# Investigation of Empathy-like Behavior in Social Housing

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**Abstract**

The sensation of pain in the human body has been very well defined. Emotional loci in the brain have also been researched and uncovered. Literature and observed human behavior strongly suggests a link between the neural mechanisms of pain and emotion. The perception of pain to an individual is unique to a specific set of circumstances, with regards to environmental, genetic, and social factors despite the concise sensory system. This phenomenon combined with the expanding comprehension of mirror neurons leads to the conclusion that emotion plays an important role in the perception of pain. Specifically, empathy, or the ability to relate to the emotional experiences of another, may alter the perception of pain. Because recent literature has shown that rodents are able to demonstrate empathy, and knowledge that rats and humans exhibit high similarity in neural structures pertaining to emotion and nociception, an experimental model assessing the influence of empathy on pain-related behavior was created. Empathy was hypothesized to influence nociception in socially-housed versus isolated rats, through the use of a localized inflammatory model. Animals were randomly housed in isolation or socially, in cages of 4. Depending on treatment group, each animal was injected with Complete Freund's Adjuvant or sterile saline in the left hindpaw. Three parameters were measured- body weight to quantify overall well-being, paw thickness to measure edema, and paw withdrawal latency, as a quantification of pain-like behavior. Behavior was also qualitatively reported. Data were collected weekly for 8 weeks following injection and a series of inferential analyses were conducted. No significant difference between any isolated or socially housed group was found, although many trends were uncovered to suggest value in the original hypothesis.

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## I. Introduction

### A. Opening Statement

"But we were burdened with like weight of pain, as much or more we should ourselves complain," William Shakespeare, Comedy of Errors.

### B. Pain and Nociception

Pain is a difficult sensation to accurately define. Like vision, there is an understood physiological framework, but genetics, personal experiences and even cultural standards affect an individual's unique perception of pain. No two people see exactly the same thing; no two people feel exactly the same pain. Pain is referred to as a complex, unpleasant sensory experience that signals current, impending or perceived tissue damage. Painful stimuli trigger behavioral and mental processes that promote withdrawal from immediate and continuous noxious stimulation (Sternbach, 1968). It is the intention of a large body of research to elucidate which factors can contribute to alleviation of pain, such as drug therapy, physical modifications and social influences. However, despite the ongoing investigations, knowledge of certain, general pain-related physiological processes has yet to be clarified.

Although it has been stated that there are emotional and cognitive factors that influence a person's distinct symptomology, nociception is the neurobiological term used to describe the processing of noxious stimuli by the body. Pain was previously thought to

be a separate facet or subcategory of the sensation of touch, but research now supports that nociception should be considered an exclusive sensory system.

Nociceptor is the term that denotes both the peripheral free-nerve endings, as well as the neurons that relay information into the central nervous system (CNS). They are unique in that they are pseudounipolar, possessing one cell body, one peripheral axon and one central axon (Gebhart et al., 2009). Nociceptors are found cutaneously, somatically and viscerally in the body and respond to noxious chemical, mechanical and thermal stimuli. Nociceptors have a resting membrane potential (RMP) between -60 millivolts (mV) and -90 mV. The polarized RMP results largely from the unequal distribution of ions across the cell membrane, and is maintained by the sodium ($Na^+$) –potassium ($K^+$) - ATPase pump. When homeostasis of a nociceptor is disrupted by a noxious stimulus, potentials are generated via activation and opening of transient receptor potential (TRP) ion channels, specifically the vanilloid channel subtype (TRPVs). Calcium ion ($Ca^{+2}$) or sodium ion ($Na^+$) fluxes through the open channels and causes membrane depolarization, and the change in membrane voltage leads to the firing of an action potential in the nociceptor and transmission of an electrochemical signal (Breedlove & Watson, 2013).

TRP channels are a family of 28 sensor proteins that specifically respond to a many distinct physical and chemical signals. Each TRP channels possesses six transmembrane segments and one uniform "TRP Box" segment. These channels collectively bear similarity with established $K^+$ channels but crystallography of the entire protein has not yet accurately pinpointed the exact structure (Zheng, 2013). TRP channels play a role in cell development, fertilization, and senses such as sight, smell, taste and hearing. The first TRP channel was discovered in a mutant strain of *Drosophila*

*melanogaster* (Montell & Rubin, 1989). TRPV channels are the six most specific for nociceptive signal transduction and are also responsive to heat. These particular receptors were discovered in expression cloning experiments in response to the compound capsaicin, the chemical component of spicy foods that produces a sensation that is perceived as burning and painful. TRPV1 is a suggested tetrameric calcium-selective protein and is responsive to heat (Caterina et al., 1997). In addition to its susceptibility to noxious temperature and chemicals like capsaicin, it is also activated by extreme changes in hydrogen ion concentration (pH) on both the acidic and alkaline end of the spectrum, and is also weakly voltage gated. TRPV1 is fascinating in that it is specifically geared to respond to these distinct chemical and physical stimuli, but it can be activated in a polymodal manner with a combination of these stimuli and with other native antagonists and agonists to produce the varying human nociceptive sense (Zheng, 2013). The range at which TRPV1 can respond to changes in voltage is relatively non-attainable in a physiological sense at neutral pH and non-noxious temperature, but the temperature-gating mechanism of activation is affected by this principle. The voltage activation threshold is significantly lowered under elevated temperature, which grants the temperature-mediated activation for which these receptors are characterized. Inflammatory factors such as bradykinin and many prostaglandins, which are released in the response to tissue damage, also increase sensitivity of TRPV1 channels (Venkatachalam & Montell, 2007). TRPV1 channels are activated at temperatures above 40°C, whereas the other TRPV heat-responsive receptors (TRPV 2-4) are activated at other unique ranges of heat. For these reasons, these channels fall under the classification of "thermoTRPs" (Zhong et al., 2012) along with other distinct TRP channels that

respond to cold. Activation of the thermoTRP channels 2-4 produces an uncomfortable burning sensation, which can be described as a feeling of pain.

There are two subtypes of physiological pain in the body: fast pain, synonymous with acute, sharp and pricking sensations; and slow pain, described as aching or throbbing pain, that typically is longer lasting. Neither of these phenomena are known to weaken over prolonged stimulation, and in some cases, the sensation becomes progressively stronger, a condition known as hyperalgesia. Under normal physiological conditions however, pain is typically felt at a level contingent with the rate of tissue damage that is occurring in both fast and slow sub-classifications of the sense. The distinction between fast and slow pain is a result of the two types of afferent neurons, Aδ and C nociceptors. Aδ-neurons have thinly myelinated axons and transmit action potentials at rates between 6-30 meters-per-second (m/s). C-fibers lack myelin and possess slower nerve conduction velocities, ranging 0.5-2 m/s. Both neurons project into the dorsal horn of the spinal cord where Aδ-fibers utilize glutamate as their primary neurotransmitter, and C fibers use a combination of glutamate and substance P. Activation of both of these nociceptor subtypes contribute to the initial response to a noxious stimulus, as well as to the continual behavioral modifications that promote healing.

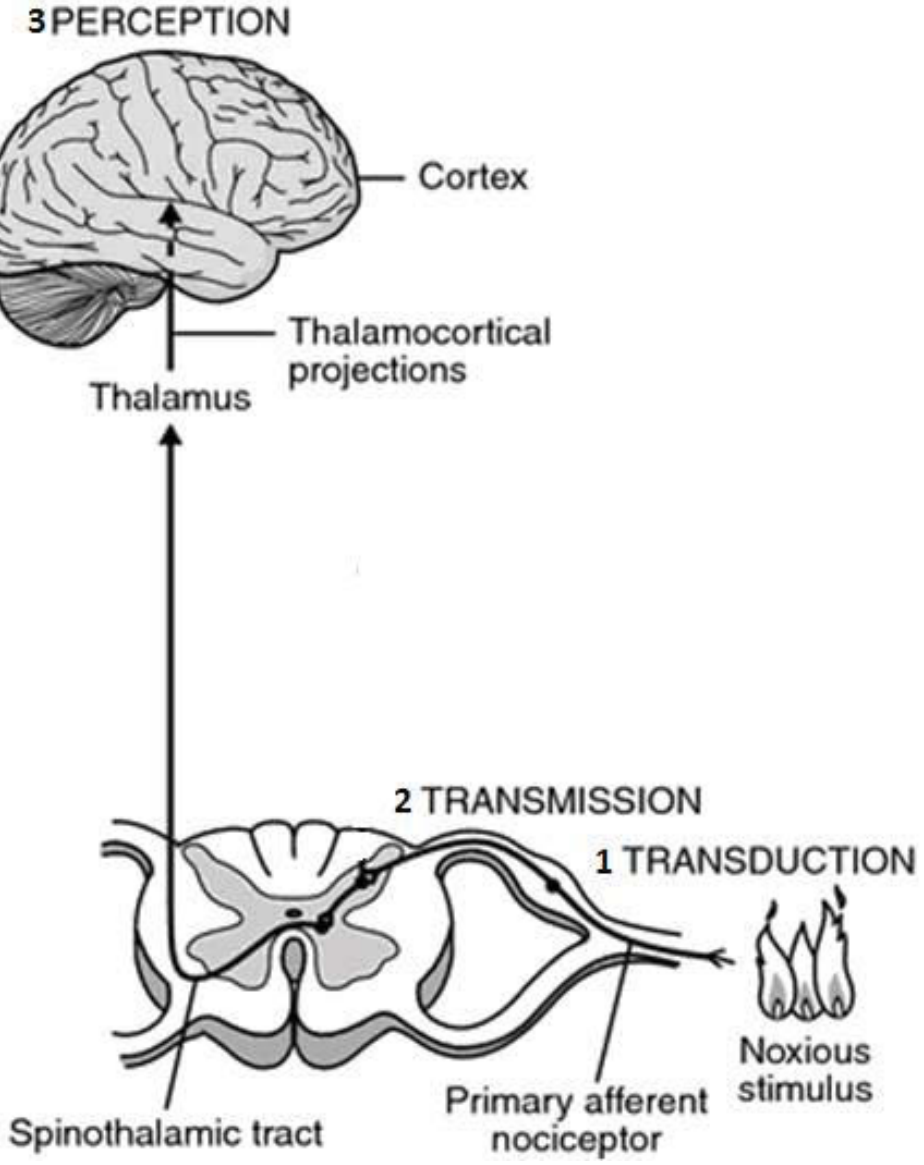
Pain is transmitted to the brain via the anterolateral system (ALS), also referred to as the spinothalamic pathway (Fig. 1). Aδ and C-fibers each subserve a component of the ALS, the neospinothalamic tract and the paleospinothalamic tract, respectively. Aδ-fibers transmit mechanical and acute thermal pain to the lamina marginalis of the dorsal horn of the spinal cord, where they synapse onto second-order neurons. The axons of these

secondary neurons immediately decussate through the central commissure of the spinal cord and ascend within the anterolateral column as the neothalamic tract, before terminating in the ventrobasal complex of the thalamus. A small proportion of the fibers of this tract end in the reticular areas of the brainstem as well as the posterior nuclei of the thalamus. From both of these thalamic zones, third-order neurons carry the signal to the basal ganglia and somatosensory cortex. Aδ-fibers alone can localize the affected area to within ten centimeters, but combined with tactile receptors of the medial-lemniscal system, the localization is nearly exact. (Hall & Guyton, 2011).

