performed on the initial position of the paw and the resulting change in position separately for the rostral-caudal and lateral-medial directions for Von Frey and needle stimulations. Within a single rat (Figures 9A, B, E and F), there appears to be a negative slope trend, for both coordinate directions and methods of mechanical stimulation, which suggests the pattern of response from Figure 8. The initial position of the paw significantly affected the change in position of the paw in the lateral-medial (Figure 9G, $r^2=0.2$, $p=0.04$, slope=-0.2 mm/mm, n=178 trials, Pearson Correlation and Linear Regression) and rostral-caudal (Figure 9C, $r^2=0.4$, $p<0.0001$, slope=-0.4 mm/mm, n=178 trials, Pearson Correlation and Linear Regression) directions after stimulation with the Von Frey. The initial position of the paw when stimulated with the needle also displayed a significant effect on the change in position of the paw in the lateral-medial (Figure 9H, $r^2=0.5$, $p<0.0001$, slope=-0.6 mm/mm, n=101 trials, Pearson Correlation and Linear Regression) and rostral-caudal (Figure 9D, $r^2=0.4$, $p<0.0001$, slope=-0.5 mm/mm, n=101 trials, Pearson Correlation and Linear Regression) directions. These results show that when the rat spontaneously placed the paw rostrally (more forward), the paw moved caudally (or more backward) in response to stimulation. Similarly, when the paw was initially caudal, the paw would move rostrally in response to stimulation. A similar pattern was observed when the rat placed its paw within the lateral-medial direction.

