EFFECTS OF SOCIAL ISOLATION AND HOUSING SUPPLEMENTATION ON NEUROPATHIC NOCICEPTION IN RATS

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Neuropathic Nociception in Rats

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ABSTRACT

Although only limited data have been published regarding the effects of environmental variables on pain, the available information suggests that the perception of pain in humans and nociception in rodents is affected by external surroundings. To better understand how the environment alters nociception, this study examined the effects of social isolation and cage complexity on the severity and duration of nerve injury-induced nociceptive behaviors. Male, Sprague-Dawley rats were used in this study, and researchers blinded to the housing treatment groups collected all data. Baseline behavioral measurements, including paw withdrawal latency to a heat stimulus and paw withdrawal threshold to a mechanical stimulus, were taken at the onset of the investigation. Rats were randomly assigned to the four housing treatment groups: (1) one rat per cage, standard cage conditions, (2) one rat per cage, supplemented cage conditions, (3) three rats per cage, standard cage conditions, and (4) three rats per cage, supplemented housing conditions. Both the standard and supplemented cage conditions provided food, water, and bedding; however, the supplemented cage condition also included objects that increased the cage complexity (polycarbonate shelters, rodent toys, things to gnaw and chew, etc.). Following a six-week period in their assigned housing condition, pre-nerve injury behavioral data were collected, and then each rat underwent the partial sciatic nerve ligation surgery. Post-surgical behavioral data were collected at 1, 7, 14, 21, 28, and 35 days. The data indicate that social interaction and supplementation decrease nociceptive behaviors.

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CHAPTER 1

I. Introduction

A. Pain

Pain is an integral part of everyday life. It can function as a reflex in response to a painful stimulus or serve as an indication of a problem within the body (Craig, 2003). Pain is broadly defined by the International Association for the Study of Pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Physiologic pain is a direct result of tissue damage and functions as an adaptation to alert an organism of noxious stimuli by triggering negative affective responses. This type of pain is short-term and dissipates when the underlying cause is resolved. Pain, however, becomes pathological when it is persistent or chronic and serves no apparent purpose.

Chronic pain has been defined as pain that persists longer than the temporal course of natural healing and is associated with dysfunction of the nervous system (Thomas, 2001). This type of pain is a result of long-term changes in the nervous system, a process called neuroplasticity. Neuroplasticity is the ability of neurons to change their function, chemical profile, or structure and can affect transmitters, receptors, ion channels, or the neurons themselves (Woolf and Salter, 2000). Chronic pain-related neuroplasticity is associated with sensitization of both peripheral and central neurons which leads to increased pain sensation (Woolf and Salter, 2000). This form of neuroplasticity is mainly controlled by the phosphorylation of a variety of receptors and

ion channels, which dramatically change the functional characteristics of the cell (Woolf and Salter, 2000).

Patients with persistent pain conditions experience different measures of pain intensity, degree of interference with lifestyle, as well as psychosocial variables (Turk and Rudy, 1988, 1990). Three groups of chronic pain patients have been identified based on incorporation of cognitive, affective and behavioral information: dysfunctional, interpersonally distressed, and minimizers/adaptive copers (Turk and Rudy, 1988). As there are different types of chronic pain, different forms of treatment are employed to yield the best benefit to the patient (Turk and Rudy, 1988). Because nociception operates on different levels, not all treatment plans for chronic pain patients are identical.

Neuropathic pain is a subtype of chronic pain in which nerve injury induces changes that lead to permanent alterations of basal nociceptive sensitivity. Clinical examples of these types of pain include diabetic neuropathy, postherpetic neuralgia, reflex sympathetic dystrophy and phantom limb pain (Jameson, 1996). Analgesic drugs are effective against physiologic pain but have been proven to lack substantial efficacy for the management of chronic or pathologic pain.

Pain research is imperative due to pain's prevalence as well as its expense. Millions of Americans suffer from chronic pain, and according to the National Institute of Health, costs associated with pain reach 100 billion dollars per year due to both direct and indirect factors. Pain also affects ones' ability to work, disrupts their daily activities and lowers quality of life.

B. Neuroanatomy & Neurophysiology

There are different kinds of cutaneous receptors that transduce external stimuli. Upon activation of a nociceptor, a cutaneous pain receptor, a depolarizing current is generated by certain transducer proteins. In nociceptors, the opening or closing of sodium (Na^+) and potassium (K^+) ion channels converts the stimulus to a change in membrane potential. This change in membrane potential results in the production of graded potentials, including excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) that can either increase or decrease the amount of action potentials that are produced, ultimately resulting in the sensation of pain.

In neurons the resting membrane potential, which is established by ionic concentration gradients, ranges from -60 mV to -90 mV where the concentration of Na^+ is greater in the extracellular fluid surrounding the neuron and the amount of potassium K^+ is greater in the intracellular fluid. Gradients of these ions are maintained by Na^+/K^+-ATPase pumps actively moving 3 Na^+ out and 2 K^+ into the neuron. Changes in membrane potential due to the opening of ion channels can elicit an action potential if the threshold of -50 mV is reached. Action potentials are generated by the openings of voltage-gated Na^+ channels, after which Na^+ ions influx resulting in cell membrane depolarization (Haines, 2002).

Nociceptors are free nerve endings that respond to noxious stimuli including chemical, mechanical, or thermal, all of which can cause tissue damage. Nociceptors depolarize and yield an action potential with the application of a noxious stimulus. Nociceptor activation is modulated by local chemical messengers that are released upon tissue injury including K^+, histamine, prostaglandins, cytokines, serotonin and substance P (SP). The presence of these chemicals can either activate or desensitize nociceptors by

raising or lowering their threshold of activation, changing transducers, or increasing nociceptor membrane excitability (Woolf and Salter, 2000). Therefore the presence or lack of the aforementioned chemicals can alter pain response, potentially leading to changes in pain perception. Dorsal roots carry information into the spinal cord from cutaneous and other receptors. Nociceptors activate two different pathways including a reflexive response that is integrated at the spinal cord or through ascending pathways to the higher center of the central nervous system (CNS), which actually elicit the feeling of pain. Signals from the nociceptors are carried to the CNS in three different types of sensory fibers; A- β, A-δ, and C fibers.

A-β fibers, with their tranducers found on cutaneous mechanoreceptors, are heavily myelinated fibers capable of the fastest conduction velocity among the sensory fibers (Haines, 2002). A- δ fibers are thinly myelinated nociceptors that transmit action potentials in response to acute pain associated with temperature and mechanical stimuli (Haines, 2002). A- δ fibers can also be further divided into two categories, type I or type II, by whether they are fast or slowly adapting and also whether they have a high or low threshold for firing. Unlike the A fibers, C fibers are unmyelinated, which slows their conduction velocity (Haines, 2002). C fibers respond to both noxious and non-noxious heat stimuli, histamine, and non-noxious touch (Haines, 2002).

If enough peripheral nociceptors are activated, primary afferent neurons synapse at laminae (I, II, V) of the spinal cord dorsal horn (Basbaum and Jessell, 2000). The afferent nociceptor then synapses with a dorsal horn neuron and is capable of producing a postsynaptic output. Fast excitatory synaptic transmission is controlled by glutamate binding to alpha-amino-3-hydroxy5-methyl-4-isoxazolepropionic acid (AMPA) and

kainate receptors (Woolf and Salter, 2000). Upon excitation, inhibitory neurons are activated, releasing both glycine and gamma-aminobutyric acid (GABA) which function to avoid over-excitation (Chery, 1999). Nociceptor activation by noxious stimulation generates fast excitatory EPSPs that function to signal such factors as the onset, duration, intensity and location of the stimulus (Woolf and Salter, 2000). If the noxious stimulus is sustained for a long period of time or if the stimulation is strong enough, neuromodulators and glumatate are released resulting in temporal summation by slow EPSPs (Duggan, 1990; Sivilotti, 1993).

