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Affiliative Behavior and Empathetic Response:

Sex Differences and Neuroendocrine Factors

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Abstract

This study investigated the ability of mice to distinguish the emotional state of other mice as an indication of empathetic behavior. The ability to distinguish emotional states was examined by using an overt pain stimulus on a mouse and measuring subsequent approach behavior by another mouse to the mouse in pain. This was used as a model for the affiliation and empathy of one mouse for another, presenting a novel paradigm for measuring affiliation and empathetic approach behavior towards another animal. The ability to identify the pain state of another mouse was examined in both female and male mice. It was found that female mice were better able than males to identify the pain state of another mouse, and, therefore spent significantly more time in proximity to the cagemate in pain than with an unaffected cagemate. Approach behavior in males was not affected by the pain state of another mouse. Since the hormone oxytocin has been shown to play a role in affiliation, its role in the ability to identify emotional states was investigated through a pharmacological manipulation in female mice where female mice were injected with oxytocin. The data from the oxytocin manipulation were inconclusive but suggested further work is necessary to investigate the role of this hormone in empathetic behavior in mice. Future research can use this novel paradigm to further investigate sex differences in empathetic behavior and the role of affiliative hormones in empathetic responses.

Affiliative Behavior and Empathetic Response: Sex Differences and Neuroendocrine Factors

Humans have evolved by relying on social bonds for survival. Without such relationships, immune systems weaken, depression sets in, and humans are more likely to die from numerous maladies. These social relationships, and social behaviors stemming from such relationships, are essential for the continued existence of humans and their social groups (usually containing related members).

Experiencing the emotional state of others is an adaptive strategy made possible by these social relationships. The emotional state associated with the perception of pain serves as a warning sign that something is wrong. Pain is, therefore, adaptive, since without this warning, an animal would be unaware that what it is experiencing is harmful to its own survival. Likewise, sensing another's pain state has evolved, since, by understanding the pain another is experiencing, animals are able to learn what to avoid for themselves. All animals, including humans, rely on these bonds and the understanding of others' emotional states to give cues regarding what is dangerous and what is safe; if one chimpanzee starts a distress call, the other chimpanzees in its group recognize this call and know it means to hurry to safety.

The social behavior of empathy, and empathy for pain in particular, therefore, becomes a key mechanism in the survival of an individual. Current research is investigating empathy as a biological and social behavior in humans and animals to better understand empathy, and to investigate the influence of social context on such empathy. Specifically, animals that are in the same social group show more empathetic behavior to each other than to animals not within their social group, which may point to a need for attachment to another animal for the experience of empathy. The development of a better understanding of humans' (and other animals) ability to empathize, and knowing what influence social context may play on this ability to empathize,

could be applied in future research to the understanding of disorders in which empathy and other social behaviors are distorted, such as in autism.

Empathy

Empathy is a difficult term to define because of on-going disagreement in the literature about its definition and its nature. There are numerous definitions stemming from different views regarding the nature of empathy as either an emotional or cognitive process. The approach taken here is based on the Perception-Action Model of empathy suggested by Preston and de Waal in 2002. This model focuses on the relationship of the object (animal being observed) to the subject (animal experiencing empathy) and the influence of the object on the subject's emotional state from previous associations and relations between the two (Preston & de Waal, 2002). Thus, the relationship between the object and subject can influence the empathy experienced by the subject for the object.

This model differs from previous models in that it focuses on the process of empathy and not the empathetic response, while also focusing on the interdependence and interrelational aspects of empathy (the relationship between the object and the subject). A previous theory proposed by Hoffman (2000) did not address how the object could influence the subject. Hoffman's theory stated that empathy is "any process where the attended perception of the object's state generates a state in the subject that is more applicable to the object's state or situation than to the subject's own prior state or situation." Thus, Hoffman defined empathy as a representation of state of another being that elicits a similar response in the observer. This theory suggests that empathy is a one-directional process, which is not supported by other research.

With the Perception-Action Model, Preston and de Waal propose that empathy is not simply a one-directional process, but instead is influenced by the target of empathetic response.

This model allows for the object to have an influence on the empathy of the subject (such as their relationship with the subject) and therefore points to empathy as a bi-directional process. While this definition does not encompass empathy entirely, it does serve as a good basis for further study.

An important aspect of this interrelational view of empathy is the process of vicarious learning, the importance of which is illustrated in both the epistemological (as in the role of empathy in learning for survival) and social need for empathy (De Vignemont & Singer, 2006). When something is learned vicariously, it is learned through the observation of others and not by doing something one's self. If an animal is able to learn about pain vicariously, e.g., if an animal sees someone else in their social group fall from a rock and break its leg, the animal will learn to avoid the same situation without having to experience the pain themselves. This vicarious learning process relies on the activation of similar systems in the animal observing the pain (or other activity) as in the animal in pain, and therefore suggests that empathy evolved because of its ability to create a faster determination of one's own behavior from viewing another's.

Vicarious learning reveals the adaptive significance for the development of empathy and the first role of empathy, the epistemological function of such empathy. Several studies have suggested that empathy is important for mother-child bonding and pair bonding, as well as survival of an animal, because of the need to learn about the environment and to understand the state of others (Hemanth and Young, 2006). In terms of learning about the environment, if an animal in social groups does not gain information about the environment from other animals in its social group, the animal will have less chance of survival than the other animals, because it would be unable to successfully interact with their environment as well as the animals around them. In understanding the state of others, just as it is adaptive to feel pain, it is adaptive and

necessary to feel the pain of others. Seeing another animal killed or hurt needs to be recognized as something they would like to avoid, since they could experience the same result. As per the definition of empathy proposed by Preston and de Waal, this recognition of another's state is a part of empathy.

The second role of empathy is the social role, which research has tied to morality, altruism, justice, prosocial behavior, and cooperation (De Vignemont & Singer, 2006). Evidence suggests that individuals with deficits in empathy are more likely to be aggressive and antisocial towards others (Hoffman, 2000) and that empathy plays a large motivational role to cooperate and help one another. Studies have found that socially isolated individuals are two to three times as likely to die in a nine year period from various diseases (Berkman, 1995). This increase in death rates could stem from an inability to interact successfully with the environment or a lack of reliable social support if the individual is compromised. Either way, the success of an individual relies on interactions with its social group.

Pain

While pain is unpleasant, in most forms it is adaptive for an individual since it signals real or impending tissue damage. Unpleasantness is pain's defining characteristic and serves as a warning system to the organism experiencing noxious stimuli (Price, 2000). This knowledge then allows the person or animal feeling the pain to adapt its behavior to prevent further injury or death. This role is illustrated by the official definition of pain from the International Association for the Study of Pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." The premise of the current research is that this warning system also influences the behavior of others observing the pain in terms of motivating the avoidance of such stimuli.

As well as being an adaptive sensory experience for an individual, pain behaviors are designed to elicit help for the individual in pain. In terms of evolution and the perspective of inclusive fitness, animals protect related animals because their genes are most similar to their own (and therefore their genes will continue to be passed on even if they die). Therefore, related animals would respond to the pain produced behaviors and help relatives to ensure the survival of their own lineage. Thus, the ability to recognize distress and respond empathetically, particularly with animals that they are related or attached to, would have evolved survival benefits for those animals (and the genes that produce this form of recognition), since animals that are able to recognize this distress will have their genes passed on more readily. Empathy for the pain of others has consequently become a key social phenomenon in survival of not just an individual, but the future of the individual's genes.

To understand the underlying mechanism that produces overt pain behaviors, some understanding of the pain pathway is needed. Nociceptors are neurons that are activated by noxious stimuli at the peripheral terminals (located in skin, joins, and muscles). Noxious stimuli depolarize the nociceptor, which transmits the signal to second order neurons in the spinal cord dorsal horn, which is divided into six laminae. Laminae II of the dorsal horn is called the substantia gelatinosa and contains interneurons that modulate the activity of the ascending pathway. These neurons, which exist entirely in the dorsal horn of the spinal cord, synapse with pain transmission cells that project into the brain. The activity of these ascending cells from the spinal cord to the brain provides the brain with information about the degree of tissue damage from the periphery. These tracts pass through the brainstem and midbrain before terminating in the thalamus. The thalamus then sends neurons to the cortex and limbic structures (Melzack & Wall, 1996). This process of creating central nervous system activity from stimuli of potential

tissue damage is termed nociception. By observing that signals are sent to the cortex and limbic system, it can be seen that pain is not simply a sensory process, but is also a cognitive and emotional one.

Many theories have attempted to explain pain processing, though most have not adequately explained certain aspects of pain. Specifically, the earliest pain theories provided no explanation of the tenuous link between nociception and pain; there can be pain in the absence of nociception and nociception that does not lead to pain. Theories also did not account for psychological factors, individual differences, and changes in pain over time (Melzack & Wall, 1965). Because of the deficiencies in previous theories, a theory of pain processing called the Gate Control Theory (GCT) was proposed by Melzack and Wall in 1965 that attempted to explain these phenomena.

The main contribution of GCT is the emphasis on modulation. GCT proposed the existence of modulatory circuitry in the substansia gelatinosa of the spinal cord dorsal horn that takes into account the input from both large diameter fibers (responding to low intensity non-noxious stimuli) and small diameter fibers (nociceptors responding to high intensity). This spinal cord mechanism is referred to as the "gate". Generally, large diameter fiber activation can reduce the output of a pain transmission cell. This means that GCT is able to explain why innocuous stimuli, such as rubbing a sore knee, can reduce pain; the activation of more large diameter fibers reduces the output pain transmission cells send to the brain so there is less pain. (Melzack & Wall, 1996).

This theory also takes into account descending output from the brain, which can be either inhibitory or excitatory and can change activity of the pain transmission cell through descending modulation. The phenomenon of Stimulation-Produced Analgesia, where stimulation of certain

brain areas leads to a decreased feeling of pain (Mayer, Wolfke, Akil, Carder, & Liebeskind, 1971), illustrates this descending modulation**.** This phenomenon is confirmed to be through descending modulation since in lesion studies of the dorsal lateral funiculus (downward projecting axons at the level of the spinal cord); stimulation of the same brain areas will not produce a decreased feeling of pain. Thus, descending modulation in the form of psychological variables, the environment (such as social groups) and events may influence the perception of pain, since the brain is able to inhibit pain transmission.

Neurons in the cingulate cortex have been associated with pain and descending modulation (Hutchinson, Davis, Lozano, Tasker, & Dostrovksy, 1999). While it has been known for a long time that the cortex participates in pain perception, Hutchinson was able to show that there are specific neurons in the cortex that respond to pain and affect the perception and intensity of pain in an individual. The cingulate cortex may be, therefore, a major player in the descending modulatory pathway suggested by GCT since these specific neurons are influencing the perception of pain in an individual. This circuitry system of modulating pain in one's self could also play a role in perceiving pain in another, since the most likely place for a neuronal system of empathy to develop through evolution would be in a similar place in the observer's brain.

Human Empathy Research

Empathy plays a role in modulating how individuals view the pain of others, and individual differences in empathy seem to influence the responsibility of an individual towards helping others in pain. People who are rated higher in empathy are likely to report that a person they are observing (previously coded for showing some pain) was experiencing more pain than people who rated low in empathy (when judging the same people) (Mutso, 2007). In addition,

those who reported a high level of interdependence (identification of others as part of one's self) felt more responsibility to help others in pain. These results suggest that individual differences in empathy influence the experience of seeing others' pain and the motivation to alleviate others' pain. These results also suggest two types of empathy that were previously not distinguished from each other: the experience of seeing someone in pain and the feeling of responsibility to help the other. This distinction fits well with Preston and de Waal's Perception-Action Model of empathy that focuses on differences in how a person perceives another, modulated by their own empathetic state and relationship with the other.

