Neurobiological Mechanisms by which Stress Prevents Empathy

by

Sivaani Sivaselvachandran

A thesis submitted in conformity with the requirements for the degree of Master of Arts

Department of Psychology
University of Toronto

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Abstract

Mice have the capacity to transmit pain status amongst familiars, which is understood to represent empathy in rodents. Recent findings show that the stress of a social interaction with an unfamiliar mouse can block empathy, while inhibiting the endocrine stress response of unfamiliar mice can evoke it. Experiments were conducted on naïve male CD-1 ® mice and found higher pain responses when familiars were tested together as opposed to with strangers. Western blot analysis demonstrated higher glucocorticoid activity in empathy involved regions (prefrontal cortex and anterior cingulate cortex) of mice in the unfamiliar condition compared to the familiar condition. The administration of Metyrapone (to block stress response) before testing resulted in higher pain responses in unfamiliars compared to isolated mice however no significant difference was found between Metyrapone pretreated unfamiliars and saline pretreated unfamiliars. These findings begin to uncover the neurobiological mechanisms by which stress prevents empathy using rodent models.
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<td>AMY</td>
<td>Amygdala</td>
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<td>BNST</td>
<td>Bed Nucleus of the Stria Terminalis</td>
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<td>CM</td>
<td>Cagemate Condition</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>ISO</td>
<td>Isolated Condition</td>
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<td>ISO-MET</td>
<td>Isolated Condition with Metyrapone Pretreatment</td>
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<td>ISO-SAL</td>
<td>Isolated Condition with Saline Pretreatment</td>
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<tr>
<td>MET</td>
<td>Metyrapone Drug (also known as 2-methyl-1,2-di-pyridyl-1-propanone)</td>
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<td>mPFC</td>
<td>Medial Prefrontal Cortex</td>
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<td>P-gluc</td>
<td>Phospho-Glucocorticoid Receptor (Ser211) Antibody</td>
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<td>PVN</td>
<td>Paraventricular Nucleus</td>
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1.1 Rodent Empathy and Neuroscience

Empathy is the sharing of emotional states ranging from simple mimicry and emotional contagion to evolutionarily more complex constructs such as perspective taking and targeted helping (see Preston and de Waal, 2002 for a review). In this literature review, “empathy” is referred to as the ability to share or relate to the affective state of another. The extent to which different animal species engage in various forms of empathy has been debated, but since empathy-related behaviours have been observed in species ranging from mice to elephants, the debate should no longer be about whether animals have empathy, but the mechanisms that engage it. This review will discuss different levels of empathy, as it exists in rodents. It will then delve into the social modulation of fear and pain as models of empathy including a component on stress. Then taking a more neuroscience perspective, this review will cover the neural mechanisms of empathy in rodents while looking at the pain matrix and affective pain pathways in detail. This is followed by a section on the limited findings of genes and empathy linking this altogether in the importance it has to the Autism Spectrum Disorders (ASDs). Due to the complexity and the methodological difficulties associated with studying empathy, neuroscience has not historically played a significant role in empathy-related research. However, the recent development of rodent models to study empathy-related and prosocial behaviour provides opportunity for identifying the neural mechanisms of empathic behaviour, which have profound implications for the treatment of social deficits and deviance in people.
1.2 Empathy as it appears in Rodents

The existence of empathy in lower order animals such as rodents has greatly been questioned in the literature. While some species are capable of various levels of empathic behaviour, it is generally found that levels requiring more cognitive abilities such as perspective-taking and prosocial behaviour are reserved for higher order species such as humans and non-human primates. As such de Waal discusses this concept in terms of the Russian doll in which the inner core represents lower empathy manifestations such as contagion with each of the doll’s outer layers representing increasingly complex forms of empathy such as prosocial behaviour (de Waal, 2008). Similarly Singer and Lamm describe empathy in terms of levels such that the bottom level contains elementary building blocks necessary for social communication (e.g., emotional contagion and mimicry) with each subsequent level progressively adding to increasingly complex social behaviours (eg. perspective –taking)(2009). The focus will now be on empathy as it appears in rodents including the sharing of pain and fear status, which encompasses the contagion type of effects studied in current rodent-empathy literature.