The paleospinothalamic tract is an evolutionarily older feature of the nervous system in comparison to the neospinothalamic tract. Peripheral nociceptive C fibers of this system terminate in laminae II and III of the spinal cord dorsal horn, collectively referred to as the substantia gelatinosa. Here, these afferents synapse onto second order neurons, whose axons pass through lamina V and decussate via the central commissure, to ascend in the anterolateral column in parallel with the neospinothalamic axons. These second-order neurons send collateral axon branches to multiple areas of the brain, including the thalamus, reticular nuclei in the medulla, pons and mesencephalon, and the periaqueductal gray. Preclinical data supports that these mesencephalic areas play a role in the suffering elements of pain, even when the somatosensory cortex is disabled (Hall & Guyton, 2011). From terminations in the brainstem, short third-order neurons relay signals to the intralaminar and ventrolateral thalamus, hypothalamus and basal ganglia. These signals are poorly localized, with conscious recognition only for a whole body part, as opposed to a specific region.

**Figure 1. The Spinothalamic Tract (Anterolateral System)**

Step 1 is Sensory Transduction, Step 2 is Transmission and step 3 is Perception. The primary afferent nociceptor represents either an Aδ or C fiber, which is stimulated by a noxious stimulus during signal transduction (Step 1). The nociceptive neuron terminates in the dorsal horn, synapsing onto the second order neuron, which then transmits the signal to the thalamus (Step 2). From the thalamus, third order neurons carry the information to relevant brain structures including the basal ganglia and somatosensory cortex, which allows for pain perception by the individual (Step 3). Adapted from *Postoperative Pain Management* (Ferrante & VadeBoncoeur, 1993).


3 PERCEPTION
Cortex
Thalamocortical projections
Thalamus
2 TRANSMISSION
1 TRANSDUCTION
Noxious stimulus
Primary afferent nociceptor
Spinothalamic tract

Nociception occurring through the ALS is very outlined. Like all neurons, it is finite in that afferent neurons either fire or do not, fluctuating only by the rate of firing of action potentials in response to stimulus strength. In short, *nociception* is a linear three-neuron-sequence used to convey a simple signal, indicating to the brain the fact that tissue damage has occurred and the bodily location of this damage. Mutations in the SCN9A gene can physiologically affect this ability. It belongs to the SC (sodium channel) gene family, and is responsible for the α-subunit of NaV1.7 sodium channel, which are found extensively in nociceptors. Altered or defective forms of this gene can lead to defective formation or functionality of the channels and subsequent signal transduction, thereby causing a degree of congenital insensitivity to pain, and inihibition of nociception (Drenth & Waxman, 2007).

In contrast to nociception, pain has more complex connections and functions in the brain. The degree of pain perception and feeling for an individual can span a large range due to a plethora of factors aside from the initial noxious stimulus. These influential factors impact interoception, or the ability of the body to consciously perceive sensory signals. While interoception is not exclusive to the sense of pain, it is important to describe and understand pain as a highly variable sense. There are multiple components to the perception of pain, including the emotional, psychological and social components in addition to the physiologic, being some of the most effectual in pain mediation. Not only does pain (particularly fast pain) cause reflexive withdrawal from detrimental sources, it also induces recuperative behaviors such as sleep, self-nurturing with food or water, inactivity, hygienic practices and other mechanisms that isolate the affected tissue to promote healing (Breedlove & Watson, 2013). While these longer-

lasting behavioral components are facilitated by slow-pain, both types of pain also provide a social signal to others. Not only does withdrawal and debilitating behavior signal to others to avoid the dangerous stimulus, it also elicits recuperative behaviors from them as well. A healthy individual may perceive the pain of a damaged cohort and act to defend or treat their injury, which contributes to pain mitigation for the injured individual. Evolutionarily, this may explain why individuals with frequent pain may display depression and/or express catastrophizing, which is irrational internal beliefs that a situation is or will be essentially worse than is realistic (Sullivan et al., 1995). This would recruit more aid, which could maximize recovery. In terms of clinical treatment, this legitimizes discussions of social influence and empathy on the perception of pain.

**C. Empathy**

Emotions are "a special class of psychological processes and states connected with instincts, needs, and motives," and "a form of reflecting the biological quality of [a] stimulus [and] its usefulness or harmfulness for the organism" (Simonov, 1986). Alternately, emotions are "states produced by reinforcing stimuli," meaning both positive and negatively reinforcing stimuli (Rolls, 1999). These loquacious definitions are more delicate, and essentially more complex than their pedestrian conceptualization as feelings. Happiness, sadness and anger are simple recognizable examples. Because emotions parallel a level of wellbeing in an individual, they are also very strong social signals to others.

In common vernacular, the terms sympathy and empathy are often interchangeable. However, they are etymologically and neurobiologically different.

While sympathy is denoted as matching the emotional state of another, empathy occurs at a much deeper psychological level, in the forms of both cognitive perspective role taking and affective reactivity to others (Davis, 1994). The term empathy derives from the German antique phrase, "Einfuhlung" or projecting oneself into perceivable surroundings. An accurate definition of the current accepted view of empathy is, "an observer's reacting emotionally because he [or she] perceives that another is experiencing or is about to experience an emotion" (Stotland, 1969 in Davis, 1994). Developmental psychologist Jean Piaget asserts that empathy is a cognitive function, as it develops throughout life. Very young children, in his theory, fail to dissociate self from others, and therefore involuntarily demonstrate empathy, evident through the tendency of infants to naturally cry in response to other distressed babies. This subconscious mimicry even persists throughout adulthood. Known as "the chameleon effect," humans have a tendency to unintentionally imitate posture, facial expression and mannerisms (Carr et al., 2003). Some experts proclaim that there must be an evolutionary basis for empathy, or else it would not exist in present populations (Davis 1994). Elaborating, altruistic behavior involves promoting another's welfare even at one's own disadvantage, and is viewed as a manifestation of empathy. Expression of altruistic behavior is logically analogous to Charles Darwin's *Theory of Evolution* and is characterized as "kin selection". Principles of this concept suggest that organisms in close contact and also caregiver/receiver-type circumstances are likely familial, and therefore display high genetic similarity. If an entity is to display generous, protective or nurturing behavior to another genetic counterpart, their shared genetic lines are more likely to survive and prosper, even at the temporary expense of the initial altruist. In more modern context,

empathy combined with cognitive reasoning allows an individual to potentially be attracted to or dissuaded from beneficial or hazardous stimuli, correspondingly. This, in turn, promotes advantageous decision-making and a higher level of fitness and safety.

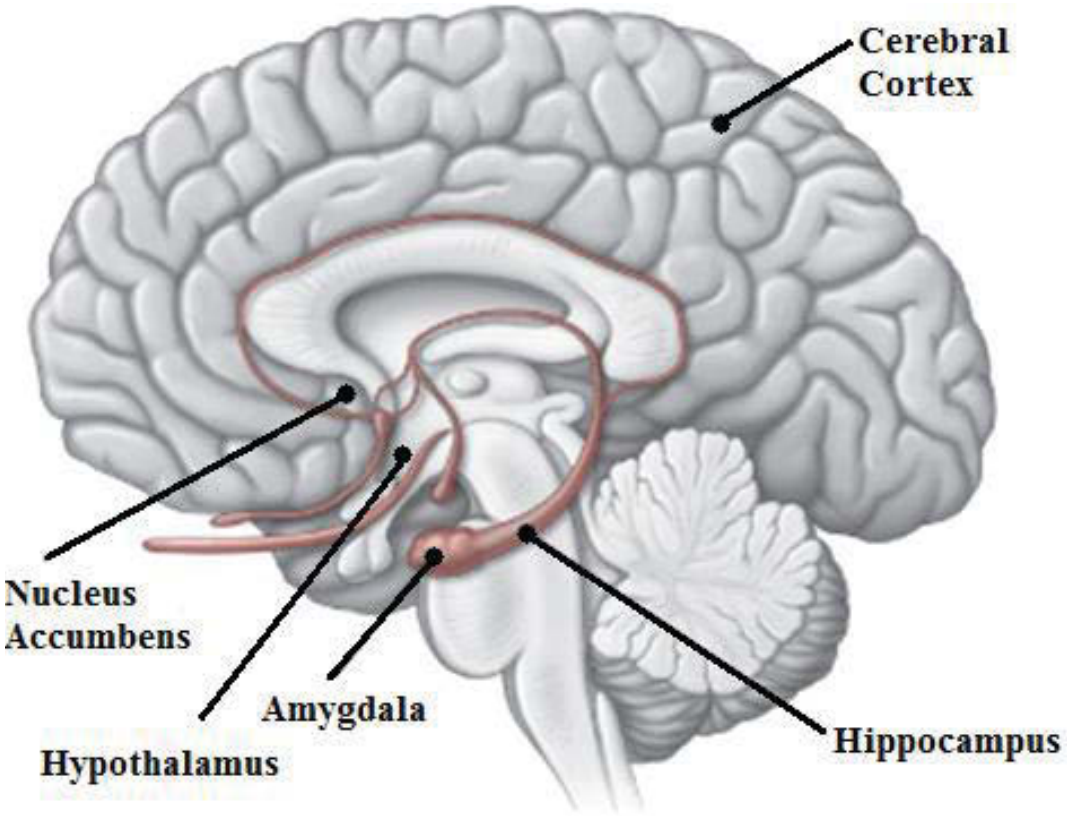
To study empathy as a behavior, it is important to recognize those brain structures involved in its processes, which can be achieved through experimentation and imaging techniques. However, assigning function to structures in the brain resembles constructing a puzzle in which each piece fits a multitude of places. Identifying structure relative to function is more a tangible task, meaning that some functions process in more than one location. There are pain centers, emotion centers and even regions suggested to play large roles in empathic behavior. Some are distinct areas whereas others overlap. The physiology of pain, as discussed previously, involves a neural circuit and the thalamic and sensory areas. The areas associated with emotion and more specifically, empathy, are still being researched and precisely defined.

Emotions and motivational responses are processed in the limbic system, a collective term referring to multiple structures surrounding the basal cerebral regions. The limbic system (Fig.2) encompasses the hypothalamus, portions of the basal ganglia and thalamus, amygdala, hippocampus, and paraolfactory area, all surrounded by the limbic cortex. The hypothalamus is highly involved in this system, sending and receiving signals from the brainstem and peripheral neurons, as well as the thalamus, cerebrum, and the anterior and posterior pituitary glands, which function to regulate and control much of the endocrine system. Electric stimulation studies have deduced that the lateral and ventromedial hypothalamic nucleus and the medial forebrain bundle are the main "reward centers," with less potent correlates in the amygdala and septum (Hall & Guyton, 2011).

