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# Rat hind limb nociceptive withdrawal response depends on initial paw position but not stimulus location

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# Rat Hind Limb Nociceptive Withdrawal Response Depends on Initial Paw Position but Not Stimulus Location

An Honors Program Project Presented to
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by Kimberly Morgan Seamon

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# II. Acknowledgements

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# III. Introduction

Escape and withdrawal responses are essential behaviors that animals must possess in order to survive in an environment in which they are under the threat of predation and injury. Escape responses, in which animals move their entire body away from the threat, have been studied extensively in various invertebrates (Card, 2012). For example, in the crayfish, a mechanical tap elicits one of two distinct escape responses dependent upon which end of the animal the stimulus is applied. Mechanical tap stimulation applied to the rostral end results in simultaneous bending of all abdomen segments, causing backwards movement away from the stimulus. In contrast, a caudal tap causes bending of only rostral abdomen segments, resulting in a sudden jump upwards accompanied by rotation of its hind end away from the stimulus (Edwards et al. 1999). In cockroaches, a puff of wind causes the animal to first orient its body away from the source of wind and subsequently run in that direction (Camhi et al. 1978). Withdrawal responses, in which the animal withdrawals part of its body away from a potential threat, are also present in invertebrates. For example, crickets respond to a noxious heat stimulus applied to their tarsi by withdrawal (Cleland et al. 2015). Vertebrates also exhibit withdrawal responses to diverse stimuli from skin, joint, and muscle (Schomburg 1990; Baxendale 1981).

## A. Nociceptive Withdrawal Reflex

The focus of this research is the mammalian withdrawal reflex known as the nociceptive withdrawal reflex (NWR). The NWR is a rapid withdrawal movement of some part of the body in response to noxious (actual or potential tissue-damaging) stimuli, which may protect the body part that is being withdrawn (Sherrington 1910). Some common examples of noxious stimuli are heat, such as touching something very hot, pressure, such as pinching your skin with tweezers, or chemical, such as eating a spicy food (Cleland and Gebhart, 1997), or extreme cold, such as sudden immersion in very cold water (Hensel et al. 1974).

The NWR was initially studied extensively in the early 1900's by C.S. Sherrington. Based on studies in spinalized cats, he showed that noxious stimulation of the skin or afferent nerves in hip, knee, and ankle joints evoked widespread stereotyped flexion of the limb. The resulting limb flexion arose from excitation of flexor muscles and simultaneous inhibition of extensor muscles acting on the ankle, knee, and hip (Sherrington 1910). More recently, the NWR has been shown to occur in both human legs (Andersen and Sonnenborg 1999) and arms (Serrao et al. 2006) as well as in rats (Schouenborg and Kalliomaki 1990), mice (Thelin and Schouenborg 2008), and rabbits (Clarke and Harris 2004).

## B. Mechanisms of the NWR

### a. Nociceptors

Nociceptors contain a single process originating from the cell body in the dorsal root ganglion which bifurcates and extends a peripheral axon to innervate the skin and a central axon to synapse on second-order neurons in the dorsal horn of the spinal cord. The central axon enters the spinal cord through the dorsal root and divides into branches that innervate multiple spinal segments in the rostral and caudal directions (Dubin and Patapoutian 2010).

The axons that transmit cutaneous nociceptive information to the central nervous system can be classified into two main groups: C-fibers and Aδs. Initial, fast-onset pain is mediated by Aδ nociceptors whose axons are myelinated and support faster conduction velocities; C-fibers are small in diameter and unmyelinated, therefore mediating slow-onset pain and supporting slower conduction velocities (Dubin and Patapoutian 2010). Fast-onset pain is characterized by a sharp, intense discomfort following stimulation, while slow-onset pain is a more gradual, aching sensation. Conduction velocities for Aδ-nociceptive units average 12 m/s with a range of 3-23 m/s while conduction velocities for C-fibers average 0.8 m/s with a range of 0.4-1 m/s (Leem et al. 1993). Aδ branches generally cluster in small areas creating a small receptive field, while C-fibers are more broadly distributed having much larger receptive fields (Dubin and Patapoutian 2010). In the rat, the receptive fields for Aδ- and C-fiber nociceptive units were distributed evenly over the paw skin innervated by each nerve and could be activated by noxious stimulation of the cutaneous surface of the paw (Leem et al. 1993).

The plantar surface of the paw in the rat receives extensive nociceptive innervation through the sural and plantar nerves. Of the sural Aδ-units, 68% of all sensory afferents were nociceptors, and all of those innervating the plantar surface of the paw were identified as nociceptors. The sural C-fiber units were made up of 44% nociceptors, while the plantar C-fiber units were composed of 77% nociceptors (Leem et al. 1993).

Although heat activates both Aδ and C-fiber sensory afferents, nociceptive information resulting from noxious skin heating at a low rate is likely mediated preferentially by C-fibers. Upon heating the hind paw skin of the rat at a relatively low rate of 0.9°C/sec, C-fibers were initially activated around 8-10 seconds after the onset of heating, while Aδ afferents were largely unaffected. In contrast, a high rate of skin heating (6.5°C/sec) activated both classes of nociceptors, but activation of Aδs was more intense and occurred after shorter onset. At the high rate of heating, Aδs were activated within 2 seconds of the onset of the stimulus, which was a similar latency to the withdrawal of the paw at 2.5 seconds. A similar effect was seen at the low rate of heating with activation of C-fibers around 10 – 12 seconds after the onset of the stimulus, also corresponding to the withdrawal latency (Yeomans and Proudfit 1996). Variation in activation of Aδs and C-fibers depending upon the rate of heating allows the pathway which is preferentially activated to be experimentally controlled by changing the intensity of the stimuli. A more intense stimulus producing a shorter latency of response could be used to activate Aδs, while a less intense stimulus resulting in a longer latency of response could be used to activate C-fibers.

### b. Spinal Cord

The NWR is a spinal reflex, as animals with severed spinal cords still exhibit the NWR in response to noxious stimuli (Sherrington 1910). Actions by nociceptors have been demonstrated as di/tri synaptic, with excitatory actions mediated through a minimum of one or two spinal interneurons and the corresponding inhibitory actions through a minimum of two spinal interneurons (Eccles and Lundberg 1959). Nociceptors that innervate the tail of the rat terminate in superficial Rexed laminae I and II of the dorsal horn (Grossman et al. 1982) and nociceptive afferents that innervate the skin of the leg and paw terminate in the dorsal horn from caudal L5 to caudal L2 (Woolf and Swett 1983). Neurons involved in transmitting information regarding the withdrawal reflex of a single muscle are present in the deep dorsal horn (Schouenborg et al. 1995).

### c. Modulation

Neural pathways can be modulated by influences that change the properties of the pathway without directly producing a response. The transmission of nociceptive information within the spinal cord is modulated by descending pathways originating in the brainstem. These influences on spinal nociceptive processing rely on neuronal pathways originating in the medulla, specifically the rostroventral medulla (RVM), including the medial nucleus raphe magnus (Gebhart 2004). These descending influences can either inhibit or enhance spinal nociceptive processing. Descending inhibitory influences were seen to descend the spinal cord in the dorsolateral funiculi, while facilitatory influences descended from the RVM in the ventral/ventrolateral spinal cord (Gebhart 2004). In the decerebrate state, there is often complete suppression of the NWR, but upon severing the spinal cord, the excitatory action to flexor nuclei and inhibitory action to extensor nuclei is released. Therefore, an overall tonic inhibitory effect is produced by the brainstem, modulating the activity of both flexor and extensor spinal pathways (Eccles and Lundberg 1959).

In order to identify the level of the brainstem responsible for modulation, Holmqvist and Lundberg (1961) made transverse lesions in a caudal progression in the brain stem and recorded the effects of these lesions on the NWR. When a low pontine lesion was made, there was a release of the inhibitory affects to extensor motoneurons. The release of the excitatory path to flexor motoneurons was not observed until a more caudal lesion was made in the medulla. Thus, it can be concluded that different aspects of the NWR are modulated by pathways originating from various locations within the brainstem; inhibitory pathways lie in the rostral region of the medulla, while excitatory pathways lie in the caudal region.

### d. Long Loop Reflexes

In addition to playing a role in the NWR by modulating the reflex in the spinal cord through descending pathways, the brain may contribute to long loop NWRs. Long loop reflexes transmit sensory information back to the brain before descending pathways convey the information to the appropriate motoneurons (Lee and Tatton 1975). Studies of the stretch reflex show that in response to sudden upper limb displacement in monkeys, three early peaks of electromyogram (EMG) activity were recorded at 10 to 12 milliseconds, 32 to 35 milliseconds, and 45 to 60 milliseconds. The first component was timed appropriately with a monosynaptic stretch reflex, but the two later components were thought to be mediated by long-loop reflexes (Lee and Tatton 1975) through the cortex. Later studies (Evarts 1973) showed that corticospinal neurons were activated as the arm of a monkey was stretched, suggesting that these neurons were playing a role in the withdrawal response. Although these results were obtained for the stretch reflex, they raise the possibility that the NWR may include long loop components.

There is indirect evidence that neurons in the RVM may play a role in mediating a long loop NWR. Thermal stimulation was used to provoke the NWR in rats and the activity of single cells in the RVM and the EMG of muscle activity in hind limb flexors were recorded. A similar pattern was seen between the rate of activity in the ON and OFF cells present in the RVM and the muscle EMG recordings following noxious stimulation. Since neurons in the RVM are known to project to the spinal cord, the similar pattern of activity between these neurons and the EMG recordings suggests that these neurons could be involved in a long loop reflex that contributes to the withdrawal response (Devonshire et al. 2015). `

### C. Dependence of the NWR on Stimulus Location

Sherrington (1910) studied the NWR by electrically stimulating different nerves in the limbs of spinalized cats and visually observing muscles in order to determine which ones were activated during the response. He discovered that patterns of muscle contraction and relaxation from various nerves were generally the same regardless of which part of the limb the stimulus was applied. Although the reflexes primarily consisted of excitation of flexor muscles and inhibition of extensor muscles, the magnitude of the responses varied depending on which nerve was stimulated. Sherrington labeled the variation in relative magnitude “local sign” (Creed and Sherrington 1926).

The concept of a stereotyped nociceptive withdrawal reflex began to be challenged in the early 1950s and 1960s (Hagbarth, 1952; Grimby, 1963). Hagbarth electrically stimulated smaller branches of nerves in the decerebrated cat hind limb and found that the resulting reflex varied with the location of the nerve which was stimulated. In one branch of the nerve, he observed excitation of flexors and inhibition of extensors, as was seen by Sherrington, but in the other branch he saw the opposite effect (excitation of extensors and inhibition of flexors). In order to explain the results obtained by Sherrington, the excitation of flexors and inhibition of extensors would have likely been the stronger response, so when the entire nerve was stimulated, flexion would dominate and mask the weaker excitation of extensors.