Mimicry is best described as an individual’s tendency to imitate another’s behaviour without the individual’s awareness or intent. Emotional contagion is when subjects match an emotional or arousal state with that of another (Preston and de Waal, 2002). These two simple empathic behaviours are primitive and most commonly reported in rodent literature. However, skepticism in the empathy field on whether mimicry and emotional contagion significantly contribute to the expression of empathy still exists. It can be argued that one cannot truly experience empathy for another without mimicking or being able to share the other’s experience (Singer and Lamm, 2009). For example, people who reported higher levels of empathetic feelings were also more likely to mimic others’ facial expressions showing that mimicry of behaviour is related to
internal feelings of empathy (Sonnby-Borgstrom, 2002). Another study found that participants whose behaviours were mimicked by another were more helpful and generous towards others, indicating that physical mimicry engages prosocial and altruistic behaviours (van Baaren et al., 2004). Interestingly, neuronal activation patterns suggest that emotional contagion engages brain areas important for pain empathy. Participants who observed a “loved one” receiving a pain stimulus showed increased brain activation in areas that are typically associated with the affective but not the sensory component of pain (e.g. anterior insula and rostral anterior cingulate cortex (ACC)) indicating that participants were mimicking their loved one’s emotional pain. In fact, self-reported empathy levels were associated with the amount of activation in pain-associated areas, which suggests that more mimicry equates with greater empathy behaviour (Singer et al., 2004). These studies demonstrate that state and behavioural mimicry can be a useful tool for inferring empathy-related feelings in humans.

1.2.1 Foundational Studies

Early studies set in the mid 1900s set the foundation for empathy-related research and many of the studies that are conducted today. A seminal study in affective psychology demonstrated that rhesus monkeys refused a food reward in order to prevent a conspecific from receiving an electric shock (Masserman et al., 1964). One of the first rodent empathy-related experiments conducted, used rats that were required to press a lever in order to obtain a food reward (Church, 1959). Then, in a twisted change, the lever was fixed to a floor in the neighboring cage and every time the rat would push the lever, the rat in the other cage would get an electric shock. Subjects previously shocked during the conditioning phase of the experiment showed the greatest decline in lever pressing compared to the other group (maintained for 10 days post-experiment). A no-shock control group did not experience this decline in lever pressing. The interpretation of this is
that rats that previously experienced shocks were very empathetic to the rat in the other cage (i.e. they did not want to shock them). This was the first study to look at the shared experience of pain in rodents and implied that a rat could recognize that its own negative experience was being shared by a conspecific.

Shortly thereafter, (Rice and Gainer, 1962) conducted one of the first prosocial behaviour studies in rodents, where they trained rats to press a lever to lower a distressed social partner that was suspended in the air. It was found that rats reliably pressed the lever to lower the squealing rat but would not press the lever to lower a Styrofoam block. This was seminal in demonstrating that rats are capable of helping behaviours that alleviate the distress of conspecifics.

1.2.2 Theory of Mind

A higher form of empathy known as ‘theory of mind’ is the ability to distinguish that the perspective of another individual is different from your own. This is also known as “empathic perspective-taking” (Decety and Svetlova, 2012). Unfortunately, since perspective taking in most species is almost impossible to assess, there is virtually no convincing evidence demonstrating that rodents or other nonhuman animals have the ability to take another’s perspective. The one exception is nonhuman primates, who have shown behaviours that imply perspective-taking such as deception (le Roux et al., 2013).