Having empathy for another's pain enhances one's own perception of noxious stimuli. This influence of empathy on one's own perception of pain is seen through a study in which participants, who were induced to have high empathy for an actor (where the actor told a story about how his girlfriend recently died), rated painful stimuli applied to themselves as more intense and unpleasant (than their own pain ratings under baseline conditions). Participants who were induced to have low-empathy for an actor (where the actor told a story of how he cheated a blind man) did not experience such enhancement (Loggia, Mogil, & Bushnell, 2007). In both cases, participants then observed the actor in pain and then rated their own perception of pain. Thus, it is the experience of empathy itself that is hypothesized to modulate pain perception in one's self (in humans) and not just the observation of pain behaviors.

Researchers have postulated that, in fact, the primary function of empathy for pain in humans is to help in the formation and continuation of social bonds. Animals survive and reproduce within social relationships and, in doing so, make strong bonds among themselves. Empathy strengthens social bonds in three ways (at the human level): it coordinates the action of

individual to allow fast response to threats, it helps understand others' thoughts and intentions, and it signals solidarity (Anderson & Keltner as cited in Preston & de Waal, 2002).

It is reasonable to hypothesize that certain brain areas would have become specialized in processing the pain of others as well as the pain of oneself in forming an empathetic response. It is also reasonable to assume that these specializations would develop in related areas of the brain, since they are related processes. Some of the common brain areas studied in functional magnetic resonance imaging (fMRI) research on pain are the anterior cingulate cortex and the insula (both parts of the established "pain pathway" and limbic system); studies show significantly greater activation in both the perceived pain of others and the perceived pain of self when compared to neutral stimuli. Different areas of these regions, however, are activated depending on the condition of the pain (self vs. other).

Research showing that these overlapping systems are activated when experiencing one's own or other's pain was performed by Singer, et al. in 2004. This study involved romantic pairs, in which the brain activity of the female partner was measured when a painful stimulus was applied either to her or to her romantic partner's hand. In the condition when she was in pain (the "self" condition), the posterior insula, sensorimotor, and caudal anterior cingulate cortex were significantly more activated than when her partner was in pain; when her partner was in pain, the anterior and posterior anterior cingulate cortex and rostral anterior insula were significantly more activated than in the "self" condition. From these results it was concluded that there are indeed separate, but related, systems that are activated in self vs. other pain. Thus, there seems to be an activation pattern in the brain that accounts for "understanding" the pain states of others.

Further work has corroborated the findings that the anterior insula shows significantly greater activation in the right hemisphere when imagining other-pain (as compared to a neutral

condition), while both hemispheres showed significantly greater activation with self-pain (as compared to the neutral condition) (Jackson, Rainville, & Decety, 2006). This partial cerebral overlap between perceived pain in another and perceived pain in oneself suggests that the pain representation of others depends somewhat on the activation of self-representation of pain, but not to the extent that it invokes pain sensory pathways. This difference in activation areas helps support the hypothesis that one of the ways to differentiate self-pain and empathetic feelings is that a partial sensory response is activated when imagining self-pain. This differentiation is very important, because animals need to be able to distinguish between an empathetic response and their own distress (Jackson et al., 2006).

The activation pattern acts like a "mirror" system of empathy, and researchers have suggested that it is related to the mirror systems previously found in monkeys that have been implicated as a neural circuit for empathy. When observing another monkey perform a task, the observer monkey's neurons in the inferior frontal gyrus (frontal lobe) fire in a similar fashion as if the observer monkey was performing the task itself (Iacoboni & Lenzi as cited in Preston & de Waal, 2002). This possible mirror system suggests a new organization for the anterior cingulate cortex and insula, given that self-pain conditions are associated with more caudal activation in this region, while other-pain conditions produced a consistently more rostral response in the same brain regions. Because of the distinction, researchers have suggested an organization of these regions based on the subject of the experience and argue that the self vs. other distinction is a continuum instead of being separate entities (Jackson, Brunet, Meltzoff, & Decety, 2006), and that this organization is represented anatomically in the rostral-caudal axis of the anterior cingulate cortex.

Not only is there different activation in the brain regions when imagining others in pain than when experiencing oneself in pain, studies have also found that people rated possible self-pain as more painful than possible other-pain. Since more of the pain pathway (thalamus, cingulate cortex, and limbic system) is activated when thinking of self-pain than other-pain, this may indicate that the entirety of the pain pathway is also involved in the intensity of sensation of pain and is influenced by the self or other state of the proposed pain (Jackson, Brunet, Meltzoff, & Decenty, 2006). While empathy for the pain of others is adaptive, it is even more adaptive to understand one's own pain. Thus, brains have evolved the tendency to represent "self-pain" as more intense then **"**other-pain".

Both perceived self-pain and perceived other-pain was rated higher than rating the perceived pain in an artificial limb (Jackson, Brunet, Meltzoff, & Decety, 2006). In fMRI work, it was demonstrated that this difference is comparable to the self vs. other pain condition, in that viewing an artificial limb in a painful situation, less of the pain pathway was activated than for other-pain (which was less than self-pain). These results indicate that brains have evolved to not only represent self-pain as more intense than other-pain, but that it has also evolved to distinguish between "real" possible pain and "unreal" possible pain. Having empathy for inanimate objects would not aid in survival and reproduction, so there is no reason for such a capacity to evolve.

Animal Empathy Research

Because of the adaptiveness of empathy, there is reason to hypothesize that correlates of this adaptation may be observed in non-human animals. There is evidence in support of this hypothesis. In a study with rhesus monkeys, subjects were trained to pull two chains to deliver different amounts of food to themselves. The researchers then altered the situation so that if the

subject monkey pulled the chain paired with more food, another monkey that the subject could see would be shocked. Two-thirds of the monkeys avoided pulling the chain that would provide a shock to the other monkey. In fact, of the remaining third, one monkey stopped pulling any chain for five days and another monkey stopped pulling any chain for twelve days (Massermann, Wechkin, & Terris, 1964). These monkeys would endure starvation to avoid witnessing the shock of another animal and this effect was enhanced by the familiarity of the shocked monkey. Thus, these monkeys responded to eliminate the suffering of another animal to the point of self suffering, which suggests an empathetic response.

While it could be argued that the subject monkeys stopped pulling the chain to simply avoid witnessing the event, other research suggests that this is not the case. In another study with rhesus monkeys, the subjects pushed a bar to avoid witnessing a shock to another rhesus monkey. The response was then extinguished by unpairing the bar pressing and shock so that the monkey would start pressing the bar again. The response could not be reinstated by viewing live rats receiving shocks or monkey puppets made to imitate a pain response (i.e., other live animals or fake animals of the same species). It would, however, be reinstated by pictures of real monkeys receiving shocks and was stronger with pictures of familiar monkeys than unfamiliar monkeys (Miller, Murphy, & Mirsky, 1959). Thus, after learning the consequence of a shock, the monkeys responded to eliminate the suffering of the other monkey but were not responsive to artificial or unfamiliar stimuli. This stimulus-specific empathetic response therefore demonstrated an empathetic response, instead of simply a desire to avoid witnessing an event.

While these studies were done in monkeys (primates being more closely related to humans than mice) the results have been replicated in rats and pigeons. One such study (Church, 1959) (in a similar experimental setup as described before in the rhesus monkeys) found that rats

decreased pressing a bar to stop another rat from being shocked. These results demonstrate that these simpler animals may have the capacity to identify emotional states of others, or at least behave in ways that are consistent of an empathetic response.

Based on results of the research with primates and earlier studies in rats and pigeons, researchers have started to study empathy in animals previously thought not to have a complex enough nervous system to make empathetic responses, (since empathy for pain would also be adaptive for these animals). Some of the most recent and influential work probing empathy in rodents is by Langford et al (2006).

These researchers discovered that mice show an enhancement in pain sensitivity when exposed to cagemates in pain. They argued that this observation demonstrates empathy in rodents. In this study the experimenters used a model of visceral pain (injection of 0.9% acetic acid) to produce pain behavior (writhing) in the absence (isolated condition) or presence of another mouse. There were two experimental conditions when there was another mouse present, either the second mouse was also injected: BW ("both writhing" condition), or one was not injected: OW ("one writhing" condition). The other mouse was either a cagemate or a non-cagemate. It was found that all mice exhibited greater pain behavior when they were with cagemates than when they were in the presence of non-cagemates, and that cagemates in the BW condition showed increased pain behavior when compared to the isolated mouse condition. Thus, the social relationship between the two mice modulated pain sensitivity in these mice.

In addition, mice that had observed their cagemates in pain continued to show enhanced pain sensitivity after a time delay (Langford et al., 2006). Therefore, this pain sensitivity is enduring and not just a result of an interaction of a mouse's own pain with another mouse's pain. These results were interpreted as empathy, because mice were responding to the situation

differently when they were familiar with the other mouse. It was also interpreted as empathy, since this hyperalgesia when in the presence of another mouse in pain points to a representation in the mouse's brain as similar to the brain of the other mouse. Therefore, mice seem to show empathetic behavior.

These findings can be interpreted in light of the Perception-Action Model of empathy of Preston and de Waal (2002), given that there is a priming of somatic responses similar to that of the object (the other mouse), and that the relationship between the subject (one mouse) and object (other mouse) modulates the empathetic response. This pain pathway priming would be adaptive because, as stated earlier, understanding the sensory experiences of others may prepare animals for environmental threats that could impinge on their sensory systems.

Although many researchers have argued that empathy is unique to humans, these studies suggest that other animals may experience a similar emotional/behavioral state, albeit in a "low" form, since their response may be at a pre-cognitive level. If it is true that mice can experience empathy (as suggested by Langford et al., 2006), then one would expect them to be able to distinguish overt emotional states in other mice. One method of studying this would be to observe their behavior and see if how they respond is consistent with this interpretation of their behavior.

A measurement that could be valid to evaluate emotionally based behavior in mice would be a use of the overt pain method from Langford et al. (2006) combined with a measure of affiliation between the animals. As of yet, no work has been done tying the social phenomenon of affiliation to such an empathetic response in mice. If the animal were to affiliate more when the other animal is in pain, this may demonstrate an ability to distinguish emotional states in

other mice and provide a demonstration of low-level empathy as well as a replicable model of rodent empathetic behavior.

Affiliation

In order to measure affiliation and use it as a means of observing empathy in animals, its meaning must be examined. Affiliation is often defined as association, connection, and attachment with another individual. Animals that live in groups show affiliation for one another, and group living is generally thought of as an evolutionary adaptation which benefits all the animals in the group. For example, through living in groups there are more animals to watch for danger or to confuse predators. Because of the adaptiveness of living in groups, group living has become an important strategy for survival for many species of animals. As reported earlier, these social relationships are also essential in humans for an individual's health and survival. Studies have found that socially isolated individuals are two to three times as likely to die in a nine year period from various diseases (Berkman, 1995).