Pain is transduced by the somatosensory system, which includes the interaction of three separate systems: an exteroreceptive system, a proprioreceptive system and an interoreceptive system. The proprioreceptive system controls movement and balance, and the interoreceptive system controls and processes information about the internal environment of the body. The exteroreceptive system is the afferent component that senses external stimuli and regulates signal transduction. The exteroreceptive system can further be divided into three divisions: transduction of mechanical stimuli, thermal stimuli, and nociceptive/painful stimuli.

There are two major ascending somatosensory pathways that transmit signals from the external environment into the central nervous system. The dorsal-column medial lemniscus system carries information about touch, and the proprioception and the anterolateral system (ALS) transmits information about pain and hot and cold temperature. The ALS is composed of three different tracts, the spinothalamic tract (STT), the spinoreticular tract (SRT), and the spinomesencephalic tract (SHT; Haines, 2002). The SRT mediates alertness and arousal to the stimuli and the SHT regulates eye

movements towards the stimulus. The STT mediates the localization of painful or thermal stimuli with its cell bodies located in the I and V laminas of the dorsal horn (Haines, 2002). At these areas the primary afferent sensory fibers synapse with second order neurons. These neurons then transmit action potentials up the spinal cord and synapse in the ventral posterolateral nucleus of the thalamus, after which the action potentials are transmitted to the postcentral gyrus of the cerebral cortex, the ventrolateral medulla, the parabrachial nucleus, the periaquaductual grey (PAG) brainstem reticular formation and the limbic structures via third order neurons (Haines, 2002; Craig and Dostrovsky, 1999). These structures can either inhibit or facilitate nociception and pain perception (Tracey and Dunckley, 2004).

Structures in the brain, referred to as the pain matrix, that are active during noxious stimulation include the anterior cingulated cortex, the insula, the frontal cortices, the primary somatosensory cortex, the secondary somatosensory cortex and the amygdala (Ingvar, 1999). The pain matrix can further be separated into a medial and lateral system. The lateral system deals with determining both the location and intensity of noxious stimuli, and the medial system mediates the cognitive-evaluative components of pain (Bushnell et al. 1997). The insula serves as a connection between the medial and lateral systems, integrating information received from both systems (Peyron et al., 2002). Central processing via the aforementioned brain structures of incoming signals from the spinal cord results in conscious perception of an event being painful (Brooks and Tracey, 2005). Therefore, the brain and spinal cord not only modulate, but also are capable of creating pain perception.

Another pathway that plays a pivotal role in nociception is the descending inhibitory control (DIC) pathway which begins in the cortex, limbic system and hypothalamus and has a stimulatory synapse in the PAG in the midbrain which then project to the medulla oblongata and the pons (Haines, 2002). Neurons from the PAG that project to the medulla have a stimulatory effect on neurons of the rostral ventromedial medulla (RVM), particularly to serotonergic neurons in the nucleus raphe magnus (NRM; Vanegas et al., 2004). Neurons that project from the PAG to the locus ceruleus (LC) in the pons stimulate norepinephrine release. These NRM and LC neurons then project down the spinal cord and terminate in laminae II and III of the dorsal horn, having the effect of inhibiting action potentials of both primary and secondary afferent neurons that transmit pain (Vanegas et al., 2004). Mechanisms by which the descending neurons suppress pain transmission are by the release of serotonin and norepinephrine into the dorsal horn as well as activation of enkephalinergic interneurons, stimulating the release of endogenous opioids. The opioids have the effect of decreasing neurotransmitter release by inhibiting primary afferent neurons which decreases the amount of calcium (Ca^{2+}) in the presynaptic axon terminal. The mechanism by which descending neurons can inhibit secondary afferent neurons is by increasing K^{+} efflux, hyperpolarizing the cell and yielding an inhibitory postsynaptic potential (Vanegas et al., 2004).

C. Hypothalamic-Pituitary-Adren (HPA) Axis

The HPA axis is involved in regulating the body's levels of glucocorticoid stress hormones. The HPA axis is also activated at times when an animal feels pain, functioning

as a survival mechanism for the animal to escape a threat. In the case of chronic pain, the HPA axis is activated indefinitely, acting as a constant stressor (Blackburn-Monro, 2001).

Corticotropin-release hormone (CRH) is produced in the periventricular nucleus (PVN) in the hypothalamus and initiates a stress response (Chapman, 2008). Corticotropin-releasing hormone (CRH) promotes sympathetic nervous system activation, enabling an animal to undergo an appropriate sympathetic response at needed times. Nociceptive signaling acts directly on the PVN to increase CRH levels (Chapman, 2008). CRH is released into the median eminence and enters the portal circulation to the anterior pituitary. There, it stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH) into systemic circulation where it stimulates cortisol release at the adrenal cortex.

Cortisol's effects in the body last for hours and allow for the repair any damage that may have occurred after a stressful stimulus (Sapolsky, 1998). Cortisol increases blood sugar and pressure, suppresses the immune system, and operates via negative feedback at the level of the PVN and anterior pituitary to decrease levels of CRF, hence ACTH and cortisol. Cortisol also functions to help hinder the sympathetic nervous system and return the body to a normal state (Sapolsky, 1998). Excess stimulation of the PVN via nociceptive signaling that occurs in the case of chronic pain can result in dysregulation of the HPA axis and ultimately decreased pain thresholds (Chapman, 2008). Both ACTH and cortisol levels are elevated in conditions of chronic pain (Chapman, 2008).

C. Neurotransmitters

Neurotransmitters are chemical signals secreted by neurons that influence other neurons and effecter tissues. Neurotransmitter synthesis occurs either in the nerve cell body or the axon terminal. The release of neurotransmitters into the synaptic cleft occurs by exocytosis, aided by calcium (Ca^{2+}) and is triggered by the generation of an action potential at the axon hillock where the summation of inhibitory postsynaptic potentials and excitatory postsynaptic potentials ensue. The axon terminal membrane has voltage-gated Ca^{2+} channels that open during depolarization during which Ca^{2+} ions move into the presynaptic axon terminal. The Ca^{2+} ions then initiate exocytosis of the neurotransmitters by binding to regulatory proteins on the presynaptic membrane. The neurotransmitters then diffuse across the synaptic cleft and bind with receptors on a target cell which initiates a response within the cell.

These responses result in graded potentials that can trigger action potentials. These graded potentials can either be classified as excitatory or inhibitory, depending on whether the neurotransmitter causes depolarization or hyperpolarization, respectively. The greater the stimulus, the greater the excitatory postsynaptic potential (EPSP) magnitude, resulting in higher excitation of the neurons. Nociceptor activation by noxious stimulation generates fast EPSPs that function to signal the onset, duration, intensity and location of the stimulus (Woolf and Salter, 2000). If the noxious stimulus is sustained for a long period or if the stimulation is strong enough, neuromodulators and glumatate are released resulting in temporal summation by slow EPSPs (Duggan, 1990; Sivilotti, 1993). The neurotransmitters primarily involved in nociception are glutamate and substance P, which are co-localized in the primary affect terminals of the spinal cord superficial lamina (Woolf, 1994).

1. Glutamate

Glutamate, a proteinogenic amino acid, is involved in cellular metabolism, having the characteristic of being the most abundant amino acid in the diet (Meldrum, 2000). Glutamate is the main excitatory neurotransmitter of the CNS and influences virtually all neurons of the neuroendocrine hypothalamus producing having a plethora of effects within the body. Glutamate binds with membrane receptors, which results in depolarization, producing an excitatory response in the postsynaptic cell.