Because empathy is considered to be a prosocial behavior, reward-involved areas are closely associated with this behavior. The hippocampus plays a large role in learning and memory formation. Relating to situations of others requires recollection of the circumstances, justifying the hippocampal importance in empathic thought. The amygdala is referred to as the "fear center" in the brain and is also where facial recognition occurs, which is crucial to distinguishing the distress or delight of another entity. Hence, the amygdala plays a role in empathy as well. The limbic cortex is said to exist as an association area for the control of behavior, and many recent studies specifically identify the orbitofrontal cortex and cingulate cortex as empathy-related, but their overall functions are still being examined (Hall & Guyton, 2011).

**Figure 2. Structures of the Limbic System**

Some of the empathy-related structures of the limbic system in the brain, including the amygdala, hippocampus, hypothalamus and nucleus accumbens. These loci play a role in the emotional component of pain and many prosocial behaviors Adapted from "Overcoming addiction: A path toward recovery" (Shaffer, 2011).


Cerebral
Cortex
Nucleus
Accumbens
Amygdala
Hypothalamus
Hippocampus

Giacomo Rizzolatti first discovered, using functional magnetic resonance imaging (fMRI) and other brain-imaging techniques, that certain areas of the brain that would activate in monkeys performing an action would also activate when observing another animal performed the same action. Through further study of animals and humans using fMRI, he uncovered more about these regions, and dubbed them "mirror neurons" (Winerman, 2005). Recent research has suggested that mirror neurons were somewhat selected for throughout human history, and they are suggested to have developed in the human brain because they promote sensorimotor associative learning and development, and grant higher levels of social cognition and action understanding (Cook et al., 2014). Mirror neurons are highly useful in the field of psychophysiology, because they allow scientists to visualize which neural structures are being utilized under certain conditions, and whether others can perceive and respond to these conditions on a neurological level. The same is true for studying empathy. With various imaging techniques, research has found that primary locations for empathy in the brain are specific areas of the cerebral cortex, including the orbitofrontal, superior temporal, inferior frontal, anterior midcingulate, and some somatosensory cortical areas, as well as the insula and the amygdala (Carr, 2003). With these studies, in addition to diverse animal research, the neural mechanism for empathy is being uncovered.

Principles of psychology are deeply intertwined with the neural basis for empathy, especially in the evidence of psychopathy. By definition, psychopathic individuals are characterized by lack of remorse, lack of affect, shallowness and insensitivity (Decety et al., 2013). In other words, a lack of empathy. These symptoms are only prevalent in one percent of the general population but rates of psychopathy are much higher in prison

populations, which are ideal targets for study. Investigations have yielded that pathologic individuals' brains display sensitivity to self-harm but fail to display normal patterns in response to the pain of others. The higher the level of psychopathic behavior in an inmate correlated with the activation of atypical regions, or lack of activation at all, in fMRIs. This evidence suggests that disruption of normal activity in any or a combination of the described neural regions leads to absence of empathic behavior, suggesting a conceivable neurological link.

The aforementioned tendency of babies to cry in response to other babies' distress is an example of "emotional contagion," which supports a neural foundation for empathy. Emotional contagion is characterized by the observation of a behavioral change in an individual involuntarily eliciting the same behavior in the observer. Thus, it is a reflexive behavior based on a common experience. A recognizable example is yawning (Panskepp & Lahvis, 2011). Emotional contagion, on a more intricate level, involves affective "state-matching" of other individuals, which can therefore incite altruistic behavior reflecting a common memory or knowledge, similar to kin selection. Evidence that these functions are effective exists in somatoform disorders, specifically feigned distress syndrome or malingering. Somatoform disorders are characterized by asserting or exaggerating pain that cannot be explained through medical diagnosis. Often, these false behaviors exist "for attention," or to escape responsibility, especially in children. It is innate, due to the empathy centers in the brain, to respond to the pain of others, and the exaggeration of pain would, in theory, recruit more aid. Thus the prevalence of these conditions supports the neural basis for empathy.

Additionally, individuals with congenital insensitivity to pain (CIP) contribute to the foundation of knowledge. Individuals with CIP are very rare, and studies have shown that CIP patients, while they cannot relate to others in terms of describing and understanding painful experiences, the same areas in their brains are activated when being shown others under painful circumstances or distressed expression. Rather than sharing the actual painful response, they showed arousal in the anterior insula and anterior midcingulate cortex equivalent to that of a healthy pain-responsive person, indicating the functioning of mirror mechanisms (Danziger et al., 2009). CIP responses also exceeded normal responses in the posterior cingulate cortex and other midline areas, indicating that these structures play a role in empathic emotional perspective-taking as well.

Krahe et al. (2013) determined that 26 independent studies involving the social modulation of pain unanimously concluded that positive social interactions between participant and social partners (in pain and neutral) showed a positive correlation with analgesia. In addition to human studies, there are many animal models using a variety of species suggesting a neural basis for empathy. Most studies found that animals display empathic behavior, refrain from intentionally causing detrimental effects to others, and respond in a positive manner to receiving emotional support from others. Sixty-seven percent of rhesus monkeys abstained from pulling a feed lever that applied an electric shock to a bystander monkey, preferring starvation to deliberate cruelty (Masserman, 1964). Mice demonstrated increased pain-like behavior (wriggling/flailing) in the presence of another aching companion, and were quicker to retreat from a heat source following the observance of another mouse doing so (Langford, et al., 2006). Rats flee

from a group of flies, even flies incapable of biting after observing other rats uncomfortable while being bitten, suggesting they understood the others' uneasiness (Panskepp & Lahvis, 2011). Thus, multiple rodent studies have shown that unaffected animals recognize and react behaviorally to the pain of other animals in a multitude of situations, and even act to prevent the cause of such pain. These findings contribute to the discussion that specific brain structures underlie emotional empathic behavior.

**D. Rat Behavior**

In order to properly observe and document whether empathic-like behavior exists in the rats, typical interactions between male rats of the same species require definition, as well as knowledge of how changes in levels of certain behaviors indicate higher emotional processing. Rats are very social creatures. In the wild, they nest in burrows of up to seven animals, and form complex burrow systems to accommodate colonies of up to hundreds of individual rats (Hanson, 2012). This lifestyle is very distinct from and unlike that of shrews, lemmings and opossums; species that spend the majority of their life, aside from mating, in seclusion. In the wild, male rats exhibit mild agonistic behavior like chasing and biting toward each other in order to establish social hierarchies that frequently result in one dominant male and many other male (and female) subordinates. These social ladders are mainly an aid for survival, and typically are more extreme and relevant in high population densities, populations with fluctuating availability of food and water, or populations with limited female members. These patterns can also exist between members of opposing groups for the purpose of defending territory (Whishaw et al., 1999). Aggressive behaviors are typically not seen in low

population densities, colonies with constant adequate resources or in captivity. Also, agonistic behaviors are not regularly demonstrated until after six months of age (Hanson, 2012). This tendency is pertinent because all research in the current experiment was conducted using animals younger than six months of age.

Proximal rats, especially of the same age, are very social in their behaviors. Rats demonstrate non-aggressive play fighting that can include tackling, biting, wrestling, and chasing, especially during puberty. This serves to create and strengthen ties between individual animals, in the wild or captivity, and can occur at any stage in development, although its frequency peaks during the juvenile stages. In play fighting, as opposed to agonistic behavior, no malicious intent is present and no distress is exhibited by either or any of the playmates. Aggressive behavior is distinct from play in that agonistic rats typically seek to nip or bite another in the rump or hind region, and to inflict pain and superiority over the opponent. In play, rats enact a wide array of movements and interactions with a vague intent to thrust the snout into the neck of the other, who tries to avoid it (Whishaw et al., 1999). Again, playful rats do not generally cause intentional harm.

Rats are social in their sleep patterns as well. Although some specimens may sleep alone, rats often sleep in a "rat pile" in the same nest area. In the wild, this pattern exists to preserve body heat in colder environments, but it also builds positive interactions and promotes unity within a group (Hanson, 2012). In a controlled environment, such as a laboratory cage, temperature has less influence over an animal's need for warmth, therefore cagemates essentially make a decision of whether to sleep alone or near others. For this reason, nesting or sleeping with another animal is indeed a

social act. In turn, an increase in group nesting can be viewed as empathetic behavior, especially in the case of injured rats. Increased warmth and quality rest would promote healing, which is why sleeping arrangements are relevant to research involving pain.

Grooming is probably the most definitive social behavior that can be a manifestation of empathy. In addition to autogrooming, or self-grooming, rats frequently demonstrate allogrooming, or the grooming of others. Autogrooming typically starts with rotatory movements of the paws around the snout and nose, then lateral movements about the face, and then swipes caudally down the fur on either side of the body. Allogrooming of another individual rat usually occurs as nibbling or licking in the head, face and body regions that cannot be reached by the rat itself. This habit serves to strengthen relationships between members of a group and spread a common unifying scent (Whishaw et al., 1999). Because this ritual is wholly altruistic, serving no purpose of benefit to the allogroomer, an increase of this behavior can be viewed as an empathetic demonstration.

**E. Complete Freund's Adjuvant**

Complete Freund's Adjuvant (CFA) is a regularly used adjuvant system in experimental research. Jules Freund developed this product in the 1940s, containing mannide monooleate emulsified in paraffin oil and surfactant (Billiau & Matthys, 2001). Unlike Incomplete Freund's Adjuvant (IFA), CFA contains heat-killed *Mycobacterium tuberculosis* and is designed to generate an immune response from continuous antigen release (Billiau & Matthys, 2001). The presence of the bacterium attracts immune cells such as macrophages, which produces inflammation at the inoculation site, and in lymph

nodes and high level of antigen in circulation leads to an increase in responsiveness of T-lymphocytes (Billiau & Matthys, 2001). In experimental scenarios, concentrations of the solution components are often altered to produce stronger responses. CFA produces such a pronounced reaction, but can cause side effects such as granulomas, arthritis and ulcers. For this reason, CFA is often utilized for initial treatment, and IFA is then used in follow-up treatments to prolong the effects of the primary response (Billiau & Matthys, 2001). To compare the level of nociceptive behavior between isolated and socially-housed rats in the present experiment, a single dose of CFA was administered to specific rats to generate an inflammatory, or painful, response. The rats that were not assigned CFA treatment received a dose of sterile saline under the same anesthetic conditions.

