Effects of Social Housing on Conditioned Place Aversion

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ABSTRACT

Although pain represents a source of considerable loss to both economic productivity and quality of life for millions of people worldwide, its underlying causes and sources of modulation are not always well understood. The present study sought to identify differential changes in behavior in response to long-lasting inflammation among rats housed in groups consisting of diverse ratios of inflamed to uninflamed individuals. Behavioral changes were quantified using a model of conditioned place aversion (CPA), with an intraplantar injection of complete Freund's adjuvant (CFA) producing inflammation in the left hindpaw for use as a noxious, conditioning stimulus. It was hypothesized that housing with uninjured cagemates might reduce the effect of conditioning in injured individuals, or vice versa. Persistent inflammation was induced in the CFA-injected individuals, and significant CPA was achieved between baseline and day 15 among subjects on average. However, significant differences were not found in developed place aversion between CFA- and saline-injected (control) individuals, nor was there evidence of a significant difference in conditioning between different housing groups. The conditioning power of inflammation is believed to have been masked by the initial stress of anesthesia and injection. Further analysis of the data revealed the existence of a "floor effect", whereby subjects only developed significant aversion if the noxious stimulus was paired with the specific floor of the conditioning apparatus that was initially preferred at baseline. No additional aversion was measured in response to conditioning on the initially aversive, non-preferred floor. This effect can be linked to the biased design of the CPA apparatus, in which subjects exhibited a significant mean preference for the "circle" floor at baseline compared to the "square" floor.

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Chapter 1

I. Introduction

A. Pain

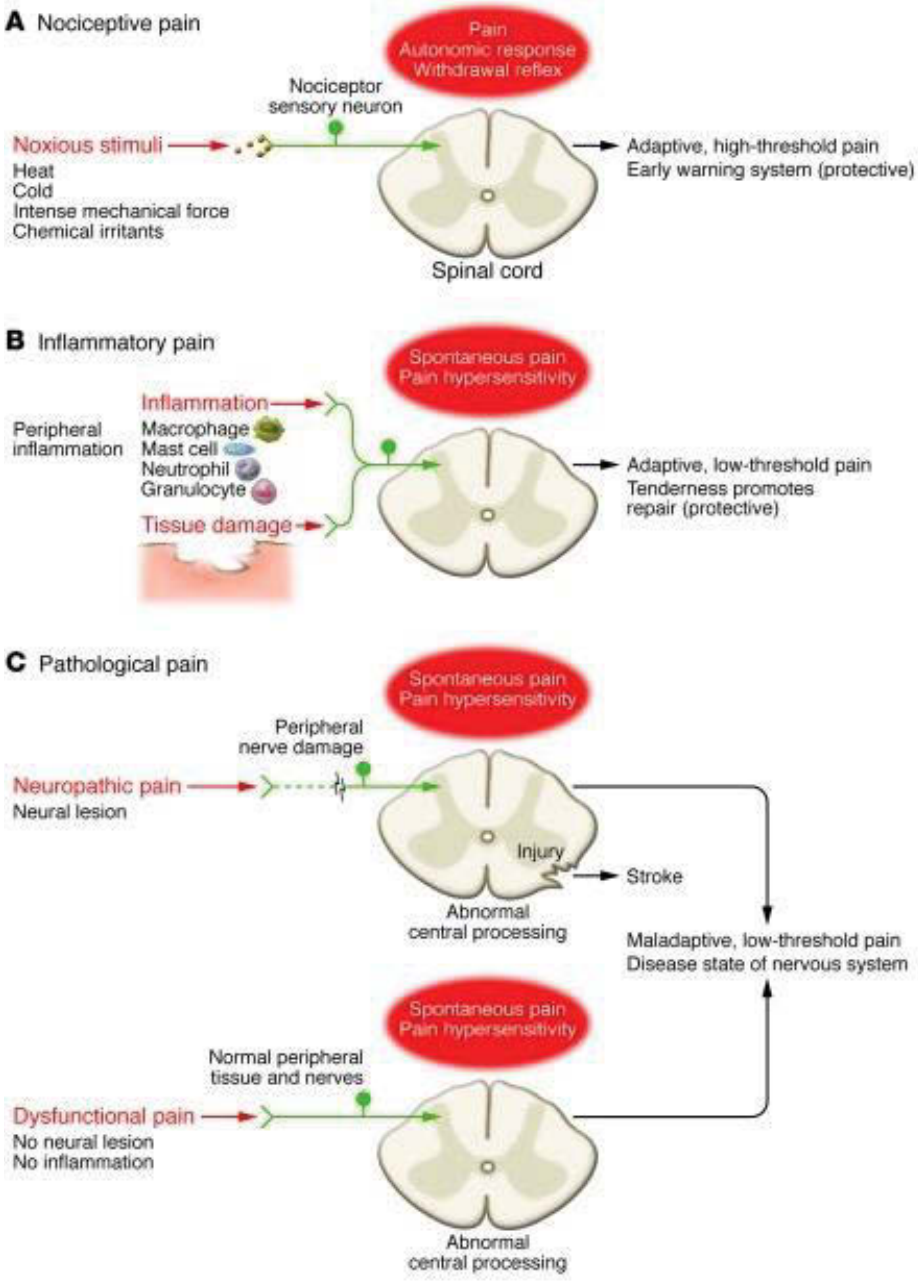
In his 2010 article published in *The Journal of Clinical Investigation*, Clifford J Woolf asks the question "What is this thing called pain?" (Woolf, 2010). On its surface, the question seems obvious; after all, to any organism experiencing it, pain represents an immediately recognizable sensation whose resolution often consumes present thought. However, defining pain and its specific causes in descriptive terms is not so intuitive. Pain can last for moments or years, originate from wounds both physical and emotional, and have causes that are obvious or apparently nonexistent. What is perhaps the most widely cited definition of pain was developed by The International Society for the Study of Pain, which states that pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP Taxonomy). The crux of this definition, and indeed any definition of pain, is the fact that, no matter its other characteristics, pain is unpleasant. This unpleasantness has consequences which affect not just individual health, but many other aspects of society. For instance, one study concluded that the treatment of and productivity lost due to pain cost the United States over $600 billion dollars in 2008. The study also found that 100 million American adults were affected by persistent pain in that same year (Gaskin & Patrick, 2012). Because of the widespread, negative influence of pain on health and the huge costs associated with its treatment, pain research is and will remain an area of profound medical importance.

In his aforementioned article, Woolf groups pain into three separate categories:

nociceptive pain, inflammatory pain, and pathological pain (Woolf, 2010; Fig. 1). Nociceptive pain serves as a protective and adaptive mechanism by providing an intense, rapid reaction to noxious stimuli such as tissue damage or extreme heat or cold. This type of pain is immediately brought to the forefront of attention in order to minimize tissue damage resulting from exposure to the pain-inducing stimulus. The second type of pain, inflammatory, is also adaptive and often follows nociceptive pain. Inflammatory pain is aptly named, as it is triggered by inflammation generated mainly by the immune system in response to infection or tissue damage. Once activated, inflammatory pain causes hypersensitivity in the affected tissue, thereby discouraging further damage and promoting healing. Unlike the first two types of pain, the third form does not serve an adaptive purpose; rather, pathological pain results from dysfunction or damage in the nervous system. Because there is no specific stimulus triggering pathological pain, the cause of this pain is often unknown. However, pathological pain is believed to result from sensitization in the peripheral and/or central nervous systems (PNS & CNS, respectively) and a resultant lowering of the threshold necessary to generate a pain response (Latremoliere & Woolf, 2009). While this is sometimes due to explicit nerve damage, at other times it occurs through nervous system dysfunction following the subsidence of an inflammatory response to tissue damage. Regardless of the type, all pain is dependent on the same basic processes occurring in and around neurons.

Figure 1: The Three Types of Pain

A) Nociceptive pain is an acute response to a noxious stimulus, which triggers a withdrawal reflex in order to prevent tissue damage. B) Inflammatory pain is slower in onset and longer in duration than nociceptive pain. It is brought on through the release of chemical signals by immune cells or damaged tissue in order to lower pain threshold and promote healing. C) Unlike with the first two types of pain, pathological pain does not serve an adaptive role, instead resulting from neurological damage or chronic dysfunction. Adapted from “What is this thing called pain?” (Woolf, 2010).