Hagbarth’s results (1952) also provided evidence that the nature of the nociceptive withdrawal response was fundamentally determined by the area of skin of the limb to which the stimulus was applied. Each of the extensor muscles studied was inhibited when most parts of the limb were stimulated, but could be excited from an area of skin mainly localized over the muscle itself. Conversely, each of the flexor muscles studied was excited from most parts of the limb but inhibited when the skin localized to the antagonist extensor muscle was stimulated.

Similarly, Grimby (1963) found that painful electrical stimuli applied to the plantar surface of the human foot produced reflex EMG that depended on the stimulus position, and was patterned to provide optimal withdrawal of the foot away from the noxious stimuli. Specifically, it was observed that as the stimulus moved from the hollow of the foot to the hallux ball, the reflex provoked gradually shifted from muscles that caused plantar flexion to those that evoked dorsiflexion. As the stimulus was shifted from the hollow of the foot to the heel, the resulting reflex shifted from dorsal to plantar flexion. As the stimulus was shifted from the medial to the lateral side of the plantar surface, the ankle movement gradually changed from inversion to eversion. This study provided further evidence for the dependence of the withdrawal response on the location of the stimulus and suggested the reflex is designed to directly withdraw the stimulated body part away from the stimulus.

As research on the NWR continued into the 1990’s, a more detailed organization of the nociceptive cutaneous receptive field of the hind limb muscle was described that further confirmed the dependence of the NWR on the stimulus in lightly anesthetized, decerebrated rats with intact spinal cords (Schouenborg and Kalliomaki 1990), following spinalization in the decerebrated rat (Schouenborg et al. 1992; Weng and Schouenborg 1996), and in the decerebrated cat (Levinnson et al. 1999). Most importantly, each muscle or muscle group in the reflex pathway was found to have different cutaneous receptive field corresponding closely to the skin area that would be expected to be withdrawn by that muscle group during contraction. Based on these findings, Schouenborg et al. (1992) concluded that an appropriate movement response would be formulated by activation of specific muscles in order to withdraw the limb directly away from the stimulus.

Previous studies on the NWR relied on recording electrical signals directly from nerves or muscles, so that the resulting movement was only implied from the patterns of EMG or motoneuron activity. Preliminary research by Clarke and Harris (2004), who recorded movements of the limb in the intact, lightly anesthetized rabbit in response to noxious mechanical stimuli, discovered a reflex movement pattern similar to that observed by Schouenborg and colleagues. As the location of the stimulus was varied along the rostral-caudal axis of the plantar surface of the paw, a corresponding change in movement was observed. Pinch of the rostral portion of the paw evoked a large flexion at the ankle joint, but as the stimulus was moved to the midsole, an extension of the ankle joint was observed before flexion. As the stimulus was moved to the caudal portion of the paw, a large flexion of the ankle joint was produced. Although the reflex elicited at the knee joint was consistently flexion, unaffected by which portion of the paw was stimulated, it appeared that there was a transition of ankle joint movement from flexion to extension as the stimulus was moved from the rostral to caudal end of the paw. From these results, Clarke and Harris concluded that the movement of the paw was affected by the location of the stimulus.

Although a majority of previous studies had been explored using various non-human mammals, in the late 1990's the NWR began to be studied extensively in humans. Andersen and Sonnenborg (1999) utilized electrical stimulation of the human foot sole which provided evidence for an organization in which each muscle had its own skin specific receptive field, similar to that found by Schouenborg and Kalliomaki (1990). Depending on the location of the stimulus, different patterns of EMG and directions of movement provided the most efficient withdrawal of the limb. For example, stimulation of the rostral portion of the foot resulted in dorsal flexion, while stimulation of the caudal portion evoked plantar flexion. Similarly, stimulation of the medial side resulted in inversion, while stimulation of the lateral side caused eversion. Sonnenborg and colleagues (2000) found that the inhibition pattern was the same as the excitation pattern, but reversed; whenever an extensor muscle was excited, the complementary flexor muscle was inhibited and similarly whenever a flexor muscle was excited, the complementary extensor muscle was inhibited. Further studies found that the reflexes elicited from the dorsal foot also depended on the stimulus location similar to those obtained by stimulation of the plantar foot (Sonnenborg et al. 2001).

In order to resolve the conflicting results represented by the findings of Sherrington and Schouenborg, Cleland and Bauer (2002) conducted studies on intact and spinalized rats by applying heat stimuli to the tail and measuring the direction and speed of the withdrawal response. In the spinal rat, it was observed that evoked responses were directed partially away from the stimulus but also showed a bias for movement in the ventral direction.  The movement away from the stimulus was also observed in intact rats, but the ventral bias was changed to a preference for movement in the dorsal direction. This change in response could be due to reflexes in intact rats being mediated by long-loop reflexes and modulation from the brainstem. The results suggested the rat uses a hybrid withdrawal strategy that is intermediate between the results obtained by Sherrington and Schouenborg et al. (1990); while responses were limited to the ventral directions in the spinal rat and dorsal directions in the intact rat, the number of response directions observed was greater than the single response observed by Sherrington (1910) and depended partly on stimulus location.

### D. Dependence of the NWR on Initial Position

While stimulus location has been found to have a significant effect on the NWR, other factors may also influence on the NWR; one of which is the posture of the limb or the part of the body to which a noxious (Brown 1911; Andersen and Sonnenborg 2003; Richard et al. 2015; Decchi et al. 1997; Serrao et al. 2006; Kim et al. 2007) or non-noxious (Baxendale et al. 1981; Knikou et al. 2006; Onushko et al. 2013) stimulus is applied. The initial position of the hind limb was initially found to have an effect on the scratching and hopping reflex in rabbits (Brown 1911). Later, Baxendale (1980) found that the initial posture of the knee in decerebrated cats influenced the non-nociceptive flexion withdrawal reflex, acting to limit hyperextension and hyperflexion of the joint. In humans, the posture of the lower limbs while standing (Decchi et al. 1997; Andersen and Sonnenborg 2003) and during walking (Richard et al. 2015) altered the reflex evoked by a painful electrical stimulus. While subjects were standing, ankle extensors played a dominate role and complete withdrawal of the limb was rarely observed; weight was simply shifted from the stimulated food to the one which was not stimulated in order to maintain balance (Decchi et al. 1997; Andersen and Sonnenborg 2003). During walking, flexion at the knee and hip joints was the primary response with flexion at the hip aiding in maintenance of balance and walking movement depending on the current posture. Additionally, the posture of the upper limbs in humans during rest and movement plays a role in modulating the NWR (Serrao et al. 2006). In humans with spinal cord injury, components of hip proprioceptors play a role in determining the reflex produced in response to simultaneous electrical stimulation and imposed movement of the leg using a motorized apparatus (Kim et al. 2007). Similarly, the effects of hip posture on the NWR were also observed in humans with spinal cord injury following a non-noxious stimulus (Knikou et al. 2006; Onushko et al. 2013). Knikou and colleagues found that flexion of the hip decreased the prominence of the flexion reflex, while extension of the hip produced the opposite effect. Additionally, extension of the hip was seen to enhance reflexes at the knee, while flexion of the hip amplified the tonic component in response to Achilles vibration (Onushko et al. 2013).

Taken together, these studies provide evidence for the influence of the initial posture of the limb on the withdrawal response to noxious or non-noxious stimuli.

The posture of the limb prior to stimulation likely has an effect on the NWR as a result of various proprioceptors. Proprioceptors are a component of the somatic sensory system that serve to sense how our body is positioned and moving in space. Muscle spindles, also known as stretch receptors, are located within a fibrous capsule among several types of specialized skeletal muscle fibers. These spindles and their associated Ia afferents are examples of proprioceptors that serve to transduce changes in muscle length. Another proprioceptor present in skeletal muscle is the Golgi tendon organ, which monitors muscle tension. Joint receptors are responsible for responding to changes in the angle, direction, and velocity of movement in a joint. These mechanosensitive axons are present in the connective tissue of joints, especially within the fibrous tissue surrounding the joints and ligaments (Bear et al. 2015). These three components of proprioception may play a large role in modulating the NWR.

## E. Specific Aim

There have been numerous studies in non-human animals on the effect of stimulus location on the NWR and at least a few studies that address the role of initial posture. However, all of these studies were conducted in anesthetized, decerebrated, or spinalized mammals. Since descending modulation and long loop reflexes may influence the NWR, the anesthetized spinalized/decerebrated animal may not be an adequate model of normal animals. Therefore, the primary aim of this study was to determine the effect of the stimulus location and initial paw position on the direction and magnitude of the nociceptive withdrawal response to heat stimulus delivered to various locations on the plantar surface of the paw of *intact, unanesthetized* rats.

Based on previous research, it was hypothesized that stimulus location and initial position of the paw would both have an effect on the NWR.

# IV. Methods

Male Sprague Dawley rats (n=36 with an average weight of 421.7g ± 100.8g) were bred from rats obtained from Harlan (Indianapolis, IN). Male and female rats were housed in separate cages with constant access to food and water and were exposed to 12 hour light/dark rotations (light 8:00-20:00) to imitate their natural environment. The room in which the rats were kept was inspected daily in order to ensure an adequate temperature between 68-79 °F, humidity between 30-70%, and to check for cleanliness and availability of food and water. The animal care facility and protocol were approved by the James Madison University Institutional Animal Care and Use Committee.

## A. Rat Preparation

Rats were placed in an acrylic box and lightly anesthetized by exposure to a mixture of 2-5% isoflurane in 100% oxygen. Anesthesia was necessary in order to shave the hind end of the rat and to mark the bottom of the paw. After the rat was successfully anesthetized, hair clippers were used to remove all of the hair on the posterior half of the rat, which aided with visualization of the movement of the paw.

Following shaving, five stimulus locations were marked on the plantar surface of the hind left paw using a black permanent marker. The points were approximately 1 mm in diameter and were organized with three points proceeding rostral-caudally and three proceeding medial-laterally (Figure 1). The stimulation sites were marked for two reasons; to improve absorption of the heat stimulus via black ink and to limit the stimulus to the marked areas, as unblackened areas absorb heat far less quickly.

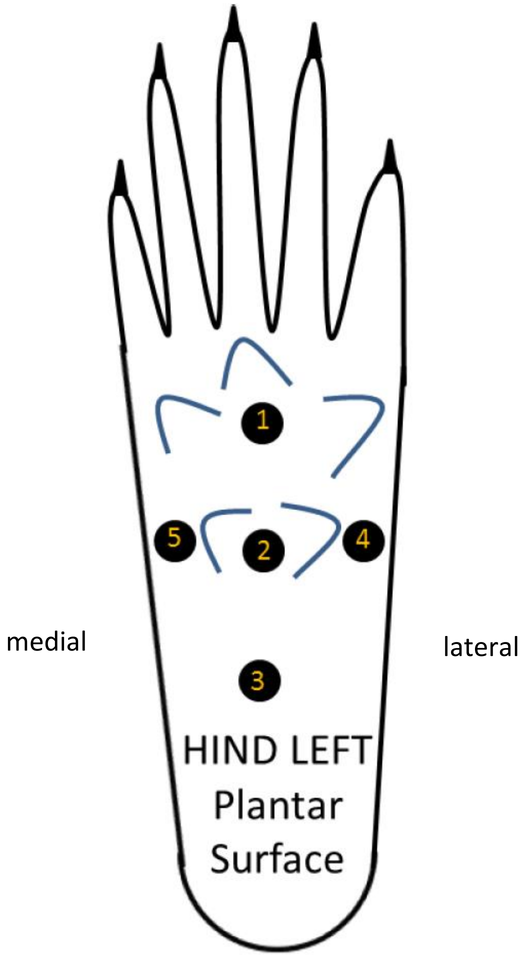

Figure 1. **Stimulation locations.** Five points (approximately 1mm in diameter) on the plantar surface of the hind left paw were marked with a permanent black marker and stimulated with a laser. All five points were used for stimulation while points 1, 2, and 3 were also used for tracking movement of the paw. Point 2 was used to represent the approximate center of mass (COM) for determining the distance and magnitude of movement. Points 1 and 3 were used to determine the initial and final angle of the paw.