Scientists that believe rodents cannot experience empathy argue that higher cognitive processes are needed for such abilities. However, there is research to suggest that rats have higher-level processes such as metacognition, which is the ability to control one’s own cognitive processes, especially when learning (i.e., thinking about thinking). Metacognition has been explored using a rat model of operant conditioning that examined decision-making in rats based on the value of information they received (Kirk et al., 2014). Rats were trained to press a lever that would give
them an immediate food reward, but pressing the lever would also tell them where more food was hidden. In a maze with 2 arms (a light would turn on in the arm of the maze containing food), lever pressing was reduced if the lever stopped delivering an immediate food reward, even though it still provided information about where other food was located. However, if the maze contained 8 possible paths instead of just 2, the rats would continue to press the lever at high rates even after the immediate reward was stopped. Presumably this was because in the 2-arm maze there was a 50% chance of choosing the right arm by simply guessing, but in 8-arm maze pressing the lever provided more valuable information and therefore it was more advantageous to continue pressing. Of course these are inferences and do suggest that rats show information-seeking behaviour—they may be simply assessing the benefits of performing certain behaviours, which are considered higher-level processes.

1.3 Social Modulation of Fear and Pain as Models of Empathy in Rodents

1.3.1 Social Modulation of Fear

Even though tests to assess whether rodents experience higher forms of empathy have yet to be developed, the more easily measured empathy-related behaviours of emotional contagion and prosociality have been developed (Ben-Ami Bartal et al., 2011; Langford et al., 2006). Emotional empathy (or emotional contagion) is the experience of others’ feelings or states. Emotional contagion has been observed in rodent studies by testing the degree of fear or pain transmitted between mice and rats. Since the core feature of empathy in rodents is the ability to share affective experiences, the majority of studies investigating rodent empathy have examined the neuronal correlates of observational fear learning (Chen et al., 2009; Jeon et al., 2010; Kavaliers et al., 2001b). This requires the distress of another to be recognized in order to engage empathy-related circuitry. One of the first demonstrations of observational fear learning consisted of
placing a demonstrator mouse in a cage with biting flies while an observer mouse watched the demonstrator in distress (Kavaliers et al., 2001a). After 24 hours, the observer mouse was exposed to flies whose biting appendages were removed. The observer mice who did not have an actual experience with biting flies still showed similar distress-like behaviours (i.e., self-burying avoidance) previously displayed by the demonstrator mice and enhanced analgesic responding. This indicates that mice have the ability to recognize distress in a conspecific possibly via the social transfer of stress odours, which modulate behavioural responding and pain sensitivity. In a different social observational fear protocol, an observer animal is conditioned for context-dependent fear by observing the behaviour of a demonstrator animal receiving a foot shock and the magnitude of the fear response of the observer is positively influenced by the animal's familiarity with the demonstrator (Jeon et al., 2010).

1.3.2 Social Modulation of Pain

While social observational fear learning has been at the forefront of empathy-related rodent research, the direct transfer of emotional information encoded by painful stimuli is a much more salient approach to examine the neuronal correlates of rodent empathy. This approach does not involve learning and instead examines the pain responses of rodents when they are engaged in various social and observational groupings. Mice tested for pain sensitivity in the presence of a cagemate (also in pain) display significantly higher levels of pain behaviour than mice tested in isolation or stranger pairs (Langford et al., 2006; Martin et al., 2015). In fact, pain behaviours are not only amplified but they can be modulated such that licking in the formalin assay is reduced when mice observe another mouse in pain, but not as much pain (Langford et al., 2006). In a subsequent study, it was demonstrated that the observed difference in pain modulation seen between familiar and unfamiliar mice was mediated through a testosterone-based mechanism.
that initiated a form of social stress-induced analgesia (Langford et al., 2011). There was no change in pain sensitivity when male mice were paired with female conspecifics or castrated male conspecifics and it was concluded that the severity of social threat modulates pain sensitivity such that hyperalgesia is produced in mild social conditions while analgesia emerges with more severe stress.

Similarly, a study has shown that pain responses demonstrated by one rat can modulate the pain threshold in a naïve observer rat. In this study, a rat was exposed to a conspecific in pain due to a bee venom injection, which evoked bilateral mechanical hypersensitivity and an enhanced flinch reflex in the naïve observer rat (Li et al., 2014). These observations were seen only in familiars, as strangers do not demonstrate the increased pain response.