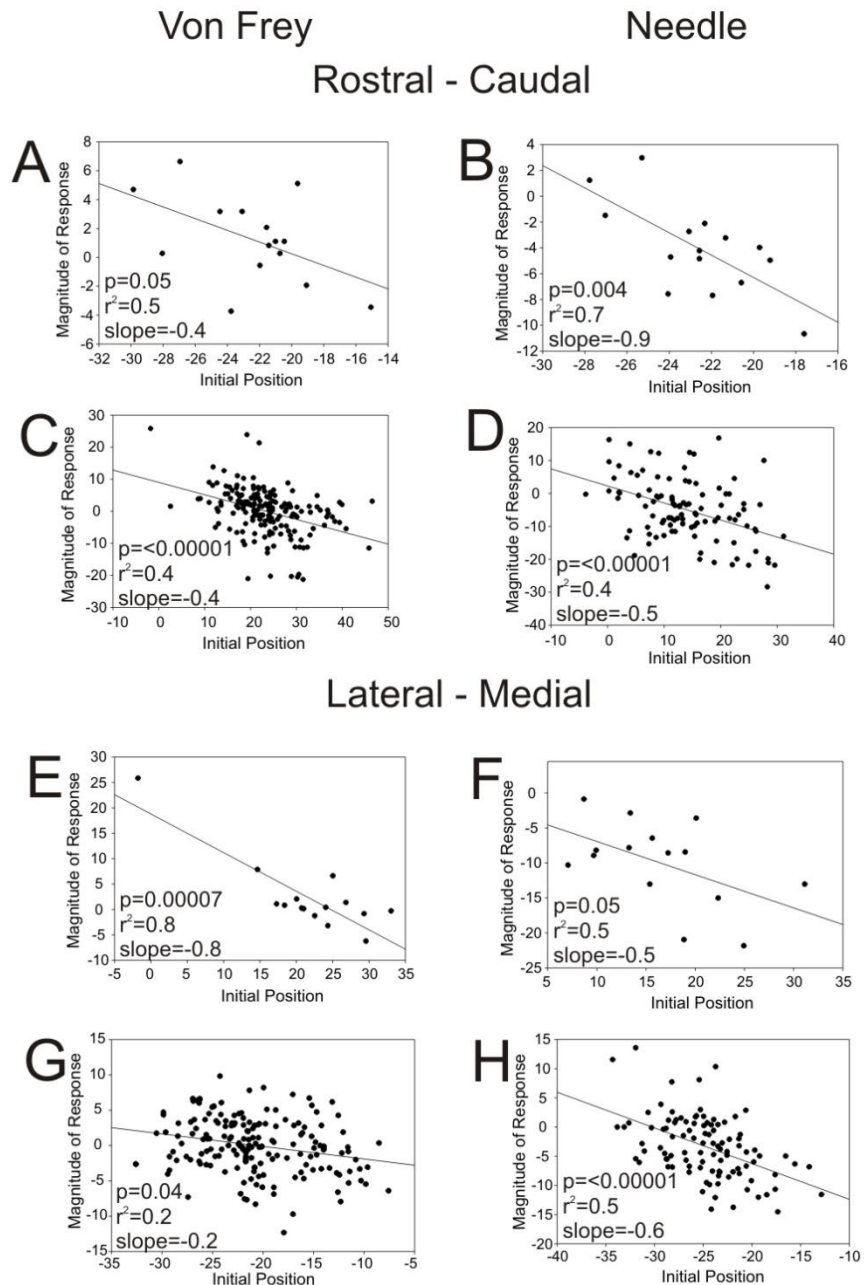


Figure 9. The effect of initial rostral-caudal and lateral-medial paw position on the magnitude of the NWR. Plots **A** and **B** represent a single exemplary experiment from a single rat, each with 15 trials. The linear regression line has negative slopes for all four panels (slopes=-0.4, -0.9, -0.4, -0.5). Altogether with 17 rats, the initial position of the paw significantly affected the change in paw position along the rostral-caudal direction when the paw was stimulated with the Von Frey (**C**, $r^2=-0.4$, $p<0.0001$, slope=-0.4 mm/mm, $n=178$ trials, Pearson Correlation and Linear Regression) and the needle (**D**, $r^2=-0.4$, $p<0.0001$, slope=-0.5 mm/mm, $n=101$ trials, Pearson Correlation and Linear Regression). Plots **E** and **F** also represent a single exemplary experiment from a single rat, each with 15 trials. Altogether with 17 rats, the initial position of the paw significantly affected the change in paw position along the lateral-medial direction when the paw was stimulated with the Von Frey (**G**, $r^2=-0.2$, $p=0.04$, slope=-0.2 mm/mm, $n=178$ trials, Pearson Correlation and Linear Regression) and the needle (**H**, $r^2=-0.5$, $p<0.0001$, slope=-0.6 mm/mm, $n=101$ trials, Pearson Correlation and Linear Regression). The coefficients of determination (r^2) for scatter plots **C**, **D**, **G** and **H** are low.

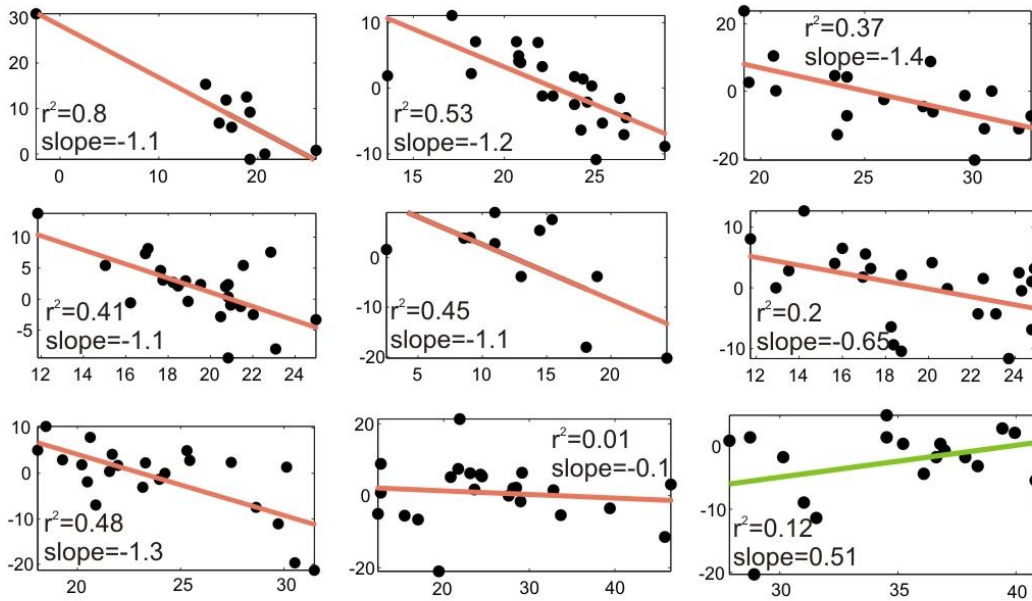
Although statistically significant, the coefficients of determination (r^2) were fairly low (rostral-caudal=0.4, 0.4; lateral-medial=0.5, 0.2). These low r^2 values could have been due to differences between rats, such as the weight or posture the animal adopted in the acrylic box. In order to eliminate the variation between rats, each rat was analyzed individually.