Prior to beginning the experiment, the rats recovered for 45 minutes in order to eliminate any influence the anesthesia might have had on their behavior.

### B. Experimental Setup

The rat was placed on a leveled glass plate (2’X1’) supported by two horizontal steel bars and constrained on top of the platform by an acrylic box in order to encourage the animal to remain in place during and between trails (Figure 2). The box contained a cutout on the back, left side providing space for the hind left paw to move following stimulation without being inhibited by coming in contact with the acrylc enclosure. The box also contained a small cutout in the back and holes on all sides so that the tail could fully extend and the rat was able to breathe. The platform was raised 51 cm from the ground so that the video camera, light emitting diode (LED) lights, and laser apparatus would fit underneath.

Two high-power, low heat, LED (SugarCube; Vergennes, VT) lights were positioned below the platform for illumination. The lights were triggered to turn on at the same time the stimulus was delivered, providing identification of the video frame at which the stimulus began. A 980nm infrared laser diode (BWTEK; Wilmington, DE) was positioned beneath the plate to deliver the heat stimulus. The laser stimulus used to provoke the withdrawal response was delivered at various watts, depending on the stimulus site. Some areas of the paw required higher wattage in order to provoke an adequate response, possibly due to thicker skin, such as the rostral and caudal end. The intensity of the stimulus was varied in order to obtain latencies between 1-3 seconds at each stimulation site. A red low power targeting laser was used so that the heat stimulus could be aligned and correctly applied to the intended location. The laser was focused using a large condenser lens to a diameter equivalent to that of the black marked location on the paw (~1mm).

A conventional video camera (Sony HDR-CX130) placed beneath the glass plate was used to record the animal’s paw movement. In some experiments, a second camera was placed to the left of the rat and used as a second source of video footage of the movement. The focus, exposure, and zoom on the cameras were manually adjusted prior to each experiment. The cameras recorded movement in pixels, thus it was necessary to covert pixels to millimeters in order to accurately interpret distance of movement. To do so, following each experiment, one frame was recorded with a ruler placed on the glass to serve as a known distance marker in order to perform this calibration.

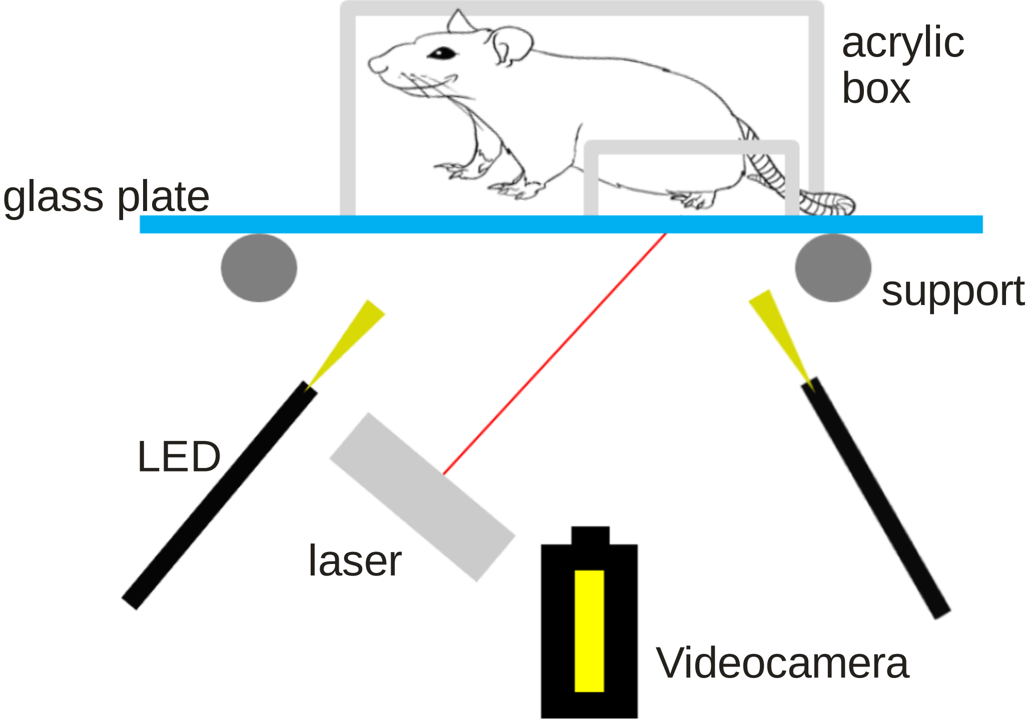

Figure 2. **Experimental setup.** The rat was placed on a glass plate supported by two steel bars and enclosed in an acrylic box. Two LED lights were directed at the rat and used for illumination. A video camera was placed beneath the platform, directly below the rat so that the entire animal was in the frame. The laser was positioned beneath the platform and adjusted so that the appropriate location was stimulated.

### C. Experimental Protocol

There were a total of 36 experiments performed, 19 of which I completed while the remainder were carried out by my colleagues. Each experiment consisted of fifteen trials, with individual locations stimulated three times. The order at which each location was stimulated was determined randomly prior to each experiment. Randomizing the stimulus location decreased any relative effect that the order at which the points were stimulated might have on the response. After each location had been stimulated once, the process with the same sequence was repeated twice.

The experiment began by placing the rat on the glass plate and allowing it to acclimate. Following orientation of the rat in the correct direction, the acrylic box was placed around the rat and the platform was shifted so that the entire rat was in the field of view of the video camera beneath. The conventional video camera (60fps) was set to record, the laser was positioned so that the red dot was placed over the desired location, and the stimulus was delivered using a foot-activated switch. After the animal had responded by moving its hind limb, the video recording was stopped. The animal was then allowed to rest for at least four minutes before the next stimulus was delivered in order to minimize the influence of repeated stimulation.

Following completion of the experiment, the video file was transferred to a computer and converted to a series of “jpg” image files using TMPGEnc Video Mastering Works 5 for compatibility with subsequent analysis software. The LED lights were set to turn on at the start of the stimulus, therefore the onset of illumination was used to determine the frame in which the application of the stimulus began. Video was then cut five frames before the onset of the stimulus and five frames following termination of the response.

The images were analyzed using ProAnalyst (Xcitex; Cambridge, MA) in order to track the initial and final position and angle of the paw. The entire movement of the limb during the response was not tracked as this is not possible at 60 fps. Prior to tracking, a Cartesian coordinate system was produced for each individual rat by setting the origin at the urinary orifice. The positive Y axis was aligned with the center of the rat in the rostral direction and the positive X axis was set to the medial (Figure 3 viewed from above).

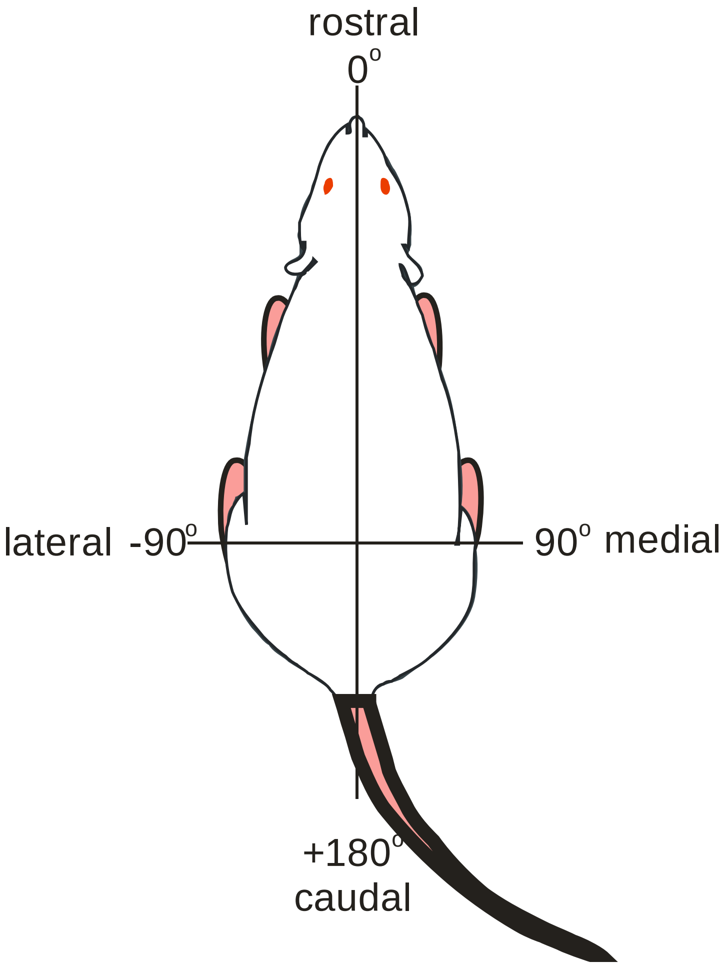

Figure 3. **Cartesian coordinate system.** A Cartesian coordinate system was defined for each individual rat prior to tracking the initial and final position of the paw. The urinary orifice was used as the origin with the axes and direction as shown in the figure.

The withdrawal response was usually characterized by a rapid removal of the paw from the plate and quick replacement of the paw on the surface. Following replacement, the rat left its paw in place on the plate for an extended period of time. In order to determine how stimulus location and initial position affected the response, the position of the first, second (representing the approximate center of mass), and third location were determined prior to the onset of the stimulus and immediately following the placement of the paw back on the glass. This was completed by recording the X and Y coordinates of these three points prior to and following the movement.

The coordinates from ProAnalyst for each trial were exported to an Excel spreadsheet, where the direction and magnitude of the vector connecting the initial and final locations of the COM of the paw were calculated. The coordinates of stimulus points one and three were used to calculate the angle of the paw before and after the movement.

Analysis and graphing was conducted using SigmaPlot and custom programs in Matlab written by Corey Cleland. Significance (α) was set at a p-value of 0.05 and error bars are standard error of the mean unless otherwise stated.  Parametric statistics were used if the data were normally distributed (Shapiro-Wilk test) and non-parametric statistics were used for data that were not normally distributed. Circular statistics were used for directional data.