There are two different types of glutamate receptors: ionotropic and metabotropic (Meldrum, 2000). Ionotropic glutamate receptors can be further categorized into three groups: (1) N-methyl-D-aspartate (NMDA) receptors (2) AMPA, and (3) kainate receptors (Meldrum, 2000). Although all of the receptors have ion channels permeable to cations like Ca^{2+} and Na^{2+}, the degree of permeability depends on receptor subtype. When glutamate binds to AMPA receptors, which are thought to mediate physiological and baseline nociception, an EPSP is generated (Dickenson et al., 1997).

NMDA receptors are both ligand and voltage-gated. Upon glutamate binding, there is an influx of Ca^{2+} to the postsynaptic cell, triggering certain second messengers. However, Mg^{2+} blocks the NMDA channel pore from opening, and even if glutamate and glycine are bound to the receptor, excitation will not occur (Dickenson et al., 1997). When the cell membrane has been depolarized first by excitation of a different excitatory receptor, like AMPA, Mg^{2+}, dissociates from the channel, allowing it to open. With Mg^{2+} gone, glutamate binds to its receptor and produces an excitatory response via Ca^{2+} influx.

G-proteins also have the ability to either stimulate or inhibit certain enzymes, called effector enzymes, within the cell membrane. This can produce either an increase or decrease of second messengers, therefore affecting the postsynaptic cell membrane. Because of this, postsynaptic nerves are able to become either more or less excitable, depending on what second messengers are present. This excitability, or lack thereof, influences the degree to which a pain response is evoked. There are also receptors coupled to G-proteins called metabotropic (mGlu) receptors that have the ability to modulate neuronal activity. These receptors are divided into Groups I-III . Group I receptors are positively coupled to the enzyme phospholipase C, or PLC (Wall and Melzack, 2006). PLC induces the hydrolysis of phosphatidylinositol 4-5-biphosphate, which yields inositol triphosphate (IP_3) and diacylglycerol, or DAG (Datar et al., 2004). The intracellular increase of IP_3 releases Ca^{2+} from the endoplasmic reticulum. DAG activates protein kinase C (PKC) that phosphorylates the protein resulting in altered function. Groups II and III receptors are negatively coupled to adenylyl cyclase (Meldrum 2000 and Neugebaur et al., 1994). Group I G-proteins induce the opening of K^+ channels, causing K^+ efflux and membrane hyperpolarization.

2. Substance P (SP)

Substance P is an 11-amino acid tachykinin, a family of neurotransmitters responsible for numerous effects in the body (Hökfelt et al., 1975). SP's excitatory effects influence the CNS and can alter pain perception (Datar et al., 2004). There are three different types of tachykinin receptors: neurokinin-1 (NK-IR), neurokinin-2 (NK-2R) and neurokinin-3 (NK-3R; Datar et al., 2004). Upon noxious stimulation, SP is released in the spinal cord by primary afferent fibers (Boehmer et al., 1998). After

binding to a NK-IR receptor on the postsynaptic membrane of neurons on laminas I and X of the dorsal horn, SP activates a G-protein coupled receptor and PKC. PKC then phosphorylates the NMDA receptor, increasing its ability to become excited by removing the Mg^{2+} block. This increases glutamate's ability to produce an excitatory response, potentially leading to increased transmission of a pain response by increasing excitability of dorsal horn neurons (Kidd & Urban 2001). Glutamate binding to the receptor causes in influx or Ca^{2+} and Na^{+}, increasing excitability and therefore causing hyperalgesia, which is an above average pain response (Kidd and Urban, 2001). It is in this manner that SP can enhance glutamates excitatory effects.

D. Qualitative and Quantitative Measures of Pain Behavior

Hyperalgesia and allodynia, sensory phenomena, are characterized as changes in pain perception due to an increased sensitivity to either noxious or non-noxious stimuli (Cervero and Laird, 1996). Both are controlled by changes in the central processing of sensory input where peripheral nociceptors send signals to the CNS after stimulation. Hyperalgesia can develop due to cutaneous or nerve-injuries and occurs due to increased sensitization of nociceptors (Cervero and Laird, 1996). Hyperalgesia acts as a protective mechanism to the organism that is experiencing it by preventing the individual from stimulating the injured area. Therefore hyperalgesia promotes the healing of an area.

Nerve injury leads to increased sensitization of both peripheral nociceptors and central nociceptors, which are located in the spinal cord dorsal horn, resulting in hyperalgesia. As a result of central sensitization, discharges from nociceptive afferents act to increase excitability of spinal cord neurons. The neurons that are most involved in

the phenomena of central sensitization are referred to as 'wide dynamic range' (WDR) neurons. Post-injury intensity based hyperalgesia is caused by central sensitization (Dougherty et al. 1994). Central sensitization is a result of injury in afferent neurons which cause increased excitability in dorsal root neurons, thereby lowering a subject's pain threshold (Woolf, 1983; Woolf and Thompson, 1991; Dubner and Ruda 1992; Coderre et al., 1993). An increase of excitability in dorsal root neurons occurs due to an increase of both glutamate and SP from primary afferent fibers, activating second messenger systems that cause an influx of Ca^{2+} and phosphorylation of proteins (Kidd and Urban, 2001). Whether central or peripheral sensitization occurs in the nervous system, changes in behavioral manifestations of nociception ensue. In areas of hyperalgesia, low intensity mechanical stimuli and warmth are capable of inducing pain response (Cervero and Laird, 1996).

Allodynia can be defined as the perception of pain due to a stimulus that does not normally invoke pain (Mersky and Bogduk, 1994). It involves a deviation of a sensation from how one normally feels. For instance, a normally non-noxious stimulus becomes painful, losing specificity of a sensation (Mersky and Bogduk, 1994). The degree of pain felt depends on the history of an affected area as well as degree of the stimulus. Allodynia is mediated by changes in afferent neurons caused by peripheral nociceptors and is mediated by the activation of low threshold mechanoreceptors with A-β afferents (LaMotte et al., 1991; Cervero and Laird 1996). Like hyperalgesia, allodynia can be viewed as a protective mechanism, aiding an individual in the healing process by discouraging stimulation of an injured area.

Allodynia can be classified as secondary hyperalgesia and is activated by low threshold mechanoreceptors with A-β afferent nerve fibers. Allodynia is caused by a change in how different sensations, like touch, are evoked by mechanoreceptors (LaMotte et al., 1991). Mechanical stimulation in areas of allodynia induces localized vasodilation, or axon reflexes, as well as touch-evoked pain sensations. Axon reflexes play an important role in allodynia in that they are both consequences of the same mechanism. Studies have shown that by blocking A-fibers, neither allodynia nor the axon response could be induced, implying that the source of allodynia is centralized rather than peripheral. Mechanistically, this is caused by certain presynaptic mechanisms, involving interaction between primary afferent neurons and the spinal cord (Rudomin, 1990).

Allodynia and hyperalgesia demonstrate that pain perception can depend on not only the magnitude of noxious stimuli stimulating an area, but also the history of that area (Cervero and Laird, 1996). Touch evoked pain, stimulated by low intensity mechanoreceptors in a previously injured area, show an important mechanism by which allodynia and hyperalgesia act, *i.e*. changing how one perceives stimuli. This is a result of a dysfunction of normal touch perception rather than a new pathological change.

E. Partial-Sciatic Nerve Ligation (PSNL)

A preclinical model has been developed and widely used to mimic certain neuropathic pain disorders in humans. In rats, half of the left sciatic nerve is ligated near the upper thigh, after which rats develop mechanical allodynia and hyperalgesia that are dependent on sympathetic outflow (Seltzer, 1990). After sciatic nerve ligation,

sympathetic axons invade the dorsal root ganglion (DRG), making a connection between the sympathetic nervous system and neuropathic pain by giving an anatomical substrate for sympathetically maintained pain (Devor et al., 1996). Ligation results in immediate and irreversible interruption of nerve conduction as well as Wallerian degeneration, which degrades axons distal to the nerve injury. Peripheral nerve damage can ultimately lead to spontaneous discharge, increasing activity of the dorsal horn neurons. This allows non-nociceptive peripheral nerves to influence the ascending pain pathways (Cervero and Laird, 1991).