Individual correlations for each of the 17 rats in the rostral-caudal (Figure 10) direction resulted in higher r^2 (10 of 17 $r^2 > 0.4$) values. Importantly, of a total of 17 rats, with combined Von Frey or needle stimulations, 15 rats had a negative slope relating to the change in position in the rostral-caudal directions. Similarly, individual correlations for each of the 17 rats, with combined Von Frey or needle stimulations, in the lateral-medial (Figure 11) direction resulted in higher r^2 (6 of 17 $r^2 > 0.3$) values, and 15 rats had a negative slope relating to the change in position in the lateral-medial directions.

The frequency distribution of the individual slopes (Figure 12) from each rat for the rostral-caudal (mean=-0.71) and lateral-medial (mean=-0.65) directions show more negative slopes, compared to positive slopes. In order to test the effect of the paw's initial position on the withdrawal response, the slope means were compared to a hypothesized population mean of 0, which is the value distinguishing between negativity and positivity. The change in position of the paw showed a strong dependence on the initial position of the paw in the rostral-caudal ($p=0.0001$, $n=17$, one-sample t-test) and lateral-medial ($p=0.00008$, $n=17$, one-sample t-test) directions. There was no significant difference between the method of stimulation in the rostral-caudal ($p=0.2$, $n=17$, Mann-Whitney test) and lateral-medial ($p=0.96$, $n=17$, t-test) directions.

Rostral - Caudal

Von Frey



Needle

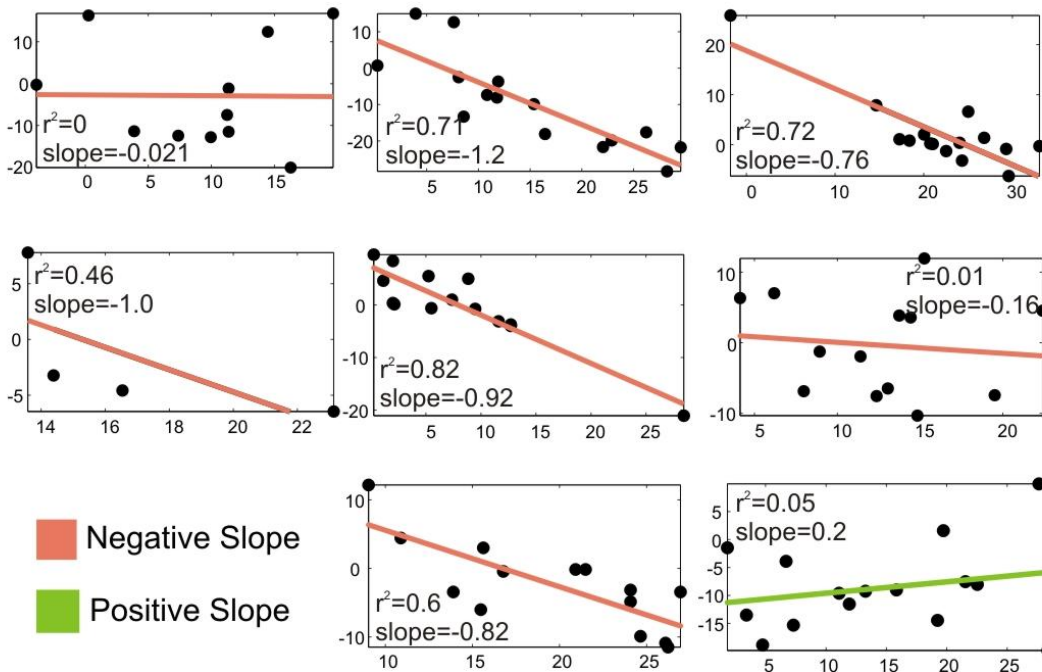


Figure 10. Relationship between initial rostral-caudal paw position and final paw position for individual rats. Each individual rat is shown on a separate graph, and the r^2 value and slope for each trail are displayed above the graph. There were negative slopes for 8 of the 9 rats that were stimulated with the Von Frey, and negative slopes for 7 of the 8 rats that were stimulated with the needle.