A Nociceptive pain
Pain
Autonomic response
Withdrawal reflex
Nociceptor
sensory neuron
Noxious stimuli
Heat
Cold
Intense mechanical force
Chemical irritants
Adaptive, high-threshold pain
Early warning system (protective)
Spinal cord
B Inflammatory pain
Spontaneous pain
Pain hypersensitivity
Inflammation
Peripheral
inflammation
Macrophage
Mast cell
Neutrophil
Granulocyte
Tissue damage
Adaptive, low-threshold pain
Tenderness promotes
repair (protective)
C Pathological pain
Spontaneous pain
Pain hypersensitivity
Peripheral
nerve damage
Neuropathic pain
Neural lesion
Injury
Stroke
Abnormal
central processing
Maladaptive, low-threshold pain
Disease state of nervous system
Spontaneous pain
Pain hypersensitivity
Normal peripheral
tissue and nerves
Dysfunctional pain
No neural lesion
No inflammation
Abnormal
central processing

B. Neurobiology of Nociception

1. Neuronal Signal Transduction

Before the brain can perceive the sensation of pain, noxious stimuli must first be converted into a signal capable of traveling through the PNS and into the CNS. The initial step in this process occurs through the activation of specific nociceptive neurons appropriately called nociceptors. The nerve endings of these cells can be found in the skin, as well as in deeper tissues such as viscera and muscle. Nociceptors, as with other neurons, have a resting membrane potential, or a difference in electrochemical charge between the inside and outside of the cell. This resting membrane potential (-60 to -70 mV) is the result of the differential concentrations of several ions in and around the cell, most notably sodium (Na^+, higher outside the cell) and potassium (K^+, higher inside the cell; Purves et al., 2001). The necessary gradient of these two ions is maintained by the Na^+/K^+ ATPase pump, which continuously transports 3 Na^+ out of the cell and 2 K^+ into the cell. Because the cell membrane is much more permeable to K^+, there is a constant flow of K^+ out of the cell along its concentration gradient. This flow of K^+, along with the concentrations of other intra- and extra-cellular ions, results in the resting membrane potential (Purves et al., 2001). Short-term alterations to the membrane permeability of specific ions, which change the membrane potential, serve as the basis of neuronal communication.

Noxious stimuli come in three general forms: mechanical, thermal (heat or cold), and chemical, none of which can be directly perceived by the CNS (Purves et al., 2001). Therefore, the noxious stimuli must be transduced into an electrical signal through alteration of the membrane potential. Nociceptors have free nerve endings which express

a variety of receptors specific to noxious stimuli. When exposed to a particular noxious stimulus, receptors specific to that stimulus are activated and open ion channels, allowing Na^+ to flow into the cell. This increase in positive charge causes a local depolarization (movement toward 0 mV) of the cell membrane potential. When the noxious stimulus is sufficient to depolarize the membrane at the axon hillock to threshold, voltage-gated Na^+ channels in the cell membrane open, resulting in a large depolarization of the cell known as an action potential (Purves et al., 2001).

After transduction of the noxious stimulus into an electrical stimulus, the resulting action potential travels along the axon of a nociceptive neuron toward the CNS. These axonal fibers are associated with two types of nociceptors; the first, referred to as Aδ fibers, are lightly myelinated and have a rapid conduction speed of about 20 meters per second (m/s). These fibers are responsible for the transmission of sharp, immediate pain, primarily resulting from mechanical or thermal noxious stimuli. The other type, called C fibers, are unmyelinated, and therefore have a slower conduction velocity of less than 2 m/s (Purves et al., 2001). These fibers transmit more generalized, burning pain signals from all types of noxious stimuli. While the nerve endings of Aδ fibers can have more than one type of receptor, C fibers always do, and are therefore referred to as polymodal nociceptors. Both nociceptor fiber types have their neuron cell bodies located in the dorsal root ganglia (Purves et al., 2001).

Upon reaching the axon terminal within the CNS, the depolarization induced by the action potential opens voltage-gated calcium ion (Ca^{2+}) channels. The subsequent influx of Ca^{2+} into the axon terminal causes the fusion of synaptic vesicles containing neurotransmitter with the plasma membrane. Neurotransmitter is thereby released into the

synaptic cleft, where it binds to receptors located on the postsynaptic cell and causes a chemical or electrical change within the target cell. In this way, signals are communicated between neurons. While there are myriad varieties of neurotransmitter, glutamate is the primary excitatory neurotransmitter, and thus is paramount in the electrochemical communication of nociception. Glutamate binds to both ionotropic and metabotropic receptors on the postsynaptic cell (Latremoliere & Woolf, 2009). Ionotropic receptors mediate intracellular changes by acting directly as an ion channel, whereas metabotropic receptors effect change through a second messenger. In regard to nociception, ionotropic receptors are most important for relaying of the afferent signal, whereas metabotropic receptors are more important for neuronal modulation and plasticity. Ionotropic glutamate receptors are divided into three subtypes: N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainate receptors. The NMDA receptor is primarily a Ca^{2+} channel, while the other two are primarily Na^{+} channels (Latremoliere & Woolf, 2009). While all three of these receptors produce excitatory postsynaptic effects, typically only the AMPA and kainate receptors produce a strong initial response after glutamate release. This is because the NMDA receptor is both ligand- and voltage-gated. After glutamate binding, the NMDA ion channel is still blocked by a magnesium ion (Mg^{2+}) plug. However, with sustained postsynaptic depolarization, the Mg^{2+} will dissociate from the ion channel, allowing entry of Ca^{2+} into the cell. As a result of the unblocking of the NMDA receptor ion channels, the postsynaptic neuronal membrane requires less additional stimulation by glutamate to trigger subsequent action potentials. Therefore, the cell is now described as hypersensitive because it relays a nociceptive signal in response to the release of less

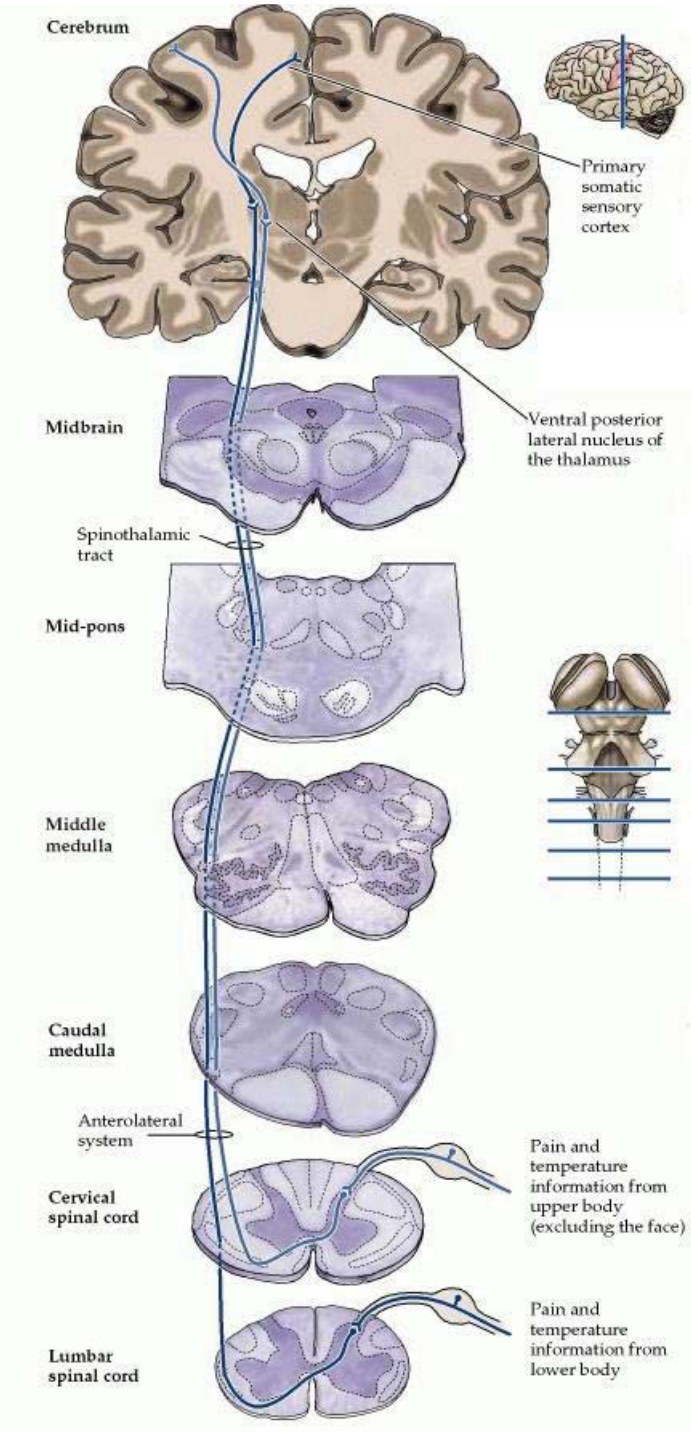
glutamate (Basbaum et al., 2009; Latremoliere & Woolf, 2009).

2. Anterolateral System

The axons of both nociceptive fiber types terminate in the gray matter of the dorsal horn of the spinal cord, which is organized into distinct regions called laminae. Aδ fibers terminate specifically in laminae I and V, whereas C fibers terminate in laminae I and II (Basbaum et al., 2009). In the laminae, axon terminals synapse with the dendrites of second order neurons, called spinothalamic tract neurons. Glutamate is released from the nociceptor axon terminal and binds to the aforementioned receptors on the spinothalamic neurons, causing an excitatory postsynaptic potential. The axons of the second order neurons then decussate within the spinal cord and ascend somatotopically to the brain in the anterolateral quadrant of the spinal cord (hence the other name for this CNS pathway, the anterolateral system). Afferent signals originating lower in the body ascend more laterally, while signals from higher in the body (but below the head) ascend more medially (Basbaum et al., 2009). After traveling through the brain stem, spinothalamic tract neurons synapse on third order neurons located in the ventral posterior lateral nucleus of the thalamus. Axons from these third order thalamocortical neurons then terminate in the primary somatosensory cortex of the parietal lobe. This is the chief pathway responsible for carrying information regarding pain location and intensity to the brain (Fig. 2). However, other pathways located within the anterolateral system, such as the spinoreticular and spinomesencephalic tracts, also convey nociceptive information. These tracts terminate in the pontine reticular formation and the periaqueductal gray of the tegmentum respectively, and mediate general cortical arousal in response to pain and activation of descending pain modulation pathways (Lynn, 1992).