# V. Results

The nociceptive withdrawal response resulting from heat stimulation applied the plantar surface of the paw in intact, unanesthetized rats was characterized by rapid removal and replacement of the paw on the glass surface. The left panel of Figure 4 shows two video frames taken from a camera positioned to the left of the rat, demonstrating the position of the paw prior to the delivery of the stimulus and during the response. Figures 4A and 4B show video frames from the same trial taken from a camera positioned directly beneath the rat, illustrating the position of the paw before and after the movement. In Figure 4B, the shaded region represents the initial position of the paw. Figure 4C shows how the direction of movement of the COM of the paw, $\theta_1$, and the magnitude of movement, d, were quantified. In this example, the direction was 143 degrees and the distance was approximately 8 mm. Figure 4D shows how the change in foot angle, $\theta_2$, was measured via the angular change in axis determined by stimulus points 1 and 3. In this example, the change in angle was about 15 degrees.

Figure 5A shows responses from each trial (n=36, n=652 trials) represented as a vector. When the magnitude and direction of all responses are represented individually, it is apparent that all directions are represented. The frequency histogram of the response directions suggests a preference for movement in the caudal-lateral direction (Figure 5B).

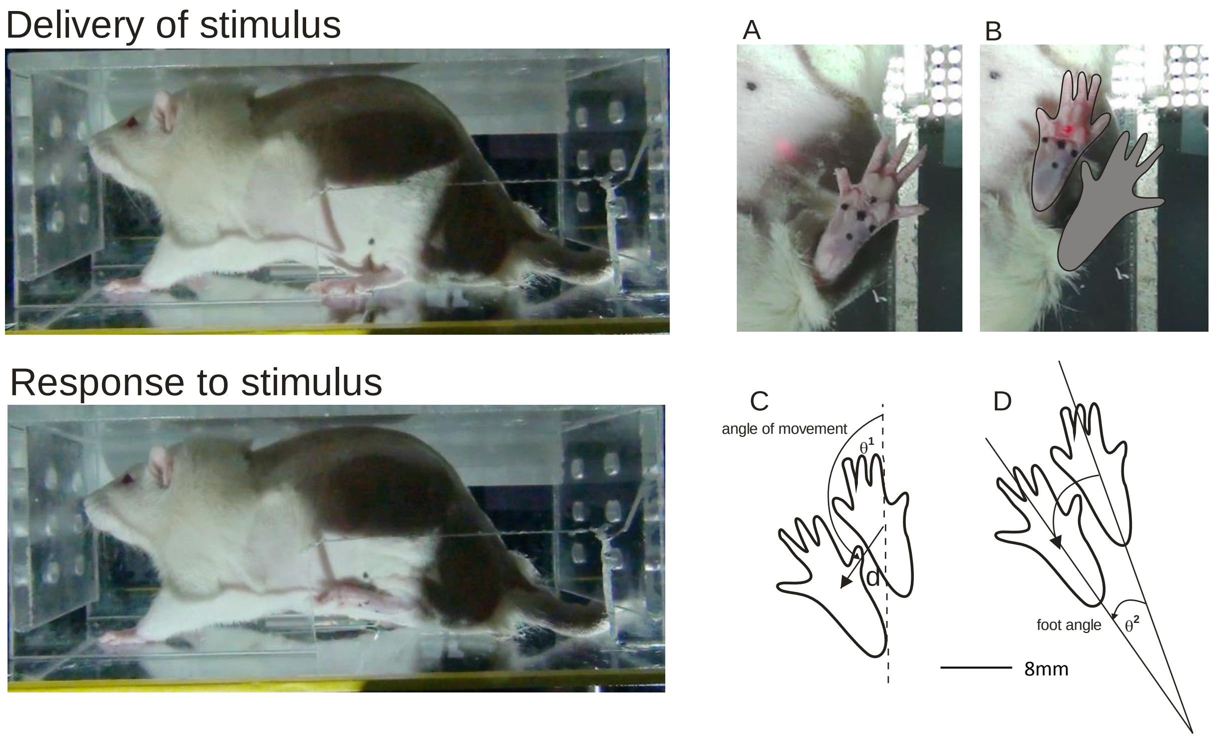

Figure 4. **Translational and rotational movement of the hind limb.** Video recording from the rat's left side illustrates the NWR evoked by heat stimulation of the plantar surface of the hind left paw. Typically the withdrawal and replacement of the paw occurred within 50 ms. Video from below the animal shows the initial position of the hind limb before the stimulus (A) and following the stimulus-induced movement (B) (the initial limb position prior to the stimulus is represented by the shaded region). Paw movement following stimulation was measured in two ways in order to determine the magnitude of the response: (C) translation and (D) rotation. The illustration shows the initial and final locations of the approximate center of mass (COM) of the paw viewed from above the rat. $\Theta_1$ represents the angle of rotation as measured from the dotted vertical line and "d" indicates the distance the paw moved. In this example, the direction was 143 degrees and the distance was approximately 8 mm. D) The change in foot angle from initial paw position to the final paw position after movement is represented by $\theta_2$. In this example, the change in angle was about 15 degrees.

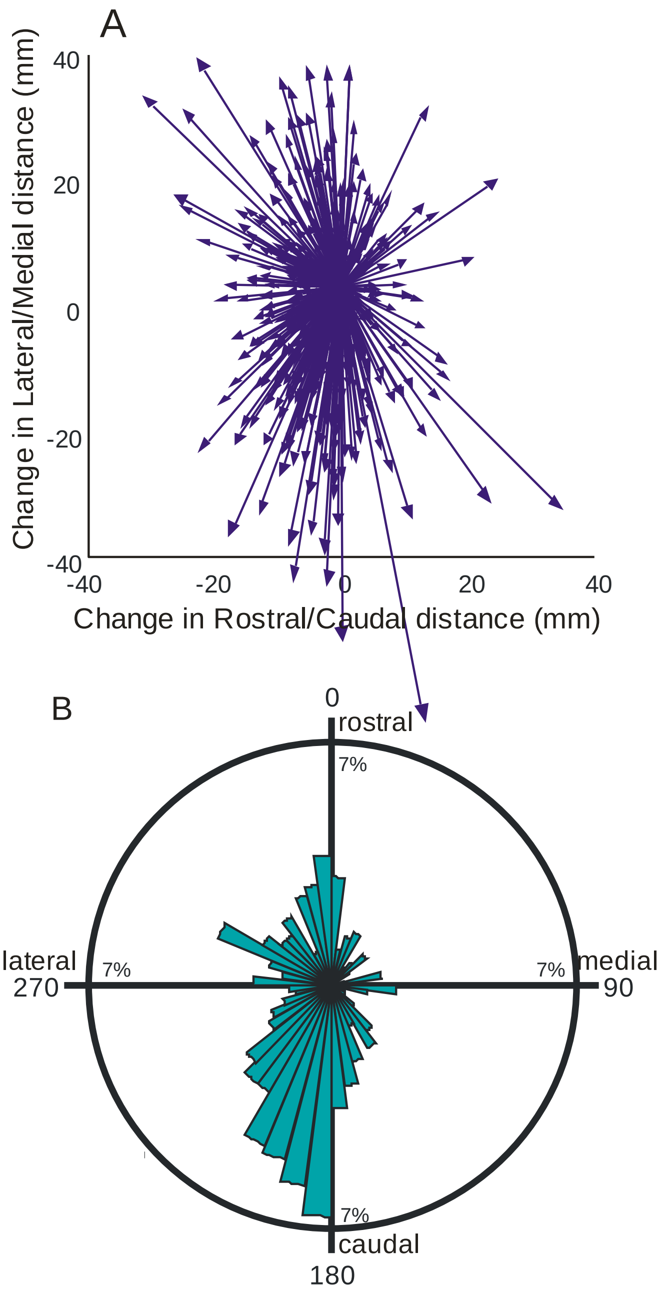

Figure 5. **Changes in response distance.** A) Each trial (n=36, n=652) is represented by a vector, showing the relative direction and magnitude of movement of the hind left paw in response to stimulation. All directions appear to be represented. B) Direction of movement is represented in a frequency histogram. Although all directions are represented, it is apparent that there is a preference for movement in the caudal direction and to a lesser extent in the lateral direction.

### A. Dependence on Stimulus Location

The first question addressed by this study was whether or not the stimulus location on the paw determined the translational (direction and magnitude) and rotational (change in paw angle) aspects of the withdrawal response. Stimulation at each location produced a variety of responses with almost all directions represented (Figure 6A). When comparing the direction and magnitude of translational movement at each stimulus location to one another, it appears that the responses were generally the same, regardless of what point was stimulated. Similarly, the change in angle of the paw was similar among all stimulus locations (Figure 6B).

In order to determine if stimulus location had a significant effect on the withdrawal movement, the response vectors in polar coordinates (Figure 6A) were converted to their rostral-caudal and lateral-medial components, which allowed each component to be aligned with the stimulus points in the appropriate direction. The change in rostral-caudal position is shown in regard to the rostral, center, and caudal stimulus locations (Figure 7A), while the data for the change in lateral-medial position is shown in regard for the lateral, center, and medial stimulus locations (Figure 7B).

Median values for change in rostral-caudal distance (Figure 7A), lateral-medial distance (Figure 7B), and angle (Figure 7C) are shown with respect to the five stimulus locations. The change in distance in the rostral/caudal position (P=0.07, n=391) and the change in distance in the lateral/medial position (P=0.16, n=391) were not significantly affected by the location of the stimulus (Figures 7A and 7B). The data for change in angle are shown with respect to all five stimulus locations (Figure 7C). Similar to rostral-caudal and lateral-medial position, the stimulus location did not have a significant influence on the change in angle of the paw (P=0.58, circular Watson-Williams F-test, n=652) (Figure 7C).

Contrary to our hypothesis, it can be concluded that stimulus location did not have a significant effect on the NWR, raising the question of what other factors might play a role in mediating this movement.