Lateral - Medial

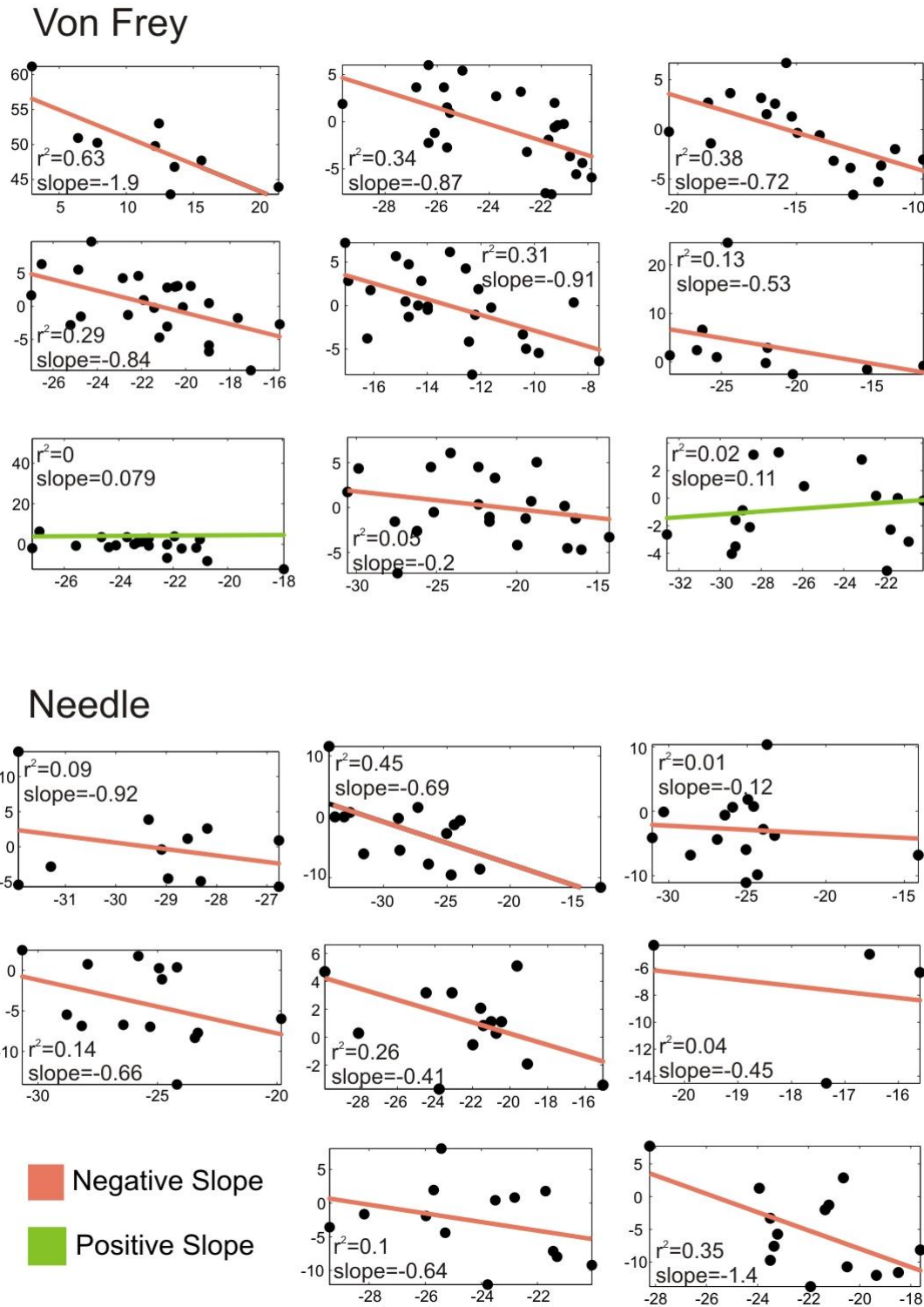
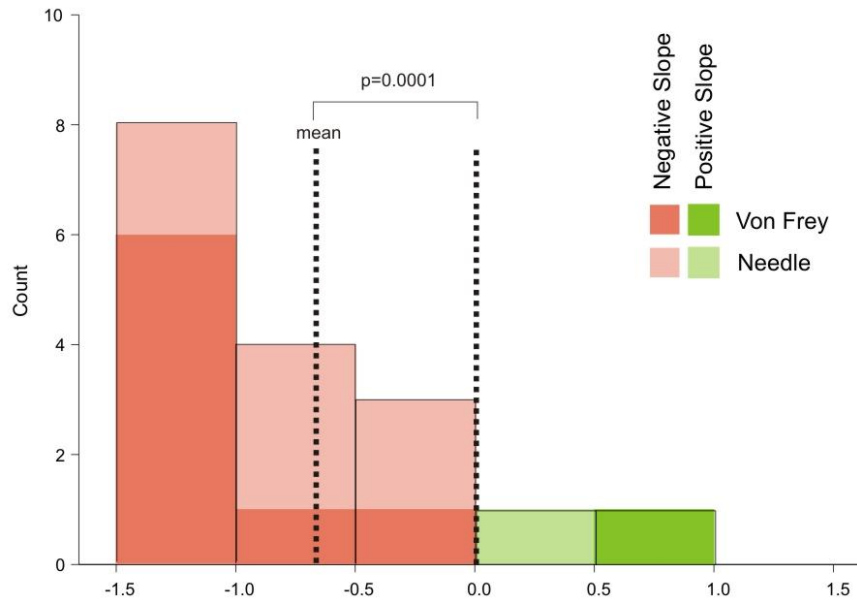