Wallerian degeneration occurs after nerve injury in both the central and peripheral nervous system. Wallerian degeneration is associated with increased amounts of nerve growth factor (NGF) in the distal portion of the nerve, which is transported to uninjured axons, resulting in sympathetic sprouting (Ramer et al., 1996). Research shows a positive correlation between the onset of degeneration and the onset of neuropathic pain (Sommer and Myers, 1995). If Wallerian degeneration is delayed, then so are the changes in pain perception including both hyperalgesia and allodynia. Nerve ligation triggers an increase in the rostral ventromedial medulla (RVM) released cholecystokinin (CCK). This creates a positive feedback loop between the RVM and the dorsal horn, which maintains allodynia and hyperalgesia of the ligated limb (Heinricher et al., 2001). Due to these results, this model has been determined relevant for syndromes relating to causalgiform pain that are triggered by partial nerve injury and maintained by sympathetic activity such as nerve bruise or gunshot-induced nerve injury (Selzter, 1990).

F. Environment

Pain is the result of a complex interaction between certain genetic and environmental factors. Although much research has gone into certain genetic factors that influence pain, more research needs to be performed to reveal how the environment affects nociception. Certain factors that can be analyzed in nociception research are diurnal effects, diet, as well as social and housing variables. By studying the effects that environment has in preclinical studies, inferences can be drawn from the findings that may be applied clinically to better understand the affects on human pain perception.

Clinical studies support the hypothesis that nociception can be influenced by a variety of emotional, physiological, and social factors, e.g., for example after gall bladder removal surgery, patients felt less pain if they were given a natural scene to view while they were recovering compared to the patients who did not receive the view (Ulrich, 1984). The patients that had the natural scene view had shorter postoperative stays as well as less negative evaluations from nurses and a decrease in pain medication as compared to the patients that did not have this view. This finding supports the inference that pain is a condition that needs to be dealt with on multiple levels.

Rodents are commonly used in research because environmental variables can be easily manipulated to determine whether or not they influence behavioral activities. By changing the environment of an animal, one can alter normal responses, and measure the results. The changes in behavior observed in rodents can provide insight regarding human behaviors. A study done regarding environmental factors involved rats living in standard cages, including only bedding, food and water, and in cages with a variety of items. In addition, rats were further separated into cages that had groups of rats, as well as cages that housed isolated rats. The rats that were housed in the social cages with items showed

dendritic branching within the visual cortex, indicative that housing environment has the ability to affect neuromorphology (Benefiel et al., 2005).

In addition to affecting neuromorphology, supplementation has also been found to impact learning and memory (Wood et al., 2006). In a study, rats were divided into two treatment groups -- socially isolated and environmentally enriched. The environmentally enriched rats were housed in groups of four-five and were provided with toys acting as supplementation. The socially isolated rats were housed one rat per cage with no such supplementation. The rats that lived in enriched housing showed an increase in learning and memory under certain conditions (Wood et al., 2006). The increase in cognitive behavior was measured using feeder cue testing where housing supplementation led to an increase in training ability.

It has also been shown that the lack of supplementation can lead to maladaptive behaviors, skewing the data that is to be collected. In addition to lack of supplementation, other factors such as placement of cages in housing facility, and increased noise or light levels may negatively affect behavioral studies (Vissers et al., 2003). All of these variables can increase the animal's stress levels, thereby influencing behavioral data (Vissers et al., 2003). By measuring an animal's levels of cortisone in the blood, one can determined the degree to which the animal is experiencing stress induced anxiety behavior (Homes et al., 2005). In one study, 21 day old male pups were weaned and placed into groups of either social or isolated mice. Levels of anxiety were then measured using a free exploration test and less anxious behavior was categorized by an increased amount of time spent in novel compartments. The mice that were housed

socially were to lessen stress by having an increased time spent in novel compartments, supporting the complexity of rodent behavior (Homes et al., 2005).

By changing housing supplementation, one study was able to support the hypothesis that an enriched environment can result in enhanced locomotor activity in rats that had undergone nerve injury (Lankhorst et al., 2001). In this study the objects of enrichment included a running wheel, climbing frames, tubes and shelters. Post-surgery, the rats that were in the supplemented housing evidenced an increased recovery rate compared to the rats that were not housed with supplementation (Lankhorst et al., 2001). Enhanced recoveries were measured by higher scores in locomotor tests with higher scores being synonymous with quicker recovery rates. It was found that the animals housed in standard conditions reached a recovery plateau after three weeks whereas the animals housed in supplemented environments continued to recover throughout the entire experiment (Lankhorst et al., 2001).

Whether or not rats are housed socially has also been found to influence behavioral pain research. In one study, rats underwent sciatic nerve ligation surgery and were divided into different groups based on if they were from genetic lines that displayed either high or low autotomy, which is the removal of a body part of an organism, by the organism, after injury. If the animals that were less prone to autotomy were housed with the animals that were more prone to autonomy, they were more likely to display autotomy than if they were housed with animals that were not prone to autotomy (Raber and Devor, 2002). This shows how social interaction is capable of changing normal genetic behavior.

Pain can be influenced by numerous variables, including environmental factors. Much research has been devoted to pain; however, more needs to be done in regards to how environment can influence nociception. By researching the potential effects that environment has, greater insight will be gained on how pain operates on a physiological as well as psychological level. Understanding such factors can lead to better pain treatment. This research project will aim to examine whether environmental factors influence neuropathic pain, *i.e.*, whether housing rodents individually or socially as well as providing them with or without supplementation will affect nociceptive behaviors.

CHAPTER 2

II. Methods

A. Animals

All studies were performed on male Sprague-Dauley rats 30-35 days old that were obtained from Charles River Laboratories Inc. (Wilmington, MA). Four groups of rats were used in these studies (n=5 per housing condition), totaling 20. The rats were kept on a 12/12 hour day/night cycle, with lights off at 10:00 A.M. and lights on at 10:00 P.M. The animal facility and testing laboratory was held at a temperature of 20 ± 1 º C. Food (Lab Diet 5P00 Prolab RMH 3000 PMI Nutrition International Brentwood, MO) and water was provided to the animals *ad libitum*. The IACUC approved the methods of this experiment.

B. Housing Conditions

After a one week acclimation period, the rats were randomly assigned to a treatment group. Rats were housed in polycarbonate cages measuring 20" long x 16" wide x 8 ½" tall. Included in the housing were food, water and bedding (Aspen pine shavings and Bed-O-Cobb). The rats were either assigned to social conditions (three rats per cage) or nonsocial conditions (one rat per cage). In order to increase cage complexity, rats assigned to supplemented housing had, nylabones (4.5" x 1.25"), crawl balls, rat tunnels, rodents retreats, balls, igloo shelters, barbells, dental balls, and chew toys, rotated each week for novelty.

Four housing treatment groups were examined: (1) supplemented, social (SUP/S); (2) supplemented, nonsocial (SUP/NS); (3) standard, social (ST/S); and (4) standard, nonsocial (ST/NS)

C. Partial Sciatic Nerve Ligation (PSNL)

Under 3% isoflurane and aseptic conditions the right sciatic nerve was exposed at high-thigh level. The sciatic nerve underwent partial injury by freeing it from surrounding connective tissues at a site near the trochanter. Forceps held the nerve in place while an 8-0 silicon-treated silk suture was inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle, and was tightly ligated so that the dorsal 1/3-1/2 of the nerve diameter was trapped in the ligature. The wound was then closed using dissolvable sutures on the internal musculature wounds and externally with wound clips. Wound clips were removed post-surgical day 7.