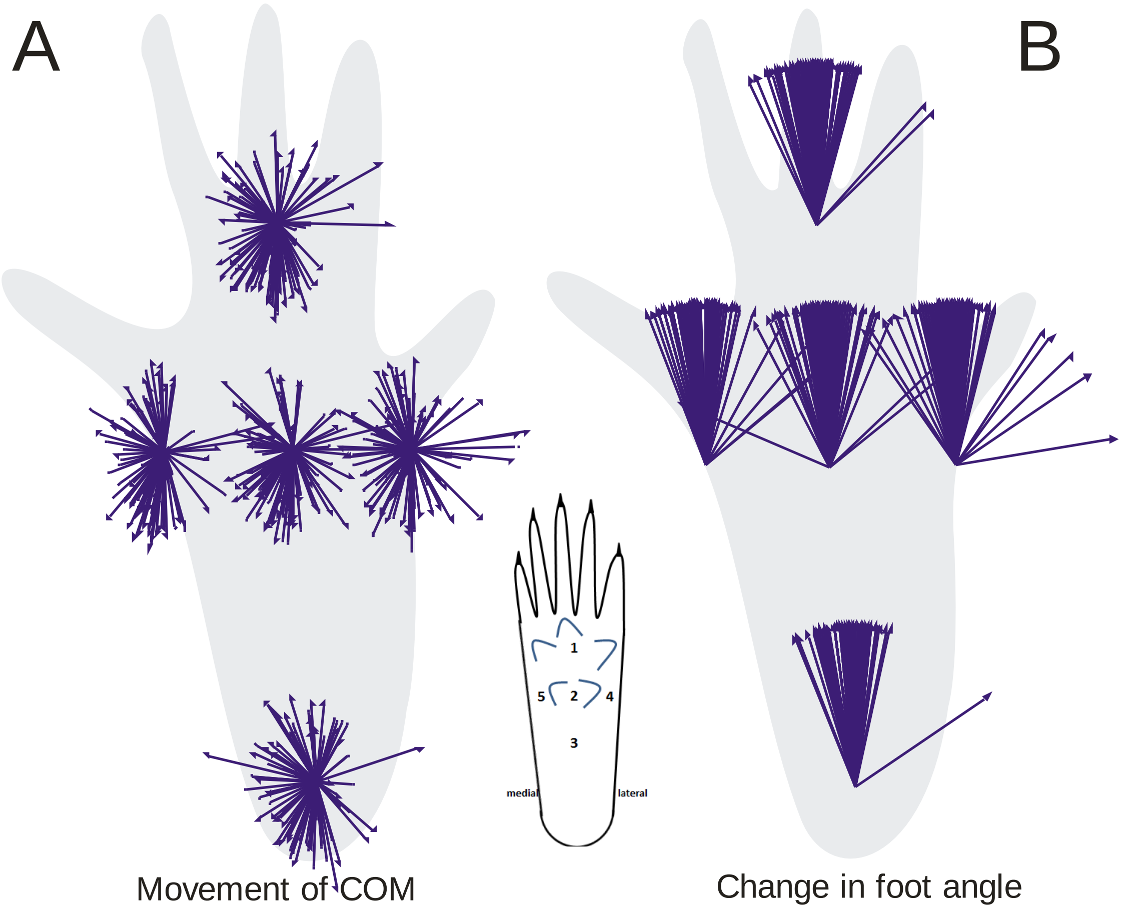

Figure 6. **Magnitude, direction, and angle of movement at each stimulation site.** Each trial at each of the five stimulation points is represented as a vector (n=36, n=652 trials). Movement of the COM (A) is described by the magnitude and direction of the movement of the paw based on initial and final position of the paw following stimulation. It appears that movement occurred in all directions at each stimulation point. Change in foot angle (B) measured prior to and following the movement also appears to occur in all directions at all stimulation sites. Unity magnitudes were set for the angle vectors, as only the change in angle was measured.

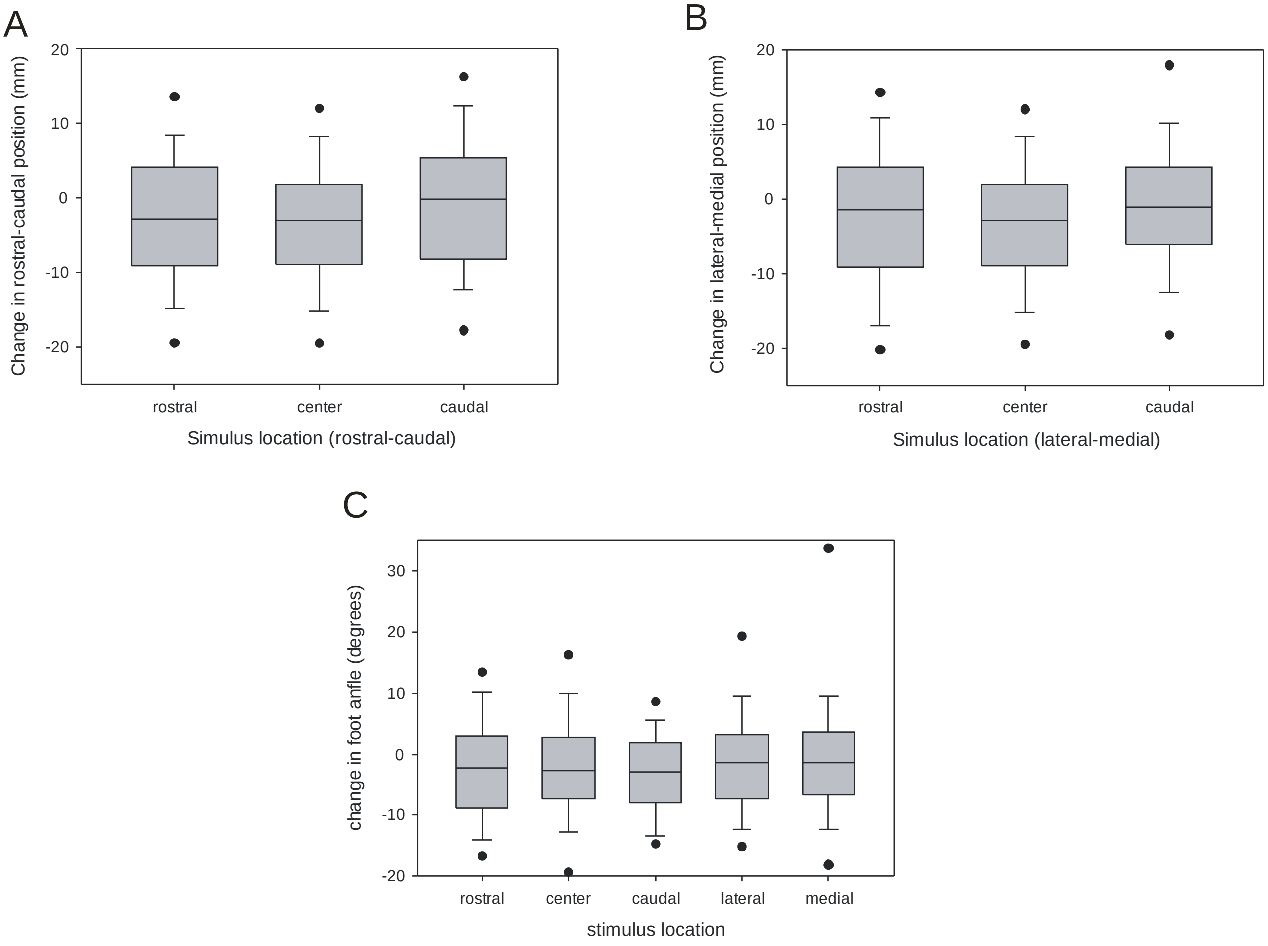

Figure 7. **Dependence of change in position and angle of response on stimulus location.** The data from each trial are represented as boxplots with the median represented by the line in the center of the box, the upper and lower boundaries of the box indicating the 75th and 25th percentile, the whiskers demonstrating the 90th and 5th percentile, and the dots showing the 95th and 5th percentile. A) The change in movement in the rostral/caudal direction for the rostral, center, and caudal stimulus locations did not significantly depend on stimulus location (P=0.09, n=391, Kruskal-Wallis test). B) Similarly, the change in position in the medial/lateral direction for the lateral, center, and medial stimulus locations did not significantly depend on stimulus location (P=0.16, n=391, Kruskal-Wallis test ). C) The mean change in angle for each stimulus location also did not significantly depend on stimulus location (P=0.58, n=652).

### B. Dependence on Initial Position

Since stimulus location did not have a significant effect on the withdrawal response, other variables that may be responsible. The way in which the rat positioned itself varied between experiments, leading to the idea that the initial posture of the paw might be responsible for the observed variation in the withdrawal response. In order to test whether the initial position of the paw had an effect on the NWR, it was first confirmed that various initial positions of the paw were in fact adopted by the rat prior to delivery of the stimulus (Figure 8).

In order to determine if initial position of the paw had a significant effect on the withdrawal response, a regression analysis was performed on the initial position of the paw (COM) and the change in position of the paw (COM) in the lateral-medial and rostral-caudal directions (Figures 9A and 9B). There was a significant relationship between initial lateral-medial position and movement in the lateral-medial direction ($R^2$=0.11, P<0.0001, slope=-0.5) as well as between initial rostral-caudal position and movement in the rostral-caudal direction ($R^2$=0.22, P<0.0001, slope=-0.6) (Figures 9A and 9B). These results show that when the paw is initially back, it tends to move forward; when initially forward, it tends to move back. Similarly, when the paw is initially right, it tends to move left; when initially left, it tends to move right. A regression analysis was also performed comparing the initial angle and final angle of the paw and it was found that final angle may significantly depend on initial angle ($R^2$=0.28, P<0.0001, slope = 0.6 deg/deg) (Figure 9C).

Although statistically significant, the $R^2$ values from the previous correlations were fairly low ($R^2$=0.11, 0.22, and 0.28). These low $R^2$ values could have been due to differences between rats used in each experiment such as weight, gender, or posture of the animal adopted in the acrylic box. In order to eliminate this variation, each rat was analyzed individually. When the data for each of the 36 rats are displayed on separate graphs, the resulting individual $R^2$ values of statistically significant relationships (n=26, 25, and 25) were much higher ($R^2$=0.47±0.16, 0.52±0.20, 0.36±0.17) (Figures 10, 11, and 12). More importantly, 33 of 36 experiments had a negative slope when examining the change in position in the lateral-medial direction (Figure 10) and 35 of 36 had a negative slope when examining the change in position in the rostral-caudal direction (Figure 11). Similarly, 32 of 36 graphs of individual experiments examining the initial and change in angle of the paw contained positive slopes (Figure 12). Of the 8 graphs with differing slopes, only one was statistically significant.

The individual slopes from each experiment were compiled onto a single graph for change in lateral-medial position, rostral-caudal position, and final angle (Figure 13). Those experiments which had positive slopes in regard to change in position in the rostral/caudal and lateral/medial are displayed as thick lines, while those that had a negative slope for initial vs. final angle are displayed as thick lines. In order to test the effect of initial positon of the paw on the withdrawal response, a one-sample T-test was performed by comparing each slope to a hypothesized population mean of 0. In the lateral-medial direction ($P<0.000001$, Slope=-0.67±0.42) (Figure 13A), as well as the rostral-caudal direction ($P<0.00001$, Slope=-0.87±0.60) (Figure 13B), the initial paw position significantly influenced the change in position of the paw in that particular direction. In addition, the initial angle of the paw determined the final angle of the paw ($P<0.000001$, Slope=0.48±0.36) (Figure 13C).

### C. Multiple Regression

It is possible that the effects of stimulus location on the withdrawal response were obscured by the significant effects of initial position. To account for this possibility, multiple regression analysis was performed in which the effects of both initial position of the paw and stimulus location were determined together. The change in position of the paw in the lateral/medial direction (P=0.51) and in the rostral/caudal direction (P=0.07) were still not significantly affected by the stimulus location. Similarly, it remained that stimulus location did not significantly affect the change in angle of the paw (P=0.15).