Figure 2: The Spinothalamic Tract

Nociceptive fibers enter the dorsal horn of the spinal cord, where they synapse onto second-order neurons. The axons of these second-order neurons immediately decussate before traveling up the anterolateral portion of the spinal cord to terminate in the thalamus, where they synapse onto third order neurons. These then project to the primary somatosensory cortex. Adapted from "Neuroscience 2nd Edition" (Purves et al., 2001).


Cerebrum
Primary somatic sensory cortex
Midbrain
Ventral posterior lateral nucleus of the thalamus
Spinothalamic tract
Mid-pons
Middle medulla
Caudal medulla
Anterolateral system
Cervical spinal cord
Pain and temperature information from upper body (excluding the face)
Lumbar spinal cord
Pain and temperature information from lower body

3. Inflammatory Pain

Up to this point, the pain-inducing stimulus activating the nociceptive pathway has been assumed to be of external origin. However, this is applicable only to nociceptive pain. A second, related type of pain, called inflammatory pain, has its own internal triggers which often follow the subsidence of nociceptive pain. When tissue damage has occurred, nearby immune cells and nociceptors, as well as the damaged tissue itself, release a complex mélange of chemicals which result in the inflammatory response (Basbaum et al., 2009). This response is characterized by pain, swelling, and loss of function, with the overall goal of promoting tissue repair and healing. Inflammation can also be triggered through the activation of immune cells by foreign agents, such as bacteria. The number of factors known to play a role in the genesis and modulation of inflammation are legion, but many of them are similar in that they bind to nociceptors to elicit hypersensitivity (Basbaum et al., 2009). Furthermore, activation of nearby nociceptors results in the release of additional chemical factors that reinforce inflammation. One such example of a factor released by nociceptors is the neuropeptide substance P. While substance P is produced and released by C fiber nociceptors in response to many different stimuli, it is of particular importance to the inflammatory response (Latremoliere & Woolf, 2009). Pro-inflammatory endogenous factors such as nerve growth factor (NGF) have been shown to bind to nociceptors and increase both the expression and release of substance P. After release, substance P acts on other cells to promote swelling, vasodilation, and immune cell activation, as well as to nociceptors themselves to cause hypersensitization. Specifically, substance P binds to neurokinin-1 metabotropic receptors, triggering a rise in intracellular levels of the second messengers

inositol triphosphate and diacylglycerol through the activation of phospholipase C. These second messengers stimulate the release of intracellular stores of Ca^{2+}, depolarizing the cell (O'Connor et al., 2004). This depolarization also aids in the activation of NMDA receptors by facilitating the removal of the Mg^{2+} plug (Latremoliere & Woolf, 2009). Together, these effects elicit the lowering of the nociceptive response threshold and the subsequent protection of the injured tissue from further exposure to injury-inducing stimuli. Behaviorally, this increased sensitivity to potentially noxious stimuli is termed hyperalgesia.

4. Pathological Pain

As mentioned previously, both nociceptive and inflammatory pain serve the adaptive function of avoiding tissue damage. In the case of nociceptive pain, a rapid, intense pain signal elicits a matching response, helping to limit initial bodily harm. Inflammatory pain, on the other hand, seeks to limit further harm by evoking behavioral changes in regard to the affected area. The final type of pain, pathological pain, is different from the first two types in that it has no adaptive purpose. Rather, it arises from damage to neurons themselves or from neuronal dysfunction following sensitization due to tissue damage. A well-known example of pathological pain due to nerve damage is phantom limb pain, in which a patient senses pain localized to an amputated body part (Purves et al., 2001). While the cause of nerve damage is often obvious, the cause of pathological pain due to nerve dysfunction is often unknown. Following tissue damage, inflammation results in a lowered action potential firing threshold in nearby peripheral and/or spinal neurons. However, this change in threshold sometimes fails to reverse itself after the inflammation has subsided, resulting in continued hypersensitivity to noxious

stimuli or even a sensation of pain in response to normally benign stimuli, a condition known as allodynia. The exact mechanisms for this pathological sensitization are unknown, but numerous receptors have been implicated in its onset, including NMDA, AMPA, metabotropic glutamate, and substance P receptors (Latremoliere & Woolf, 2009).

C. Effect of External Factors on Pain

While pain is generally thought of as an internal response to a stimulus, external factors can in fact have a significant role in both pain's development and resolution. Examples of external variables associated with pain include stress, mood, social interactions, and environmental enrichment. The influences of these psychological, social, and environmental influences have been examined most thoroughly in preclinical studies, but have also been demonstrated to a lesser extent in clinical investigations. Nevertheless, the reality of the phenotypic behaviors associated with pain response being an amalgamation of not only internal biology (genotype) but also external factors (environment) is well established by relevant literature.

Mood and stress are perhaps the most intuitive factors affecting nociception, as they often have obvious origins in and/or effects on the CNS. For instance, one study demonstrated that stress induced through a forced swim test in rats caused an increase in both immediate (30 minutes) and delayed (48 hours) nociception following hindpaw injection with formalin, a model of nociceptive pain (Quintero et al., 2003). Furthermore, this study showed that there were concurrent changes in gene expression among the sensory spinal neurons of rats with induced hyperalgesia, but not in sham-injected or naive rats. Another study demonstrated the effect of stress on rats with carrageenan-induced localized inflammation, finding that each consecutive round of non-nociceptive

stress caused correspondingly greater increases in hyperalgesia in these rats (Rivat et al., 2007). The correlation between stress and pain is not limited to animal models, as several clinical studies have yielded similar results. A review of stress on healing concluded that increases in stress were correlated with longer healing times, which were themselves positively correlated with increased sensations of pain (Solowiej et al., 2009). Another study compared the incidence of pain in the year following psychological distress related to new employment, concluding that increased distress was predictive of musculoskeletal pain across several sites (Nahit et al., 2003). A different study concluded that increased stress and "daily hassles" are associated with several common pains, including headache, backache, and stomachache (Sternbach 1986). Finally, positive mood has been associated with decreased same-day and subsequent-day pain in patients with sickle cell disease (Gil et al., 2004).

A second external factor influencing the course of nociception can be found in the state of the physical environment surrounding the subject in question. In rat models, studies comparing standard housing conditions with enriched housing conditions have shown that environmental enrichment through introduction of wheels, tunnels, and other interactive objects decreases the duration of hypersensitivity following carrageenan-induced knee inflammation and complete Freund's adjuvant-induced hindpaw inflammation (Gabriel et al., 2010; Tall, 2009). These changes in nociception were not due to changes in anxiety-like behavior as measured by the elevated plus maze test between housing groups. Similar results have been demonstrated in mice with surgically-induced chronic pain. Three months after surgical nerve injury, mice housed in enriched conditions demonstrated significantly reduced mechanical and cold hypersensitivity as

well as reduced levels of substance P compared to mice housed in impoverished conditions (Vachon et al., 2013). Clinical research into housing environment has been less conclusive, but there is some evidence that this has an effect on patient outcomes. A systematic review found that factors such as access to sunlight, windows, and pleasant odors showed predominantly positive influences on patient well-being (Dijkstra et al., 2006). Another study examined the effect of being on the bright or dim side of a hospital wing in 89 spinal surgery patients, concluding that the patients exposed to greater amounts of sunlight exhibited reduced stress, pain, hourly analgesic use, and overall pain medication cost (Walch et al., 2005). Although these studies do not represent conclusive evidence on their own, they are supported by the more robust findings of preclinical research.

Of greatest pertinence to this study, nociception has also been shown to be influenced by the effect of social interaction. In addition to the previously discussed effect of environmental enrichment, social enrichment was likewise correlated with a decrease in the duration of hypersensitivity (Gabriel et al., 2010). Furthermore, following surgery, social housing exhibits benefits compared to individual housing. Mice that had undergone surgery and were subsequently housed socially displayed smaller changes in heart rate and behavior compared to mice housed individually (Van Loo et al., 2007). Recovery from a severe spinal cord injury has been shown to be positively influenced by social enrichment as well. In fact, socially housed animals displayed far less gray matter loss in the injury area, possessed higher levels of brain-derived neurotrophic factor, and had reduced mechanical hypersensitivity than animals housed alone (Berrocal et al., 2007). In regard to human pain, social factors are less well understood, although there is

evidence that social support reduced pain reported by women with breast cancer (Goodwin et al., 2001).