The modulation of pain behaviour by the presence of a conspecific has been explained, however another interesting approach to looking at empathy-related behaviours can be through the examination of social approach behaviours (Langford et al., 2010). In this paradigm, there was a mouse placed in the middle of an alley apparatus with each end containing a closed compartment holding a mouse either in distress due to acetic acid injection or an uninjected mouse. It was found that females were more likely to approach a cagemate in pain compared to an equally familiar mouse which was not in distress. This effect was not evident in males who did not distinguish between the distressed and non-distressed cagemate or females with stranger mice. This heightened response to social approach in females could be related to a heightened affiliation mechanism in females. The “tend and befriend” model proposed by Taylor et al. describes that under conditions of stress, female mammals have a tendency to care for young and engage in social interactions (2000). The exposure to the distressed mouse can be viewed as a
mild stress for the observer mouse which increased social approach or in other words empathy-like behaviours towards the distressed conspecific.

1.3.3 Stress on Social Modulation of Pain
Stress significantly impacts empathy and it has recently been shown that the pharmacological blockade of the glucocorticoid stress response enabled the expression of pain empathy in mouse and human strangers (Martin et al., 2015). Conversely, 15 minutes of restraint stress was sufficient to block the expression of pain empathy in familiar mice. Thus, an enhanced stress response prevented emotional contagion for pain in mice and people. While we believe that increased stress blocks empathy-related behaviours in mice, others have found no such relationship (Li et al., 2014). Conversely, an increased stress response has been found to underlie distress in social observational learning. The repeated witnessing of a conspecific in pain triggers freezing responses in rats, a behaviour that eventually decreases and is replaced by spontaneous yawning (Carrillo et al., 2015). In this context, yawning is hypothesized to reflect increased distress as a coping strategy. Blockade of the HPA axis blocks yawning in rats that have previously witnessed a cagemate receive repeated footshocks indicating that the observation of a conspecific in distress may evoke the stress response and enhance emotional responsiveness.

1.4 Neural Mechanisms of Empathy in Rodents
There is quite a bit of research done in terms of empathy and the brain in humans especially with the use of functional imaging (Ruckmann et al., 2015; Singer et al., 2004; Völlm et al., 2006). Most of these findings, however, are more large-scaled leaving many important molecular questions unanswered. Rodents express fear by freezing, usually in response to a foot shock used in conditioning procedures (Martin et al., 2015; Martin et al., 2011). However, shock-naïve mice learn to freeze by simply observing a conspecific receive repetitive foot shocks, a mechanism
promoted by social learning (Jeon et al., 2010; Jeon and Shin, 2011). Social and observational fear learning is assumed to underlie the basic processes of empathy because they represent affective communication and the sharing of emotional states. In fact, observer mice have higher fear responses when observing mating partners or siblings in distress compared to stranger mice (Jeon et al., 2010), suggesting that social transfer of fear in rodents is possible and stronger among familiars. The neural mechanism underlying the social transfer of fear remain largely unknown, however, the ACC an area of the brain involved with pain and affect has emerged as a likely source. The lateral pain system and medial pain system are the two primary pain-processing pathways that relay signals via the thalamic nuclei of the central nervous system (Price, 2000). Social fear learning appears to be mediated in part through the ACC, which belongs to the medial pain system as inactivation of the ACC with lidocaine significantly disrupts social fear learning. Furthermore, an ACC-limited deletion of Cav1.2 Ca\(^{2+}\) channels in mice impaired social fear learning and reduced behavioural pain responses demonstrating the functional involvement of the affective pain system and Cav1.2 channels of the ACC in observational social fear (Jeon et al., 2010).

The transmission of shared emotional states is not only limited to fear stimuli, as vicarious pain behaviours elicit hypersensitivity in observer rats. A brief social interaction with a rat in pain (injected with bee venom) evokes bilateral mechanical hypersensitivity and an enhanced bee venom paw flinch reflex in observer rats. In line with the preponderance of other empathy-related studies in rodents, these results are only true for cagemates as noncagemates do not demonstrate a social modulation of pain. Interestingly, it is not only the behavioural responses that are altered by observing a cagemate in pain because cagemate observers also have increased neuronal activity in the dorsal horn of the spinal cord as measured by cFos immunoreactivity. In
addition, the social enhancement of pain responses can be ablated with bilateral lesions of the medial prefrontal cortex but not the amygdala or entorhinal cortex (Li et al., 2014).