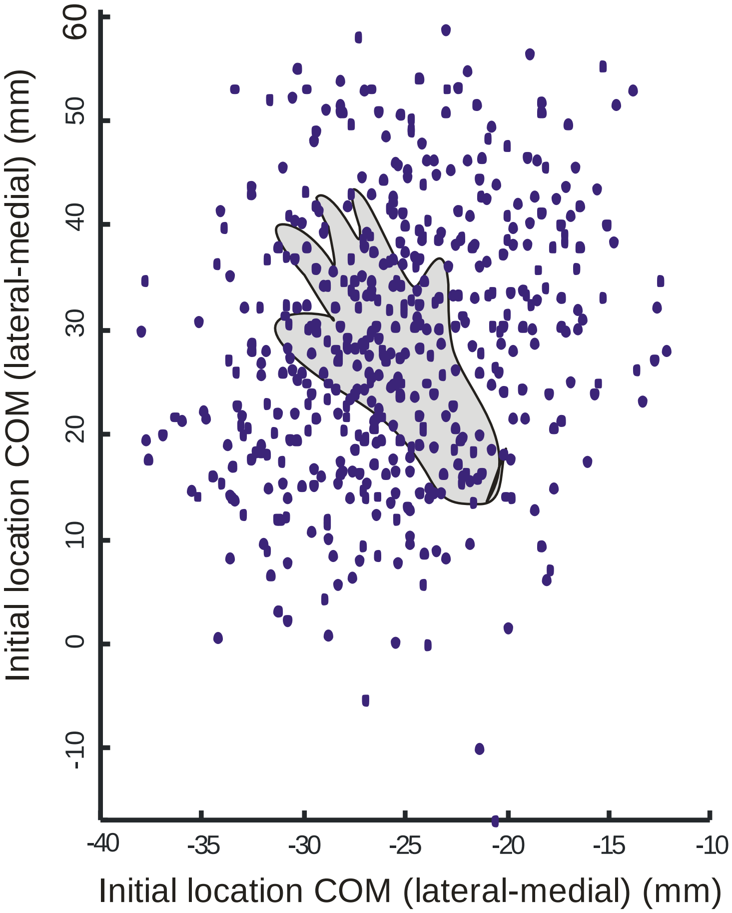

Figure 8. **Initial locations of the paw prior to stimulation.** The initial locations of the COM of the paw are displayed in the lateral-medial and caudal-rostral plane, showing there was a wide distribution of initial locations of the COM of the paw adopted voluntarily by the rat.

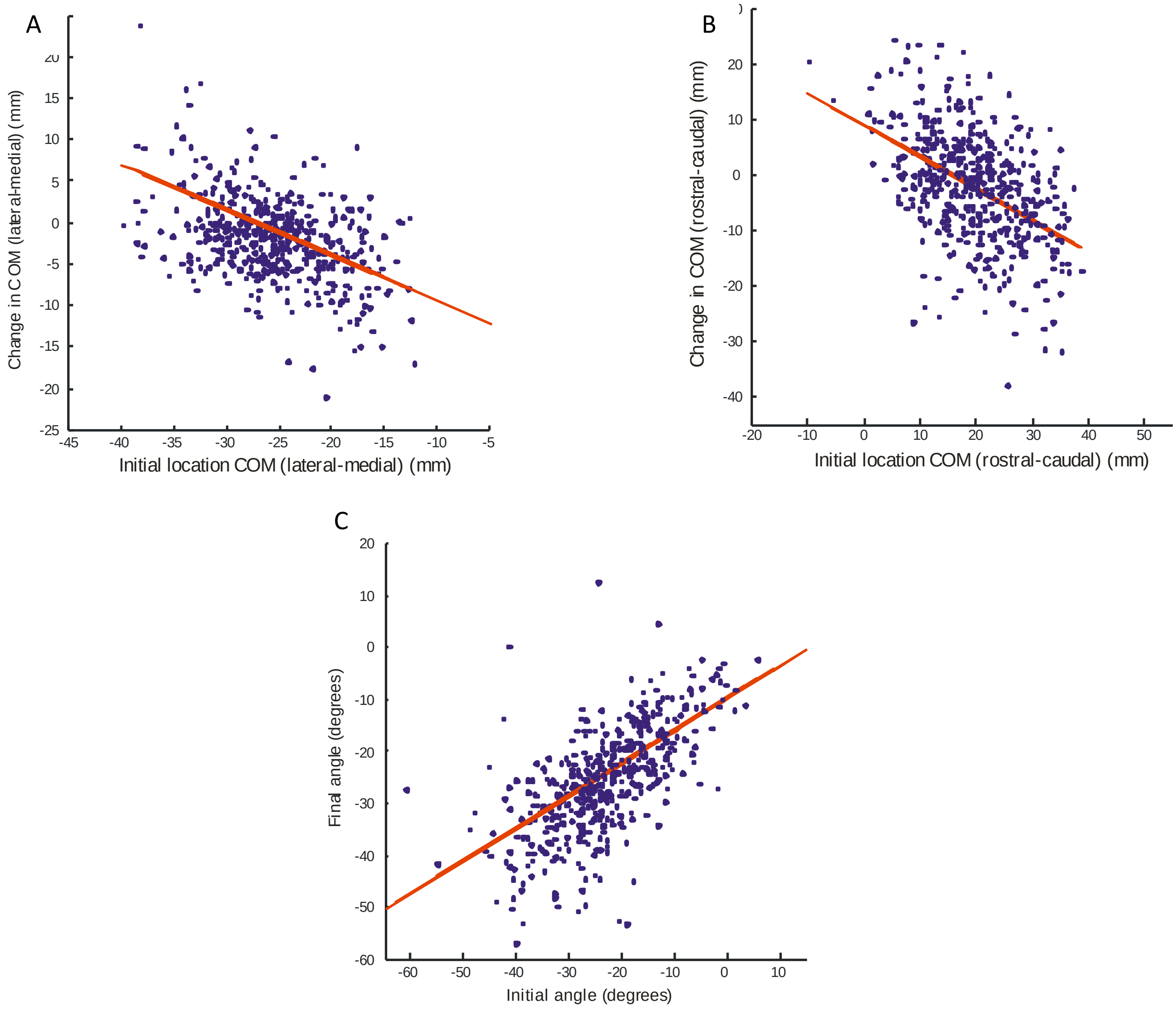

Figure 9. **Initial rostral-caudal, medial-lateral, and angular paw position influences final paw position.** The initial location of the paw significantly influenced the change in position of the paw in the lateral-medial direction (A) ($R^2$=0.11, P<0.0001, slope=-0.5 mm/mm) as well as in the rostral-caudal direction (B) ($R^2$=0.22, P<0.0001, slope=-0.6 mm/mm). The initial angle of the paw also significantly influenced the final angle of the paw ($R^2$=0.28, P<0.0001, slope = 0.6 deg/deg).

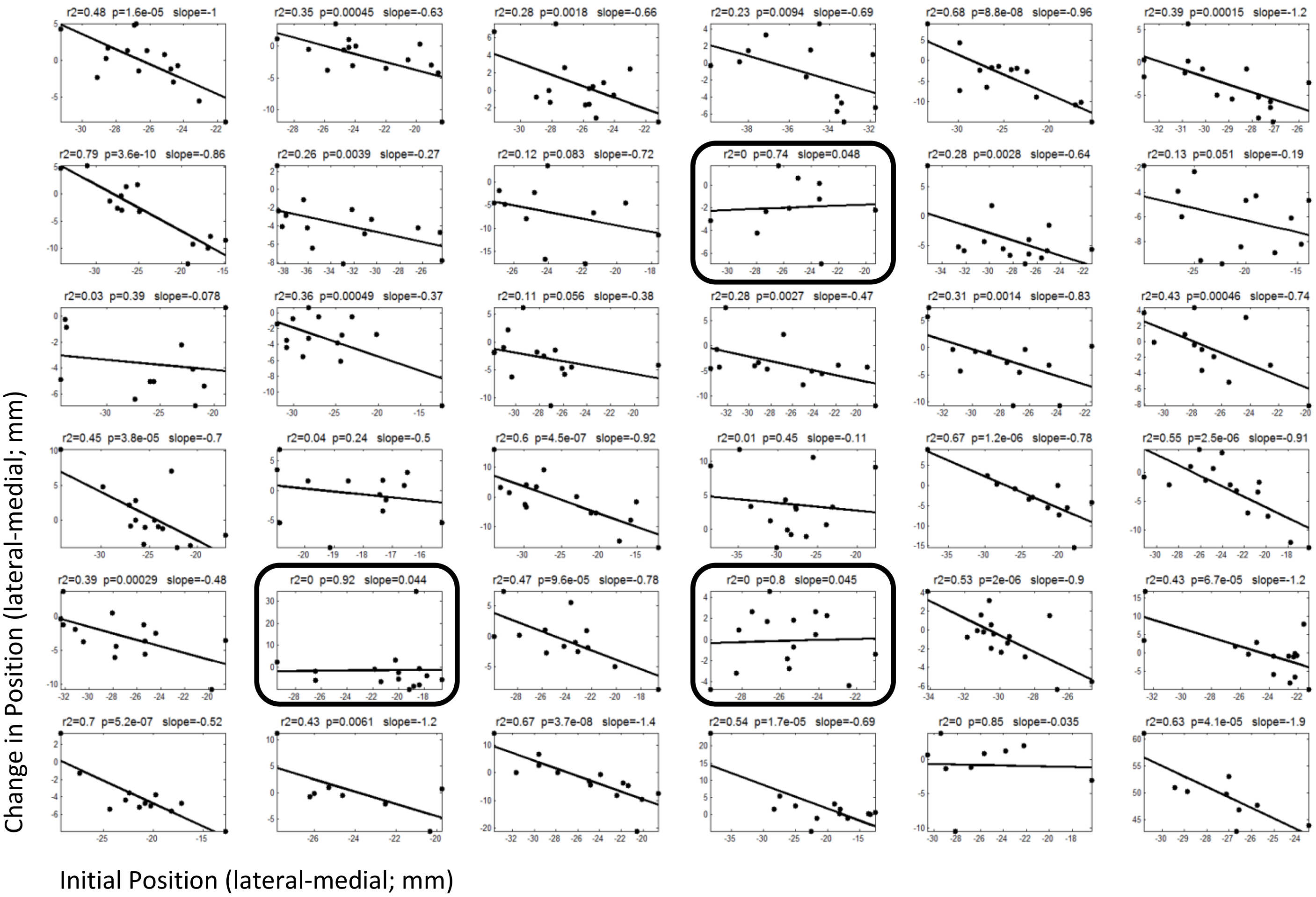

Figure 10. **Individual rats show initial lateral-medial paw position influences final paw position**. Each individual rat is shown on a separate graph, further demonstrating the dependence of change in position of the paw on initial position of the paw. The $r^2$ value, p-value, and slope for each trail are displayed above the graph. There were negative slopes for 33 of 36 rats, with those that had a positive slope indicated by a box. The average $R^2$ value for all significant experiments (n=26) was 0.47±0.16 and the average slope for all rats was -0.67±0.42. These values demonstrate the significant influence of the initial lateral-medial position on the change in lateral-medial position of the paw.