Interestingly, the transfer of lesion-specific nociceptive changes has been demonstrated in sham-lesioned rats that are housed with lesioned littermates. Rats which underwent a sham operation adopted the specific changes in pain threshold associated with the particular lesion administered to their cagemates. N-acetyl-l-aspartyl-l-glutamate (NAAG) and quinolinic acid (QUIN) represent two NMDA receptor agonists with different mechanisms of action. Hippocampal lesions with NAAG in newborn rats have been shown to result in increases in both tail-flick and paw withdrawal latencies later in life. Conversely, hippocampal lesions induced through the administration of QUIN have been shown to produce slightly decreased latencies in these nociceptive measurements. Sham-lesioned rats housed with NAAG-lesioned rats exhibited the same increases in hindpaw pain threshold as did cagemates which had actually been lesioned. Likewise, sham-lesioned rats housed with QUIN-lesioned rats adopted the unique changes in hindpaw pain threshold which matched their cagemates' lesion-induced changes (Yamamotova et al., 2007). Paw withdrawal threshold is not the only behavioral response shown to be transferable between familiar rats; indeed, another study demonstrated that pre-exposure to a fear-conditioned rat facilitated subsequent learning, exploratory behavior, and aversive conditioning in naive rats (Knapska et al., 2010). Knapska speculates that this transfer of fear and subsequent arousal could represent a basic form of empathy. Apparent displays of empathy have also been demonstrated more recently in rats given the ability to free a cagemate from imprisonment, regardless of strain (Bartal et al., 2014). The present study seeks to reproduce the effect of social interaction with

conditioning using conditioned place aversion (CPA) through induced hindpaw inflammation.

D. Conditioned Place Aversion

Conditioned place preference began as a model used to quantify the rewarding effects of a particular stimulus. This is accomplished by pairing the reward with a specific context and then examining the effect of pairing on preference for that context after removal of the reward. The modern version utilizing differences in time spent between compartments has been applied to a wide variety of substances since the initial development of this model (Rossi & Reid, 1976). While the conditioned place model was originally designed for measuring preference, later studies began to use it for examining aversion to noxious stimuli. The earliest studies applying this model of aversion to pain did not appear until the late 1980s, and it was not until the early 2000s that significantly increased interest in this method of pain research began to appear in the literature (Tzschentke, 2007). One of the most often-used forms of conditioned place aversion (CPA) involves inducing inflammation in the plantar region of the hindpaw as the aversive stimulus. This methodology has yielded several important insights regarding the mechanisms and modulation of nociception. One study utilizing this method showed that inflammation reduced the reward generated by morphine administration by decreasing activation of limbic area opioid systems (Narita et al., 2005). Furthermore, Johansen et al. demonstrated that CPA could be abolished by a lesion of the rostral anterior cingulate cortex, even though the lesion did not alter nocifensive behaviors (Johansen et al., 2001). The role of glutamate and its receptors has also been examined with inflammatory CPA. Glutamate and NMDA receptor antagonists injected into the anterior cingulate cortex

eliminated inflammation-induced CPA, whereas an AMPA/kainate receptor antagonist did not (Johansen & Fields, 2004; Lei et al., 2004). More recently, research regarding the periaqueductal gray has shown its importance in CPA as well (Sun et al., 2014). These findings, along with many others not mentioned, display the worth of the inflammatory model of CPA in rats in regard to the interactions between pain, conditioning, and neurobiology.

E. Specific Aims

The purpose of the present study was to determine if and to what extent co-habitation with injured subjects influenced the development of conditioned aversion. To accomplish this, a model of conditioned place aversion through complete Freund's adjuvant (CFA)-induced inflammation was employed. Conditioning occurred from inflammation onset through recovery, while periodic behavioral measurements examined any effect of housing group on development of aversion. The experiment sought to determine the following: first, whether CFA-induced pain affected conditioning between control groups (groups housed with all CFA- or all saline-injected subjects); second, whether CFA-induced conditioned aversion was reduced in subjects housed within a healthy social group or vice versa; and third, to what extent the first two questions were true across varying ratios of healthy to inflamed group members. Results were expected to offer evidence either for or against the transfer of inflammation-induced conditioned aversion between cagemates, potentially indicating empathetic behavior.

Chapter 2

II. Materials & Methods

A. Animals

All research was conducted using male Sprague-Dawley rats obtained from Charles River Laboratories (Wilmington, MA). Upon arrival, rats were 28-32 days old and weighed approximately 100 g. Two sets of 24 rats were used during the course of the study, with a total $n = 48$. Rats were kept on a 12/12-hour light/dark cycle, with lights off at 10:00 AM and lights on at 10:00 PM. Both the animal housing facility and the research laboratory were kept at a constant 20° ± 1° C. Food (Lab Diet 5P00 Prolab RMH 3000 PMI Nutrition International, Brentwood, Missouri) and water were supplied *ad libitum*.

B. Housing Conditions

All rats were housed within polycarbonate enclosures measuring 20" long x 16" wide x 8½" tall, with four rats per home cage. Along with the aforementioned food and water, each enclosure was supplied with bedding consisting of aspen pine shavings, which was changed weekly. Following arrival at the animal facility, all rats were randomly assigned to one of five test groups (Fig. 3) by the animal care technician and allowed to acclimate to their new environment for one week before handling.

C. Conditioned Place Aversion Box

Behavioral data were collected using a conditioned place aversion (CPA) box (Fig. 4). The CPA box consisted of a metal rectangular box, 24” long x 12” wide x 12” tall, with rigid, clear plastic sheets nestled within the metal walls of the box. In the center of the box, a divider made of two plastic sheets could be raised or lowered in order to separate the box into two equally-sized compartments. One of two distinct patterns (red

squares or orange stripes) was placed between the plastic sheets and the metal walls of each room with the aim of providing a visual cue distinguishing one room from the other. In addition, each of the two rooms within the box had its own distinct flooring, with one consisting of a metal mesh supported by a square grid (referred to as the "square" side) and the other consisting of a black plastic plank filled with small circular holes (referred to as the "circle" side). This served to further distinguish each room from the other by providing a tactile cue. Metal screens were placed over the tops of the rooms in order to prevent the rats from climbing out during conditioning.

D. Baseline Behavioral Assessment

Rats were habituated to the laboratory and investigators during three consecutive days of handling (5 minutes per rat per day) in the week following acclimation to the animal facility. One day prior to injection (day -1), baseline behavioral data were collected for each rat using the CPA box. Each rat was placed within the starting room with the center divider raised and allowed to freely roam between the two rooms for 10 minutes (600 seconds). Time spent in each room was carefully recorded, with a transition between rooms being designated by the crossing of all four paws over the center line. This represented a baseline measurement for preference between the two rooms. The box was wiped down with a solution of 70% ethanol between each rat. All rats were initially placed in the circle side of the box (starting room) during behavioral measurements on days -1, 15, and 50. This procedure of data collection was repeated on days 15 and 50 post-injection.

F. Inflammation Model

Under brief isoflurane inhalation anesthesia (3%), an intraplantar injection (100 µl)

of 100% complete Freund's adjuvant (CFA) or 0.9% sodium chloride (NaCl, control) was administered to the left hindpaw in the area located on the plantar surface between the foot pads. Depending upon the assigned housing group, each home cage contained between 0 and 4 rats subjected to injection with CFA and subsequent development of inflammation (Fig. 3).

G. Edema Qualification

Dorso-ventral paw thickness measurements were made using a caliper in order to assess the degree of edema in the hindpaw. Data were collected immediately prior to anesthesia and intraplantar injections at day 0 and prior to conditioning at weekly time points post-injection through day 49.

H. Conditioning

Equal numbers of CFA- and saline-injected rats were randomly assigned for aversive conditioning within only the square or circle side of the CPA box (n = 24 each). Following injection, each rat was placed within its assigned conditioning room (square or circle) for 30 minutes. The center divider was lowered during this time, preventing movement between rooms. For each rat, the 30-minute trial was repeated weekly through day 49, following recording of weight and paw thickness (Fig. 5).

E. Conditioned Aversion Measurements

On days 15 and 50 post injection, the aforementioned behavioral assessment procedure was repeated for each rat and times were similarly recorded. By measuring the change in the amount of time spent in each rat’s assigned conditioning room over the course of the study, the effect of conditioning was determined.

Figure 3: Treatment Distribution by Group

The number of rats per home cage within each treatment group which received hindpaw inflammation through injection with CFA and the number which received an injection of a saline control. This distribution was repeated for each cohort, with two E groups within each, for a total $n = 48$.

Treatment Group	Total Rats per Home Cage	Number of Rats with Localized Hind Paw Inflammation per Home Cage	Number of Rats without Localized Hind Paw Inflammation per Home Cage	Total Number of Rats in Each Treatment Group
C (100% CFA)	4	4	0	8
D (75% CFA)	4	3	1	8
E (50% CFA)	4	2	2	16
F (25% CFA)	4	1	3	8
G (0% CFA)	4	0	4	8

Figure 4: The Conditioned Place Aversion Box

All conditioning and behavioral measurements were conducted using the conditioned place aversion box, shown in this photo with the center divider down. The left room utilized red as a visual cue and a metal screen floor as a tactile cue (referred to as the "square" floor), whereas the right room had orange visual cues and a plastic floor (referred to as the "circle" floor).