Little is known, however, about the neurobiological mechanisms underlying such social relationships (Young, Lim, Gingrich, & Insel, 2006). While some studies have determined that human sociosexual processes are influenced by the neuropeptides oxytocin and vasopressin, most research in this area has been completed with rodents (Depue & Morrone-Strupinsky, 2005). Animal models have been useful for the investigation of neurobiological mechanisms for affiliation in general, as well as to investigate the role of the hormones underlying this behavior.

Oxytocin and Vasopressin: Affiliative Hormones. Oxytocin and vasopressin play a large role in affiliation. These two closely related neuropeptides (Bielsky & Young, 2004) are found only in mammals and are two of the most highly conserved hormones across species (Argiolas & Gessa, 1991)**.** The peptides are transcribed from adjacent genes and differ by just two amino

acids are produced in discrete regions of the brain and are both released centrally and peripherally (Bielsky & Young, 2004). Although peripheral oxytocin and vasopressin act as endocrine hormones (involved in uterine contraction and blood pressure regulation), they also act as neurotransmitters in the brain. In general, oxytocin and vasopressin synthesis is present throughout limbic and autonomic brain regions and this distribution suggests a possible role in behavior related to survival of an animal, such as affiliation with others.

Injections of oxytocin facilitate mother-infant interaction, partner preference, off-spring preference, and nonsexual social contact (along with socio-sexual functions) in voles (Argiolas & Gessa, 1991). Vasopressin is found to be tied to mating, paternal care, and partner preference in male prairie voles (a monogamous species) (Young, Wang, & Insel, 1998). Much of the work by Young and colleagues has been done studying the mechanisms of social attachment and affiliative behavior comparing monogamous voles (prairie voles) to non-monogamous voles (montane voles). Affiliation between adults can be viewed as a measure of social approach, and this is measured as latency time to approach another vole and the amount of time spent in social contact (Lim & Young, 2006).

This social approach (in particular, pair bonding) is measured by placing cohabitating male prairie voles in a 3-chamber testing area where a cohabitating female prairie vole is tethered in one chamber of the three, and a stranger female (not cohabitating with the male) is tethered in the other chamber. The male is placed in the center and allowed to freely move around. If the male spends more than twice as much time near his partner rather than near the strange female, he is considered to be showing a partner preference. This process is also done in reverse with the females roaming free and the males tethered (Hemanth & Young, 2006). While both prairie voles and montane voles show social approach, only the monogamous prairie voles

show a partner preference in this paradigm. Further research investigates the causal mechanism of this difference between prairie and montane voles.

Monogamous prairie voles were found to have a higher density of oxytocin receptors in the nucleus accumbens than the non-monogamous voles (who do not show partner preference). Additionally, site-specific injections of oxytocin antagonists in female prairie voles prevent the partner preference previously seen, pointing to a direct effect of oxytocin in producing this response in females. Male prairie voles also have a higher density of vasopressin receptors (V1a) in the ventral pallidal area (the major output to the nucleus accumbens, both of which are tied to reward). Therefore, oxytocin and vasopressin may be facilitating affiliation and social attachment in the monogamous prairie voles through the reward pathways, and that oxytocin plays a role in female prairie vole attachment and vasopressin plays a role in male prairie vole attachment (Young et al., 2001).

These brain regions, the ventral pallidum and the nucleus accumbens, are involved in the mesolimbic dopamine reward system, which, as Young suggests, could mean that pair-bonding activates the same reward circuitry as naturally reinforcing stimuli (Young & Wang, 2004). Reward depends on the mesocorticolimbic dopaminergic system, which consists of dopamine-producing neurons in the ventral tegmental area that projects to the nucleus accumbens and other forebrain areas and projections of the nucleus accumbens to the ventral pallidum (Klitenick in Young & Wang, 2004). Dopamine's release in this circuit has been shown to be involved in natural reward (such as the response to food). The release of dopamine in the nucleus accumbens has been shown to be critical in the formation of the partner preference in prairie voles (Young & Wang, 2004) in a receptor specific manner. Only the activation of D2 (not D1) in the nucleus accumbens of females is critical, while the blocking of this receptor blocks pair bonding (Liu &

Wang, 2003). It is interesting to note that human studies support the findings that reward circuits may be involved in attachment. An fMRI study examined brain activation of subjects viewing a picture of someone with whom they were "deeply in love". These researchers found that the regions of activation were very similar to those suggested in the vole model, the nucleus accumbens and ventral pallidum (Aron et al., 2005).

While dopamine release is important in pair-bonding formation, there is a question of why pair bonds are not naturally observed in other animals that show dopamine release associated with mating. The suggestion from current research is that the answer lies in the interaction between oxytocin, vasopressin, and the dopamine reward system.

Throughout this system, sexual dimorphisms have been found (Bielsky, Hu, & Young, 2005). Female prairie voles, when given an oxytocin antagonist in the nucleus accumbens showed no preference for their partners blocked partner preference induced by D2 receptor activation. The blocking of D2 receptors, however, prevents the partner preference induced by oxytocin (Liu & Wang, 2003). These results indicate that dopamine and oxytocin are acting concurrently and the interaction is necessary for pair bonding in females. Studies have also shown this interaction in males, but at the V1a receptor in the ventral pallidum. The ventral pallidum is an important structure in the dopaminergic reward pathway, which receives input from the nucleus accumbens. While the nature of the interaction between the dopamine system and the V1a receptor is not yet clearly understood by researchers, the interaction suggests that affiliation is tied to reward.

Some researchers believe the V1a receptor in males has similar properties to the oxytocin receptor in females. Mice that lack the gene for the V1a receptor had lower social affiliation and higher levels of anxiety than wildtype male mice (Bielsky, Hu, Szegda, Westphal, & Young,

2004). Recent work has shown no behavioral difference between V1a receptor female knockout mice (mice lacking the gene for a V1a receptor) and non-mutant mice. These findings not only suggest that vasopressin is indeed more important for social behavior in the male mouse (and not in the female mouse), but also implicate the V1a as a causal mechanism for such behaviors--a higher density of vasopressin fibers in the male system compared to the female system.

While vasopressin is associated with pair-bonding in males, the infusion of vasopressin into the ventral pallidum of non-monogamous male montane voles did not facilitate pair-bonding and affiliation (Young et al., 1999). These data suggest that V1a receptor distribution patterns, which were shown to be denser in the ventral pallidum of monogamous voles, may account for the social differences of these animals (Hemanth & Young, 2006). However, the V1a activation in the ventral pallidum is unlikely to be the only mechanism in the formation of a pair-bond. Hemanth and Young (2006) suggest that social bonds may be determined by a bi-dimensional system, one that elicits adverse feelings when social bonds are disrupted and another that elicits gratifying feelings in the presence of the other bonded animal (Hemanth & Young, 2006). These researchers hypothesized that V1a receptor mediated reward mechanisms may be important for the formation, and maintenance, of these bonds.

The V1a receptor has also been shown to be necessary and sufficient for normal social recognition (a simple ability to recognize another mouse as familiar) (Bielsky, Hu, Szegda, Westphal, & Young, 2005). In previous research, male mice with a null mutation in the V1a receptor exhibited impairment in social recognition and high anxiety (Bielsky et al., 2004). Nevertheless, a follow-up study found that the re-expression of the V1a in the lateral septum rescued social recognition in mice deficient for the V1a receptor. This finding demonstrates that the V1a receptors in the lateral septum, along with those in the ventral pallidum, are critical in

the neural processing of social stimuli and determining complex behavior in response to such stimuli (Bielsky et al, 2005).

Since oxytocin and vasopressin are shown to be critical in social behavior, a question arises to what differences would be seen in animals with a lifelong absence of one of these neurotransmitters. Female oxytocin knockout mice were used to determine the approach and affiliation of mice with a lifelong absence of oxytocin. Approach and affiliation were differentiated in this study by that approach measured curiosity for a new object or mouse and social affiliation was measured by recognizing and preferring a mouse that they knew. Results established that, while the approach of these mice towards other animals in general was generally not abnormal they did not show social affiliation for known mice (Crawley et al., 2007). These data supported the previous evidence that oxytocin plays a very specific role in social affiliation and memory, but is not crucial for generalized social approach. Knockout mice, however, are limited in what they can tell researchers about normal mice, so further work must be done with non-knockout mice.

The overall conclusions to be drawn from previous research on the role of these affiliative hormones and social behavior are that oxytocin and vasopressin play different roles in pair-bonding in males and females, with oxytocin being important in affiliative behavior in females and vasopressin being important in affiliative behavior in males. As Young and colleagues suggest, this affiliation measures an attachment to another individual. Currently, no research has attempted to determine the influence of affiliation on empathetic behavior. Furthermore, as of yet, no work has been done to investigate the role of these relevant “social neuropeptides” in terms of a possible empathetic response. The next step in research needs to address the role of affiliation, and its affiliative hormones, in the empathetic response of mice

observed by Langford et al. (2006). Since behavior towards others in pain may serve as a model for empathetic behavior, and there are differences between males and females affiliative behavior, one would expect there to be sex differences in this empathetic behavior.

Sex Differences

In general, there are many rodent sex differences in emotionally related behavior, including the response to environmental threats (Archer, 1975). Taylor et al. (2000) suggest that fight-or-flight may account for the physiological response to such environmental threats (i.e. stressors) for males, but females' behavioral response can be characterized by a "tend-and-befriend" strategy rather than a fight-or-flight response strategy. Tend-and-befriend refers to the nurturing of offspring and affiliating with social groups under conditions of stress. Attachment is often understood as a stress-related biobehavioral system at the heart of maternal bonding and group bonding, and female stress responses have likely evolved not only to protect the female herself, but also to protect the female's offspring.

Due to this tend-and-befriend strategy, one might predict that females would show a tendency to affiliate with one another when under stress, a hypothesis supported by much data. For example, crowding has been found to calm female rats, and female rats housed together live 40% longer than female rats housed alone (Brown, 1995 as cited in Taylor et al., 2000). This group housing has also been found to buffer the negative effects of stress on adult neurogenesis in rats (Stranahan, Khalil & Gould, 2006). In addition, and of particular interest to the present study, prairie voles have shown that female prairie voles under conditions of stress show selective preference for their same-sex cagemates (Carter, 1998).

There is also evidence for this affiliation response under stress in human studies. It is well known and well supported in the literature that females under stress seek social support to a

higher degree than do males under stress. Adult females maintain same-sex close relationships to a greater extent than males, use their social support more frequently in times of stress, and report more benefits from this contact with female friends and relatives (Taylor et al., 2000). When stressful situations arise, females often rely on their social networks for child care and exchange or resources. This reliance on social networks has been well studied in disadvantaged African-Americans and has been shown to be important in providing both physical and emotional support (Taylor et al., 2000). Affiliative behavior studies have shown that oxytocin may enhance affiliation in humans, and Carter suggests that oxytocin may be the mechanism for many instances of social attachment, including adult pair bonds and friendships (1998). Research has shown that while there is no overall sex difference in oxytocin levels in humans, female gonadal hormones (estrogen) enhance the effects of oxytocin (McCarthy, 1995). This difference in female gonadal hormone levels could lead to more social attachment in human female networks, since oxytocin's effects are greater in females.

In rodents, however, the oxytocin levels in females are greater than in males. Combined with the research suggesting that oxytocin affects social attachment (Young et al., 2001), the sex differences in response to threatening stimuli (Taylor et al., 2002) and more social attachment in female networks, research suggests that there may be a sex difference in empathetic response when an affiliated mouse is in pain, which has not yet been examined.