**F. Specific Aims**

To summarize, humans and rodents have been shown to demonstrate empathy, which is evident from behavioral and imaging studies. There are both specific and broad regions in the brain that directly pertain to and regulate the level of empathic processing in both humans and other species. The aim of the present study was to explore the influence of empathic behavior on the severity of nociceptive behavior. This influence was determined through investigating the relationship of group housing on inflammation and nociceptive behaviors. In doing so, the hypothesis was tested whether rats with localized inflammation, living together with rats without inflammation, would display differences in their pain-related behavioral responses, degree of inflammation, general health and frequency of empathy-related behavior. The parameters of examination included body weight, paw withdrawal latency and paw thickness, following an injection

of Complete Freund's Adjuvant (CFA) into the hind paw for an inflammatory (painful) model or sterile saline as the control. The goal was to determine if rats display empathy, provide a social support to conspecifics, and whether this social support has a positive effect. A qualitative behavioral-assessing component was included to ensure that the observed effects were indeed due to interactions between animals and not a confounding variable. These discoveries may illuminate potential future therapeutic techniques for living conditions and treatment techniques for chronically pained individuals.

Specifically, the experimental hypothesis was that whether injured rats receiving empathic social support of higher magnitude from non-injured rats would display less pain-like behavior, as measured by higher paw withdrawal latency times, due to the empathic influence of healthy rats. Based on this idea, the injured rats in cages with lower ratios of afflicted rats were expected to display the least amount of pain-like behavior. In addition, it was hypothesized that affected rats receiving social support from other affected rats would also fare better, and therefore display less pain-associated behavior, than injured isolated control rats, because of the influence of empathy. This means that rats affected with inflammatory agent in cages of higher ratios (cages with more affected than non-affected rats) would experience higher paw withdrawal latency times when compared to an isolated affected control group. The group displaying the most pain-like behavior and highest paw withdrawal latency time was expected to be those affected and in isolation, because they would experience the effects of the inflammatory model but without the empathic social support experienced by socially housed animals. In comparison to the saline control groups, an unaffected social group was expected to display higher paw withdrawal latency than the isolated saline control group, and the

social group's level of pain-like behavior compared to the social groups was used to draw conclusions regarding which social ratios in fact yield the most alleviating and beneficial effects. These inferences are useful for speculation regarding possible future treatments and therapies.

During the behavioral observation of the rats in their home cage environments, baseline occurrences of playing, digging, self-grooming and eating and drinking were indicators of activity level and general well-being. Allogrooming and moments of non-specific contact between one or more rats were considered an indication of voluntary interaction and therefore empathy. These behaviors were compared with the numbers of occurrence after injection and following recovery. Also, comparison of the frequency of certain behaviors in individual cages with level of pain-like behavior in that specific cage may yield further interesting support of the base hypothesis. These observations are strictly qualitative.

## II. Methods

### A. Animals

All studies were performed on male Sprague-Dawley rats (n = 72), 27-32 days old upon arrival (Charles River Laboratories, Inc., Wilmington, MA). The animals were kept on a 12/12-hour dark/light cycle beginning at 10:00 A.M. and 10:00 P.M., respectively. The animal facility, surgical and behavioral testing laboratories were maintained at 20-23° C. A diet of pellet food (Lab Diet 5P00 Prolab RMH 3000 PMI Nutrition International Brentwood, MO) and water was provided *ad libitum*. All rats received the same housing conditions, in polycarbonate cages measuring 20" long, 16" wide and 8½" tall. The home cage bedding consisted of aspen pine shaving and Bed-O-Cobb, and it was changed weekly by animal facility staff. No additional supplemental materials were provided in the home cage environment. Cages were housed on shelves either 6", 3' or 6' from the floor. All procedures were approved by Youngstown State University's Institutional Animal Care and Use Committee (Protocol #03-14).

### B. Home Cage Housing Groups

After a one-week acclimation period to the animal care facility, animals underwent habituation sessions with the experimenters for one hour on three consecutive days. During each session, rats were transported from the animal care facility to the behavioral testing laboratory where they were handled individually for approximately ten minutes per rat. Animals were then randomly assigned to either a control group (saline injection), or a treatment group (CFA injection). The control and treated animals were then placed into different housing groups, as detailed in Table 1. Group A consisted of

one rat per cage with localized hind paw inflammation, and Group B consisted of one rat per cage with saline injection. The individually housed rats served as controls to demonstrate the results of the inflammation without home cage social influence. The remaining groups of four rats per home cage served to test home cage social influences of differing magnitudes and its effects on localized inflammation. Group C consisted of four rats all with localized inflammation. Group D consisted of three rats with localized inflammation and one rat with saline injection. Group E consisted of two rats with localized inflammation and two rats with saline injection. Group F consisted of one rat with localized inflammation and three with saline injection. Group G consisted of four rats per cage with saline injection. Home cage placement on the shelves in the animal care facility was random. After each data collection point or experimental procedure, home cages were randomly placed on the shelves in the animal care facility to reduce any social influence between cages.

**Table 1. Home Cage Housing Environments**

The control and experimental treatment groups, including the number of rats per home cage, and the numbers of rats with both saline and Complete Freund's Adjuvant (CFA) in each respective cage. This experimental design was repeated three times with separate rats, and the total number of rats per treatment group is also represented. The total experimental sample size was n = 72. The rows below each group indicates the percentage of affected rats in each cage. These percentages will be used to refer to rats in the Results and Discussion sections.

| Treatment Group Name | Total Rats/ Home Cage | Total # of Rats with Localized Hind Paw Inflammation | Total # of Rats without Localized Hind Paw Inflammation | Total # of Rats in each Treatment Group throughout the Experiment |
|---|---|---|---|---|
| A | 1 | 1 | 0 | 6 |
| | | 100% CFA | | |
| B | 1 | 0 | 1 | 6 |
| | | | 100% SAL | |
| C | 4 | 4 | 0 | 12 |
| | | 100% CFA | | |
| D | 4 | 3 | 1 | 12 |
| | | 75% CFA | 25% SAL | |
| E | 4 | 2 | 2 | 12 |
| | | 50% CFA | 50% SAL | |
| F | 4 | 1 | 3 | 12 |
| | | 25% CFA | 75% SAL | |
| G | 4 | 0 | 4 | 12 |
| | | | 100% SAL | |

**C. Experimental Design**

Following familiarization to the investigators and behavioral testing laboratory, baseline behavioral data was obtained prior to housing assignment (14 days prior to injection). Pre-treatment behavioral data was collected one day before injection and post-injection data was collected once per week following injection (7 days, 14 days, 21 days, 28 days, 35 days, 42 days, 49 days, and 56 days) until behavioral measurements returned to baseline level. The experimental timeline is depicted in Table 2. Body weight was also recorded at each behavioral data collection session to ensure normal health and well-being. All data were collected between 10:00 A.M. and 3:00 P.M., during the animals' active (dark) phase.

**Table 2. Experimental Design and Timeline**

This table depicts the experimental design for this study, showing what procedure or collection occurred at each time point. Each row represents approximately seven days. The arrival period was to allow animals to acclimatize to the new environment and decrease the influence of stress regarding travel and novel surroundings. Randomized housing assignment occurred on Day -14. Only the qualitative behavioral observation was conducted on Day -7, which served as baseline. Day -1 included a weight, paw thickness and paw withdrawal latency measurements. The quantitative results on Day -14 and Day -1 served as baseline. Day 0 included weight and paw thickness measurements following the injection procedure. Qualitative behavioral observation occurred on Day 7 in addition to the other data collection. All post-injection behavioral data collection sessions consisted of weight, paw thickness and paw withdrawal latency measurements. All paw thickness measurements had returned to baseline levels by Day 56, when qualitative behavioral observation was conducted again.

| Day | Action |
|---|---|
| -24 | Arrival |
| -17, -16, -15 | Habituation |
| -14 | Baseline Behavior<br>Assignment to Home Cage Housing Environment |
| -7 | Qualitative Home Cage Behavioral Observation |
| -1 | Pretreatment Behavior |
| 0 | Injection |
| 7 | Post-Injection Behavior<br>Qualitative Home Cage Behavioral Observation |
| 14 | Post-Injection Behavior |
| 21 | Post-Injection Behavior |
| 28 | Post-Injection Behavior |
| 35 | Post-Injection Behavior |
| 42 | Post-Injection Behavior |
| 49 | Post-Injection Behavior |
| 56 | Post-Injection Behavior<br>Qualitative Home Cage Behavioral Observation |

**D. Injection Procedures**

All hind paw injections were performed under isoflurane anesthesia (3%) and aseptic conditions. Animals assigned to receive a localized inflammation were injected with 100 microliters of 100% Complete Freund's Adjuvant (CFA) using a sterile 28-gauge syringe. Control animals received an injection of 100 microliters of 0.9% sterile saline solution, also with a sterile 28-gauge syringe. Injections were administered subcutaneously to the central plantar surface of the left hind paw, as depicted in Figure 3. Prior to injection, each paw was cleaned with an ethanol solution (70%) and animals were monitored upon waking from anesthesia. Each subject was under anesthesia no longer than five minutes.

**E. Animal Body Weight**

Animal body weight was measured using a standard laboratory scale, which was sterilized using a 70% ethanol solution following each measurement. Animal body weight can be viewed as a manifestation of health and well-being. Sprague-Dawley rats reach adolescence at approximately 35 postnatal days and early adulthood at approximately 63 postnatal days and are considered mature adult after approximately 98 postnatal days (Sengupta, 2013). The rats utilized in the study were between 27 and 32 postnatal days old upon arrival, and the experiment lasted approximately 80 days. Therefore, rats were a maximum of 112 days old at the conclusion of the experiment. Since rats were still maturing during the majority of the experiment, reaching adulthood at approximately Day 42, they would be expected to gain weight until this point, and then reach a steady plateau weight. Although weight can vary between rats of the same age,

weight gain reflects typical level of health at during these stages of development. Normal weight gain suggests that no factor was altering food or water consumption, nutrient acquisition, urination, defecation or any other bodily processes that could be attributed to disease, malnourishment or other problem (Grunberg et al., 1986) and this translates to a wholesome level of health. Normal behaviors such as eating, grooming, digging, which were observed, as well as drinking, and sleeping under healthful conditions signify a high level of well-being (Horn et al., 2012).

**F. Edema Quantification**

Hind paw thickness of each hind paw was measured using a caliper placed along the dorso-ventral surfaces. Measurements were made to assess the degree of edema in the CFA versus saline-injected paws. The targeted areas for both edema measurement (Figs. 6 and 7) and paw withdrawal latency (Figs. 5 and 6) are provided.