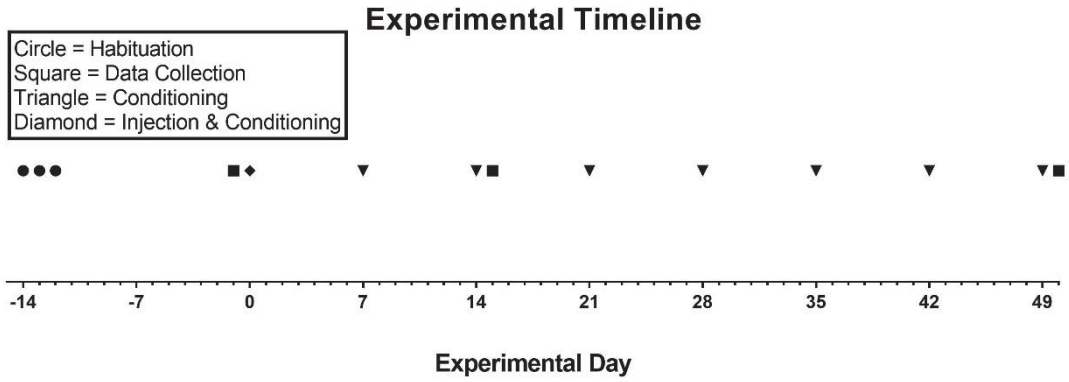
Square Floor **Circle Floor**

Figure 5: Experimental Timeline

A. Timeline of the procedures carried out during the course of the study. Circles represent habituation, squares represent behavioral data collection, triangles represent conditioning sessions, and the diamond represents day 0, which involved injection and initial conditioning.

B. Table listing the specific of days, actions, and measurements for each procedure.

A.



B.

Experimental Stage	Experimental Day	Procedures and/or Measurements
Habituation	Day -14, -13, -12 (14, 13, and 12 days prior to injection)	❖ Introduction to the behavioral testing laboratory and handling by investigators for three consecutive sessions ❖ Each home cage is randomly assigned to treatment group C – G, N=4 rats per cage
Pretreatment Data Collection	Day -1 (One day prior to injection)	❖ Pretreatment behavioral measures: conditioned place preference baseline (door open 10 min)
Injection	Day 0	❖ Baseline paw thickness ❖ Hind paw injection with CFA or saline ❖ Conditioning (door closed 30 min), randomly assigned left or right side of the box
Post-injection Conditioning	Day 7, 14, 21, 28, 35, 42, 49	❖ Paw thickness and weight recorded ❖ Conditioning (door closed 30 min), same side of the box each week
Behavioral Data Collection	Day 15, 50	❖ Conditioned place aversion measurement (door open 10 min)

I. Statistical Analysis

All data have been reported as a mean ± standard deviation. All statistical analyses were conducted using IBM SPSS Statistics software version 20. Repeated measures analysis of variance (ANOVA) tests were used to examine differences between groups in conditioned place aversion over the course of the study. Normality of data was established using the Shapiro-Wilk Test. Sphericity of data was established using Mauchly's Test of Sphericity. The outlier labeling rule was used to identify any outliers (Hoaglin & Iglewicz, 1987). A p value of ≤ 0.05 was considered significant for all testing. The mean weight of each treatment group was tracked as a measure of wellness in the test animals. The mean left paw thickness of each treatment group was tracked as an indicator of successful induction of inflammation. All graphs were constructed using GraphPad Prism software version 6.

Chapter 3

III. Results

A. Comparison of Weights among Treatment Groups

Preceding injection (day -1), the mean weight of all rats was 270 ± 33 g, whereas at the conclusion of the study (day 50), the mean weight had risen to 403 ± 31 g. Graphical examination of the data showed a gradual and continuous increase in weight among all housing groups, indicating normal animal growth with no indication of harm (Fig. 6). Furthermore, the mean weights of rats injected with CFA (404 ± 36 g) and saline (401 ± 25 g) were not significantly different on day 50 (unpaired Student's t-Test, p = 0.78).

B. Qualification of Edema

To demonstrate the effectiveness of the CFA-induced inflammation model, the thickness of the left hindpaw was measured weekly. Immediately before injection on day 0, mean left hindpaw thickness for all rats was 5.08 ± 0.66 mm. One week later, the mean left hindpaw thickness for rats injected with CFA (7.38 ± 0.54 mm) was significantly greater than that of rats injected with saline (5.06 ± 0.42 mm; $p \leq 0.001$; (Fig. 7). There was a clear deviation between the measured paw thickness of CFA rats and control rats after day 0, indicating the successful induction of inflammation. In addition, the paw thickness of CFA-injected rats showed a gradual decline toward control levels over time, consistent with the inflammatory model (Fig. 7).

Figure 6: Mean Weight of Each Housing Group over Time

The mean weight of rats in each of the five housing groups maintained steady growth over the course of the study. $N = 8$ for all groups except Group E where $n = 16$. No significant differences in weight gain were observed between housing groups.

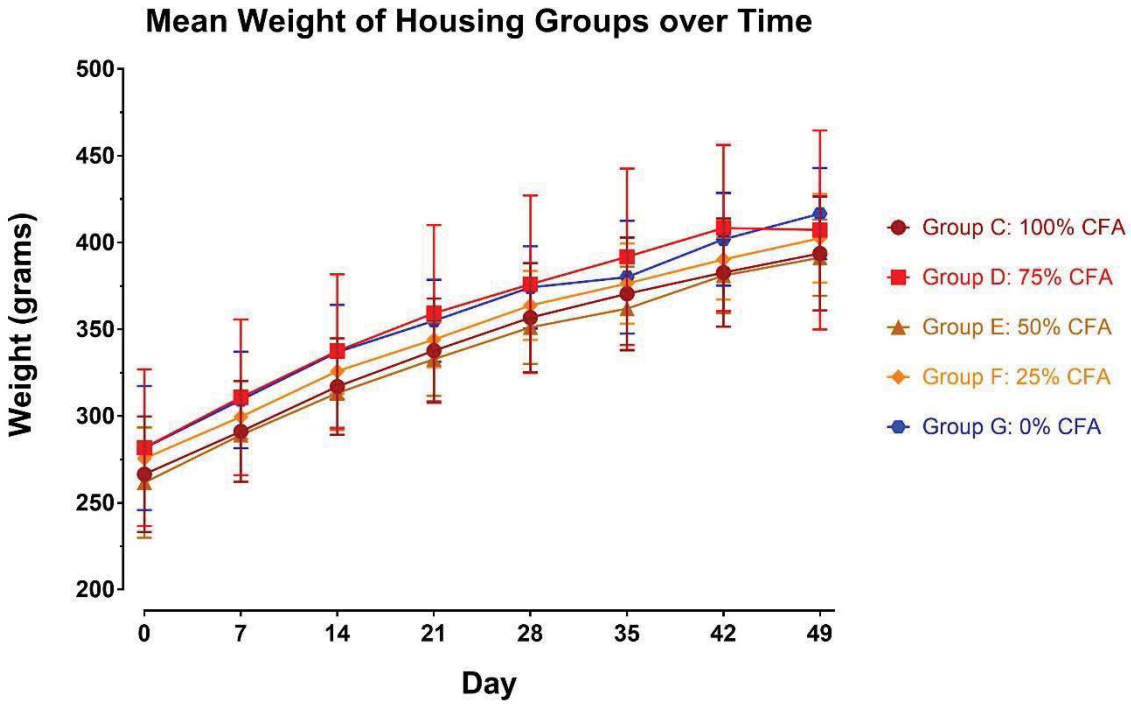
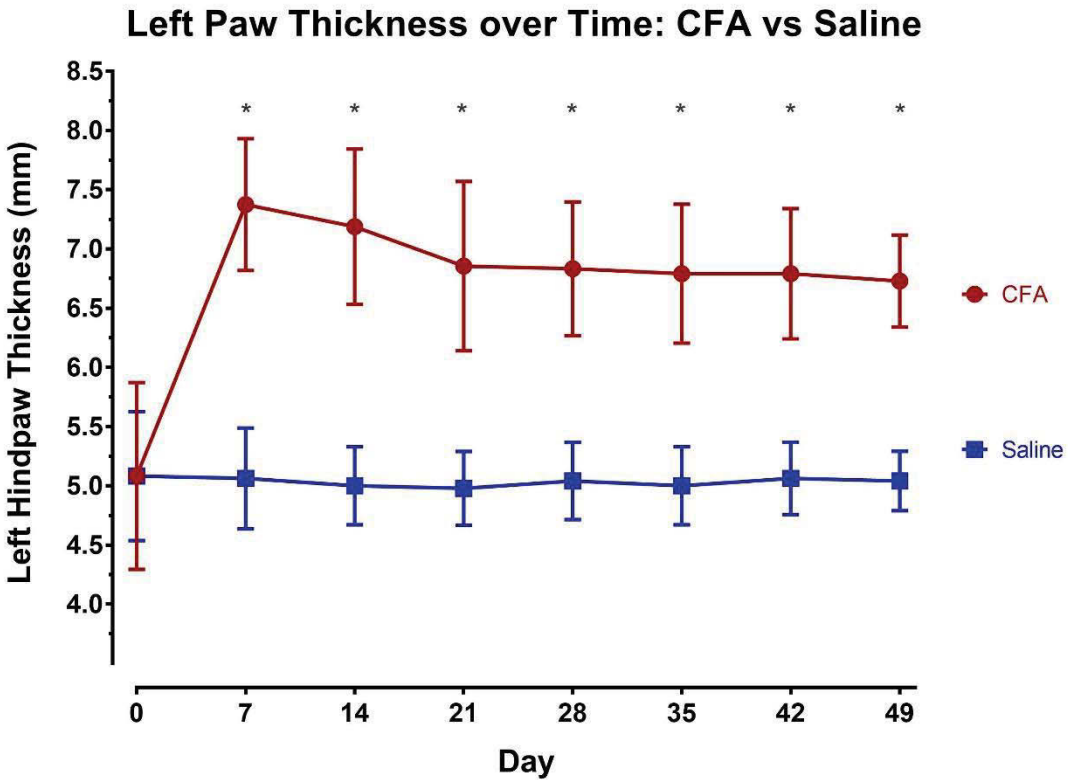

Mean Weight of Housing Groups over Time
Weight (grams)
500
450
400
350
300
250
200
0
7
14
21
28
35
42
49
Day
Group C: 100% CFA
Group D: 75% CFA
Group E: 50% CFA
Group F: 25% CFA
Group G: 0% CFA

Figure 7: Progress of Inflammation in CFA and Saline Rats

Comparison of left hindpaw thickness in rats injected with CFA vs rats injected with saline across days 0, 7, 14, 21, 28, 35, 42, and 49. The clear deviation in mean left hindpaw thickness between CFA- and saline-injected rats after day 0 showed the effectiveness of the CFA model of inflammation. As time passed, the paw thicknesses of the CFA-injected rats trended closer to that of controls, revealing the gradual decrease in inflammation. $N = 24$ for both groups. Significant differences ($p \leq 0.05$) are marked by an asterisk.