The Present Study

From previous research it is apparent that empathy for pain is important for the survival of an individual in its social group and that empathy is modulated by the familiarity and affiliation that one animal has for another. Affiliation has also been tied to the affiliative hormones, oxytocin and vasopressin, in a sex-specific manner. As of yet, no one has attempted to

use this affiliative behavior (observed through social approach) as a measure of empathy. Additionally, previous research has also not answered the question of how these hormones play a role in empathy or how sex differences in affiliation and response to stressors may influence a sex difference in empathetic response. This study plans to propose possible answers to these questions.

In an attempt to answer the proposed questions, the study will use a novel paradigm to measure empathy through social approach (as a measure of affiliation), towards familiar others who may be experiencing pain. This is the logical next step of research because of the implications of affiliation playing a role in empathy, and since their ties to affiliation are already known, allows researchers to examine the effects of oxytocin and vasopressin on empathy. The conclusion that mice experience empathy suggests that mice should be able to recognize pain states in others.

Cagemates will be used so that the affiliation between each pair of mice should not differ. The overt pain stimulus from Langford and colleagues will be used in combination with a social approach paradigm as a model for this pair bonding and affiliation, which is a novel way to measure mouse empathetic behavior. If the subject mouse approaches another mouse more when the other mouse is in pain, this will be viewed as an empathetic response.

Previous research findings lead to certain hypotheses in the proposed study. First, we hypothesize that female mice will distinguish pain versus no pain states in other mice to a greater extend than males. Furthermore, we hypothesize that male mice would not show this difference in affiliation based on the pain state of another mouse. We hypothesize this sex-specific response because of the observed sex differences in mouse affiliative behavior, response to threats, and circulating levels of oxytocin.

Since we are interested in observing sex differences in how female and males respond to another mouse in pain, we will use both male and female mice in the first part of our study to compare their empathetic approach behavior to a cagemate. As stated, for this first experiment we would hypothesize that female mice will show more affiliation with the other mouse when it is pain while the male mice will not show this difference. We would also hypothesize that females would show greater approach behavior towards familiar cagemates (regardless of condition) when compared to the males. We base this hypothesis on the fact that females affiliate more in nature than males do, and that the hormone oxytocin is tied to affiliation.

If our hypothesis of a sex difference is correct, we will investigate the role of the affiliative hormone oxytocin on empathetic approach behavior. While it is known that this hormone is tied to pair bonding and affiliation, no one has investigated its possible role in empathy. This present research will attempt to answer this question by enhancing the levels of oxytocin, so that any affiliative response would be exaggerated. We hypothesize that increasing the circulating oxytocin in females would increase the empathetic approach behavior when compared to the male mice and compared to the female mice with normal oxytocin levels. If this hypothesis is shown to be true, it could be concluded that oxytocin plays a role in empathetic approach behavior in female mice.

In summary, we propose a novel paradigm to further study the nature and existence of empathetic behavior in rodents in a situation when a familiar animal is in pain. We also propose that a sex difference in this empathetic behavior will be found, and that an increase of the affiliative hormone oxytocin will increase empathetic behavior in female mice. In phase one of the study, we first investigated if females show overall more affiliation than males, and if females are able to distinguish the pain states of another animal.

Phase One. A pilot study was performed to establish the procedure for evaluating the phenomenon of interest and to determine the existence of a baseline sex difference in approach behavior mediated by the pain state of another mouse. We hypothesized that females would show more affiliation behavior overall as well as more affiliative behavior to a mouse in pain than to one not in pain. We hypothesized that males would not show a difference and would have less overall affiliative behavior than the females. Our pilot study found that females did show significantly more affiliative behavior overall and trended towards a greater difference in affiliative behavior dependent on the pain state of the other mouse. Males did not show any affiliative behavior difference. Because of this trend towards significance, we continued with our proposed study.

Proposal for Experiments One and Two. Experiment One will expand upon the sample size of the pilot study to determine sex differences in socially mediated empathetic approach behavior. As stated, for this first experiment we would hypothesize that female mice will show more affiliation with the other mouse when it is pain while the male mice will not show this difference. We would also hypothesize that females would show greater approach behavior towards familiar cagemates (regardless of condition) when compared to the males.

Experiment Two will include a pharmacological manipulation of oxytocin in female mice. We hypothesize that these mice will show exaggerated socially mediated approach behavior, in that females with more circulating oxytocin will show greater overall affiliation with the other mouse and a significantly greater difference in approach behavior when the other mouse is in pain compared to those not in pain. We hypothesize this will occur due to the role oxytocin plays in pair-bonding and affiliation in female mice. Since this hormone plays a role in

this affiliation, it may play a role in different recognition and responses to pain states of other mice.

Implications of Research

Our research aims to lead to a better understanding of the evolutionary ancestry of empathy, as well as suggesting a purpose for which it may have evolved. This research will also establish an acceptable model for studying this form of social behavior.

Understanding low-level empathy in animals may help us understand human social behavior. Not only will this add to the understanding of the brain pathway of empathy in humans, but it could eventually point to mechanisms of social disorders (such as autism), which have an impairment in behaviors similar to those seen in oxytocin and vasopressin deficient mice. Because of the role of oxytocin and vasopressin in animal social processing and social bonding, these neuropeptides are strong candidates for dysregulation in autism (Lim, Bielsky, & Young, 2005).

Several studies have been done to support the hypothesis that oxytocin and vasopressin play a role in autism, including one that found when given intravenous oxytocin, repetitive behaviors were significantly reduced in adult humans with autism and Asperger's (Hollander et al., 2003). Possible sources suggested for this dysregulation could be a mutation in enzymes that post-translationally process oxytocin (Lim et al., 2005). Vasopressin is also a candidate for social impairments in people with autism. A study by Thompson et al. in 2004 (in Lim et al., 2005) found that intranasally administered vasopressin enhanced human facial responses, which they concluded meant that vasopressin may modulate the processing of social stimuli similar to the responses seen in rodent models.

In conclusion, a reliable rodent model may allow researchers to gain valuable insight into the neuropathology of social disorders through the investigation of neural control in social bonding and behavior. Continued studies on the oxytocin and vasopressin systems may give us more information into the mechanisms of autistic and Asperger's disorders, as well as further the understanding of comparative aspects of social behaviors in general.

Method

Subjects

Experiment One

Subjects were mice of the CD1 strain: 18 males and 39 females (obtained from Harlan, Indianapolis, IN). Subjects were group housed by sex (5 per cage) in a light and temperature controlled environment (lights on 0800; 12:12hr light: dark cycle), with food and water available *ad libitum*.

Experiment Two

For Experiment Two, 40 CD-1 strain female mice were obtained from Harlan, Indianapolis, IN. Subjects were group housed (5 per cage) in a light and temperature controlled environment (lights on 0800; 12:12hr light: dark cycle), with food and water available *ad libitum*.

Materials

Experiment One

We used a modified elevated-plus maze (15 cm x 75 cm x 6 cm) with the center portion marked as an alley. Wire mesh was placed at each end, leaving an 8 cm space behind the mesh separated from the middle alley portion at each end. Each end was covered with Plexiglas to prevent the mice from climbing out of their section. The center part of alley was split into two

bins (see Figure 1). The mouse in the alley section is referred to as the "free mouse" while the mouse behind the mesh is referred to as the "enclosed mouse". A video camera was used to record the experiment. An amount of 0.9% acetic acid was used to produce overt pain, observed as writhing behavior in the enclosed pain mice.

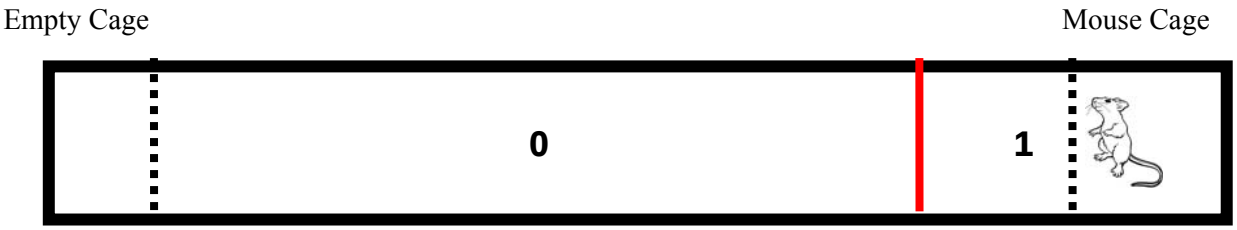

Figure 1. Set-up of Modified Elevated Plus Maze.

Experiment Two

The materials are the same as in experiment 1, with the addition of oxytocin (Sigma Aldrich, St Louis, MO) 2 mg/kg, at an injection volume of 10 ml/kg for each mouse.

Procedures

Experiment One

The free mouse was placed in the open alley section for 20 minutes to habituate. A cagemate of the free mouse was chosen to be the enclosed mouse in one of two conditions: in pain or not in pain. There were 9 male enclosed mice were in the pain condition and 9 male enclosed mice in the no pain condition. Additionally, 20 female mice were in the pain condition and 19 were in the no pain condition. To create pain in the enclosed mouse, 3 minutes prior to the end of the 20-minute habituation period the enclosed mouse was injected in the abdomen with 10 mL/kg of 0.9% acetic acid to produce writhing behavior. At the end of the 20 minutes habituation, the enclosed mouse was placed in one mesh section and the other mesh section was

left empty. The video camera then recorded the behavior of the two mice for 30 minutes. At the end of this time the enclosed mouse (if in the pain condition) was sacrificed**.**

The videos were later coded for affiliative behavior by members of the research team. Every 20 seconds the position of the free mouse was recorded as a dichotomous variable (when the free mouse was within 10 cm of the enclosed mouse or when it was not within 10 cm of the enclosed mouse). The time spent in physical contact with the wire mesh was also measured.

Experiment Two

The free mouse received a subcutaneous injection of oxytocin (2 mg/kg) immediately before being placed in the open alley section for 20 minutes to habituate. A cagemate of the free mouse was chosen to be the enclosed mouse in one of two conditions: when the enclosed mouse was in pain or when the enclosed mouse was not in pain. In this experiment, 20 enclosed mice were in the pain condition and 19 enclosed mice were in the no pain condition. The remaining procedure is the same as in experiment one.

Results

Experiment One

In the preliminary data from 18 males and 21 females, the female mice spent significantly more time on the proximate mesh (wire mesh enclosing the cagemate) (M=505.85, *SE*=46.25) than did the male mice (M=339.89, *SE*=34.89), $t(37)=2.788$, $p<0.01$. (See Table 1 and Appendix A, Figures 2 and 3).

Measurement Condition	*Sex*	*Mean*	*S.E. of Mean*	*t-value*	*p-value*
Proximity Score	F	38.81	2.98	0.50	0.62
	M	36.28	4.21		
Time on Proximate Mesh (seconds)	F	505.85	46.25	2.79	0.01
	M	339.89	34.89		
Time on Distal Mesh (seconds)	F	210.95	25.21	1.63	0.11
	M	156	21.56		
Contact Time Difference (seconds)	F	294.91	56.45	1.62	0.12
	M	183.89	34.03		

Table 1. Overall Sex Differences in Affiliation Measurements, descriptive statistics

In addition, females trended towards significance to show more affiliation behavior measured by proximity score (dichotomous proximity variable) when the enclosed mouse was in the pain (M=43.1, *SE*=4.02) than in no pain (M=34.1, *SE*=4.11), $t(19)=1.56$, p=.135. The contact time difference (time on proximate mesh minus time on distal mesh) was not near significance ($t(19)=.329$, ns). The males exhibited no difference in approach behavior in relation to the pain condition of the enclosed mouse in either the proximity score ($t(16)=.503$, ns) or the contact time difference ($t(16)=.392$, ns).