Figure 11. Relationship between initial lateral-medial paw position and final paw position for individual rats. Each individual rat is shown on a separate graph, and the r^2 value and slope for each trail are displayed above the graph. There were negative slopes for 7 of the 9 rats that were stimulated with the Von Frey, and negative slopes for all 8 rats that were stimulated with the needle.

Rostral - Caudal



Lateral - Medial

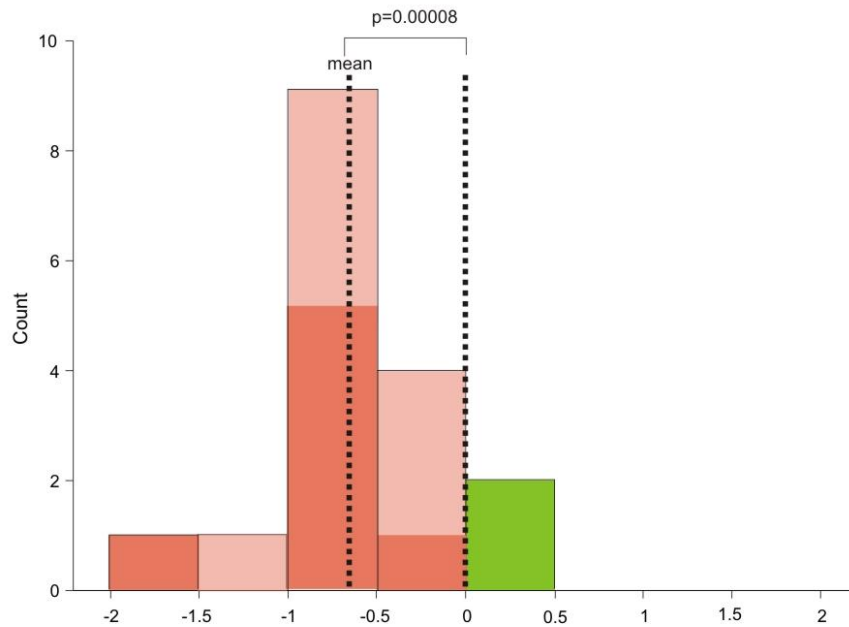


Figure 12. Combined slopes from individual rats demonstrate dependence of final paw position on initial position. Slopes from individual trials were combined and displayed on a frequency histogram, separately for rostral-caudal and lateral-medial axes. The graph shows that the number of negative slopes for the rostral-caudal and lateral-medial directions is large, compared to the number of positive slopes. The average slope is more negative than zero for the rostral-caudal (mean=-0.71, $p=0.0001$, $n=17$ rats, one-sample t-test) and lateral-medial (mean=-0.65 $p=0.00008$, $n=17$ rats, one-sample t-test) axes. There was no significant difference between the method of stimulation and the slopes in the rostral-caudal ($p=0.2$, $n=17$, Mann-Whitney test) and lateral-medial ($p=0.96$, $n=17$, t-test) directions.

B. The Determination of Information Used by the CNS

The direction of the NWR, as shown above, is influenced by the initial posture of the hind limb in intact, unanesthetized rats. There are two alternative hypotheses (Figure 13A) for the mechanism used by the CNS to determine the initial posture of the foot. In Figure 13A, the vertical dotted line corresponds to the starting position of the paw, and the arrow represents the repositioning of the paw just prior to heat stimulation. The first hypothesis is proprioceptive sensory feedback, which is supported when the change in locations lies on the negatively-sloped linear regression line, showing no dependence on the initial position of the paw prior to stimulation. The second hypothesis is corollary discharge, which is supported when the change in location does not lie on the linear regression line, showing dependence on the paw's initial position.