**G. Paw Withdrawal Latency to a Heat Stimulus**

Paw withdrawal latency (PWL) to a heat stimulus was recorded using the IITC Plantar Analgesia Meter, Model 400 (Woodland Hills, CA). Rats were placed into a vented plexiglass chamber on a glass surface at ambient room temperature. Following a 5-10 minute habituation period, a radiant heat stimulus of 44-46 °C was alternately applied from underneath to each hind paw, as depicted in Figure 4. The time from initial application to paw withdrawal was recorded in seconds. Data collection was performed by sliding the device underneath the glass surface. By aligning the stimulus to the target region, via the guide light, the heat was applied solely to the hind paw area. The thermal

stimulus was focused onto the glass to create a 4x6 mm spot on the plantar paw surface. A 20-second exposure limit was imposed to prevent tissue damage. Each paw was tested four times, with each exposure separated by at least five minutes to prevent overstimulation or sensitization of the involved neural pathways. The PWL of each paw of each rat was calculated as the mean of trials 2-4 (trial 1 was excluded to prevent irregularity of the animals' initial adjustment to the apparatus). The apparatus was sterilized with a 70% ethanol solution following data collection from each treatment group.

**Figure 3. Injection Site and Stimulus Target**

The specific target for subcutaneous injection and the black circle illustrates the area used for paw thickness measurements and for the application of the heat stimulus area during withdrawal latency measurements.

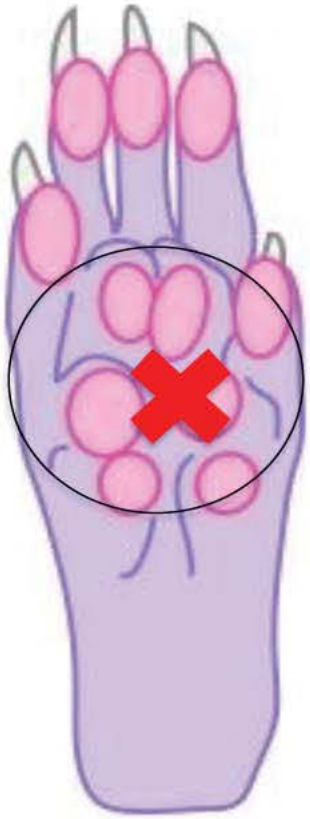


**Figure 4. Ventral View of the Typical Lab Rat**

A ventral view of the rat as viewed on the paw withdrawal latency data collection apparatus. The guide light and mirror facilitate proper alignment with the center hind paw region, as indicated by the black circles.

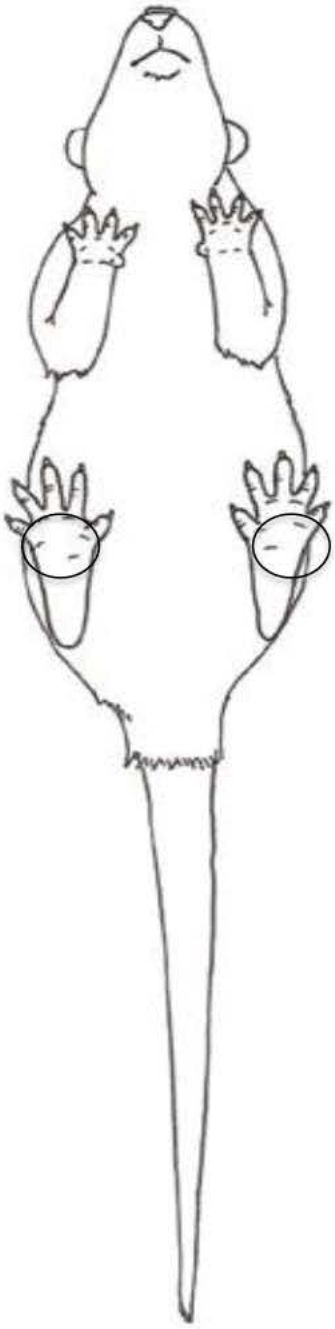


**Figure 5. Paw Thickness Target**

The dorsal surface of the rat, the left hind paw is colored red. The black bracket details the area around which the caliber is placed to measure paw thickness and assess edema quantification.

**G. Statistical Analysis**

A series of mixed Analysis of Variance tests (ANOVA) was used to analyze the effects of group housing over the course of a sub-chronic localized inflammation. Tests including Shapiro-Wilk test for normality, Levene's test for homogeneity of variance, Mauchly's test for sphericity (Lund & Lund, 2013) and tests for outliers using a g value of 2.2 (Hoaglin & Iglewicz, 1987) were conducted for each measurement to ensure that all assumptions of the Mixed ANOVA tests were met. The Greenhouse-Geisser statistic for weight, and a log-based ten normalization for paw withdraw latency, were employed to accommodate for violations of assumptions of sphericity (Tukey, 1977). ANOVA testing determined whether CFA injection caused a subchronic inflammation in selected subjects by comparing to the non-selected subjects. Further testing compared the level of overall health and wellbeing in affected versus non-affected rats via a mixed ANOVA test using the body weight measurements across time. Lastly, a difference in nociceptive behavior among affected versus non-affected rats was investigated. To achieve this, groups were compared using a mixed ANOVA test. Data were consistently expressed as mean ±standard deviation for all numeric parameters and was considered significant if $p \leq 0.05$. Significant results were investigated using the Tukey SD and Bonferroni post-hoc tests.

**H. Qualitative Home Cage Behavioral Analysis**

Qualitative data of home cage behaviors were collected for ten minutes per cage on three separate occasions. Baseline home cage behavioral observations occurred on day -7, seven days prior to injection. The second qualitative behavioral observation was

conducted immediately following PWL and thickness measures on post-injection day 7. The second observation served as the experimental data and the point of comparison to baseline. The third analysis was conducted on Day 56 in the same manner, serving as the measurement following recovery for comparison to both previous analyses. These data collection days are listed in Table 2. Subjective observations were documented by the same researcher, and specific notes of digging in the bedding, rearing against the side of the cage, eating or drinking, autogrooming or self-grooming with the paws or licking, allogrooming or licking or grooming of another animal with the paws, playful nipping or tumbling, or aggressive behavior like bites between cagemates were made. Moments of non-specific contact were also noted and defined as paw to body contact between two rats not distinctly recognizable from playing, grooming or aggression. The length of each instance of behavior was not calculated, and each measure was uniquely tallied only if the previous instance of the same behavior in the same rat was completely dissociated. The observational data was not analyzed statistically, but served as an indicator of social interactions in each respective home cage housing environment for comparison of how these interactions changed after injection.

## III. Results

### A. Qualitative Behavioral Analysis

During the qualitative analysis, each social treatment group (n = 4 rats per home cage) was observed in their home cage for 10 minutes after PWL data collection. This included home cage treatment groups C (100% CFA), D (75% CFA, 25% SAL), E (50% CFA, 50% SAL), F (25% CFA, 75% SAL) and G (100% SAL). Isolated groups (n = 1 rat per home cage) A (100% CFA) and B (100% SAL) were not assessed because no empathic behavior could be documented in singly-housed animals. These observations were made three times per cage: (1) 7 days prior to injection, (2) 7 days after injection, and (3), 56 days after injection. While general observations were being made, instances of specific behaviors were monitored. These behaviors included playfulness, grooming events, digging and nesting, eating, rearing, and moments of nonspecific contact between one or more rats. Statistical analyses were not performed because of the small sample sizes.

Overall, on the second observation day, Day 7, rats in cages C-G, the social rats in various CFA/SAL ratios, remained in rearing postures, either against the wall or ceiling of the cages, approximately 30-40% of the time. It seemed as if ambulation time, motion, and general activity levels were increased at Day 7 (after injection) in comparison to Day -7 (prior to injection). Observed behaviors were not considered to be distinct from motion, stillness, or rearing posture, meaning they could occur simultaneously or independently of movement. For example, while digging was categorized distinctly, it was not excluded when calculating general level of movement.

Rats were generally much more active during the post-injection observatory period. Cages displayed an average of 8 instances of playfulness and 9.4 digging events on Day 7 in comparison to only 4.6 instances of play and 5.8 digging events on Day -7. Only 2 instances of eating occurred in the Day -7 period, by only one rat in cage G, however there were 25 distinct eating events on Day 7, across a number of different rats in all the social cages. This overall increase in activity is unexpected because on Day 7, some rats are afflicted with the CFA. It would be expected that because of the inflammation evident in the paw edema quantification measurement, that these rats specifically in Cages C (100% CFA) and D (75% CFA 25% SAL) would be more sedentary to prevent further pain. This is not the case, since all three behaviors (digging, eating and playing) increased in both cages, supporting that the rats in these home cages were less afflicted by the CFA than would be expected. These behavioral frequencies are listed in Table 3A-C.

Moments of non-specific contact in which one or more paws or snout of one animal touched any part of another animal's body in a manner independent from grooming, playing and aggression also increased from Day -7 to Day 7 but were largely present among all cages in both intervals. There were 43 total instances for an average of 8.6 per cage on Day -7, and 54 total instances for an average of 10.4 instances per cage. Length of each specific interaction was not measured, and it is to be noted that each individual moment of non-specific contact with another rat varied. Some lasted only seconds, others lasted over a minute, but each consisted of contact with one or more paws to any part of another rat. Moments of non-specific contact in each cage are reported in Table 3D.

Grooming events, involving the licking and wiping of the face and body, were of particular interest during the observation. It was expected that rats would engage in hygienic behavior when placed back in their home cages after being handled, however two types of grooming occurred. Self-grooming or autogrooming events were prevalent, and they remained relatively constant between Day -7 and Day 7, from 24 total instances to 26. Allogrooming, or one rat grooming another, increased from 22 total instances on Day -7 (similar to the 24 autogrooming events) to 38 total events on Day 7. This is a 72% increase in allogrooming events, again noting that length of event was not measured. These measurements are displayed in Table 3E-F. Another point of interest was Cage C (100% CFA), where only 3 allogrooming events occurred prior to injection, but 17 events occurred post-injection, over a 400% increase. This demonstrates that more allogrooming occurred in the 100% CFA housing group following injection. Since allogrooming is considered to be an empathetic behavior, this observation supports the experimental hypothesis. The observed increases in playful behavior and moments of close proximity and contact also support the hypothesis, because playing is a social behavior that is not directly associated with survival. The increase in playing indicated that more socialization was occurring by choice in cages from Day -7 to Day 7. As previously discussed, temperature maintenance is not a hazard to caged rats in the controlled laboratory environment, so contact with other rats is likely by choice. These increases in close-knit behavior also indicates an increase in social behavior, which is of specific interest for Cages C (100% CFA) and D (75% CFA 25% SAL) as these cages displayed increases in instances of non-specific contact from 7 to 15 and 4 to 10 over the span of time, respectively.

Observational measurements made on Day 56 were not as strongly correlated with the experimental hypothesis as those made on the other two days. It would be expected that overall activity level would increase at this experimental interval, because the CFA effects would be less prominent, the injected paw has healed and the animals would experience less nociception. Also, at this time point, the animals had been group housed for approximately ten weeks and it would be expected that the home cage group would be bonded to each other and comfortable with behavior such as contact, playing and allogrooming. However, because the CFA effect is weakened or completely absent in the injected rats at this point, there is more similarity with regards to paw thickness, cause for pain-like behavior, and overall well-being between injected and non-injected rats in each cage, and it would be expected that specific empathy-rooted behaviors would remain constant or even decrease.