From the mean differences, it seems as though there may indeed be a sex difference in empathetic response, but our sample size must be expanded. Because we saw no trends towards significance in the male data and our female data were close to significance, the original proposal was amended to focus solely on females and their affiliative behavior (continuation of Experiment One) in an attempt to reach a level of significance. (See Table 2 for female statistics and Table 3, and Appendix A, Figures 4 and 5 for male statistics).

Measurement Condition	*Pain Condition*	*Mean*	*S. E. of Mean*	*t-value*	*p-value*
Proximity Score	*NP*	34.11	4.11	1.56	0.14
	P	43.11	4.02		
Time on Proximate Mesh (seconds)	*NP*	463.20	74.71	0.87	0.39
	P	544.64	57.31		
Time on Distal Mesh (seconds)	*NP*	188.20	24.17	0.86	0.40
	P	231.64	43.12		
Contact Time Difference (seconds)	*NP*	275.00	81.89	0.33	0.75
	P	313.00	81.29		

Table 2. Female Differences in Affiliation through various measurements, Pain vs. No Pain Condition.

Measurement Condition	*Pain Condition*	*Mean*	*S.E. of Mean.*	*t-value*	*p-value*
Proximity Score	*NP*	34.11	5.94	0.5	0.62
	P	38.44	6.23		
Time on Proximate Mesh (seconds)	*NP*	341.44	44.36	0.04	0.97
	P	338.33	56.61		
Time on Distal Mesh (seconds)	*NP*	143.89	29.54	0.55	0.59
	P	168.11	32.65		
Contact Time Difference (seconds)	*NP*	197.56	50.57	-0.39	0.7
	P	170.22	48.14		

Table 3. Male Differences in Affiliation through various measurements, Pain vs. No Pain Condition.

Experiment One. Expanded Female Sample. The sample size for females was then increased to a total of 39 females, with 20 in the pain condition and 19 in the no pain condition. After this expansion, female mice were found to show significantly more affiliation behavior measured by proximity score when the enclosed mouse was in pain (M=39.85, *SE*=3.14) than when the enclosed mouse was not in pain (M=30.84, *SE*=2.81), $t(37)=2.13$, p=0.04. Females seem to be distinguishing between the different pain states of another mouse. The measure of contact time difference was not significant ($t(37)=1.29$, ns), though the means were in the predicted direction. (See Table 4 and Appendix A, Figures 6 and 7).

Measurement Condition	*Pain Condition*	*Mean*	*S.E. of Mean.*	*t-value*	*p-value*
Proximity Score	*NP*	30.84	2.81	2.13	0.04
	P	39.85	3.14		
Time on Proximate Mesh (seconds)	*NP*	379.95	45.87	1.68	0.10
	P	488.45	45.19		
Time on Distal Mesh (seconds)	*NP*	189.79	12.93	0.46	0.66
	P	203.95	27.09		
Contact Time Difference (seconds)	*NP*	190.16	48.86	1.29	0.21
	P	284.50	54.47		

Table 4. Female Differences in Affiliation through various measurements, Pain vs. No Pain Condition. Expanded Sample.

Experiment Two

To determine if oxytocin enhanced the tendency of female mice to approach a cagemate in pain, we first performed a Mixed-Factorial ANOVA, with the independent variable being pain condition (pain vs. no pain) and the dependent variables being proximity score and contact time difference. We also used time spent on the proximate mesh and time spent on the distal mesh as dependent variables. There was no significant effect of pain condition on proximity score ($F(1,38)=.182$, ns), and no significant effect of pain condition on contact time difference ($F(1,38)=.02$, ns). There was also no effect of pain condition on time spent on the proximate mesh ($F(1,38)=1.33$, ns), but there was a significant effect of pain condition on the time spent on the distal mesh between the no pain (M=167.95, *SE*=21.84) vs. pain condition (M=111.55, *SE*=16.88), $F(1,38)=4.175$, p=.048. (See Table 5 and Appendix B, Figures 8 and 9).

Measurement Condition	*Pain Condition*	*Mean*	*Std. Error*
Proximity Score	*NP*	35.65	4.25
	P	38.55	5.31
Time on Proximate Mesh (seconds)	*NP*	334.45	45.37
	P	268.65	34.51
Time on Distal Mesh (seconds)	*NP*	167.95	21.84
	P	111.55	16.88
Contact Time Difference (seconds)	*NP*	166.50	52.09
	P	157.10	41.77

Table 5. Female Oxytocin injected mice, Differences in Affiliation through various measurements, Pain vs. No Pain Condition. Experiment Two.

Cross-Experiment Results

To examine if the drug manipulation mice differed in empathetic response from the no drug mice in empathetic response, we performed a 2x2 Factorial ANOVA with the independent variables being pain condition (pain vs. no pain) and drug condition (oxytocin injections vs. no oxytocin injections). This combined the 40 female mice from Experiment Two with the 39 female mice from Experiment One. The dependent variable was proximity score. For proximity score there was no significant main effect for drug condition ($F(1,75)=0.19$, ns), no significant main effect for pain condition ($F(1,75)=2.187$, ns), and no significant interaction between drug and pain condition ($F(1,75)=.575$, ns). This means that there was no difference between the drug mice and no drug mice for their total proximity score. (See Table 6 and Appendix C, Figure 10).

Pain Condition	*Drug Condition*	*Mean*	*Std. Error*
NP	*OT*	35.65	4.00
	None	30.84	4.10
P	*OT*	38.55	4.00
	None	39.85	4.00

Table 6. Total Proximity Score, Pain Condition by Drug Condition.

To follow up on the findings that the drug condition mice showed a significant difference in time on the distal mesh when they were in the pain vs. no pain condition, we performed a 2x2 Mixed Factor ANOVA with the dependent variables being pain condition (pain vs. no pain) and drug condition (additional oxytocin vs. no additional oxytocin). The within-subjects independent variable was side, measured by time spent on both the proximate mesh and the distal mesh. For side, within-subjects there was a trend towards a significant interaction for drug condition by side ($F(1,75)=2.32$, $p=0.13$), no interaction for pain condition by side ($F(1,75)=0.73$, ns), and no significant interaction between drug condition and pain condition ($F(1,75)=1.1$, ns). We did find an overall significant main effect of side within subjects, $F(1,75)=64.83$, $p<0.01$. This means that all of the mice spent significantly more time on the proximate mesh than the distal mesh, regardless of pain condition or drug condition.

There was a significant between-subjects main effect for drug condition, $F(1,75)=17.34$, $p<0.01$, and a significant between-subjects interaction between pain condition and drug condition, $F(1,75)=7.22$, $p<0.01$. There was no between-subjects main effect for pain condition ($F(1,75)=.00$, ns).This demonstrates that there is something significantly different happening between the drug condition and the no drug condition in terms of time spent on mesh (regardless of whether a mouse was enclosed behind it), as well as a significant difference between the drug condition animals depending on whether they are in the pain or no pain condition (from previous statistics).

To determine where the difference in "side" was (proximate versus distal mesh contact time) between the drug condition and the no drug condition in the pain vs. no pain condition, we performed a 2x2 Factorial ANOVA examining whether the difference in the drug condition vs. no drug condition was observed in the proximate mesh. The independent variables were pain

condition and drug condition, and the dependent variable was time on proximal mesh. There was a significant main effect for drug condition, $F(1,75)=9.542$, $p<0.01$, and a significant interaction for pain condition by drug condition, $F(1,75)=4.12$, $p<0.05$. There was no significant main effect for pain condition ($F(1,75=0.25$, ns). (See Table 7 and Appendix C, Figure 11). These results indicate that the mice in the drug condition spent significantly less time on the proximal mesh than the mice in the no drug condition. This decrease is observed in the pain condition of the drug mouse, but not in the no pain condition.

Pain Condition	*Drug Condition*	*Mean*	*Std. Error*
NP	*OT*	334.45	42.66
	None	379.95	43.77
P	*OT*	268.65	42.66
	None	488.45	42.66

Table 7. Time on Proximate Mesh, Pain Condition by Drug Condition.

To investigate if this difference by drug condition (finding that drug condition mice in the pain condition spend significantly less time on the proximate mesh than the mice in the no drug condition) is also seen on the distal mesh, we performed a 2x2 Factorial ANOVA with the independent variables of pain condition and drug condition and the dependent variable of time on distal mesh. There was a significant main effect of drug condition, $F(1,75)=7.72$, $p<.01$, but there was no significant main effect of pain condition ($F(1,75)=1.05$, ns) or interaction of pain condition and drug condition ($F(1,75)=2.95$, ns). (See Table 8 and Appendix C, Figure 12). These results seem to indicate the difference we see in the drug condition is based upon these animals spending less time overall on either mesh, which suggests that oxytocin affected the motor ability of these animals. Oxytocin also seems to be affecting this motor ability particularly in the pain condition, in terms of time spent on the proximal mesh.

Pain Condition	*Drug Condition*	*Mean*	*Std. Error*
NP	*OT*	167.95	20.42
	None	189.79	20.95
P	*OT*	111.55	20.42
	None	203.95	20.42

Table 8. Time on Distal Mesh, Pain Condition by Drug Condition.

Discussion

The results support the main hypothesis that females are able to distinguish between a mouse in pain and a mouse not in pain, suggesting an empathetic response in a female mouse to another cagemate in pain. Furthermore, our hypotheses that (1) females would demonstrate more affiliative behavior overall than males, and (2) that males would not show a difference in affiliative behavior based on pain state, were supported. The result from Experiment One, demonstrating that females distinguish between pain states of another mouse, led us to extend the study to investigate the possible role of the affiliative hormone oxytocin in the affiliative behavior response. Since we focused solely on females in the expansion of Experiment One, we decided to continue to focus solely on females in the pharmacological manipulation of oxytocin.

Contrary to our expectations, oxytocin-treated females did not show enhanced affiliative behavior based on the pain state of the other mouse. Oxytocin-treated females also did not differ from untreated females in affiliative behavior. Thus, the increase of oxytocin did not significantly increase affiliative behavior in female mice.

Since the results of the pharmacological manipulation did not support the original hypotheses, we performed further statistical tests to determine on what particular dimensions these animals differed in their affiliative response to a cagemate in pain. From these tests, it was discovered that oxytocin-treated mice moved significantly less around the enclosure than untreated mice. Additionally, the oxytocin-treated mice in the pain condition spent less time on

the proximal mesh than did the oxytocin-treated mice in the no pain condition, and less time on the proximal mesh than untreated mice in the pain condition. Therefore, there is a possible confound in our pharmacological manipulation, in that the oxytocin injections seemed to affect the motor ability of the mice, particularly in the pain condition.

This effect on motor behavior was apparent in the video tapes of the mice. Many of the mice chose one corner of the enclosure and stayed there without moving for long periods of time. While some mice stayed completely immobile, others demonstrated an increase in almost obsessive grooming behavior. The grooming mice also did not move from one spot. Additionally, the oxytocin-treated mice seemed to have difficulty climbing the mesh, and their back legs seemed to dangle or drag behind them.