D. Behavioral Assessments

Animals were familiarized to the investigators, the laboratory and behavioral testing equipment, three consecutive days for one hour each. Behavioral data was collected by researchers blinded to the type of housing conditions the rats recieved. Baseline behavioral data were collected after acclimation period. Pre-treatment data was collected five weeks after living in the assigned housing condition. The PSNL surgeries took place the day after pre-treatment data collection. After PSNL, post injury data was collected once a week for a total of seven weeks (Post7d, Post14d, Post21d, Post28d, and Post35d).

E. Paw Withdrawal Threshold (PWT) to Mechanical Stimuli

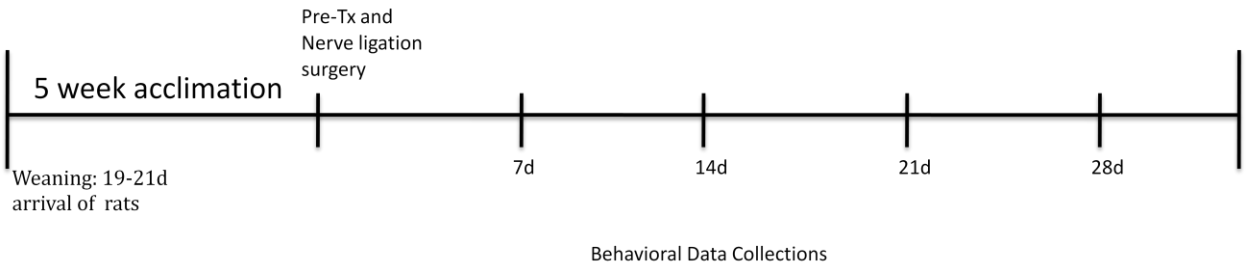
The withdrawal threshold to a mechanical stimulus was measured with a set of eight, calibrated von Frey monofilaments (0.5 - 64.0 grams force) using the up-down technique (Chaplan et al., 1994; Dixon 1980). Rats were placed in plexiglass chambers on an elevated, stainless steel mesh screen and were acclimated for 10-15 minutes. The von Frey monofilaments were applied to the plantar surface of each hindpaw between the footpads for 5-7 seconds using a slight pressure to cause a buckling of the monofilament against the surface of the hindpaws. A brisk withdrawal from the mechanical stimulus was considered a positive response. The PWT was determined in gram force units using the up-down technique.

F. Mechanical Allodynia

Mechanical allodynia was measured with a 4.0 gram Von Frey monofilament (Fecho et al., 2005). Rats were placed in plexiglass chambers on an elevated stainless steel mesh screen and were acclimated for 10-15 minutes. These tests took place directly after PWT tests. To test for mechanical allodynia the 4.0 gram monofilaments were be applied ten times with one second in between each application, using a slight pressure to cause buckling of the monofilament against the plantar surface of the injured hindpaw. A raising or licking of the paw was indicative of a positive withdrawal reflex, and the percentage of paw withdrawals was recorded.

H. Experimental Time Line

The line graph below depicts the timeline each group of rats followed upon arrival to the final testing day.



I. Statistical Analysis

Mechanical allodynia measurements were expressed as the mean ± S.E.M and analyzed using a repeated measures analysis of variance (ANOVA). Mechanical allodynia measurements were analyzed using ANOVA followed by paired T-tests and data presented as the mean ± S.E.M. Paw withdrawal threshold (PWT) was analyzed using ANOVA followed by paired T-tests and data was presented as the median ±S.E.M. A p value of < 0.05 was considered statistically significant. Given the significant difference in the multivariate means, the dependent measures were tested one at a time using univariate tests. As before, p values of ≤ 0.05 were considered to be statistically significant.

CHAPTER 3

III. Results

A. Paw-Withdrawal Threshold (PWT)

Animals were tested for PWT to a mechanical stimulus with a set of eight calibrated von Frey monofilaments using the Up-Down technique to determine if housing supplementation and social interaction influenced basal and post partial-sciatic nerve ligation (PSNL) nociceptive behaviors. The data collected is shown and divided into three graphs in Figures 1-3. Note that the greater the bar, the greater the animals' PWT, indicating a decrease in nociception.

1. Two-Way ANOVA for Supplementation

A repeated measures two-way ANOVA was computed to determine if there was a significant difference in the mean vector between subjects in the two groups where the presence was supplementation was the only difference – Supplemented/Nonsocial, and Nonsupplemented/Nonsocial. The p-value was considered statistically significant at $p = 0.031$. No significance was seen in post-hoc tests.

2. Two-Way ANOVA for Sociality

A repeated measures two-way ANOVA was computed to determine if there was a significant difference in the mean vector between subjects in the two groups where the presence of other animals in the housing was the only difference – Nonsupplemented/Nonsocial, and Nonsupplemented/Social. The p-value was not

Figure 1: Paw Withdrawal Threshold (PWT) Value Comparing Supplementation/Nonsupplementation

This figure shows a comparison of rats housed in Supplemented/Nonsocial and Nonsupplemented/Nonsocial cages. Paw withdrawal thresholds were found using von Frey filaments and employing the Up-Down technique. Data is presented for treatment groups on baseline, pre-treatment and post 7, 14, 21, 28, and 35 days. Values are represented as the mean ± SEM. Data was analyzed with a two-way ANOVA, yielding a p value of 0.031. Univariate testing indicated no significance at any time point, however a trend of increased PWT is seen in animals that were housed in supplemented cages. N = 5 rats/treatment group.

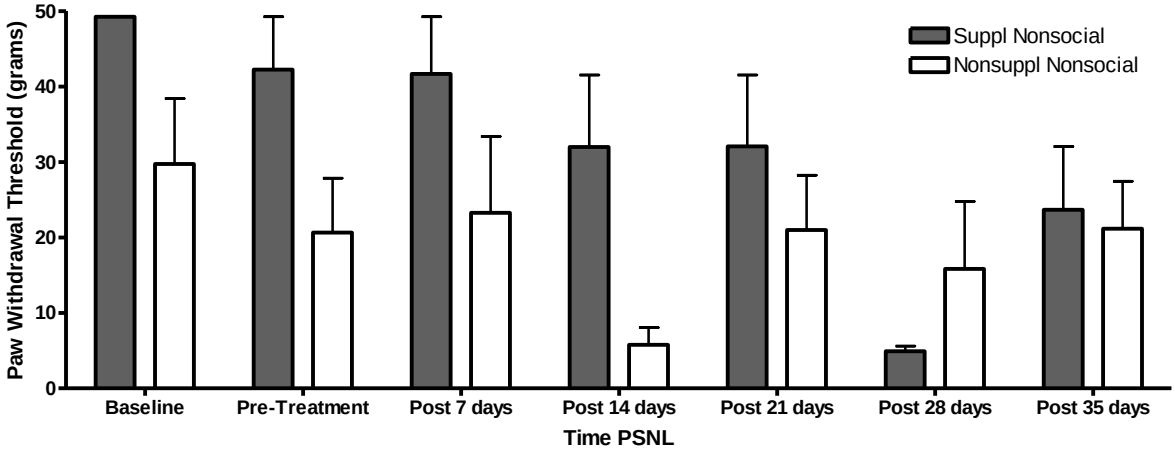
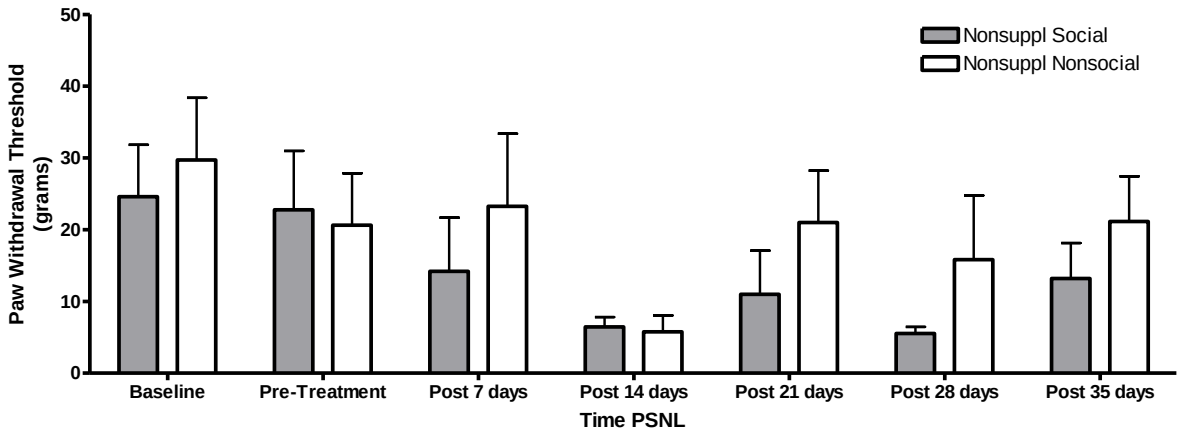