1.4.1 Pain Matrix

For one to experience pain it involves nociception that is the internal perception of bodily damage. This capacity to “orient towards events that are taking place in/to one’s own body” rather than exteroceptively is a vital element in distinguishing pain from the other senses such as vision or olfaction. Pain is known to be a rather private subjective experience evident by the variability seen in pain reports by different individuals for the same stimulus and the development of pain scales. The sense of pain is not a mere perception but rather it has a significant affective component to it. This is a crucial distinction that provides a unique opportunity for empathy research by using pain as a proxy. Painful personal experiences are known to be represented in complex neural networks also known as the ‘pain matrix’.

1.4.1.1 Physical pain vs. emotional pain

One often associates feeling pain to direct physical causes such as one’s response to being poked by a needle or hitting one’s knee. However often we find ourselves cringing when watching videos on the internet of other people during accidents or being able to feel the exact pain a loved one would experience when they fall and hit their head even though we just watched them and did not experience this ourselves. We often talk of the worst kind of pain as loss of important social bonds that may lead to ‘hurt feelings’ or ‘broken hearts’ using similar jargon as we use when we refer to physical pain (Macdonald and Leary, 2005). The feeling of pain has been reported to not only be restricted to physical sensation but to also occur as a result of observing another’s affective state (Jackson et al., 2005). There is increasing neural evidence supporting the idea that social relationships can sometimes be “painful” (Eisenberger and Lieberman, 2004;
Somatic symptoms are often observed in people suffering from the social pain of grief or bereavement (Zisook et al., 1982). An fMRI study aimed to cause “social pain” by exclusion and found that the neural circuit involved in pain processing was activated when participants were socially excluded from an online computer game (Eisenberger et al., 2003). The ACC showed increased activity with exclusion conditions and this positively correlated with self-reported distress. The authors argued that social pain is analogous in ways with the neurocognitive function to physical pain. There are now parallels being found in neuroanatomical and neurochemical processing of physical pain and the “pain” of social rejection suggesting that underlying neural mechanisms of negative social states may have been incorporated from pain circuitry. Nonhuman animal research has shed light on the common physiological mechanisms that social and physical pain share (Macdonald and Leary, 2005). The ACC is a crucial site for processing physical pain signals but specifically pain affects not pain intensity (Rainville et al., 1997; Singer et al., 2004). This activation of the ACC in relation to the affective component of pain provides support that both social and physical pain have similar neurological underpinnings.

1.4.1.2 Sensory and Affective Pain pathways can be differentiated at a neuronal level

Pain perception being not just a sensation but having affective qualities attached to it is directly supported by findings from neural work. The sensory-discriminative dimension of pain processing encodes the sensory components of a pain stimulus (eg. bodily location and intensity of stimulus) while the motivational-affective dimension contains the subjective response and preparation with respect to the pain event (Craig, 2003; Devinsky et al., 1995; Rainville et al., 1999a; Rainville et al., 1997; Rainville et al., 1999b; Sowards and Sowards, 2002). It is fascinating that neuroscientists have found translational evidence in the brain specifically in the
pain matrix of differentiation at the neuronal level of such sensory and affective pain pathways. Thus it follows that different components of pain such as the affective and sensory components are encoded in different parts of the pain matrix (Hutchison et al., 1999). However, the sensory and affective components are not necessarily separate or independent (Fernandez and Turk, 1992). It is now well known that pain does in fact have both perceptual-discriminative and affective-motivational components with neuroscientists suggesting that these two components of pain are sustained by different neural structures (Auvray et al., 2010). Morrison et al. had found differences in the response patterns of sensory and motivational aspects between the primary somatosensory cortex (SI) and ACC indicative of the distinctive roles that the sensory-discriminative and affective-motivational dimensions of pain processing play. The perceptual-discriminative aspects of pain is believed to occur in the somatosensory thalamus, primary somatosensory cortex (S1) and secondary somatosensory cortex (S2) while the neural activity underlying the affective-motivational aspects are located in the medial thalamus, amygdala and limbic cortex (Jones et al., 1992; Kulkarni et al., 2005). This illustrates how the sensory and affective component of pain translates in neural findings as these aspects of pain can be differentiated at a neuronal level.