Left Paw Thickness over Time: CFA vs Saline
Left Hindpaw Thickness (mm)
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
*
*
*
*
*
*
*
0
7
14
21
28
35
42
49
Day
CFA
Saline

C. Injection in Hindpaw is a Conditioning Stimulus

Rats were allowed to freely move between the two sides of the conditioning box on day -1 (preceding conditioning) and on days 15 and 50 (post-conditioning). Data for these days were recorded as time spent on the subsequently (day -1) or previously (days 15 and 50) conditioned floor (either circle or square for each rat). At baseline (day -1), the mean time spent by all rats on their assigned conditioning floor (either square or circle) was 311 ± 91 seconds. On day 15, the mean time spent on the conditioned floor decreased significantly to 271 ± 82 seconds ($p \leq 0.02$). By day 50, mean time spent on the conditioned floor had risen slightly to 279 ± 115 seconds and was no longer significantly different compared to day -1 ($p = 0.054$). Repeated measures mixed ANOVA testing showed a significant within-subject effect for time spent on the conditioned floor by day, indicating that rats on average did alter their behavior across the three measurement days overall ($p = 0.022$).

Surprisingly, repeated measures ANOVA testing also revealed that the type of injection (CFA vs saline) did not have a significant interaction with the observed change in time spent on conditioned side across days -1, 15, and 50 ($p = 0.212$). Graphical rendering of the two groups showed an aversive response at day 15 in saline-injected rats, followed by a return toward baseline at day 50. Conversely, CFA-injected rats showed a more moderate but increasing aversive response at days 15 and 50 (Fig. 8). Interestingly, repeated measures ANOVA testing revealed a significant interaction between assigned conditioning floor (square or circle) and conditioned aversion across each of the three days ($p = 0.001$), indicating that rats conditioned on a given floor did not respond to conditioning in the same manner. Further testing of between-subjects effects showed that

rats had a significant preference for the circle floor at day -1 (i.e., before conditioning occurred; *Means* = 348 ± 76 seconds vs 274 ± 91 seconds; $p = 0.005$; Fig. 9) and that this effect persisted over the course of conditioning ($p \leq 0.001$; Fig. 9). To determine the cause of the observed influence of assigned floor on conditioning, rats were split into two groups based on whether their baseline floor preference matched their conditioned floor. After comparing the mean change from baseline in time spent on the conditioned floor for each group, it was discovered that rats developed significant aversion only if conditioning occurred on their initially-preferred floor (Fig. 10). In other words, rats that preferred (i.e., spent more time on) the square/circle floor at baseline and were then conditioned to avoid that same floor successfully developed aversion ($n = 26$, $p \leq 0.001$), while rats that preferred the square/circle floor at baseline and were conditioned to avoid the opposite floor did not ($n = 22$, $p = 0.22$).

D. Housing Group did not Alter Aversive Conditioning

To determine whether housing group had a significant influence on the course of aversive conditioning, a repeated measures mixed ANOVA was conducted. Results indicated that housing group did not have a significant interaction with time spent on the conditioned floor across days -1, 15, and 50 ($p = 0.952$; Fig. 11). This was not surprising given the confounding aspect of the significant interaction between conditioning and assigned conditioning floor. Limited N values for multiple groups prevented retesting using only rats that had been conditioned on their initially-preferred floor.

Figure 8: Injection with CFA did not Increase Aversive Conditioning

Comparison of the mean time spent on the conditioned side for rats injected with CFA vs rats injected with saline across days -1, 15, and 50. Overall, aversive conditioning was successfully induced between days -1 and 15 ($p = 0.013$); however, injection with CFA was not found to have more conditioning power than saline at either day 15 or 50. Rats injected with saline showed a sharp aversive response at day 15, countered by recovery at day 50, whereas rats injected with CFA showed smaller, continuous decreases across the three days. $N = 24$ for each group.

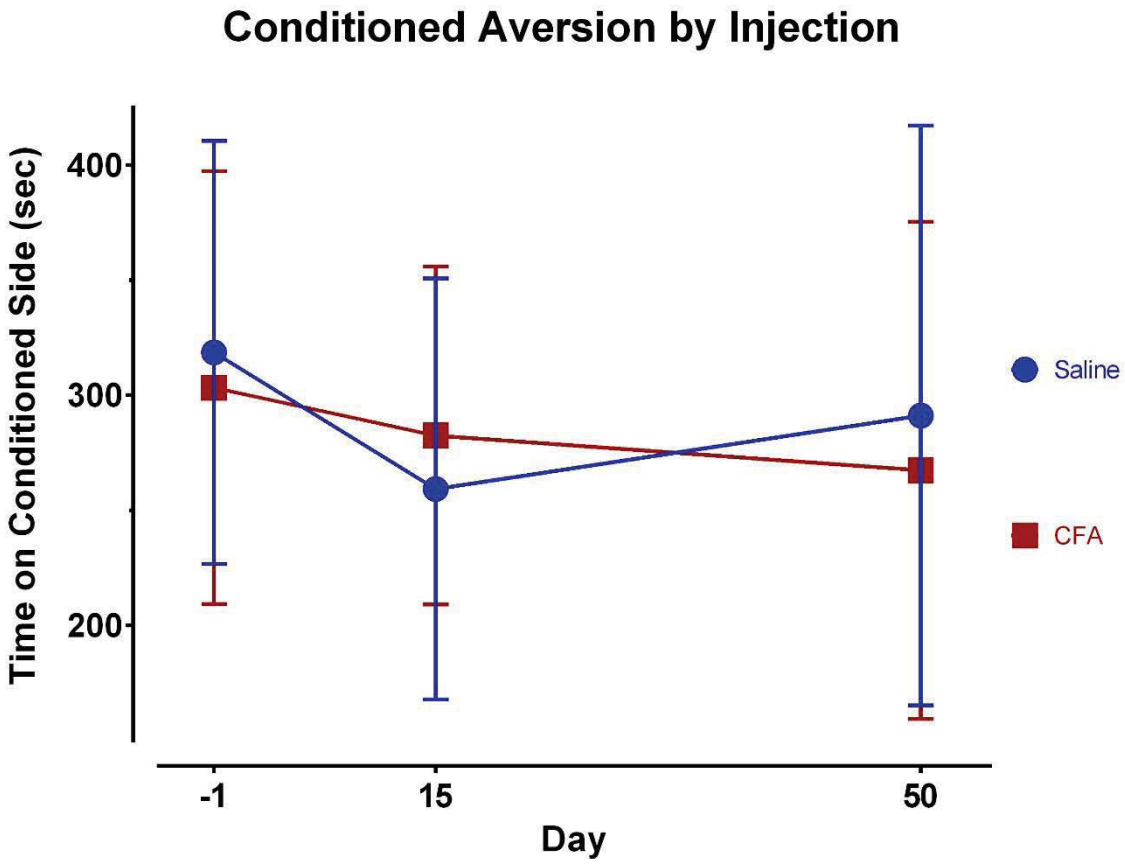

Conditioned Aversion by Injection
Time on Conditioned Side (sec)
400
300
200
-1
15
50
Day
Saline
CFA

Figure 9: Conditioned Aversion was Greater on the Square Floor

Comparison of the mean time spent on the conditioned side for rats conditioned on the square floor vs rats conditioned on the circle floor across days -1, 15, and 50. Before conditioning occurred (day -1), rats showed a baseline preference for the circle floor ($p = 0.005$). This preference persisted across days 15 and 50 (both $p \leq 0.001$), and ANOVA testing showed that the degree of aversive conditioning overall was linked to the floor type (illustrated here by the increasing divergence of the two groups). $N = 24$ for both groups. Significant differences ($p \leq 0.05$) between groups are marked by an asterisk.