Paw Withdrawal Threshold (grams)
50
40
30
20
10
0
Suppl Nonsocial
Nonsuppl Nonsocial
Baseline
Pre-Treatment
Post 7 days
Post 14 days
Post 21 days
Post 28 days
Post 35 days
Time PSNL

Figure 2: Paw Withdrawal Threshold (PWT) Value Comparing Social/Nonsocial

This figure shows a comparison of rats housed in Nonsupplemented/Nonsocial and Nonsupplemented/Social cages. Paw withdrawal thresholds were found using von Frey filaments and employing the Up-Down technique. Data is presented for treatment groups on baseline, pre-treatment and post 7, 14, 21, 28, and 35 days. Values are represented as the mean ± SEM. Data was analyzed with a two-way ANOVA, yielding a p value of 0.097. Post-hoc tests were not examined. N = 5 rats/treatment group.


Nonsuppl Social
Nonsuppl Nonsocial
Paw Withdrawal Threshold (grams)
50
40
30
20
10
0
Baseline
Pre-Treatment
Post 7 days
Post 14 days
Post 21 days
Post 28 days
Post 35 days
Time PSNL

considered statistically significant at p=0.097. Due to this, post-hoc test results were not examined.

3. Two-Way ANOVA for Interaction of Supplementation/Sociality

A repeated measures two-way ANOVA was computed to determine if there was a significant difference in the mean vector between subjects in the two groups where the presence of both supplementation and sociality was examined – Nonsupplemented/Nonsocial, and Supplemented/Social. The p-value was considered statistically significant at p=0.025. No significance was found in post-hoc tests.

B. Mechanical Allodynia

Mechanical allodynia was tested using a 4.0 grams force von Frey monofilament to determine if increased cage complexity and sociality had an effect on this nociceptive behavior both before and after partial-sciatic nerve ligation (PSNL). Data is presented as a number out of 10 consecutive allodynic stimulations that elicited a positive response in Figures 4-6. Note that unlike data from Paw-withdrawal threshold (PWT), a lower bar on the graph is indicative of a decrease in nociceptive behaviors.

4. Two-Way ANOVA for Supplementation

A repeated measures two-way ANOVA was computed to determine if there was a significant difference in the mean vector between subjects in the two groups where the presence was supplementation was the only difference – Supplemented/Nonsocial, and

Figure 3: Paw Withdrawal Threshold (PWT) Value Comparing Interaction between Supplementation/Sociality

This figure shows a comparison of rats housed in Supplemented/Social and Nonsupplemented/Nonsocial cages. Paw withdrawal thresholds were found using von Frey filaments and employing the Up-Down technique. Data is presented for treatment groups on baseline, pre-treatment and post 7, 14, 21, 28, and 35 days. Values are represented as the mean ± SEM. Data was analyzed with a two-way ANOVA, yielding a p value of 0.025. Univariate testing indicated no significance at any time point, however a trend of increased PWT is seen in animals that were housed in supplemented/social cages. N = 5 rats/treatment group.

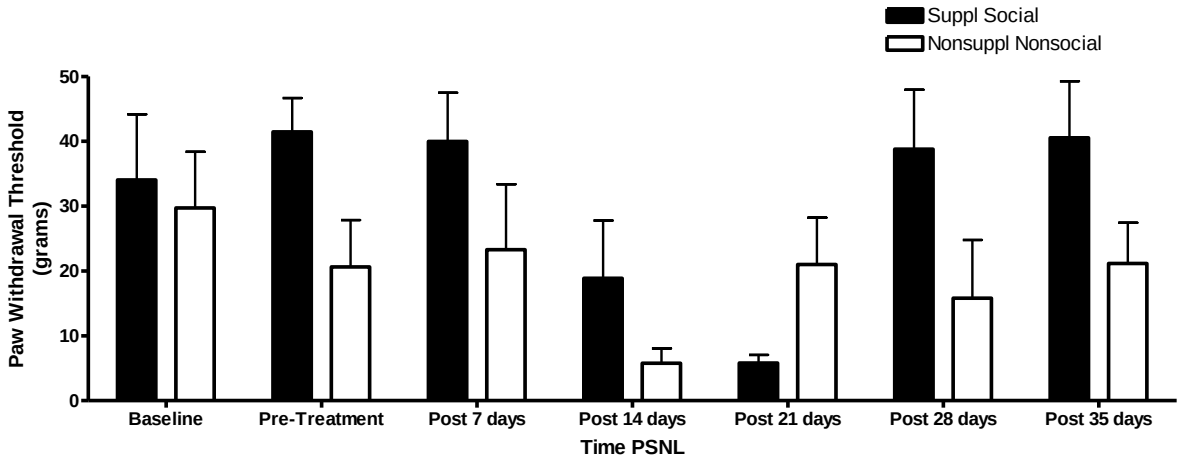
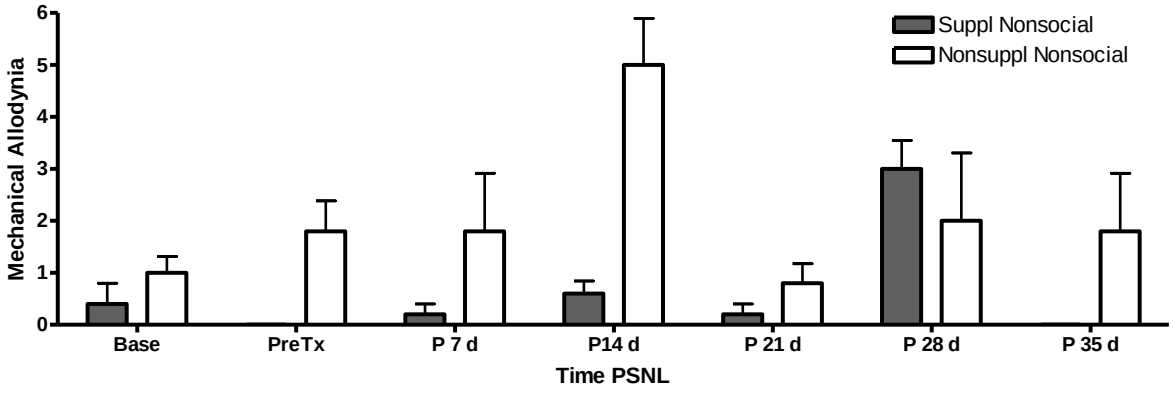

Suppl Social
Nonsuppl Nonsocial
Paw Withdrawal Threshold (grams)
50
40
30
20
10
0
Baseline
Pre-Treatment
Post 7 days
Post 14 days
Post 21 days
Post 28 days
Post 35 days
Time PSNL

Figure 4: Mechanical Allodynia Comparing Supplemented/Nonsupplemented

This figure shows a comparison between rats housed in Supplemented/Nonsocial and Nonsupplemented/Nonsocial conditions. Data was found using 4.0 grams/force von Frey filaments and applying them to the injured hindpaw ten consecutive times. Values represent how many times a paw-withdrawal was seen out of the ten allodynic stimuli. Values are represented as the mean ± SEM. Data was analyzed with a two-way ANOVA, yielding a p value of 0.056. Univariate testing was not employed due to no significance, however a trend of decreased allodynia is seen in animals that were housed in supplemented cages. Data is shown from treatment groups on baseline, pre-treatment and post 7, 14, 21, 28, and 35 days. N = 5 rats/treatment group.