1.4.1.3 Similar activation in the brain when we feel pain ourselves or observe others

Although pain happens to be a rather private subjective experience, the capacity to communicate pain to others and to experience the pain of others by observation is fundamental in social interactions and relationships. While there is significant literature on self-pain perception, the perception of others’ pain is quite limited nevertheless this research has many important psychological implications and thus is in need of further investigation. Hutchison et al. conducted a one single-cell recording study in precingulotomy patients finding that the pain-
related neurons in the ACC can discharge both during the actual sensation of pain as well as
during the observation of the same stimuli applied to another person (1999). Morrison et al.
reported similar findings demonstrating that both feeling a painful pinprick and observing
another person go through that same pain event was associated to similar activity in the right
dorsal ACC (2004). Both experiencing pain oneself and just watching signals of possible
administration of pain to a partner produced changes in the hemodynamic response in the
anterior insula (AI), ACC, brain stem and cerebellum of participants (Singer et al., 2004). These
studies support an important role of the ACC in having a role of pain perception in others due to
its activation upon the observation of others in pain.

While most pain-empathy studies stimulate some sort of pain and then measure resulting
empathy, one study did the opposite by using empathy as the independent variable and looking at
resulting pain sensitivity. Loggia, Mogil and Bushnell conducted a study in which they test
whether empathy-evoked activation in the pain network could lead to heightened pain
perception(2008). They hypothesis was based on a study conducted in laboratory mice finding
that these mice have heightened pain behavior when exposed to cagemates (Langford et al.,
2006). The participants in the present study were induced to either have a high state of empathy
towards an actor or a low state and then the pain sensitivity to heat stimuli of various intensity
was measured in the participants. Participants of the high empathy group rated painful stimuli as
more intense and unpleasant than other groups. The positive correlation between empathy scores
and pain ratings strengthen the fact that the magnitude of the empathic response induced matters
in pain perception. The researchers concluded that it was empathy itself rather than the
observation of pain behaviours as the mentioned effects were observed when the participants
watched the model receiving either a neutral or painful stimuli. They state that the cortical
activations seen in the study may not reflect specific neural correlates of empathy for pain but these activations relate to general feelings of compassion towards another in distress suggesting that the mere exposure to others’ emotional distress would lead to the sensitization of areas that are involved in the processing of noxious stimuli. This falls in line with the Perception Action Model proposed by Preston and de Waal stating that that empathy for an emotional state is mediated by the activation in the observer’s brain of the representations of the same state’s observed in another individual (Preston and de Waal, 2002).

1.4.1.4 Empathy for pain involves affective not sensory dimension of pain
The two dimensions of sensory-discriminative and affective-motivational can be manipulated independently. A study had found that only the affective component of pain changed as the perceived intensity given was kept constant when participants were put under hypnotic suggestion (Rainville et al., 1997). This study had found that the modulation of pain-related activity in the ACC had significant involvement in the affective component of pain which supports the common pain and emotion role the ACC is known to hold. Rainville and colleagues proposed that pain-related activation found in the ACC reflects a nociceptive input from a highly modifiable pain pathway and that the amount of pain-evoked ACC activation seen in an individual depends on that individual’s emotional and behavioural reaction to pain. This is a clear depiction of activation of only the areas associated with the affective-motivational aspect of the pain matrix. Also showing that a variation in activation exists clearly demonstrates that neural pathways can distinguish the two dimensions of pain.