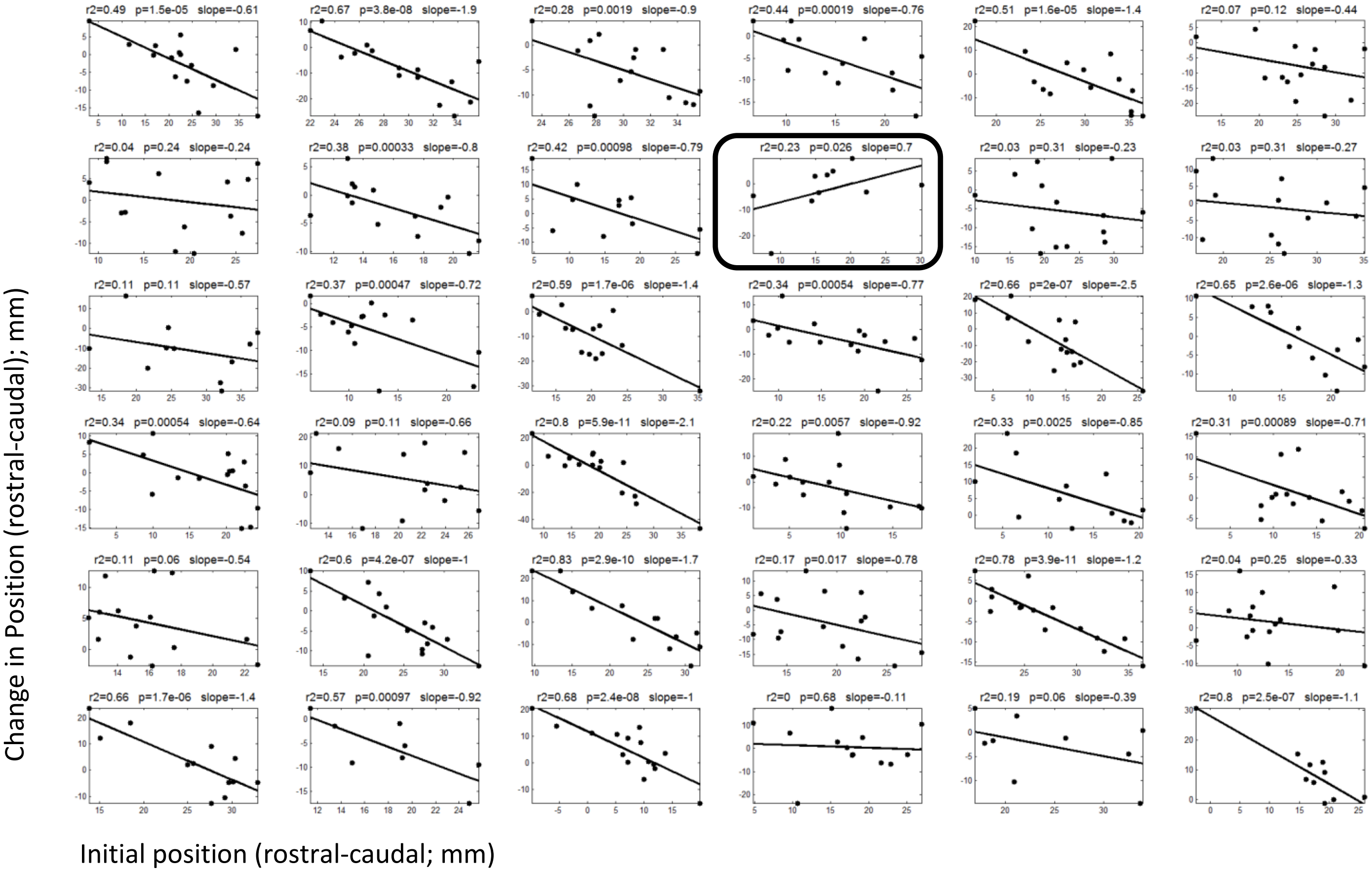

Figure 11. **Individual rats show initial rostral-caudal paw position influences final paw position**. Each individual rat is shown on a separate graph, further demonstrating the dependence of change in position of the paw on initial position of the paw. The $r^2$ value, p-value, and slope for each trail are displayed above the graph. There were negative slopes for 35 of 36 rats, with the one that had a positive slope indicated by a box. The average $R^2$ value for all significant experiments (n=25) was 0.52±0.20 and the average slope for all rats was -0.87±0.60. These values demonstrate the significant influence of the initial rostral-caudal position on the change in rostral-caudal position of the paw.

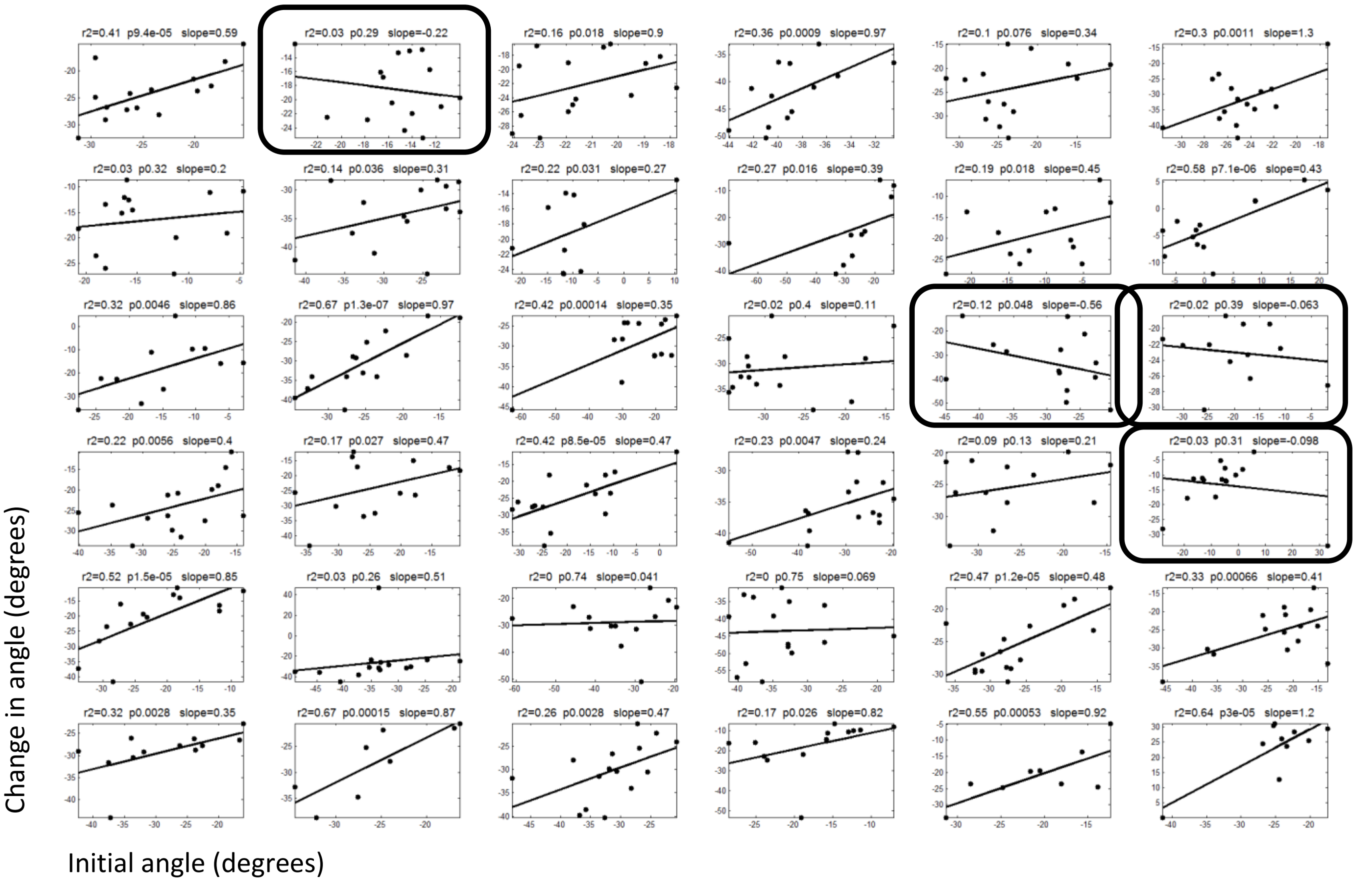

Figure 12. **Individual rats show initial angle of paw position influences change in angle of paw position**. Each individual trail is shown on a separate graph, further demonstrating the dependence of change in angle of the paw on initial angle of the paw. The $r^2$ value, p-value, and slope for each trail are displayed above the graph. There were positive slopes for 32 of 36 rats, with those that had a negative slope indicated by a box. The average $R^2$ value for all significant experiments (n=25) was 0.36±0.17 and the average slope for all rats was 0.48±0.36. These values demonstrate the significant influence of the initial angle of the paw on the change in angle of the paw.