In order to determine the mechanism used by the CNS, the proprioceptive representation of the initial position of the foot was dissociated from the corollary discharge representation of the initial position of the foot. From one individual rat (Figure 13B), the black data points represent the normal trials, and the red data points represent the movement trials. The black linear regression line is from the data of the normal trials. The perpendicular distances between two representative data points and the regression line from the normal trial data were measured to determine the type of information the CNS of intact rats utilizes. If proprioceptive sensory feedback is supported, then the distances would be similar in value to each other. If corollary discharge is supported, then the distances would be different in value to each other. In this example, the movement (red) data point is further from the regression line, which would suggest corollary discharge. Amongst the five other rats (Figure 13C), the analysis was completed likewise.

The distances from the regression line to the normal and movement trials were compared, and there was no significant difference between the two types of trials (Figure 13D, $p=0.95$, $n=210$ normal trials, $n=169$ movement trials, Mann-Whitney test). The lack of a significant difference between the two types of trials indicates that the CNS uses the proprioceptive sensory feedback mechanism to determine the direction of the NWR in intact, unanesthetized rats.

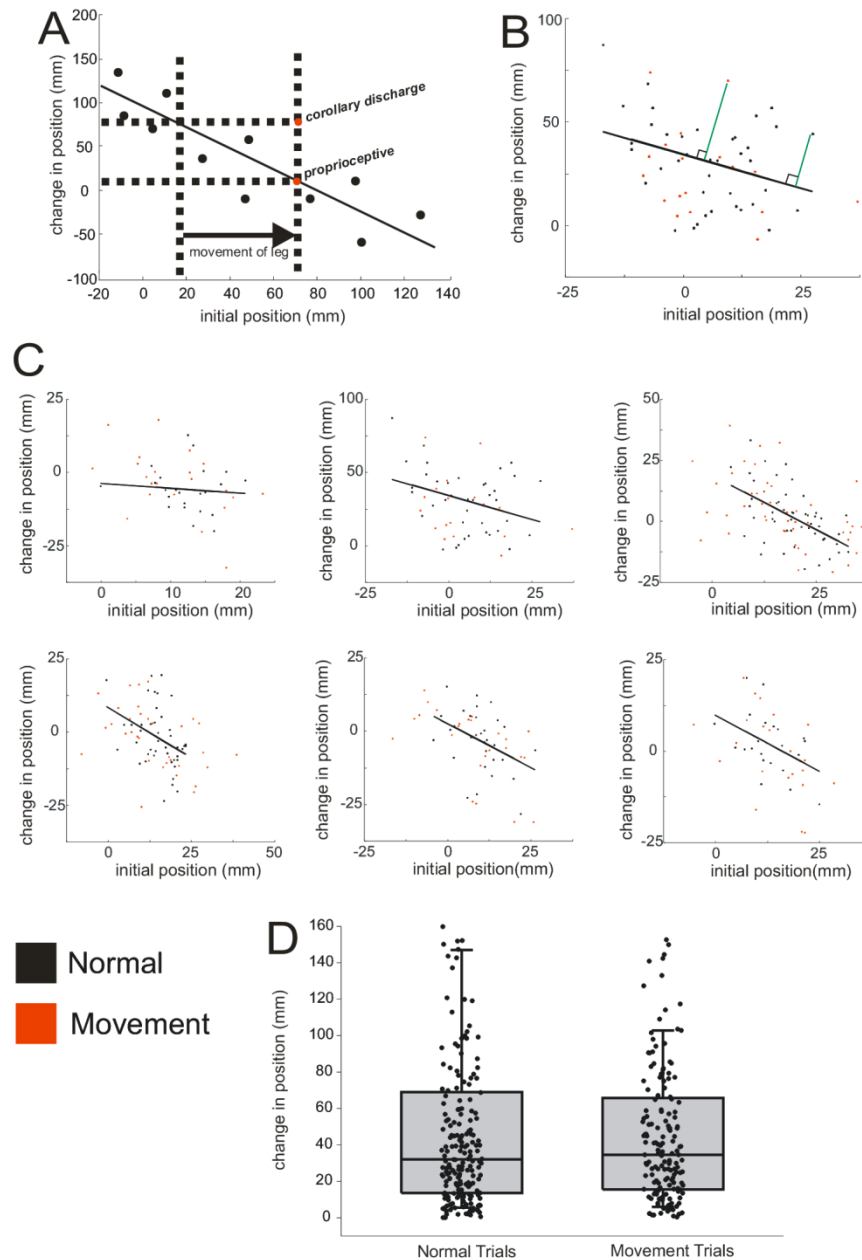


Figure 13. Determination of the mechanism used by the CNS to assess the initial position of the stimulated paw. **A** Two hypotheses for the mechanism used by the CNS are shown on the graph, using simulated data. The vertical dotted line corresponds to the starting position of the paw, and the arrow represents the repositioning of the paw by the experimenter just prior to heat stimulation. **B** One individual experiment, with a single rat, is shown on the graph. The distances were measured between two sample data points, normal (black) and movement (red) trials, and the regression line is for the normal data. In this example, the movement (red) data is further from the regression line, as would be expected for corollary discharge. **C** Each individual rat was separately analyzed, in which the regression line is from the normal data, and the perpendicular distances from the linear regression line to the normal (black) and movement (red) were calculated. **D** There was no significant difference between the two types of trials ($p=0.95$, $n=210$ normal trials, $n=169$ movement trials, Mann-Whitney test).

VII. Discussion

A. Summary of Results

a. Stimulus Location

The effect of stimulus location on the NWR in intact, unanesthetized rats was determined by applying two mechanical stimuli – Von Frey monofilament and a 30-gauge needle – to the plantar surface of the hind, left paw at five different locations. The withdrawal response generally consisted of rapid removal and replacement of the paw on the platform. In contrast to previous literature, the stimulus location did not significantly influence the direction and magnitude of the NWR in intact, unanesthetized rats.

b. Initial Position

The effect of initial position on the NWR in intact, unanesthetized rats was determined by applying a mechanical stimulus to the paw following placement in a variety of different positions adopted voluntarily by the rat, and the withdrawal movement was observed. Similar to the previous literature, the initial position of the paw prior to mechanical stimulation did significantly influence the NWR in intact, unanesthetized rats. If the paw was initially placed in the rostral direction, it tended to move caudally, and similarly, if the paw began more caudally relative to the rat's body, it would move in the rostral direction. As the paw was positioned in the lateral direction, it moved medially, and similarly, if the paw was initially positioned in the medial direction, it tended to move laterally.