General activity level in all cages generally remained constant throughout all three observations, but instances of some specific behaviors varied in comparison to prior dates. Autogrooming events remained constant throughout the experiment from 25 ± 1 total events on each day tested, which is to be expected as measurements took place after rats were handled. The general activities of playing, digging and eating, each decreased on Day 56 from Day 7. However, the empathic-related social behaviors of allogrooming and moments of contact are of interest. Allogrooming increased over the observations on Day -7, Day 7 and Day 56 from 22 to 38 to 56 events, respectively, and contact increased from 43 to 54 to 55. These increased despite the dwindling effects of CFA suggesting not only that the closely-associated cagemates were bonded closely, but also that the empathic behavior following the injections may be prolonged beyond the experimental

window. This may indicate a learned behavior that could continue through the animals' lives. While contact increased just slightly, varying by only a few instances per cage, autogrooming, which was deemed wholly empathic, increased by 147%. It increased in all cages but C, decreasing from 17 to 13 total instances. Since all four rats were treated with CFA in this case, this cage likely demonstrated the strongest evidence of CFA effects, meaning the CFA wearing off is most perceivable in this cage. This decrease in behavior can be attributed to the deterioration of CFA effect, by all four rats at once.

In conclusion, the qualitative analysis of empathic and active behaviors indeed supports the experimental hypothesis that home-cage environment plays a role in levels of empathy demonstrated among rats housed together with different ratios of other CFA-treated rats. Not only does the environment influence levels of pain-like behavior over the course of the localized inflammation, it directly impacts the frequency with which empathic behaviors are demonstrated among the experimental rats, specifically after being handled.

**Table 3. Qualitative Behavioral Analysis Results**

The summarized documented instances of specific behaviors observed in each social cage on Days -7, 7 and 56. In each cage C is 100% CFA-treated animals, cage D is 75% CFA and 25% SAL, cage E is 50% CFA and 50% SAL, cage F is 25% CFA and 75% SAL, and G is 100% SAL-treated animals. The total number of occurrences of each behavior at each time point, as well as the average number of instances among social cages are shown. Part A details the instances of friendly, non-aggressive pouncing, tackling or snubbing between two or more rats of the same social cage. Part B details the instances of digging by a single rat in the bedding during social cage observation. Part C lists eating events by a single rat from the provided diet pellets during social cage observation. Part D references the number of times per social cage that one rat came in contact with another rat's body using its paws, a motion that was not recognizable as play or aggression. Part E depicts the self-grooming events in social cages during the observatory periods. Part F illustrates the amount of allogrooming between one or more rats in social cages. Tables A-C depict behaviors that reflect overall measures of activity. Tables D-F detail empathy-related behaviors.

**A.**

| Playing | Day -7 | Day 7 | Day 56 |
|---|---|---|---|
| **C** | 9 | 12 | 5 |
| **D** | 7 | 11 | 7 |
| **E** | 2 | 8 | 13 |
| **F** | 2 | 2 | 4 |
| **G** | 3 | 7 | 6 |
| **total** | 23 | 40 | 35 |
| **mean** | 4.6 | 8 | 7 |

**B.**

| Digging | Day -7 | Day 7 | Day 56 |
|---|---|---|---|
| **C** | 2 | 10 | 5 |
| **D** | 7 | 10 | 0 |
| **E** | 5 | 9 | 1 |
| **F** | 5 | 12 | 5 |
| **G** | 10 | 6 | 3 |
| **total** | 29 | 47 | 14 |
| **mean** | 5.8 | 9.4 | 2.8 |

**C.**

| Eating | Day -7 | Day 7 | Day 56 |
|---|---|---|---|
| **C** | 0 | 5 | 2 |
| **D** | 0 | 4 | 1 |
| **E** | 0 | 2 | 7 |
| **F** | 0 | 4 | 1 |
| **G** | 2 | 10 | 8 |
| **total** | 2 | 25 | 19 |
| **mean** | 0.4 | 5 | 3.8 |

**D.**

| Contact | Day -7 | Day 7 | Day 56 |
|---|---|---|---|
| **C** | 7 | 15 | 16 |
| **D** | 4 | 10 | 9 |
| **E** | 6 | 9 | 11 |
| **F** | 17 | 12 | 13 |
| **G** | 9 | 8 | 6 |
| **total** | 43 | 54 | 55 |
| **mean** | 8.6 | 10.8 | 11 |

**E.**

| Self-groom | Day -7 | Day 7 | Day 56 |
|---|---|---|---|
| **C** | 6 | 9 | 4 |
| **D** | 2 | 8 | 7 |
| **E** | 7 | 6 | 5 |
| **F** | 4 | 0 | 7 |
| **G** | 5 | 3 | 3 |
| **total** | 24 | 26 | 26 |
| **mean** | 4.8 | 5.2 | 5.2 |

**F.**

| Allogroom | Day -7 | Day 7 | Day 56 |
|---|---|---|---|
| **C** | 3 | 17 | 13 |
| **D** | 5 | 8 | 17 |
| **E** | 4 | 4 | 7 |
| **F** | 6 | 3 | 10 |
| **G** | 4 | 6 | 9 |
| **total** | 22 | 38 | 56 |
| **mean** | 4.4 | 7.6 | 11.2 |

**B. Quantitative Data Analysis**

In the following statistical analyses and figures (6-8), responses are shown in relation to the ratios of affected versus non-affected rats. Groups A and B are isolated animals (one animal per cage) and groups C-G are housed socially (four animals per cage). In all figures, the shades of red and circles indicate those animals that were treated with CFA and the blue squares represent those animals that were treated with saline. All data were collected and organized according to each rat's specific treatment group assignment, meaning that CFA-injected rats and saline-injected control rats in the same housing group were classified separately for analytical purposes.

Animal body weights (Figure 6) show gradual consistent increase among all treatment groups. The average body weight regardless of housing group at Day 0 was 275 ± 42 g, and 424 ± 37 g at Day 56. Animal weights increased every week. A statistical analysis comparing the mean weight of the treatment groups across time found no significant difference ($F[1, 12.954] = 1.379$, $p = 0.19$). Social versus isolated housing and different affect ratios played no role in the average weight gain, degree of health, or level of well-being in the animals, as deduced by the statistic. This constant increase verified that all rats gained weight steadily and were healthy throughout the course of the experiment.

The average paw thickness of the injected hind paw of each treatment group at each time point is depicted in Figure 7. The CFA rats' mean left paw thickness on Day -14 was 5.00 ± 0.40 mm, increasing 135.8% to 6.79 ± 1.12 mm at Day 7 and gradually returning to relative normal of 5.80 ± 0.99 mm at Day 56. In contrast, left paw thickness in control rats was relatively consistent across the experiment, 5.00 ± 0.45 mm at Day -

14, 5.34 ± 0.40 mm at Day 7, and 4.90 ± 0.63 mm at Day 56. Thus, mean paw thicknesses diverged between saline and CFA injected rats abruptly after Day 0, but this difference declined with time up to Day 56. Paw thicknesses at the conclusion of the experiment are similar between all treatment groups, although higher than at the commencement. The fact that the mean paw measurement is slightly thicker at the end of the experiment versus the beginning of the experiment is attributed to weight gain in all rats, which indicates that proper care was provided throughout the experimental period.

It was concluded that there was a significant difference that occurred in average paw thickness as a result of treatment ($F[1, 72] = 3.297$, $p < 0.001$). This was expected, as cages with a higher ratio of CFA-injections to saline injections would have a higher average paw thickness because of the edema and inflammation. However, post-hoc testing determined that differences only occurred between groups between the CFA versus saline groups. No significant differences were found between treatment groups of the same injection type, indicating that housing group had no affect on paw thickness in this respect. These trends in paw thickness validate the experimental model of CFA as an inflammatory agent (Billiau & Matthys, 2001). In this procedure, the use of CFA as a temporary localized inflammation was efficacious and effects of the agent did not persist following the conclusion of the experiment.

Injected hind paw withdrawal latency (PWL) was determined for all animals over the course of the experiment (Figure 8). The average PWL for all rats at Day -14 was 12.60 sec, negligibly increasing to 13.06 sec at pretreatment Day -1, a change which could partially be attributed to familiarization with the testing apparatus. The PWL measurements of saline-injected control rats held steady throughout the study, averaging

12.25 ± 0.53 sec across all time points and 11.97 sec at the conclusion, Day 56. This consistency served as an overall control for the CFA treated groups. However, PWL measurements in CFA treated rats become more irregular with regards to the established control, as expected following the CFA injection. The inflammatory model's effects are most distinguishable at this point, becoming less discernible at later times as the edema subsides and the effects abate. The average PWL of CFA rats at Day 7 decreased to 9.96 sec with some lower values of 8.84 sec in isolated Group A, and 7.32 sec in affected rats in social Group F. The average PWL of CFA rats fluctuate at each time point, 11.14 sec at Day 14, 11.08 sec at Day 21, 11.01 sec at Day 28, 12.45 sec at Day 35, 12.01 sec at Day 42, 10.67 sec at Day 49 and 11.61 sec at Day 56, with the CFA rats of social Group F showing a response markedly lower in most cases: 6.825 sec at Day 14, 5.54 sec at Day 21, 6.445 sec at Day 28, 7.57 sec at Day 35, 8.28 sec at Day 42, and 6.68 sec at Day 49. The PWL values of affected rats in Group F are is still low at the final behavioral session, with a mean of 6.93 sec at Day 56, despite paw thickness measurements having returned to the baseline level. This indicates some factor affected the CFA rats in social group F. This is evident in Figure 8, where the responses of CFA-treated Group F rats appear to be lower than all the other experimental groups.

In order to determine if any significant interaction between housing groups with regards to display of pain-like behaviors occurred, a statistical analysis was conducted. It was concluded that there was no difference between treatments groups in left PWL across time, ($F[1, 81] = 1.181$, $p = 0.15$). This means there is a 15 out of 100 likelihood that the differences in behavioral quantification between treatment groups occurred by chance alone, which is highly likely. Despite this unexpected result, there are still many trends

and observable correspondences throughout the experiment that are notable and worthy of discussion.

**Figure 6. Rat Body Weights Over Time**

The average body weight of each experimental group over time.