Singer et al. had found that empathy for pain involves the affective but not the sensory components of pain. With the use of functional imaging to assess brain activity, volunteers were given a painful stimulus after which their experience was compared to results of when they
observed a signal indicating that a loved one had experienced the same painful stimulus. It was found that the neural substrate for the empathic experience did not involve the entire ‘pain matrix’ and that specifically it involved just the part associated with affective qualities were found to mediate empathy. Further, Singer et al. had found that people who scored higher in empathy show higher pain-related brain activity. Segregation of sensory-discriminative and autonomic-affective attributes of the pain experience is demonstrated by this study however these regions are rather interactive in terms of encoding different aspects of pain (Fernandez and Turk, 1992; Singer et al., 2004). The rostral ACC and AI seem to reflect the emotional experiences that induce our reactions to pain and may contain the neural basis for our ability to understand the pain of both ourselves and others. Overall this data suggests that although the whole pain matrix is not activated during the empathizing with another in pain, it is based on “activation of those second-order re-representations containing the subjective affective dimensions of pain”. With this study it is important to realize that the participant just observed a signal indicating that their loved one would receive a painful electric shock and not the loved one’s actual reaction to the pain stimulus. However this was still sufficient to cause empathic activations. This clearly exhibits the neural underpinnings in our ability to understand the emotional importance of a stimulus for another individual and thus demonstrates our ability to predict its likely consequences. The affective pain-related areas such as the dorsal ACC and AI of the pain matrix was engaged in a situation when there was imminent and ongoing threat of pain to both oneself and a loved one. Using pain as a stimulus in empathy paradigms gives researchers a unique advantage as pain allows for the ability to form subjective representations of feelings that can then help predict actions and responses.
Jackson et al (2005) conducted an fMRI study in which participants were shown a series of photographs depicting either hands or feet in situations that are likely to cause pain with matched neutral controls without pain events. The participants then rated the level of pain that they believed was experienced by those in the photographs. Change in cerebral activity was specifically predicted in neural regions of the affective component of pain such as the ACC and AI (Rainville et al., 1997) which was reinforced by findings of pain perception in self and others showing enhanced activation of ACC (Morrison et al., 2004) and ACC and bilateral AI (Singer et al., 2004). Jackson and colleagues found that perceiving and assessing painful situations in others was correlated to significant bilateral changes in activity of important pain processing regions such as the ACC, AI, cerebellum as well as to a lesser degree, the thalamus. The activity of ACC was strongly correlated to participants ratings of others pain implying that the ACC is modulated according to subjects reactivity to the pain of others or in other words their levels of empathy. They had also found a partial cerebral commonality between perceiving pain in another individual and experiencing it oneself. The perception of the pain of another was considered as a social stimulus that triggers a specific affective state in the perceiver from which empathy may be underlying. These results clearly demonstrate that the observation of another in distress during pain events may trigger a specific part of the pain matrix known to be involved in self-pain processing as well. The findings of the ACC and AI during perception and assessment of someone else’s pain is consistent with previous neuroimaging studies that have demonstrated the affective aspect of pain processing’s role. Also the strong correlation between the ratings and level of activity within the posterior ACC supports the crucial role of this region in the interactive and evaluative function associated with pain events. Many other neuroimaging studies further support that it is the affective components of pain but not the sensory discriminative that
are crucial to empathy for others pain (Auvray et al., 2010; Bufalari et al., 2007; Morrison et al., 2004; Singer et al., 2004).