These effects of oxytocin on motor behavior have previously been observed. High doses of oxytocin were found to cause significant sedation in male rats when the subcutaneous dose was greater than 250 μg/kg (Uvnaes-Moberg, Ahlenius, Hillegaart & Alster, 1994), and the time course for this effect was maximal within 1 hour of the injection. Thus, oxytocin, regardless of its possible role in affiliative behavior, can cause sedation in animals, particularly at high doses. Since our present work used a dosage of 2,000 μg/kg, the injection could have similar (if not significantly greater) effects on sedation in the mice in the current study. Future work should use a lower dose of oxytocin to eliminate this sedation effect in mice or use direct intracerebroventricular injections of oxytocin (to attempt to bypass the motoric effects).

Oxytocin has also been found to have different sedative effects on female rats depending on their stage in the estrous cycle. At a subcutaneous dose of 100 μg/kg, locomotor activity was decreased in rats at the diestrus stage. At a dose of 1000 μg/kg, locomotor activity was significantly decreased in rats at the metestrus and diestrus stages, when compared to a saline

injection (Petersson, Ahlenius, Wiberg, Alster, & Uvnas-Moberg, 1998). Since the estrous cycle contributes to changes in endogenous levels of oxytocin, these results indicate that the effect of oxytocin on locomotor activity in female rats may be somewhat dependent on the endogenous levels of oxytocin. Our study did not control for estrous cycle, which would have provided additional information in determining how the interaction of endogenous fluctuating oxytocin and injections of oxytocin may affect general locomotor behavior.

Simply eliminating the estrous cycle, however, is not a viable solution, since ovariectomized female rats have been shown to have increased locomotor activity when given a subcutaneous 100 μg/kg dose of oxytocin (Petersson, Eklund & Uvnas-Moberg, 2005). Since these rats do not have an estrous cycle, the experimenters concluded that oxytocin is under a strong influence of prevailing endogenous steroid hormone levels. Given that an increase in motor behavior is also an undesired effect, when using females to examine the effects of oxytocin, the estrous cycle must be monitored. From this previous research, animals should not be used while in either the diestrus or metestrus stages of the estrous cycle to eliminate the motor effects observed in the current study.

Excessive grooming behavior, as observed in the oxytocin manipulation, has also been examined in the literature. Both male and females rats significantly increase their grooming behavior when given an intracerebroventricular injection of oxytocin, with the maximum effect occurring within 45 minutes of injection (Drago, Pedersen, Caldwell, & Prange, 1986). These researchers repeated the study using a subcutaneous injection of oxytocin, and also saw a significant increase in grooming behavior. Since the grooming behavior was found to be predominantly increased in genital grooming (thought to be particularly rewarding), the researchers tested whether a dopamine antagonist would decrease this grooming behavior. This

hypothesis was supported through their research. Thus, these researchers concluded that the increase in grooming behavior seen in oxytocin-injected rats involved the dopamine reward pathway. It can also be concluded that when rats or mice are busy grooming, they do not attend to their surroundings, which was crucial for the behavior assessed in the present study.

An unexpected result in the present study was that the oxytocin-treated females avoided the other mouse more when the other mouse was in pain. While this seems to be contrary to our hypothesis, there may be an explanation in the literature. Higher oxytocin levels have been shown to affect the avoidance of other animals when the other animal has a stomach parasite (Kavaliers et al., 2003). While wildtype females distinguished between animals with parasites and those without parasites and would avoid those with parasites, female oxytocin knockout mice were unable to distinguish between males with stomach parasites and those without parasites. Wildtype female mice were able to distinguish between the two because of an odor that was emitted by the parasitic male. Thus, oxytocin seems to affect the odor recognition mechanism in female mice. While this study by Kavaliers (2003) examined mate choice in females, perhaps female mice with significantly increased oxytocin levels are able to distinguish an odor given off by a mouse in pain that is aversive to them, such as the smell of the acetic acid. If this is the case, these mice might avoid the mouse in pain more than a mouse in the no pain condition. This avoidance in the presence of an aversive odor may also explain why the drug condition mice in the pain condition spent less time on the proximate mesh than the mice in the no drug condition, since the drug condition mice may be able to distinguish an aversive odor. In general, the pharmacological manipulation of oxytocin was unsuccessful and the various confounds of this experiment need to be taken into account in future research.

Theoretical Implications. Regardless of the limitations of our pharmacological manipulation, the positive results that females are able to distinguish between the overt emotional state of another mouse and affiliate more with a mouse when it is in pain, further extends the theory that mice are able to express empathetic behavior (Langford et al., 2006). Mouse affiliative behavior, as measured by social approach, demonstrates an ability to differentiate between the emotional states of other mice. The use of our novel paradigm is validated to further investigate social approach as a measure of empathy, since it was used to successfully demonstrate a differentiation of social approach based on the emotional state of another mouse in this current study.

It could be argued that the ability of a female mouse to differentiate between the emotional states of another mouse could be based on some pleasant odor that the other mouse is emitting that females prefer (because of higher circulating oxytocin, which is tied to better scent distinction). The surprising result, however, that oxytocin-treated females spent less time associating with the mouse in pain seems to negate this possibility. If it would be argued that females, because of their higher natural oxytocin levels, are able to smell something attractive that the males are not, one would expect the females with extremely high levels of oxytocin to affiliative even more. Since this is not the case, oxytocin does not seem to play a role by pleasant odor detection. If anything, female mice with increased oxytocin may be responding to an unpleasant odor, causing a decrease in affiliative behavior.

A sex difference was found, demonstrating that females are able to distinguish between emotional states of another mouse, and males may not have this ability. This sex difference may support the premise that the overall female network behavior of affiliation, circulating oxytocin levels, and their tend-and-befriend strategy may play a role in empathetic response. Previous

work with affiliation show that female humans who identify more with their spouse, and spend more time with them, have more empathy for them (Levenson & Ruef, 1992). Since female animals show more affiliation with other females and spend more time with them than males spend with other males, female mice would be expected to show more empathy for each other than males do. This difference in affiliation could be influenced by the fact that females have higher levels of endogenously circulating oxytocin than males do. If oxytocin does play a role in empathy, as suggested, these natural differences in hormone levels may influence the sex differences that we observed.

The results of this study support the conclusion that mice demonstrate empathy (Langford et al., 2006), and in doing so, fit with the Perception-Action Model of empathy (Preston & de Waal, 2002), which states that the relationship between the object (enclosed mouse) and the subject (free mouse) would modulate the empathetic response. Extending this theory to the current study, which used cagemates, mice do seem to be responding in a way suggesting that the familiarity of the other mouse is important for the formation of an empathetic response. While Preston and de Waal originally proposed their theory in terms of human empathy, current results indicate that this theory should be extended to other animals.

Some experimenters postulate that the primary function for empathy for pain in humans is to strengthen social bonds. Empathy is said to do this in three ways: it coordinates the action of individuals to allow rapid response to threats, it helps understand others' thoughts and intentions, and it signals solidarity (Anderson & Keltner as cited in Preston & de Waal, 2002). While mice do not have the cognitive ability to "understand" others' thoughts, the observation of empathetic behavior in mice may demonstrate a similar function in the mice: the strengthening of social bonds, as well as more primitive functions, such as learning about the environment for one's own

survival. If empathy does play a role in strengthening social bonds, one would expect that after an experience of empathy (such as with the experimental paradigm in this study) mice would show increased bonding with one another. Future research could investigate the change in the level of bonding through comparing time spent together before and time spent together after empathy social approach paradigm. If it was found that mice did show increased bonding behavior after experiencing empathy, this would support the role of empathy in strengthening social bonds, and extend this theory to rodents.

Future Research. The results from this study support a new means of measuring empathetic approach behavior through a novel experimental paradigm combining social approach and an overt pain stimulus. Since oxytocin and vasopressin are tied to affiliation, by using the paradigm designed here, future research can be conducted to look at the role of these hormones in approach behavior modulated by the overt pain state of another mouse. Future work can also rely on this paradigm to further investigate the sex differences observed in the current study. Since this paradigm has been shown to be a valid measurement of empathetic approach behavior, future research could expand its use to investigate the modulation of other emotional states in enclosed mice (fear, stress, etc.) on empathetic response.

Taking into account the confounds of this study, future work should use subcutaneous injections of oxytocin at lower doses, such as 100 μg/kg and 200 μg/kg (since the current research at a dosage of 2,000 μg/kg seems to have overdosed the mice). Future research should also control for the estrous cycle of female mice, and not use female mice while in the diastrus stage of the estrous cycle, since estrogen fluctuation has been shown to interact with the injected oxytocin and cause sedation.

It would also be interesting to do a similar pharmacological manipulation in male mice. Since males do not naturally show affiliation and did not distinguish pain states of another mouse, their behavior may mimic the behavior observed in females by increasing the circulating levels of vasopressin. If found that males notably increase their general affiliative behavior and also gain an ability to distinguish between pain states of another mouse, this would offer strong support for the role of affiliative hormones in this empathetic approach behavior.

Examining how a lack of oxytocin influences this approach behavior, using female knockout mice, would expand the current research investigating the role of this hormone. If females without endogenous oxytocin do not exhibit an ability similar to intact female mice, this would offer further support for the role of oxytocin in the female empathetic behavior response. Current work in this lab is investigating this role of oxytocin through the use of oxytocin knockout mice (derived from B6 mice). Additionally, if these females were able to acquire the ability to distinguish emotional states of another mouse and show an empathetic response after being given exogenous oxytocin, the hypothesis that oxytocin plays a direct role in the ability to distinguish pain states of another mouse (or any emotional state) would be strongly supported.

Examining stress of the free mouse would be necessary to investigate through future work. If the female free mouse is stressed, one would expect them to affiliate more with the other mouse regardless of situation, since females use this tend-and-befriend strategy when in stressful situations. Perhaps the stress of the situation is exacerbated when the cagemate is in pain, which is the observed sex difference in affiliative behavior. While this would still support our results that females are able to distinguish the pain state of another mouse, it would be important to examine how the stress of the situation may be affecting the behavior we are observing. This would also aid in the examination of the observed sex differences; if females respond

empathetically because of stress, males may not do so because they are also stressed (and they have a different response to stress).

As of now, it is unclear how the female mice are distinguishing the other mouse's emotional state. It would helpful to investigate the behavior of the mouse in pain to see how its behavior is changing, and this could be done using the experimental manipulation from Langford et al (2006) while observing the behavior of the pain mouse. Future work could use both microphones and close video of the mouse in pain to investigate to what the free mouse is responding. Since mice are also very attuned to odors, some measurement of odor that the pain mouse is emitting would be useful.

Conclusions. With the help of the novel paradigm that this study has proposed to measure empathetic approach behavior in mice, future research can continue to investigate how animals are able to distinguish emotional states of other animals. In addition, this paradigm will allow an investigation of how the affiliative hormones, oxytocin and vasopressin, play a role in this ability. This new experimental technique offers a powerful model for examining empathy in mice, and provides a better understanding of social modulation of empathy. Furthermore, this novel paradigm offers an ability to investigate the influences of other states on empathy besides pain, such as fear or stress.