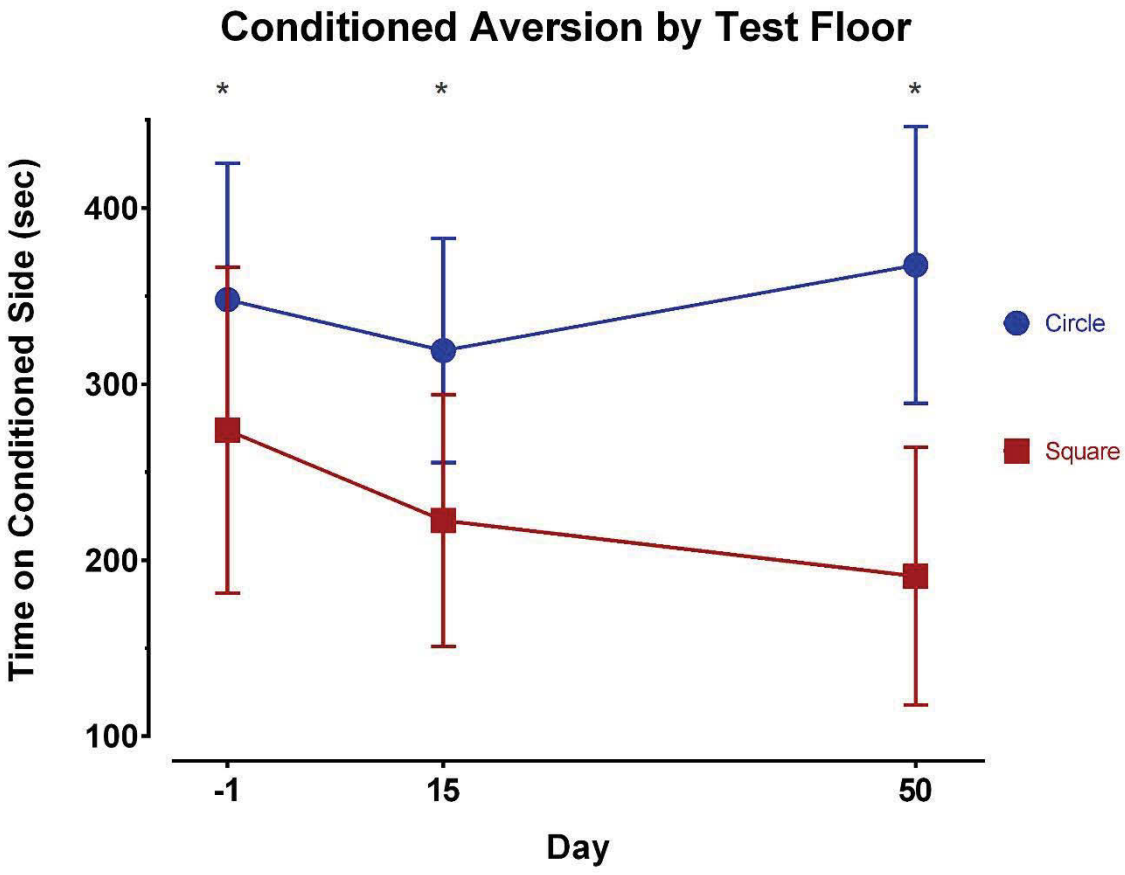

Conditioned Aversion by Test Floor
*
*
*
Time on Conditioned Side (sec)
400
300
200
100
-1
15
50
Day
Circle
Square

Figure 10: Difference in Conditioning by Baseline Floor Preference

Each rat displayed some degree of bias at day -1, preferring either the circle or the square floor. Aversion only developed if subsequent conditioning was matched to the floor that was initially preferred. The y-axis indicates the net change in time spent on the assigned conditioning floor between day -1 and either day 15 or 50. Columns and error bars represent mean changes in time ± SD. For unmatched bias/floor group, $n = 22$. For matched bias/floor group, $n = 26$. The asterisk indicates a significant difference in time between groups at day 15 ($p \leq 0.001$). Unlike the unmatched group, the matched group showed significant aversion compared to baseline for day 15 ($p \leq 0.001$; not shown) and a trend toward continued aversion at day 50 ($p = 0.051$; not shown).

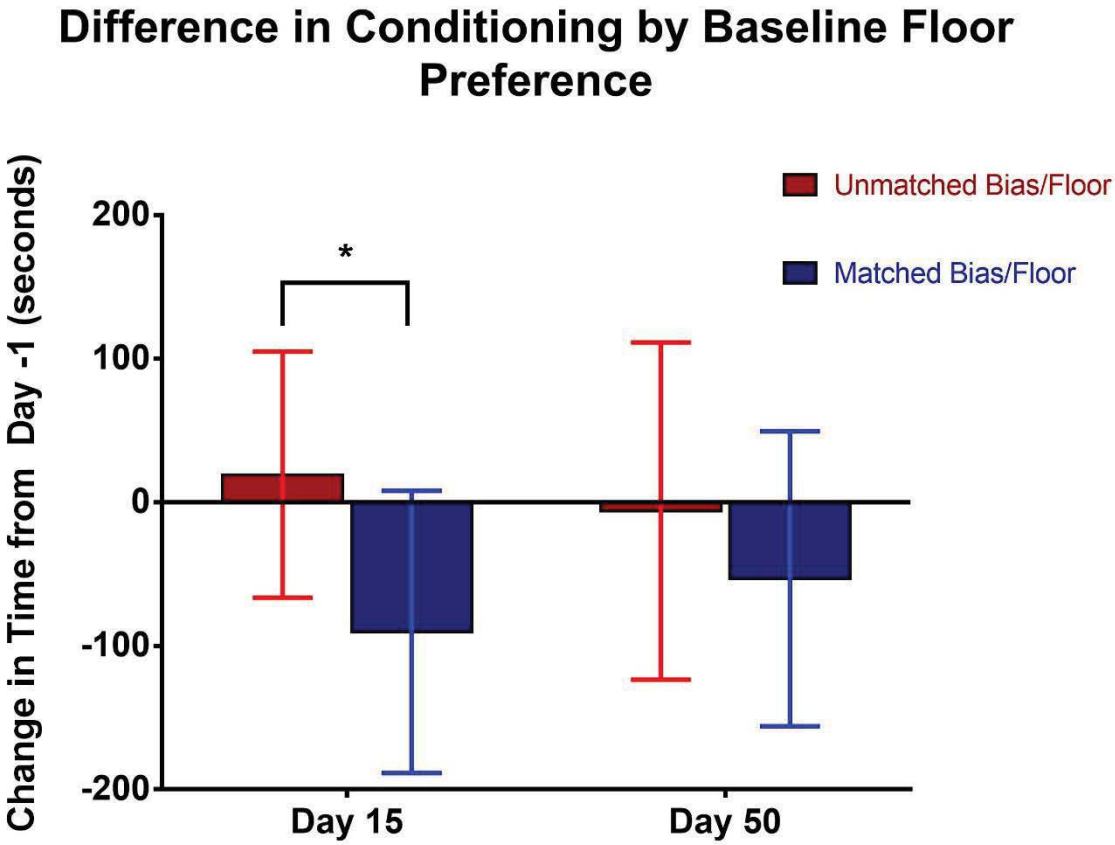
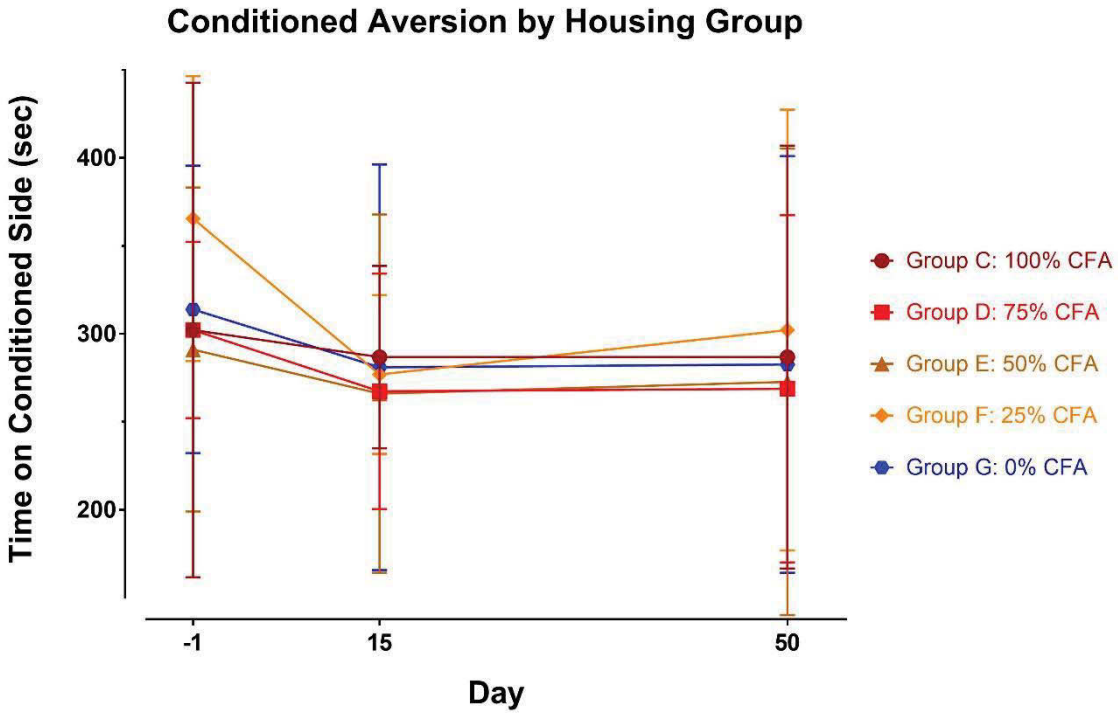

Difference in Conditioning by Baseline Floor Preference
Change in Time from Day -1 (seconds)
200
100
0
-100
-200
*
Day 15
Day 50
Unmatched Bias/Floor
Matched Bias/Floor

Figure 11: Conditioned Aversion was not Affected by Housing Group

Comparison of the mean time spent on the conditioned side for each of the five housing groups across days -1, 15, and 50. Although there was a significant overall decrease in time spent on the conditioned side (not shown), repeated measures ANOVA testing did not show any significant interaction between group and measurement at each testing day. $N = 8$ for all groups except Group E where $n = 16$.