Suppl Nonsocial
Nonsuppl Nonsocial
Mechanical Allodynia
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Base
PreTx
P 7 d
P14 d
P 21 d
P 28 d
P 35 d
Time PSNL

Nonsupplemented/Nonsocial. The p-value was determined not to be significant, but exhibited a trend of decreasing allodynia at p=0.056.

5. Two-Way ANOVA for Sociality

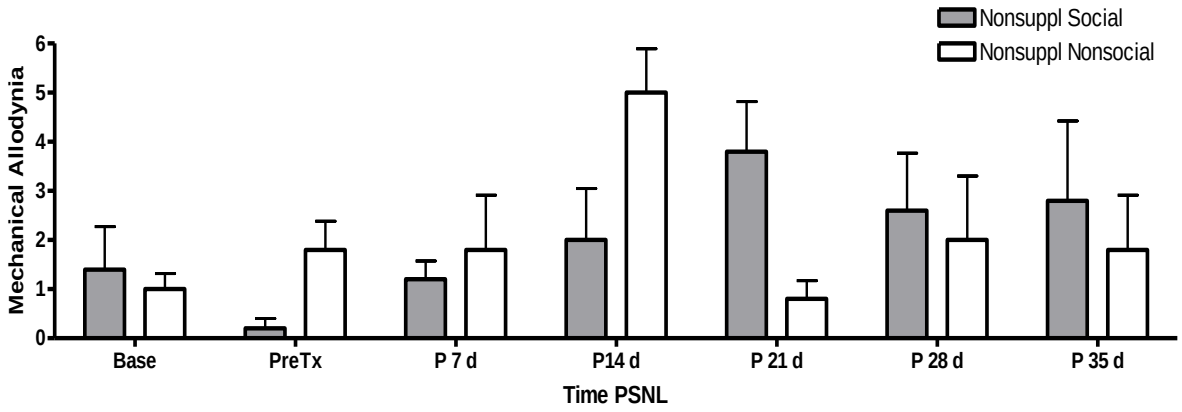
A repeated measures two-way ANOVA was computed to determine if there was a significant difference in the mean vector between subjects in the two groups where the presence of other animals in the housing was the only difference – Nonsupplemented/Nonsocial, and Nonsupplemented/Social. The p-value was not considered to be significant at p=0.98. Post-hoc tests were not examined.

6. Two-Way ANOVA for Interaction

A repeated measures two-way ANOVA was computed to determine if there was a significant difference in the mean vector between subjects in the two groups where the presence of both supplementation and sociality was examine – Nonsupplemented/Nonsocial, and Supplemented/Social. The p-value was not considered to be significant at p=0.07, but exhibited a trend of animals living in supplemented/social housing having a decrease in allodynia.

Figure 5: Mechanical Allodynia Comparing Social/Nonsocial

This figure shows a comparison between rats housed in Nonsupplemented/Nonsocial and Nonsupplemented/Social conditions. Data was found using 4.0 grams/force von Frey filaments and applying them to the injured hindpaw ten consecutive times. Values represent how many times a paw-withdrawal was seen out of the ten allodynic stimuli. Values are represented as the mean ± SEM. Data was analyzed with a two-way ANOVA, yielding a p value of 0.98. Univariate testing was not employed due to no significance. Data is shown from treatment groups on baseline, pre-treatment and post 7, 14, 21, 28, and 35 days. N = 5 rats/treatment group.


Nonsuppl Social
Nonsuppl Nonsocial
Mechanical Allodynia
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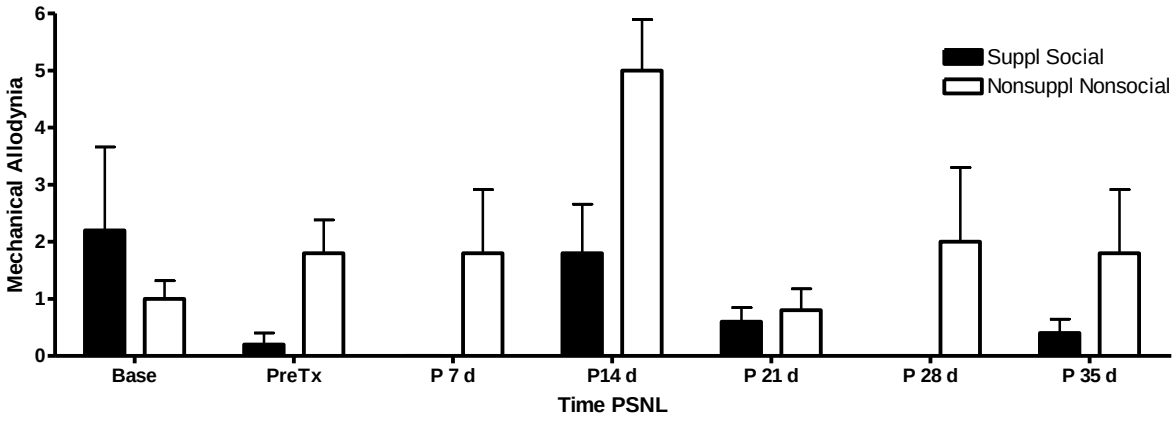
Base
PreTx
P 7 d
P14 d
P 21 d
P 28 d
P 35 d
Time PSNL

Figure 6: Mechanical Allodynia Comparing Interaction between Supplementation and Sociality

This figure shows a comparison between rats housed in Supplemented/Social and Nonsupplemented/Nonsocial conditions. Data was found using 4.0 grams/force von Frey filaments and applying them to the injured hindpaw ten consecutive times. Values represent how many times a paw-withdrawal was seen out of the ten allodynic stimuli. Values are represented as the mean ± SEM. Data was analyzed with a two-way ANOVA, yielding a p value of 0.07. Univariate testing was not employed due to no significance, however a trend of decreased allodynia is seen in animals that were housed in supplemented/social cages. Data is shown from treatment groups on baseline, pre-treatment and post 7, 14, 21, 28, and 35 days. N = 5 rats/treatment group.


Mechanical Allodynia
6
5
4
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0
Suppl Social
Nonsuppl Nonsocial
Base
PreTx
P 7 d
P14 d
P 21 d
P 28 d
P 35 d
Time PSNL

CHAPTER 4

IV. Discussion

Nociceptive behaviors can be influenced by both internal (genetic) and external (environmental) factors. By changing the environment in which an animal lives, one is capable of changing its pain perceptions. The two environmental variables that were studied in this investigation are sociality and environmental enrichment or supplementation. The presence of supplementation in an animal's housing allows it to exhibit more natural behaviors for that species such as hiding, gnawing and foraging. Supplementation has also shown to have positive effects on anxiety, cognition and recovery from brain lesions (Benaroya-Milshtein et al. 2004; Chamove 1989; Devenport et al. 1992). In relation to pain, supplementation has had the effect of increasing the antinociceptive effects of opioid drugs (Smith et al. 2003). Animals living in supplemented housing exhibited a greater sensitivity to opioid drugs than did animals living with no supplementation.