In conclusion, this model of empathy in mice will provide us with valuable insight into the social behavior of mice and allow for the roles of affiliative hormones in these social behaviors to be determined. With the proposed future work, more information on mouse social behavior will be discovered, and eventually information into the mechanisms of human social disorders, as well as additional information on understanding comparative aspects of social

behavior. Social behaviors, such as empathy and the distinguishing of emotional states, that after this current work can no longer be considered human specific.

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Appendix A

Experiment One-Sex Differences

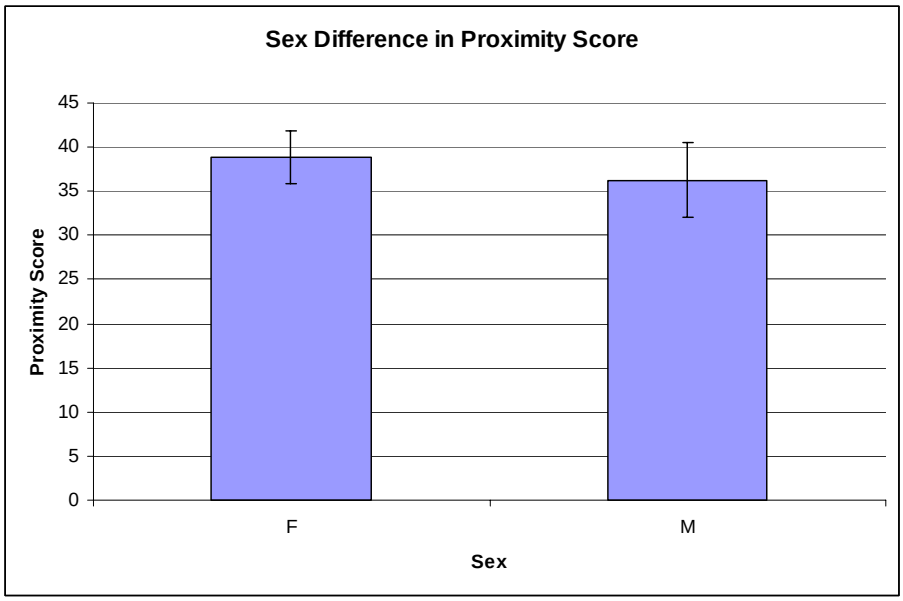

Figure 2. Sex differences in proximity score with F= female mice and M= male mice. Bars illustrate the mean proximity score for each sex. The difference between males and females was not significant.

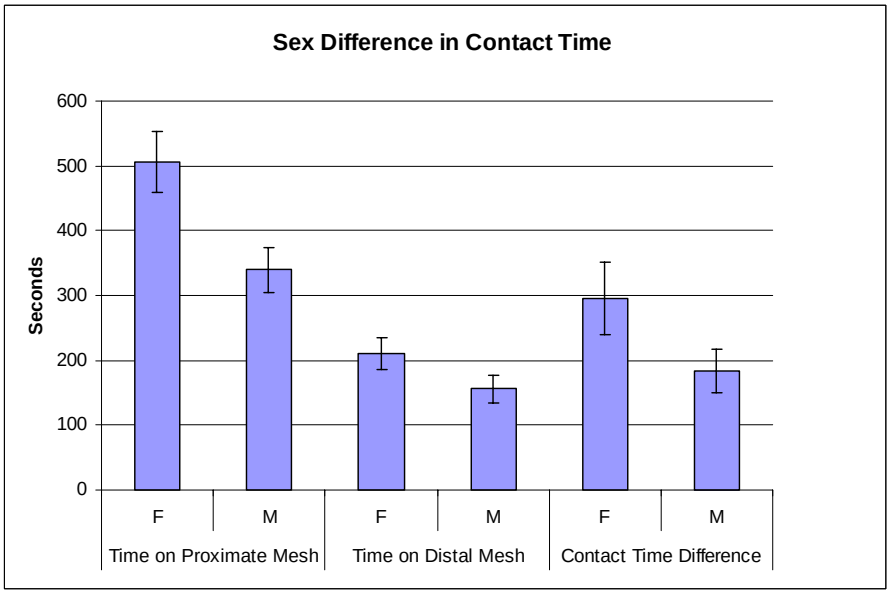

Figure 3. Sex differences in contact time with F= female mice and M= male mice. Bars illustrate the mean time (in seconds) that mice spent on the proximate mesh, on the distal mesh, and the time difference (in seconds) between time spent on the proximate and distal mesh. Female mice spent significantly more time on the proximate mesh than male mice (p= 0.01).

Experiment One-Male Results

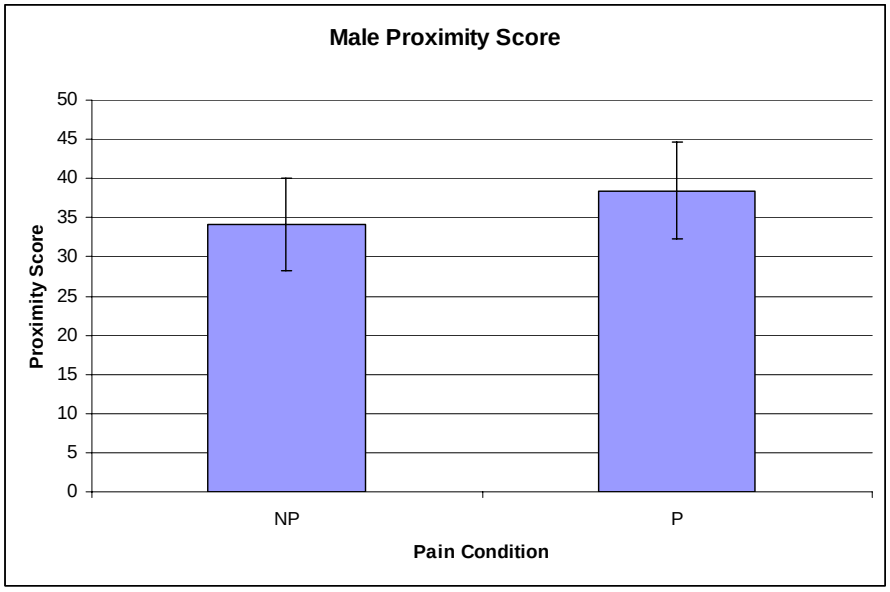

Figure 4. Male proximity scores by pain condition, with NP= no pain condition and P= pain condition. Bars illustrate the mean proximity score for each pain condition. The difference between pain conditions was not significant.

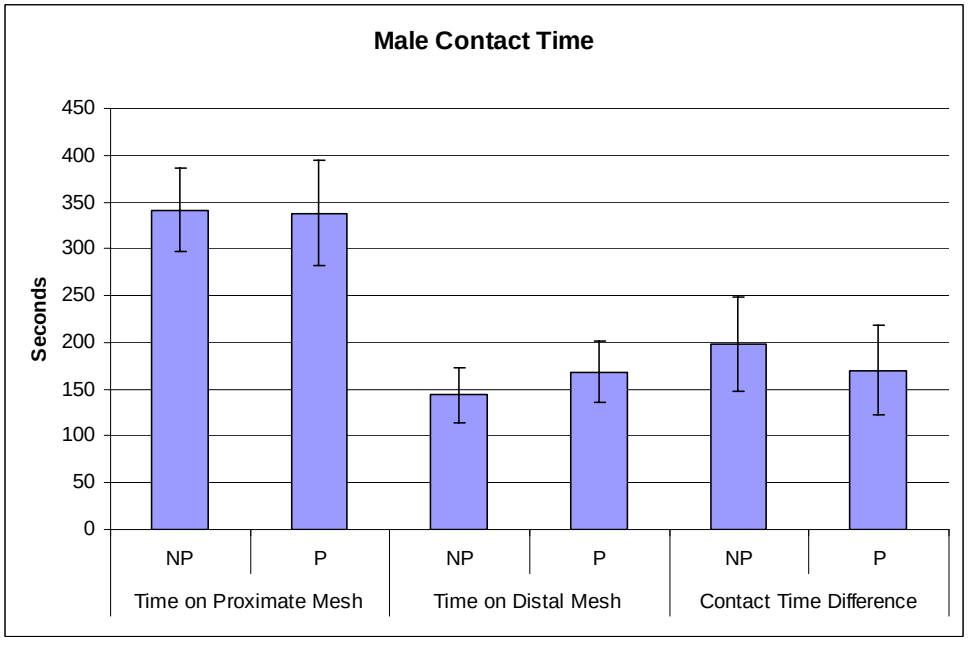

Figure 5. Male contact time, with NP= no pain condition and P=pain condition. Bars illustrate the mean time (in seconds) that mice spent on the proximate mesh, on the distal mesh, and the time difference (in seconds) between time spent on the proximate and distal mesh. The difference between pain conditions on these variables was not significant.

Experiment One-Expanded Female Sample Size Results

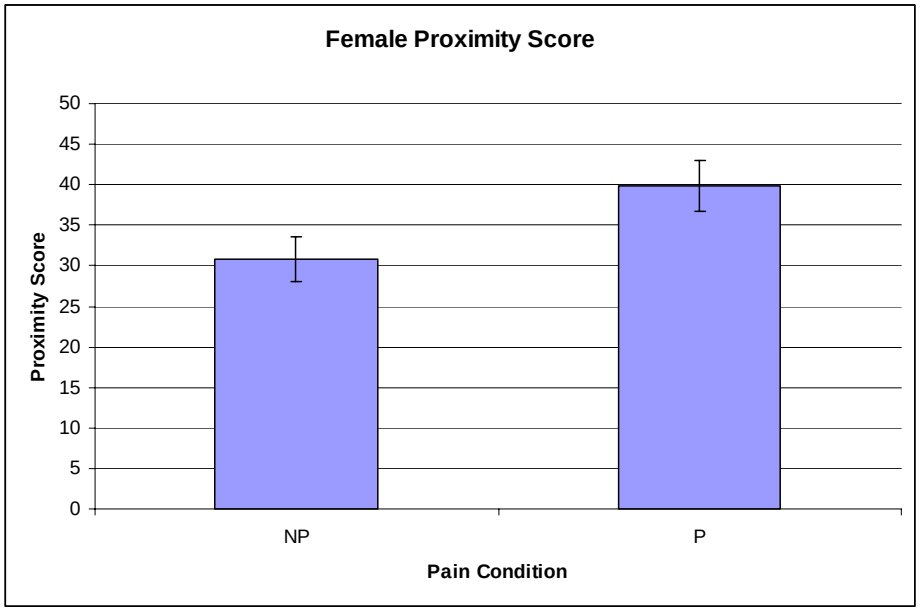

Figure 6. Female proximity scores with the expanded sample, with NP= no pain condition and P= pain condition. Bars illustrate the mean proximity score for each pain condition. Females had significantly higher proximity scores in the pain condition than in the no pain condition (p=0.04).