c. Proprioceptive Sensory Feedback vs. Corollary Discharge

The source of information used by the CNS responsible for the dependence on posture for the NWR was determined by pre-determining the position of the rat's paw before a heat stimulus was applied. Previous research has not distinguished between the use of proprioceptive

sensory feedback and corollary discharge in intact, unanesthetized mammals, such as when the mammal spontaneously positions the paw. When the distances from the regression line to the normal and movement trials were compared, there was no significant difference between the two types of trials. The lack of a significant difference between the two types of trials indicated that the CNS uses the proprioceptive sensory feedback mechanism to determine the direction of the NWR in intact, unanesthetized rats.

B. Comparison to Previous Results

a. Stimulus Location

The NWR of intact humans has been shown to depend on stimulus location. In the lower limb, electrical stimulation at locations in the lateral region of the plantar surface of the human foot evoked eversion responses, while electrical stimulation at locations in the medial region evoked inversion responses; thus in both cases, the NWR caused the area of stimulated skin to move directly away from the stimulus (Andersen *et al.*, 1999 & 2001). Another contrast to our studies is from Schouenborg (1992), who suggested that the EMG responses in the ankle muscles of spinalized rats depended on the location of mechanical and heat stimulation to the hindlimb. In contrast, our experiments suggested that there was no effect of stimulus location on the direction and magnitude of the response to mechanical and heat stimulation. One possible explanation for the difference in results could be the difference in method of stimulation, as Andersen (1999 & 2001) applied electrical stimulation to intact humans and we applied mechanical stimulation to intact rats. However, since the studies performed by Schouenborg (1992) used similar methods of stimulation, another possible explanation for the contrast of results is that Andersen (1999 & 2001) and Schouenborg (1992) measured the movement and EMG signals during the movement, which we did not necessarily do. Instead, we simply

measured the direction and distance between the initial and final positions of the stimulated paw to quantify the NWR.

Our results from the experiments testing the dependence of stimulus location on the NWR of intact, unanesthetized rats were *similar* to the results of studies with spinal cord-injured humans, in which the location of electrical stimulation of the dorsal surface of the human foot did not influence the pattern of muscle activation in the NWR (Andersen *et al.*, 2004). Sherrington's (1910) observations are in parallel to the behaviors seen in intact, unanesthetized rats; that is, the mammal withdrew the stimulated limb directly away from the delivered stimulus. Sherrington (1910) suggested that the muscle contractions and relaxations involved with the withdrawal reflex were largely independent of which nerve was stimulated, which is proportional to our findings of the independence of location of stimulation on the NWR in intact rats. However, the measurements of the NWR were varied between the two studies; our study used the direction and distance between the initial and final positions of the stimulated paw to quantify the NWR, while Sherrington (1910) observed the EMG signals of the flexor and extensor muscles in the hind limbs of spinalized cats and dogs.