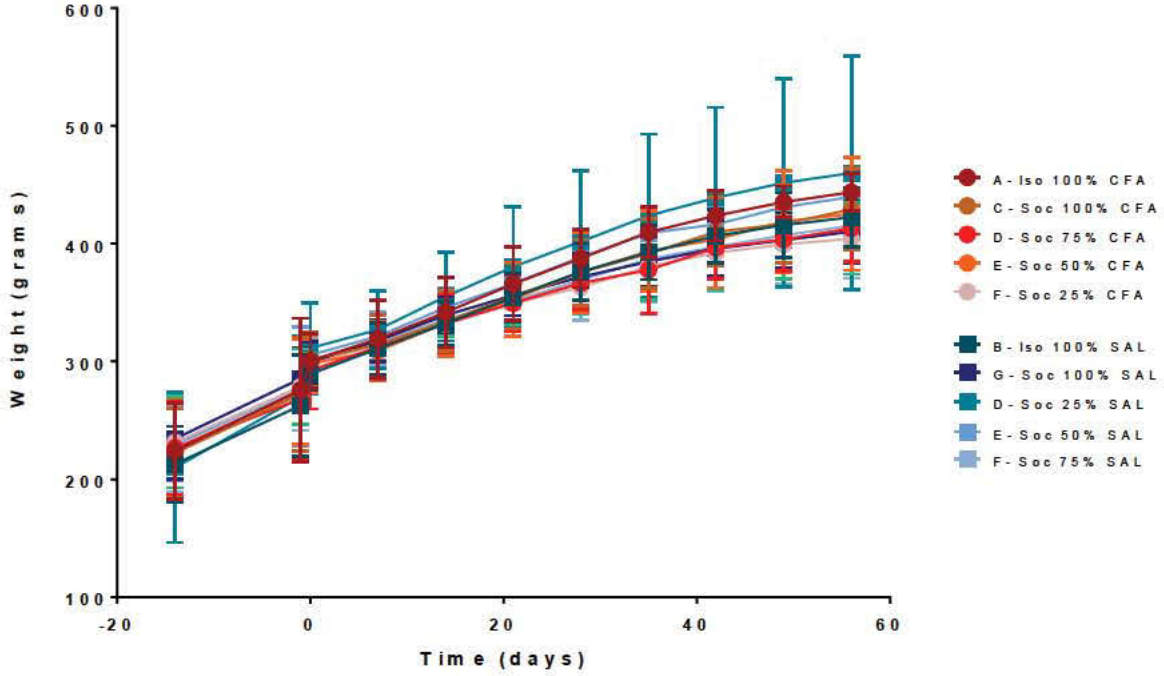

600
500
400
300
200
100
Weight (grams)
-20
0
20
40
60
Time (days)
A - Iso 100% CFA
C - Soc 100% CFA
D - Soc 75% CFA
E - Soc 50% CFA
F - Soc 25% CFA
B - Iso 100% SAL
G - Soc 100% SAL
D - Soc 25% SAL
E - Soc 50% SAL
F - Soc 75% SAL

**Figure 7. Injected Paw Thickness over Time**

The mean paw thickness of the different experimental groups over time.

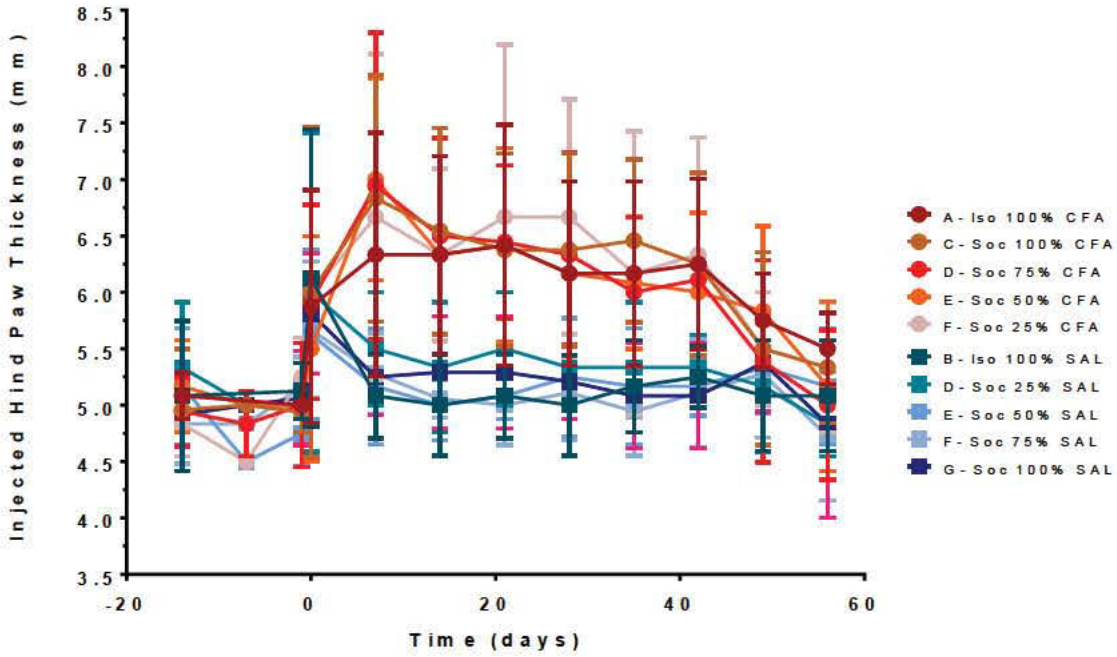
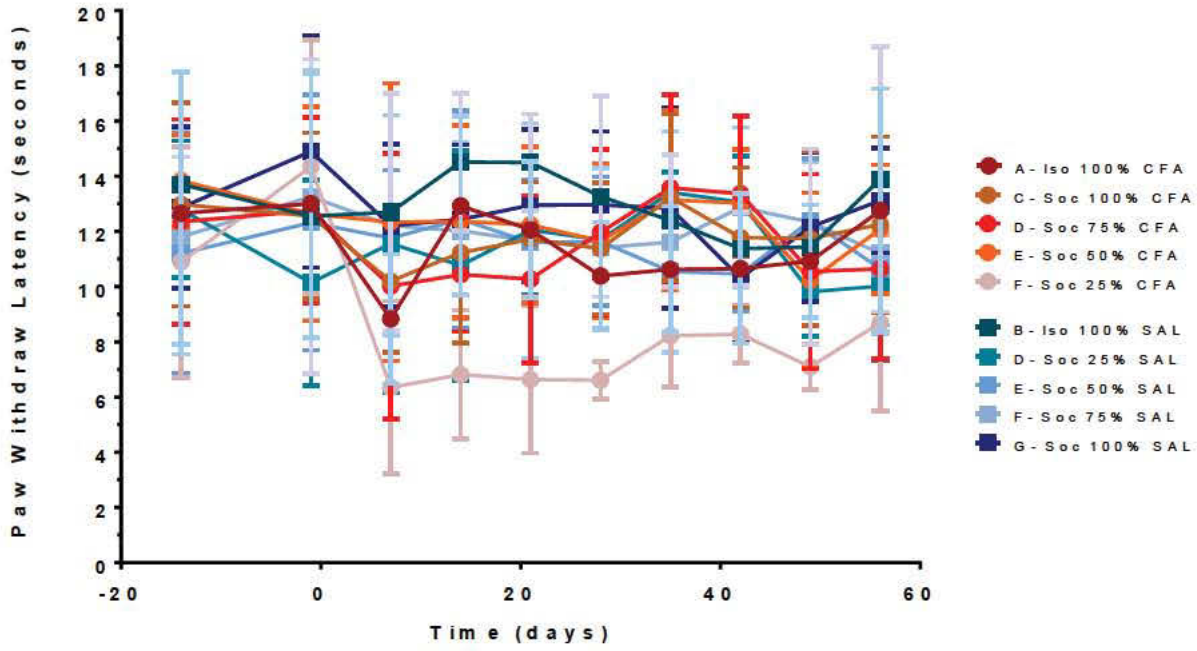

Injected Hind Paw Thickness (mm)
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
-20
0
20
40
60
Time (days)
A - Iso 100% CFA
C - Soc 100% CFA
D - Soc 75% CFA
E - Soc 50% CFA
F - Soc 25% CFA
B - Iso 100% SAL
D - Soc 25% SAL
E - Soc 50% SAL
F - Soc 75% SAL
G - Soc 100% SAL

**Figure 8. Injected Paw Withdrawal Latency over Time**

The mean paw withdrawal latency (PWL) of the different experimental groups over time. Each rat's individual measurement at each time point was an average of three independent PWL trials.


Paw Withdraw Latency (seconds)
0
2
4
6
8
10
12
14
16
18
20
-20
0
20
40
60
Time (days)
A - Iso 100% CFA
C - Soc 100% CFA
D - Soc 75% CFA
E - Soc 50% CFA
F - Soc 25% CFA
B - Iso 100% SAL
D - Soc 25% SAL
E - Soc 50% SAL
F - Soc 75% SAL
G - Soc 100% SAL

## IV. Discussion

The main aim of this investigation was to determine if social groups affected the level of nociceptive behavior displayed by Sprague-Dawley rats following a localized inflammation to the hind paw region. While no overall statistical significance was found, there are visible trends to be discussed and other principles to be considered, including the benefits of social isolation, especially on the healing process, and the advantage of similarity between like-individuals in rehabilitation, and the tendency of victims of a severe strain to develop further stresses and psychological tendencies.

With regards to the previously stated specific hypothesis, empathy-related social support was not proven to alleviate level of pain-like behavior in any of the tested social group ratios. CFA-treated rats housed with non-affected rats (Groups E and F) did not display significantly higher PWL than isolated control rats (Group B). In fact, the opposite effect was observed, particularly in the 25% CFA Group F, and a number of explanations can be offered for this, to be discussed. CFA-treated rats housed with other CFA-injected rats did not exhibit notably higher or lower levels of pain-like behavior than CFA-affected rats housed with non-affected saline rats, than isolated CFA or saline-injected rats, or than socially-housed control saline rats. On this evidence alone, empathic behavior derived from social housing does not play a role in the level of pain-like behavior demonstrated by rats affected by a localized inflammation. However, physiological principles, existing literature, observational data, and observable trends in the data still suggest a relationship to be discussed and determined in future experimentation.

Firstly, it is notable by the data that the control rats, housed in isolation, did not differ significantly from the rats housed socially in any of the parameters, regardless of injection type (CFA or saline). Social isolation has shown to be a form of stress, as it has been shown to increase pain-like responses in similar animal models, and social housing is considered beneficial to overall development and alleviating pain-associated behavior (Bravo et al., 2013). This experiment tested that notion. However, it is possible that contrary effects occurred and lead to the current results. Crowding is also considered a stressful stimuli, and crowding, or having more than appropriate number of animals per unit of space, has been shown to increase corticosterone secretion and decrease body weights of involved species, and can also affect certain behaviors. These effects are perceptible even after only one week (Chaouloff and Zamfir, 1993). The duration of the current experiment was twelve weeks, and the animals grew notably throughout (an average weight gain of ~150 g) so the effects of crowding may have become more prominent as animals gained weight and physically occupied more space in the cage. In turn, this crowding may have negatively influenced the socially housed rats and became more influential as time passed, although the crowding was not extreme enough for the animals to exhibit weight loss. Negative effects of crowding on socially housed rats would affect them such that their response to be increased and their PWL would be lowered, and more similar to the isolated rats. The social groups displaying an exacerbated level of pain-like behavior, therefore lower PWL measurements would more closely resemble the isolated groups. Group A PWL theoretically would be the lowest, because these rats were subject to the effects of both isolation and CFA and Group B PWL would be highest be highest because they were not exposed to CFA or the effects of

crowding. However, results indicated that the display of pain-like behavior in Group B was only minimally elevated compared to other groups, however not significantly, and not reliably across all time points. Also, the measurements of Group A were statistically comparable to Group B as well as all social groups. This indicates some factor was causing leveling the level of pain-like behavior exhibited between isolated and social groups, possibly due to crowding.