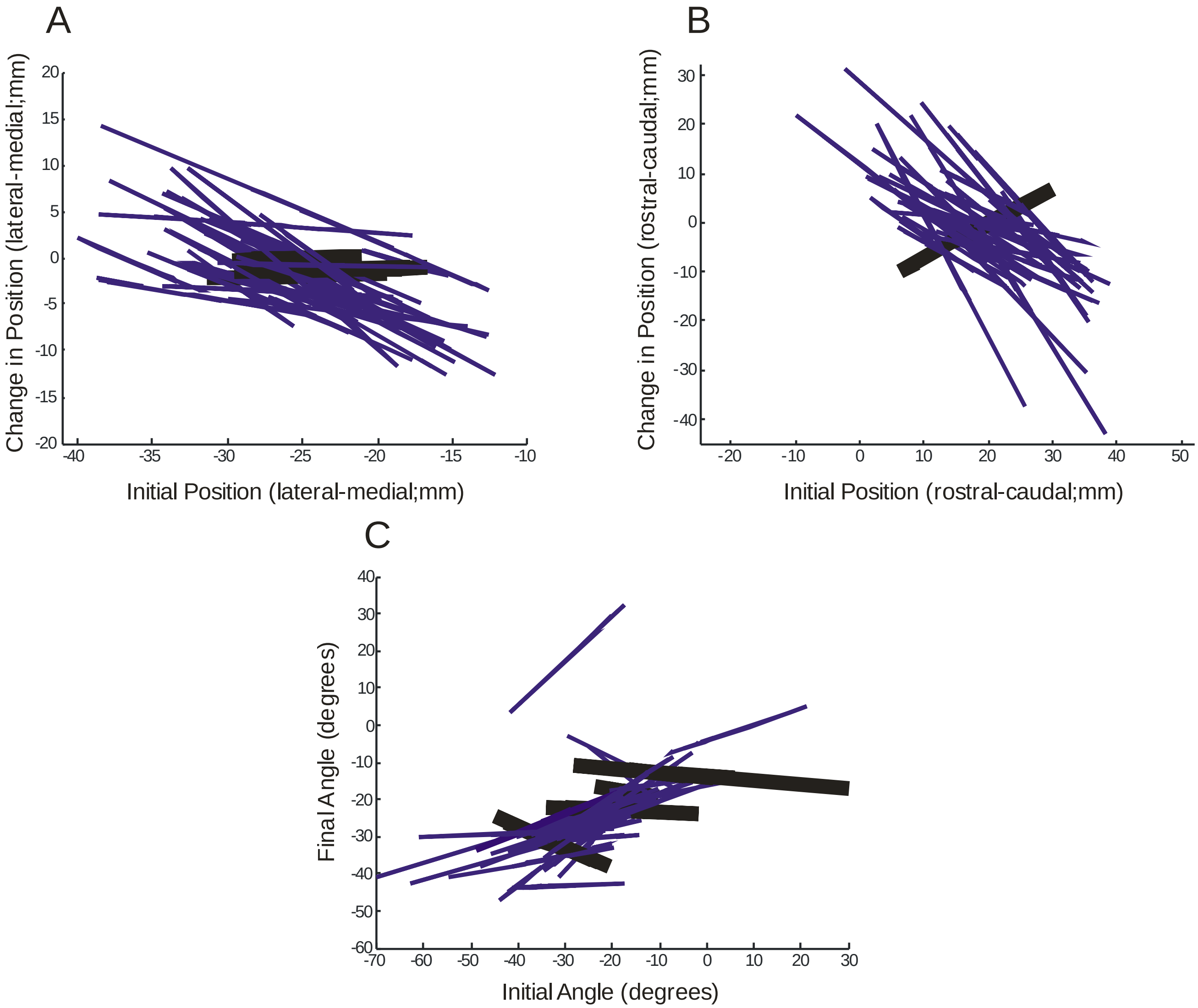

Figure 13. **Combined slopes from individual rats demonstrate dependence of final paw position on initial position**. Slopes from individual trials were combined and displayed on a single graph. The data for analysis of initial position passed the Shapiro-Wilk normality test, so normal statistics were used for investigation. Those experiments which had positive slopes in regard to change in position in the rostral/caudal and lateral/medial are displayed as thick lines, while those that had a negative slope for initial vs. final angle are displayed as think lines. The change in position of the paw showed a strong dependence on the initial position of the paw lateral-medially (A) and rostral-caudally (B) (P<0.000001, Slope=-0.67±0.42; P<0.000001, Slope=-0.87±0.60, one-sample T-test, n=652 trials). Similarly, the initial angle of the paw had a significant effect on the final angle of the paw (P<0.000001, Slope=0.48±0.36, one-sample T-test, n=652 trials).

# VI. Discussion

Previous studies have shown that the NWR is affected by stimulus location and initial posture in anesthetized, decerebrated or spinalized non-human mammals as well as in humans; however, there had been no studies regarding these variables and their effect on the NWR in intact, unanesthetized non-human mammals.

The effect of stimulus location on the NWR in intact, unanesthetized rats was determined by applying an adequate heat stimulus to the plantar surface of the hind, left paw at five different locations in order to evoke the NWR. The withdrawal response generally consisted of rapid removal and replacement of the paw on the platform. In contrast with previous literature, the stimulus location did not significantly influence the direction of movement, magnitude of movement, or the change in angle between the initial and final positions of the hind, left paw.

Although stimulus location did not have an effect on the NWR, our hypothesis was supported by the fact that the initial position of the paw prior to stimulation did significantly influence the response. In order to determine the effect of the initial position on the NWR, the paw was stimulated following placement in a variety of different positions adopted voluntarily by the rat prior to beginning each trial and the withdrawal movement was observed. 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 as the paw was initially positioned in the medial direction, it tended to move laterally. The initial angle of the paw was also seen to play a significant role in determining the final angle of the paw following the response.

## A. Comparison to Previous Studies

### a. Stimulus Location

The first purpose of this study was to determine the effect of stimulus location on the NWR in intact, unanesthetized rats. It was found that the direction of the response, magnitude of the response, and change in angle of the paw were all unaffected by which location on the plantar surface of the paw had been stimulated. This finding contradicted results found in previous studies, as it has been shown that the NWR could be influenced by which part of the limb is stimulated. Hagbarth (1955), Grimby (1963), Schouenborg and colleagues (1992), and Andersen and Sonnenborg (1999) all found that different responses could be evoked depending upon which specific area of the limb was stimulated.

Andersen and Sonnenborg (1999) studied the actual movement trajectory of the foot in humans in response to noxious stimuli delivered to various locations on the foot and showed that dorsi-flexion, plantar flexion, inversion and eversion were differentially evoked by stimuli in different locations. In contrast, this study was solely based on the initial and final position of the paw, rather than the movement that happened between removal and replacement of the paw on the platform. It is possible that different patterns of eversion and inversion were occurring as the paw was removed and replaced on the platform, but the final position of the paw remained unaffected by the stimulus location.

Additionally, studies by Grimby (1963) and Andersen and Sonnenborg (1999) were completed in humans, while my experiment was completed in rats. In rats, the NWR tends to be much stronger than the reflex seen in humans. Although the stimulus location was seen to affect the NWR in the studies completed by Grimby (1963) and Andersen and Sonnenborg (1999), the observed response was much weaker than the rapid removal and replacement of the paw witnessed in the rat.

Sherrington (1910) relied on visual observation of muscles while Grimby (1963) and Schouenborg and colleagues (1992) analyzed the NWR based on EMG recordings. Responses were inferred based on these electrical recordings of nerves of muscles, therefore withdrawal movement was never actually observed. As the present experiment looks at the actual movement of the paw, this could be another reason for the differing results.

Most importantly, studies in the past have all been completed in animals that were anesthetized, decerebrated, or spinalized. Our study was conducted in rats that were intact and unanesthetized, so the pathways mediating the response could be very different. Modulation and long loop reflexes are two components that may influence the NWR, possibly playing a role in the differing results seen in intact, unanesthetized rats compared to animals that were anesthetized, decerebrated, or spinalized.

Finally, Clarke and Harris (2004) performed an experiment in intact, lightly anesthetized rabbits, where they found that varying the stimulation location on the plantar surface of the hind limb resulted in a change in response. As the pinch stimulus was moved from the rostral to the caudal end of the paw, the response at the ankle joint gradually shifted from flexion to extension, but the response at the knee was consistently flexion. The ankle joint movement does not affect the final location of the paw, while the movement at the knee joint does. Consistent with our results, Clarke and Harris found that the movement at the knee unaffected by which part of the paw was stimulated.

### b. Initial Position

The second purpose of this study was to determine the effect of the initial position of the paw on the NWR. Relying on spontaneously variability in the initial position of the paw adopted voluntarily by the rat, it was discovered that where the paw was positioned prior to delivery of the stimulus had a significant effect on the translational (direction and magnitude) and rotational (angle) aspects of the withdrawal response.  If the paw was initially placed rostrally, it would move caudally during the response and if the paw was initially placed caudally, it would move rostrally during the response. Similarly, if the paw was initially place laterally, it would move medially during the response and if the paw was initially placed medially, it would move laterally during the response.

The finding that initial position plays a significant role in mediating the NWR is supported by previous research regarding nociceptive (Brown 1911; Andersen and Sonnenborg 2003; Richard et al. 2015; Decchi et al. 1997; Serrao et al. 2006; Kim et al. 2007) as well as non-nociceptive (Baxendale et al. 1981; Knikou et al. 2006; Onushko et al. 2013) reflexes. It was first discovered that the initial posture of the hind limb in rabbits played a role in mediating the scratching and hopping reflex (Brown 1911). Later, Baxendale (1981) found that that posture of the knee in decerebrated cats acted to influence the non-nociceptive flexion-reflex in order to limit hyper-extension of the limb. It has since been discovered that the initial position of the lower limb in humans plays a role in mediating the NWR while the subject is standing (Decchi et al. 1997; Andersen et al and Sonnenborg 2003) as well as walking (Richard et al. 2015). Extensors were activated while standing while hip flexors were primarily activated while walking, both acting to assist in maintaining balance. Similarly, the posture of the upper limbs has been seen to influence the NWR during rest and movement (Serrao et al. 2006). These findings were further supported in humans with spinal cord injury, where it was found that the response to a noxious (Kim et al. 2007) and non-noxious (Knikou et al. 2006; Onushko et al. 2013) stimulus were significantly influenced by the position of the lower limb prior to stimulation.

Although a majority of previous research on the effect of initial posture of the limb on nociceptive and non-nociceptive reflexes has been completed in humans, a similar pattern of neural mechanisms mediating the NWR is likely present in rats, resulting in similar outcomes being obtained. In agreement with previous research, our results have indicated a dependence of the NWR in intact, unanesthetized rats on the initial position of the hind, left paw prior to noxious stimulation.

## B. Mechanisms

The present results reveal that withdrawal movement is influenced by the initial position of the hind paw, resulting in changes in the direction, magnitude, and angle of response. The dependence of hind paw location and angle on initial position raises the question of what underlying mechanisms mediate this relationship. The first possibility is that the CNS is using information from limb proprioceptors. There are three types of proprioceptors located in the limb that may provide information that could influence the response. Muscle spindles, located in muscles, measure the length of muscles and indirect joint angle. Golgi tendon organs, located within muscle tendons, measure muscle force. The third type of sensor is joint afferents, located in the connective tissue of joints, which measure the angle, direction, and velocity of movement in a joint (Bear 2015). Information from these three types of proprioceptors may also be combined with the CNS. Since proprioceptors are known to modulate spinal reflexes (Onushko et al. 2013), it is possible that NWR pathways may also be influenced.

The second possibility is that the reflex is influenced by corollary discharge, also known as efference copy. Corollary discharge is an internal copy of a motor-producing command that can be sent to other areas of the brain, informing them of the forthcoming movement. Corollary discharge has been shown to be important is regulating motor output as well as interpreting incoming sensory information, so it is possible that corollary discharge is playing a role in mediating the NWR based on initial position of the paw (Nelson 1996). One critique of this explanation is that the animal's paw sometimes remained in place for an extended period of time before the stimulus was delivered, therefore reducing the possibility that the information from the corollary discharge was still active in the CNS upon stimulation.

### C. Significance

The ability of an animal to effectively withdrawal a part of its body away from potential harmful stimuli is important for survival. In order for the withdrawal to be effective, it is imperative that the animal maintains its balance. As suggested by these experiments, the posture of the animal plays an important role in mediating the withdrawal response to a noxious stimulus. Upon withdrawal of the limb, the animal must ensure that it does not tip over following movement, resulting in further damage and inability to escape the threat. Similarly, posture may be significant so that the animal is able to position itself in order to run or move away from the stimulus following withdrawal of the limb.

Unlike initial position, stimulus location was not found to play a significant role in determining the withdrawal response. This could imply that as long as the paw is moved an adequate distance away from the stimulus, it is unimportant in which direction the movement in directed. Also, since the paw of the rat is so small, the changes in where it is stimulated may have been so minute that they did not cause a change in the withdrawal strategy.

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