Correspondingly, Chrzan (2013) and Seamon (2015) also concluded that the location of heat stimulation along the plantar surface of the intact rats' hind paw did not significantly impact the direction of the NWR, and we observed a similar independence with mechanical stimulation. Despite the type of stimulation, whether electrical, heat or mechanical, the stimulus location did not show an effect on the direction of the NWR.

b. Initial Position

Similar to our studies with intact, unanesthetized rats, the NWR of intact, unanesthetized humans has been shown to depend on the initial position. After an application of electrical

stimulation to the fingers of intact humans, the direction of the withdrawal response was altered based on the initial position of the upper limb; from flexion to extension of the limb, the NWR was in the posterior-lateral direction, and then shifted to the posterior direction, in which the limb moved further away from the electrical stimulus (Peterson *et al.*, 2014). Likewise, for intact rats based on the results of Chrzan (2013) and Seamon (2015) using heat stimuli, the direction of the withdrawal response was altered based on the initial position of the lower limb; when the paw was placed rostrally, the paw moved caudally in response stimulation. This inverse relationship was also seen in reverse; when the paw was initially caudal, the paw would move rostrally in response to stimulation. A similar pattern was observed when the rat placed its paw within the lateral-medial direction.

The posture of decerebrated and spinalized mammals has been also shown to influence the NWR after the delivery of a noxious stimulus, similar to the effects of modifying the posture of intact, unanesthetized rats. In decerebrated cats, the non-noxiously evoked flexion withdrawal and crossed extensor reflexes were altered by the changes in knee joint angle, in which the extended position of the knee activated the flexor muscles' activity of the lower limb (Baxendale & Ferrell, 1981). Similarly, in spinally-injured human subjects, increase in hip extension resulted in larger torque responses in the hip (Kim *et al.*, 2007). Despite the conditions of the mammal, whether intact, unanesthetized, spinalized, or decerebrated, the initial posture of the limb had an effect on the direction of the NWR.

C. Mechanisms

Our results show that the CNS uses proprioceptive sensory feedback to assess the initial foot position, rather than corollary discharge. When the CNS uses proprioceptive sensory feedback to determine the direction of the NWR of the hind limb in intact rats, information about

the muscle length, force, and joint angles in the limb is provided (Onushko et al., 2013). When the hind limb is moved in the caudal direction prior to stimulation, the rat is aware of the change in the position of the hind limb, thus causing the rat to bring the paw more rostrally in response to the stimulation.

Our results, however, do not show the receptors responsible for proprioceptive information and from which joint the information arises. The sensory receptors in skin, joints and muscle spindles can be responsible for providing proprioceptive information to a mammal (Hasan, 1992). From previous studies of the NWR in the human lower limb, observations of larger torque responses in the ankles when the *hip* joint was extended, compared to when the hip joint was flexed, suggested that the hip primary spindle receptors increased the NWR in the lower limb (Kim *et al.*, 2007). However, the flexed and extended positions of the knee joint of decerebrated cats determined the intensity of the NWR, suggesting that *knee* joint afferent activity explained the observed pattern. Furthermore, when the joint receptors were blocked from the knee joint through the injection of a local anesthetic, there was no longer a modulation of the NWR when the knee joint angle was changed, thus supporting the role of knee joint receptors (Baxendale & Ferrell, 1981). From the previous literature, the hip, ankle and knee joints can be suggested to provide proprioceptive sensory information to the rat; therefore, determining the direction of the NWR.

D. Significance

The ability of an animal to effectively withdraw a part of its body away from potential harmful stimuli is critical to survival. However, in order for the withdrawal movement to be completely successful, the animal must also be able to maintain a proper balance of its body. The experimental results suggested that the animal's posture has a significant influence on the

NWR; upon withdrawal of the limb, the animal replaces the stimulated paw in a position that allows the body to be in a more stable posture.

In contrast to posture, stimulus location did not contribute to the determination of the direction and magnitude of the NWR. These results suggest that maintaining stable posture after a potentially damaging threat is encountered is more important for survival, than optimizing the response direction based on stimulus location. Further, there will be a minimal amount of choices of directions to respond in, thus potentially increasing the speed of the response to a noxious stimulation (Schmidt & Lee, 1999).

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