It is also notable that all environments were non-supplemented, meaning that nothing was present in the cages other than bedding. Previous studies have shown that animals housed in this manner display increased levels of anxiety in behavioral testing scenarios in comparison to animals reared in supplemental housing with stimulatory toys/shelters/etc (Turner & Burne, 2014). Even minimal levels of supplemental items have been shown to be anxiolytic and been correlated with beneficial physiological effects, like resistance to stressors or carcinogens (Yokota et al., 2013). It is possible that heightened anxiety due to lack of stimulation contributed to the effects of crowding, because of the limitation of space. It is also possible that the lack of stimulation contributed to the display of nociceptive behavior itself. These possibilities could have further intensified the pain-like responses of the social groups and caused them to more closely resemble the isolated controls.

Social support is shown to reduce susceptibility to adverse effects of stress, and better quality relationships between individuals and groups produce stronger correlations (de Jong et al., 2005). The duration of the experiment and the young age of the rats at onset, and also considering that the housing groups were in effect prior to injection, it is likely that the animals developed fairly close familial relationships. Most socially housed

animals, similar to the isolated animals, demonstrated moderate PWL measurements, averaging between 10 and 14 seconds, for each time point during the experiment. The only identifiable animals that deviated from this response range were the 25% CFA rats, or the CFA-injected rats of Group F. It was hypothesized that the singly-affected rats housed with three non-affected healthy rats would be aided by the social support of the more vigorous brethren and perceive less pain, therefore demonstrating less pain-like behavior, as evidenced by an increased PWL (compared with isolated CFA control A). Results indicated the contrary, and CFA rats of group F exhibited a consistently lower PWL compared to isolated and other social group rats at every time point following injection. This indicates that these rats perceived a higher level of pain than the other groups, although the difference was not statistically significant.

Examining the qualitative observational data alone, it appears that there is evidence to support the neural basis for empathy and its role in social influence and nociceptive effects. Examining the quantitative data alone, the experimental model is validated but no significant differences were found to connect housing group to their level of pain-like behavior, despite the apparent trends. However, examining the two sets of data together yields noteworthy findings.

There are two particular principles of interest that relate to each other and to the discussion. In theory, Day 7 following injection would be the time at which empathy-related behaviors like playing, allogrooming and moments of non-specific contact should be the most frequent in comparison to other time points, because the CFA effects are at a peak level. At this time point, it would also be logical to assure that the level of activity (digging or playing) to be decreased, as this would exacerbate the level of pain. Self-

benefiting behaviors such as eating and self-grooming should also be theoretically increased as these behaviors promote healing and health.

The CFA-afflicted rats in Group F (25%) generally (but not significantly) displayed a lower paw withdrawal latency, and therefore a high perceived level of pain than both the other experimental and control groups. It is interesting to denote that CFA and saline rats in Group F also displayed the lowest level of empathic behaviors in 66% of the measurements at Day 7, when elevation was expected. Group F only had 2 instances of play and 3 instances of allogrooming, versus the 100% CFA group C, which displayed 12 instances of play and 17 instances of allogrooming. Group C exhibited paw withdrawal latency that was not visibly nor significantly different from the isolated controls or other social groups, including 100% saline Group G. This indicated that Group C was in fact, procuring a higher level of empathic behavior than the other social groups and this behavior may have been reflected in the perception of pain. In contrast, Group F did demonstrate a lower level of empathic behavior and this paralleled their higher level of perceived pain.

Group F also displayed the highest number of digging events at Day 7, suggesting an overall increase in activity. High levels of activity in the period of onset of effects of CFA could have exacerbated nociceptive symptoms in the one affected cagemate, causing the low paw withdrawal measurement. Group F also displayed a prominently low level of self-supplementing behaviors, with only four eating events in comparison to the highest frequency of any cage (10 events), and a complete lack of self-grooming events at Day 7. This perhaps implies that a lower level of hygiene and wellbeing was being

maintained in Group F during this period, which also could have intensified pain perception and lead to the lower paw withdrawal latency in the affected rats.

After examining relationships between the quantitative and qualitative data, it is clear that while the specific experimental hypotheses are being rejected, there is a definite trend with regards to social housing and levels of nociceptive and empathic behavior in existence, although a statistical link is not evident.

In each socially-housed cage except Group F, each CFA-injected rat had one (or more) other identically-treated CFA rats in its home cage. In each Group F cage, the 25% CFA group, only one rat was injected with the inflammatory agent, and the other three rats were controls and injected with sterile saline. Because this CFA-treated rat did not have another cagemate under the same strain, perhaps another form of isolation resulted, which produced the observed behavioral changes.

This experiment tested the premise that social support would generate protective effects, with healthy rats providing support for the CFA rats. However, this supposition that these effects would occur in this experimental design may have been incidentally flawed. Research has shown that "non-supportive social relationships and competition or aggression within a group are associated with enhanced reactivity to stress" (de Jong et al., 2005). As stated, crowding may have been a factor that contributed to stress, or even aggression among the socially housed groups, although no direct instances of aggression toward handlers were observed in the testing environments or observatory sessions. Perhaps the relationships fostered in the cage F situations were, in fact, less than the supportive ideal with regard to the CFA animals, and this unsolicited combination initiated adverse consequences. Theoretically, if CFA rats did not receive the empathy-

related support that was proposed, but were still subject to the pain of the inflammatory model, and the stress of crowding and detrimental social influences, their low PWL and high level of pain-like behavior could be logical and justified. It has also been proven, in some experimental cases, that social housing is anxiolytic and advantageous to animals in a psychological sense, but it was not effectual in ameliorating physiological responses to stress (de Jong et al., 2005). So, the hypothesis may still be true, but not been demonstrated by the behavioral PWL measurement as anticipated.

The grouping of like-classified individuals together has been shown to be valuable in both animal and clinical models. One example of this is support groups. In humans, support groups revolving around psychological conditions, illnesses, addictions, and other situational categories have been shown to be successful in lowering anxiety and depression in participants, and many well-known support groups are household names. Also, a relationship between the length of participation in a support group and levels of psychosocial benefits has been established as directly proportional (Collins, 1998). This means that the more time an individual spends with like-individuals in the supportive environment, the more social benefit is reaped and able to be physiologically influencing. In addition, support groups of non-affected individuals have been shown to be successful in reducing the effects of their stress resulting from interactions with affected others. This means families and friends of affected individuals profit from coming together for social support as well. A notable example is siblings of children with cancer showing decreased levels of stress when involved in support groups (Salavati et al., 2013). Analogous to this experiment, perhaps Groups C, D and E, with more than one CFA rat each in cohort, could have developed a supportive effect and displayed increased pain resistance and

higher PWL. Or, perhaps the non-affected cagemates in Group F inadvertently procured social support from each other's company despite the suffering of the affected conspecific, leading to only CFA-treated rat of cage F without the protective support.

It is important to note that stressors commonly applied in behavioral labs, whether repeatedly or solely administered, are not typical scenarios that the animals would encounter in their every day life, in the wild or non-experimental environment (de Jong et al., 2005). The surgical anesthetic injection procedure, as well as the traumatic inflammatory injury, followed by repeated thermal application, all fall under this categorization of unnatural stressors to a Sprague-Dawley rat. Both single-application stressors like the injection and repeated stressors like the PWL quantification have been shown to incite long-term changes in physiology that influences reactions and behaviors. Rats exposed to such stressors have been known to show anxiety-like behaviors such as decreased weight gain despite the availability of resources, heightened aggression and social instability even weeks after the events (Baranyi et al., 2005). These principles perchance played a role in the experimental findings. All rats were likely subjected to the outcomes based on their experience with the laboratory models. However, as social influences generally play a role in the opposite manner, perhaps these stressors were not as prevalent in the socially housed groups, again aside from CFA rats in group F. Isolated control rats may have been more resilient to these tendencies because of their availability of space. No definite conclusions can be drawn in this regard.

In a clinical application based on this data, it would be recommendable to keep individuals suffering from similar conditions in close quarters, resembling experimental socially-housed groups, or in isolation, resembling experimental Group A, as no

significant difference or observable trend with regards to pain-like behavior was identified between these two groups. An example of this grouping may be a hospital wing or nursing or rehabilitation home. Based on visual trends only, it would not be advisable for a suffering individual to maintain residence in close proximity to healthy individuals, for example an ill person living with unaffected family member, mimicking social experimental Group F. CFA-affected rats in this social experimental group presented markedly lower paw withdrawal latency (although insignificantly), and therefore higher level of pain-like behavior which reflects higher perceived pain and a lower state of well-being, likely as an amalgamation of all the effects of the specific housing group. Of course, this discernment would vary based on a specific person's condition, personality and relationship with their cotenants.

In conclusion, statistically significant evidence was not obtained to demonstrate the idea that social empathy affected pain-like behavior. However, a number of other relationships were uncovered for further consideration. The principle still holds promise to uncover a definitive link between housing and nociceptive display. In the future, the experiment could be modified to include a greater number of animals in of each treatment group. The experimental design embodied small sample sizes, which were not conducive to accurate representative statistics. Also, the experiment was conducted over the course of a year and a half, and the weather and season could have affected both temperature and the animals' hormone levels, despite best efforts to control such variables. Some variability in measurement procedure could have unavoidably existed between different testing personnel, again despite attempts at uniform collection standard. Larger and more frequent qualitative observation periods could also be conducted, perhaps at alternate

time intervals rather than just following handling to gain a more accurate representation of behavior. This could perhaps include sleeping arrangement, as nesting in a cage is considered empathic.. Since many explanations for the unexpected results involved demonstration of anxiety in addition to the pain-like display, perhaps an anxiety-assessing component would be beneficial and illuminative in a modified future experiment. No strict conclusions can be drawn due to lack of statistical significance, but the experiment as a whole achieved the goal of uncovering what effects empathy in housing groups may have on pain-like behavior.

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## Appendix A. IACUC Protocol Approval

The approval of the protocol for the research procedures by the Institutional Animal Care and Use Committee of Youngstown State University.

Youngstown
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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