In addition to affecting behavior, supplementation has also been shown to alter physiology, namely by increasing dendritic branching in the brain, increasing the rate of neurogenesis in the dendrate gyrus of the hippocampus, increasing total brain weight, and increasing the number of synapses as well as neurotrophin levels (Henderson 1970; Greenough & Volkmar 1973; Ickes et al. 2000; Turner & Greenough 1985). Perhaps it is through these physiological alterations that supplementation ultimately affects behavior. It is important to note that this study, along with others, demonstrated that

supplementation only has affects on nociceptive behaviors after a type of trauma, not on baseline behavior.

Social interaction also plays a key role in an animals' environment. If an animal is housed in isolation, deleterious effects have been observed (Elliot & Grunberg 2005). Animals that were housed in isolated environments showed a decrease in open field locomotor performance compared to animals that were housed three/cage. This study supports the aforemented literature and suggests that animals living in supplemented and social housing have altered nociceptive behaviors, namely increased pain thresholds and decreased allodynia.

Before collecting data in relation to neuropathic pain, both baseline and pre-treatment data were collected from each housing group. Consistent with the literature, housing animals socially or in isolated conditions as well as providing them with or without supplementation did not statistically influence paw-withdrawal threshold (PWT) or mechanical allodynia during either baseline or pre-treatment. After post-sciatic nerve ligation (PSNL), however, animals housed in supplemented cages showed a decrease in allodynia and an increase in paw withdrawal threshold (PWT). It appears that the animals that were housed in supplemented housing had the most beneficial effects in regards to pain as compared to the animals that were only housed socially, with no supplementation. Although this general trend is observed, there was a statistically significant interaction between supplementation and sociality, suggesting that these two variables somehow work together to produce a result that either one alone could not.

It has been shown that stress and pain have a synergistic relationship – by increasing stress one sees an increase in pain response (Blackburn-Monro et al. 2001; Benefiel et al. 2005). Animals in one study were subjected to a stressor – a chronic-restraint, and were found to have increases in nociceptive behaviors (Gameiro et al., 2005). One way that supplementation can affect nociceptive behaviors is by altering corticosterone, adrenocorticotropic hormone and adrenaline responses in rodents (Fox et al. 2006). Environment seems to not completely diminish stress levels, as stress responses are still observed, but act as a buffer or a form of protection from excessive stress, most likely through modulation of the hypothalamic-pituitary-adrenal (HPA) axis. In a neuropathic pain model, such as the PSNL, stress and corticosterone levels increase, which may lead to anxiety and depression in the animal. It could be that supplementation acts by decreasing stress levels, and hence corticosterone, having the effect of minimizing the normal laboratory response of anxiety and/or depression models. Due to this, nociceptive behaviors would be improved.

Another way that supplementation could decrease stress is by giving the animal increased control over their environment. Supplementation allows the animals to organize their housing, giving them more control and decreasing their stress hormone levels (Wiepkeme & Koolhaas 1993). As mentioned before, supplementation increases rates of neurogenesis in the hippocampus. This brain region is heavily involved in emotionality and has been said to play a role in both the perception of fear and anxiety. Perhaps this increase in the rates of neurogenesis in the hippocampus is operating to decrease stress (Huttenen & Myers 1986).

Another mechanism through which supplementation alters stress levels is through regulation of the HPA axis (Larson et al., 2002). Animal models of chronic pain, such as the PSNL, act as an inescapable stressor, causing dysregulation of the HPA axis. Upon excessive stimulation, the normal negative feedback mechanisms of the HPA axis are disrupted (Blackburn-Munro 2001). This dysfunction of the HPA axis has been linked to an increase in likelihood for clinical depression, which can influence pain response. Clinically, patients with depression are more likely to score their pain as more severe than patients that do not have depression, suggesting that depression actually causes allodynia (Wilson et al. 2001). It could be that supplementation influences depression by regulating the HPA axis. A different mechanism through which supplementation could be improving pain response is by providing a form of exercise mild for the animal. Mild excersice has been shown to decrease nociception by decreasing stress levels (Tsatsoulis 2006). This study supports these general findings. Animals housed with supplementation had an overall decrease in nociceptive behaviors, through a means not clearly known but most likely related to a decrease in stress levels.

Whether or not an animal is housed socially or in isolation alters nociceptive behaviors. This study supports, through trends, the general positive influence that housing animals, particularly rats, socially has on behavior. In fact, studies have shown that animals housed in isolation actually have deleterious effects on behavior (Raber & Devor 1997). In these studies, the act of housing animals socially has had the effect of decreasing nociceptive behaviors. Since rats are social animals, living with other rats would have the effect of decreasing stress levels, hence nociceptive behaviors. This becomes interesting when looking at the data from this experiment, as there was a

significant interaction between sociality and supplementation. It appears that the act of housing rats socially and supplemented has a synergistic relationship, perhaps acting through a mechanism of decreasing stress hormones.

This study had the goal of determining if housing animals socially, as well as providing them with or without supplementation influenced nociceptive behaviors in a neuropathic pain model. Significant differences were found in mechanical allodynia following PSNL and trends were observed in PWT. These data suggest that supplementation greatly influences nociceptive behaviors, although there is an interaction between both supplementation and social interactions. The animals that were housed socially with supplementation exhibited the most beneficial behavioral responses.

In future studies, certain variables could be improved upon and further examined. It seems that stress-related hormones play a key role, through unknown mechanisms, in nociceptive behaviors. With that being said, it would be beneficial to the study to measure hormones such as corticosterone and adrenocorticotropic hormone throughout the experiment via blood testing. In addition, it may be helpful to expand the experiment beyond 35 days after PSNL to more clearly examine the effects environment has on behavior. Although trends were observed in PWT, significance was not found although increasing each groups' cohort size would decrease variability and ultimately lead to statistical significance. In relation to depression, it may be helpful to measure additional behavioral variables and see how closely an animal's behavior after nerve ligation compares to a depressed animal model. This could give insight into a more complete mechanism through which supplementation influences nociception. Another factor that could be examined of, is the degree to which the animals moved around the toys that

were placed in their cages, determining if supplementation actually does increase feelings of control, hence decrease stress and nociception. Another variable that may influence behavior on top of sociality and supplementation is ability to exercise. The supplementation can be seen as giving the animal mild exercise, which is beneficial, although an increase in exercise could have deleterious effects. In this particular neuropathic pain model, it would have been helpful to examine the nerve ligation after sacrifice to determine if the suture was still in place or not.

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Appendix A: Animal Use Approval Form

Institutional Animal Care and Use Committee of Youngstown State University of approval to use animal subjects throughout the course of this master thesis research project.

Youngstown
STATE UNIVERSITY

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Monday, February 04, 2008

Dr. Jill Gifford
Department of Biology
UNIVERSITY

Re: IACUC Protocol # 01-08
Title: Effects of Housing Conditions on Nociceptive Behaviors
Approval date: January 31, 2008 Expiration date: January 31, 2011

Dear Dr. Gifford:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration titled **"Effects of Housing Conditions on Nociceptive Behaviors"** and determined it should be unconditionally approved for the period of **January 31, 2008** through its expiration date of **January 31, 2011**.

This protocol is approved for a period of three years; however, it must be updated once a year via the submission of an Annual Review-Request to Use Animals form ***prior*** to its yearly anniversary date of January 31, 2009 and January 31, 2010. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Sincerely,

[redacted]

Dr. Peter J. Kasvinsky
Associate Provost for Research
Research Compliance Officer

PJK:dka

C: Dr. Walter Horne, Consulting Veterinarian, NEOUCOM
Dr. Robert Leipheimer, Chair IACUC, Chair Department of Biological Sciences
Dawn Arnolsch, Animal Tech., Biological Sciences
Cheryl Coy, Grants and Sponsored Programs