Conditioned Aversion by Housing Group
Time on Conditioned Side (sec)
400
300
200
-1
15
50
Day
Group C: 100% CFA
Group D: 75% CFA
Group E: 50% CFA
Group F: 25% CFA
Group G: 0% CFA

Chapter 4

IV. Discussion

The measurement of affective and motivational change represents a major obstacle in the use of any non-human animal model. Though the modern conditioned place preference procedure was developed in the mid-to-late 1970s to test the rewarding effects of drugs, it was not until the late 1980s that its converse, conditioned place aversion (CPA), was applied to the quantification of nociception (Tzschentke, 2007). In the intervening years, place conditioning research has shown a remarkable increase within the scientific literature, signifying its importance as a mode of investigation. Numerous studies have utilized the intraplantar injection model of inflammation to study the effect of a persistent noxious stimulus on measures as diverse as neuron receptor regulation, the rewarding effects of morphine, and the anti-inflammatory properties of cannabinoids (Carlton & Coggeshall, 1999; Narita et al., 2005; Conti et al., 2002). In addition, recent research involving rats has shown potential indications of empathy-like behavior in social situations (Bartal et al., 2014). Therefore, the present study sought to apply the model of inflammation-induced CPA to the identification of any potential group-specific differences in behavior following induction of a socially-shared injury. The hypothesis was that social housing with uninjured cagemates would reduce the conditioning effect of hindpaw inflammation brought on through injection of complete Freund's adjuvant (CFA), or vice versa.

As described in the results section, initial outcomes were positive. Inflammation was successfully induced in the left hindpaw of rats, with the mean paw thickness of CFA-injected rats significantly greater than saline-injected rats at day 7 (7.38 mm vs 5.06

mm, $p \leq 0.001$). Development of maximum inflammation sometime during the first 2 weeks post-injection and maintenance of inflammation over the 7-week study were both consistent with previous research utilizing this model (Stein et al., 1988). No significant difference in weight gain was observed between CFA- and saline-injected rats, indicating that there were no changes in overall health between groups. Following injection and initial conditioning sessions, conditioned place aversion was achieved, with the mean time spent on the conditioned side falling significantly from 311 ± 91 seconds on day -1 to 271 ± 82 seconds on day 15 ($p = 0.01$). Curiously, CFA and saline did not exhibit a significant difference in conditioning power in between-subjects testing. There are several potential explanations for this unexpected result, the most likely of which is that the combination of acute stress from anesthetization and injection masked the chronic effect of inflammation by providing the stronger conditioning stimulus on day 0. There is some support in the literature for this line of thought, as previous studies have indicated that procedural stress can inhibit place conditioning in rats (Jorenby et al., 1990). Evidence for this line of reasoning might be found in the examination of the trends presented by Figure 8, where saline-injected rats saw a possible reversal of aversive conditioning from day 15 to day 50 after removal of the initial stressors. Conversely, CFA-injected rats suggested a slow but continued development of CPA, possibly as a result of their ongoing exposure to inflammatory pain. While not significant on their own, ANOVA results regarding the effect of CFA vs saline on conditioned time did show a trend of decreasing p values from day -1 (before injection, $p = 0.54$) to day 15 ($p = 0.24$). The observed drop in values may represent the masked effect of inflammation on conditioning. Another factor potentially involved in the lack of conditioning power for

inflammation might be the young age of the rats used in the study. A previous investigation utilizing the same strain of rats found that, compared to adults, adolescent rats displayed an insensitivity to conditioning through an aversive taste (Anderson et al., 2010). Finally, a lack of ongoing association between hindpaw inflammation and the CPA box after day 0 may have blunted CFA's conditioning power. Mechanical aggravation of the injection area prior to each conditioning session may have aided in reinforcing the pairing between the aversive stimulus and the box.

While the ineffectiveness of CFA as a conditioning stimulus can potentially be explained by a number of factors, less easily explained and more confounding was the persistent interaction between assigned conditioning floor and the aversive response. Mean conditioned aversion only occurred among rats that were coincidentally assigned to be conditioned against the floor that they had preferred at baseline. Rats that were aversively conditioned on their initially less-favored floor developed no additional aversion on average. Appropriately, the opposite effect has been described before among studies examining conditioned place preference, whereby preference can only be conditioned when the stimulus is paired with the initially non-preferred room (Tzschentke, 1998). When discussing this asymmetrical conditioning effect, two factors involving experimental design must be considered: biased vs unbiased testing apparatus and biased vs unbiased stimulus assignment. A biased testing apparatus is one in which one of the tactile cues is on average significantly preferred by the test subjects at baseline, whereas an unbiased apparatus does not evoke strong preference at baseline. Similarly, biased stimulus assignment involves purposefully conditioning subjects to their non-preferred floor (in the case of CPP) or their preferred floor (in the case of CPA) in order

to ensure a strong conditioning effect. For example, one study investigated this subject using mice, finding that ethanol produced CPP on either floor in an unbiased set-up, but had the same effect with a biased set-up only when purposefully paired (biased assignment) with the non-preferred floor (Cunningham et al., 2003). The authors referred to this as a ceiling effect, as it is difficult or impossible to make subjects gain additional preference for an already preferred cue. In the case of the present study, a more appropriate term would be "floor effect", as subjects could not be made to prefer a non-preferred cue (i.e., floor) even less. Because of its easier interpretation, the preferred method of experimental design is the unbiased apparatus; however, the biased version can be useful in certain situations to distinguish between the anxiolytic and truly rewarding effects of stimuli (Tzschentke, 1998).

Considering the issues discussed above, the lack of significant results regarding variations between specific housing groups did not come as a surprise. Because the five treatment groups differed from one another in their ratio of CFA- to saline-injected rats, the lack of quantifiably greater conditioning power with CFA compared to saline made any potential group effect similarly unmeasurable. Furthermore, any alteration in behavior between groups would have had to have been relatively profound in order to overcome the confounding influence of the "floor effect". While attempts were made at performing group comparisons among only rats unaffected by this variable, the random nature of the distribution of matched to unmatched bias and floor resulted in n values that were too low to be of use. Therefore, no evidence of alteration in conditioning between housing groups was detected from the acquired data.

Any further avenues of research utilizing CPA could benefit from a number of

advantageous adjustments in future experimental design. Firstly, the conditioning power of CFA would likely be improved by some mechanism of renewed stimulus pairing each week, such as mechanical agitation of the injection site prior to conditioning. Alternatively, a stronger noxious stimulus, such as mock surgery, could be employed. A longer experimental timeline may also aid in uncovering any potential trends of saline vs CFA over time, as the paw thickness had yet to return to normal at day 49. Regardless, the most important change to the protocol would be the assurance of unbiased tactile cues prior to the start of conditioning. This would allow for less interaction within the data and easier interpretation of test results. Notably, although limited pilot data were recorded prior to the start of this study, it was obviously insufficient to judge the inherent preference by the rats of the circle floor. This inaccuracy in the pilot data was either due to too small a sample size or due to the use of adult rats in its collection.

Although the present study was unable to reach conclusive results regarding the potential for differential behavior as a result of social group, it did reveal the presence of a floor effect regarding CPA in a biased design. Furthermore, it confirmed the effectiveness of an intraplantar injection of complete Freund's adjuvant in inducing pronounced and persistent inflammation of the hindpaw in rats. Finally, it made important strides toward improvement of the conditioned place aversion model as it relates to inflammation.

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Tuesday, October 21, 2014

Dr. Jill Gifford
Department of Biological Sciences
UNIVERSITY

Re: IACUC Protocol # 03-14
Title: The effects of home cage social groups on nociception.

Dear Dr. Gifford:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration and determined it should be unconditionally approved for the period of October 21, 2014 through its expiration date of October 21, 2017.

This protocol is approved for a period of three years; however, it must be updated yearly via the submission of an Annual Review-Request to Use Animals form. These Annual Review forms must be submitted to the IACUC at least thirty days ***prior*** to the protocol's yearly anniversary dates of October 21, 2015 and October 21, 2016. If you do not submit the forms as requested, this protocol will be immediately suspended. 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.

Good luck with your research!

Sincerely,

[redacted]

Dr. Scott Martin
Interim Associate Dean for Research
Authorized Institutional Official

sm:dka

C: Dr. Walter Horne, Consulting Veterinarian, NEOMED
Dr. Robert Leipheimer, Chair IACUC
Dawn Amolsch, Animal Tech., Biological Sciences
Dr. Gary Walker, Chair Department of Biological Sciences