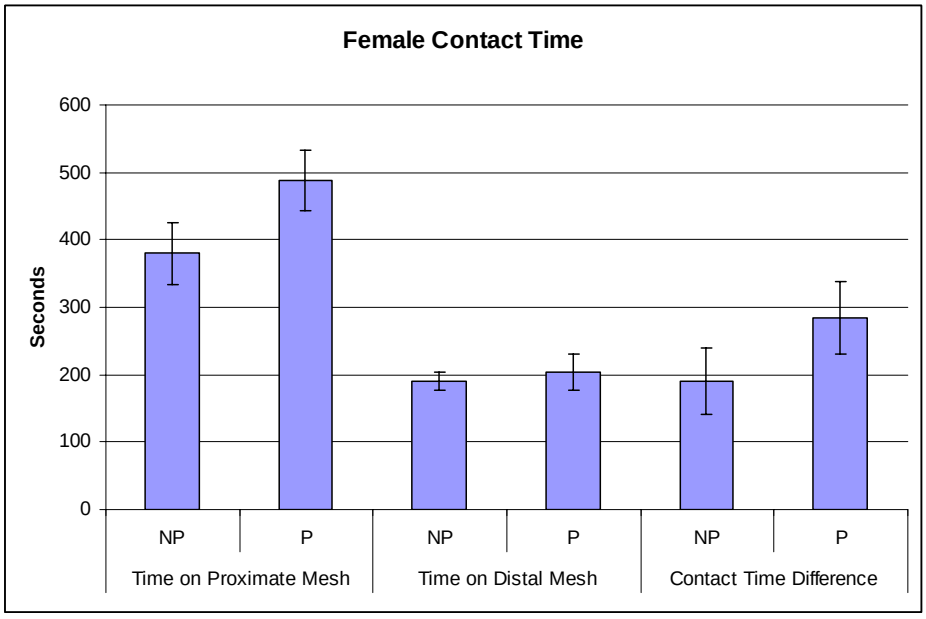

Figure 7. Female contact times with expanded sample, with NP= no pain condition and P=pain condition. Bars illustrate the mean time (in seconds) that mice spent on the proximate mesh, on the distal mesh, and the time difference (in seconds) between time spent on the proximate and distal mesh. The difference between pain conditions on these variables was not significant but trended in the predicted direction.

Appendix B

Experiment Two-OT-treated mice

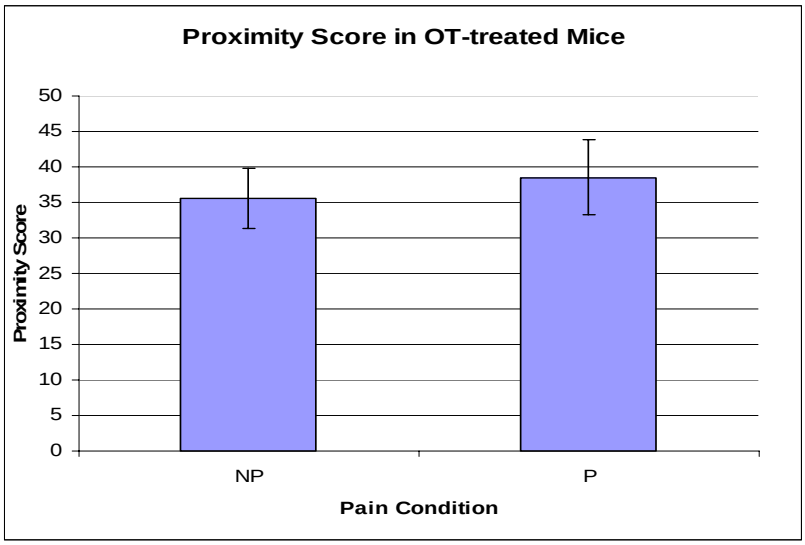

Figure 8. Oxytocin treated mice proximity scores by pain condition, with NP= no pain condition and P= pain condition. Bars illustrate the mean proximity score for each pain condition. The difference between pain conditions was not significant.

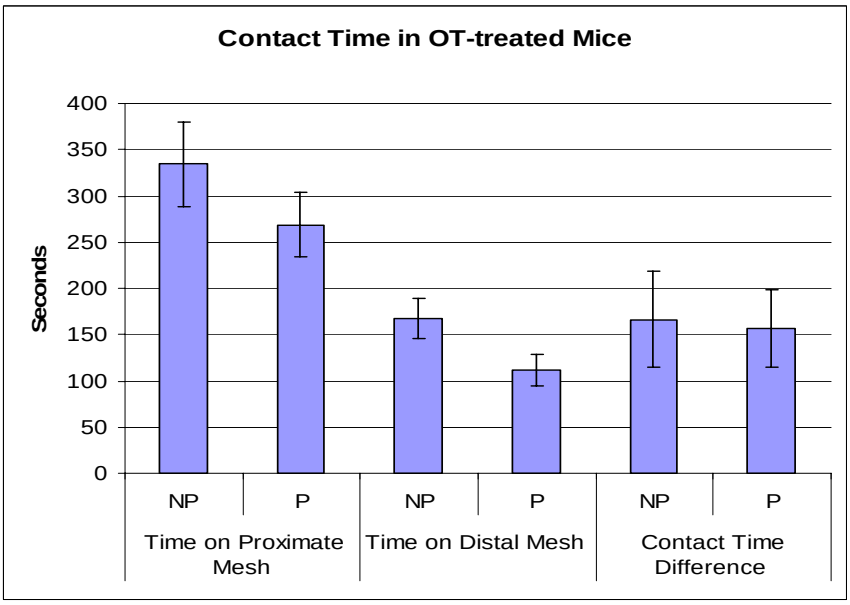

Figure 9. Contact time in oxytocin treated mice, with NP= no pain condition and P= pain condition. Bars illustrate the mean time (in seconds) that mice spent on the proximate mesh, on the distal mesh, and the time difference (in seconds) between time spent on the proximate and distal mesh. Mice in the pain condition spent significantly less time on the distal mesh ($p= 0.48$) and a trend for mice in the pain condition to spent significantly less time on the proximate mesh.

Appendix C

Cross Experimental Results

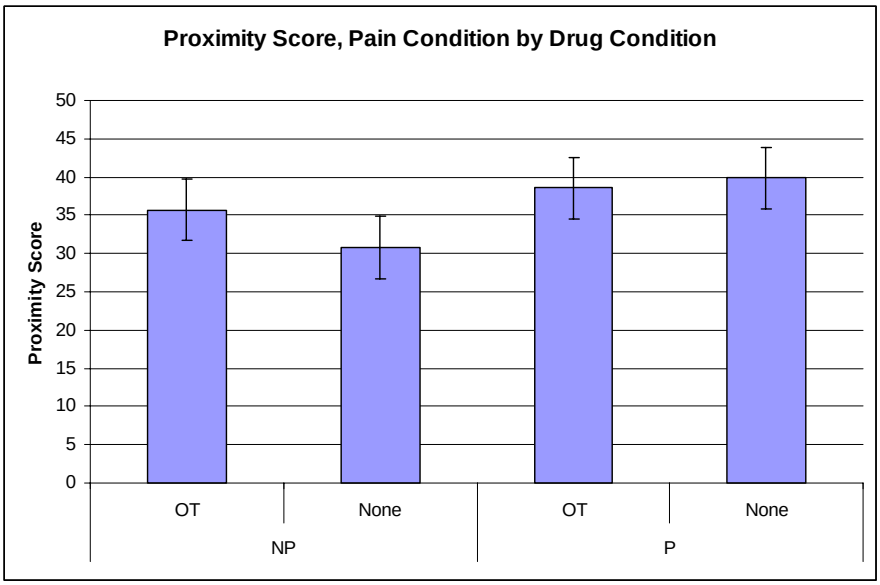

Figure 10. Proximity score, pain condition by drug condition, with NP= no pain condition, P= pain condition, OT= drug condition, and None= no drug condition. There were no significant differences in proximity score in pain condition by drug condition.

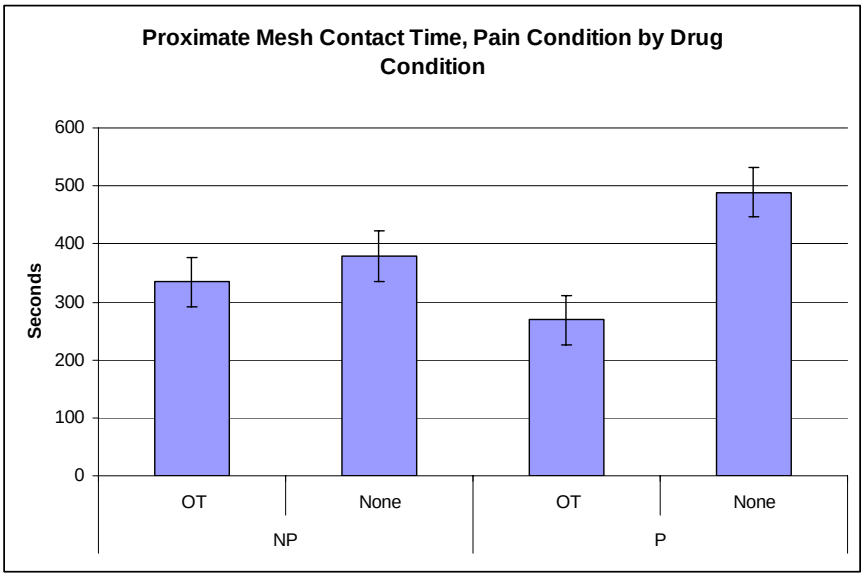

Figure 11. Contact time on proximate mesh, pain condition by drug condition, with NP= no pain condition, P= pain condition, OT= drug condition, and None= no drug condition. Mice in the drug condition spent significantly less time on the proximate mesh than mice in the no drug condition ($p= 0.01$) and this decrease is seen significantly in the pain condition, but not the no pain condition.

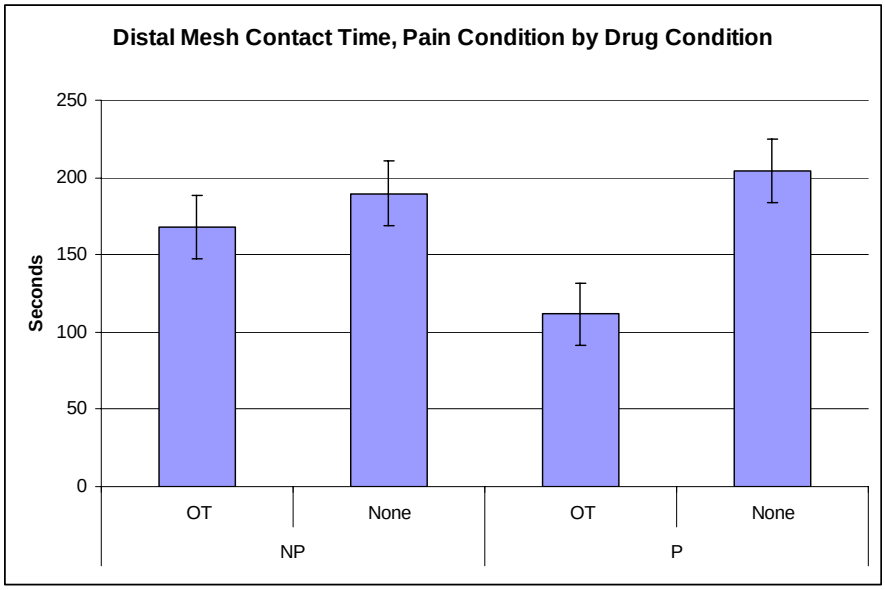

Figure 12. Contact time on distal mesh, pain condition by drug condition, with NP= no pain condition, P= pain condition, OT= drug condition, and None= no drug condition. Mice in the drug condition spent significantly less time on the distal mesh than mice in the no drug condition (p= 0.01), but there was no significant difference in this decrease by pain condition.

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