1.4.1.5 Motor link to affective pain: Another aspect of the pain matrix indicating motivation and action

Neuroscientists have shown that a given emotional state of an individual can activate corresponding representations in another individual observing that state (Decety and Jackson, 2004; Gallese, 2003; Preston and de Waal, 2002). Just as motor responses to one’s experience of pain allow for survival of oneself through motor responses such as either freezing or escape reactions, it has been found that even when just watching another individual experience a painful event, similar motor responses are activated (Avenanti et al., 2005). Studies have found that the motor cortex which is crucial for planning actions and the ACC (involved in motivation and motor planning) are both activated during the experience of pain (Farina et al., 2003; Ingvar, 1999). In the Avenanti et al. study, transcranial magnetic stimulation was used to record changes in corticospinal motor representations of hand muscles of individuals observing needles penetrating hands or feet of human models or objects (ie. tomato). Avenanti and colleagues had found a reduction in amplitude of motor-evoked potentials that was specific to the muscle that subjects observed being pricked and this inhibition correlated with the participant’s subjective rating of the sensory qualities of pain attributed to the human model and with sensory but not affective state or empathy trait measures. Avenanti et al. suggest that this indicates that empathy for pain may rely not only on affective- motivational but also on somatomotor representations. They suggest a link between visual representation of others painful experience and somatomotor representations of feeling that experience oneself. Thus it is almost as if the sensory quality of others’ pain resulted in the making of an empathic inference and resulting automatic embodiment in the motor system which may be vital for social learning of reactions of others pain. Avenanti
et al. state that experience of pain is related to action and that motor systems may also be involved in empathy for pain. It makes sense that such an experience would cause an individual to want to either escape before experiencing the same to itself or to help the other in distress. Other neural studies have found that pain systems are tightly linked to action systems that may be part of the pain matrix (Farina et al., 2003; Ingvar, 1999; Juottonen et al., 2002; Saitoh et al., 1999; Wager et al., 2004). This study illustrates that social dimensions of pain extend even to the basic sensorimotor levels of neural processing. Avenanti and colleagues essentially established a link between pain and action systems at a social level by finding possible motor correlates of watching and empathizing with others in pain demonstrating that the motor system is an important aspect in the pain matrix. By using rodent models to study empathy and socially modulated behavioural responses we are able gain a better understanding of the neural mechanisms that govern higher order and more socially complex behaviours.

1.5 Autism, Genes and Empathy

Autism Spectrum Disorders (ASDs) are characterized by impairments in sociability and social interactions. Increased reactivity of the stress response system to social stress and novel stimuli has been shown to be greater in children with autism compared to control groups (Spratt et al., 2012). Another study reported that children with ASDs had significantly elevated cortisol reactivity to a social interaction with a stranger compared to a familiar peer (Lopata et al., 2008). Children with autism who participated in a social playground paradigm had elevated cortisol activity compared to a non-autistic control group (Corbett et al., 2010). The mentioned studies demonstrate the increased stress response associated to social interactions that people with ASDs have. Studying the genetic determinants of empathy and prosocial behaviour increases
understanding deficiencies of sociality including autism spectrum disorder—of which a lack of empathy is a defining feature—and such future findings will have great impact on the mechanisms underlying psychosocial susceptibilities.

Arguably, the most fundamental of all biological factors that regulate behaviour is our genes, yet there have only been a handful of genes linked to empathy-related behaviours. The only research into the genetic determinants of empathy and prosocial behaviours has been performed using "candidate gene" association studies in humans. Published associations for only six different genes—AVPR1A, COMT, DRD4, MAOA, NOS1, and OXTR—that are relevant for trait empathy, prosocial temperament, altruism, partner bonding and cooperation have been identified (Avinun et al., 2011; Bachner-Melman et al., 2005; Israel et al., 2009; Mertins et al., 2011; Retz et al., 2010; Reuter et al., 2011; Rodrigues et al., 2009; Tost et al., 2010; Walum et al., 2008; Wu et al., 2012). However in mice, empathy-related behaviours are modulated by genetics and genetic background but the specific genes have not been identified. In particular, the BALB/cJ (BALB) mouse strain displays less social motivation and reduced social fear learning when compared with the more sociable C57BL6/J (B6) mouse strain (Chen et al., 2009). Further, since empathy-related genetic targets have yet to be identified in abundance, attention should be given to genes that have been identified in animal models of social deficiency. In this regard, a number of immune genes have been identified that correlate in a seemingly direct manner with sociability (Ma et al., 2015).

An autism model study tested the less social strain BTBR T +Itpr3tf/J (BTBR) and the more social c57BL/6J (B6) mice strain in the Transfer of Emotional Information test and Social Proximity test finding that the BTBR mice displayed asocial behaviour in both tests (Meyza et al., 2015). Specifically in the Transfer of Emotional Information test, it was also found that there
was no increase in c-Fos expression of the amygdala or prefrontal cortex in BTBR mice compared to B6 mice that did show an increase. The Social Proximity test however showed a significant increase in c-Fos expression in the CA3 field of the hippocampus and two hypothalamic regions of BTBR brains. There was also a strong activation of the periaqueductal region suggesting that the BTBR mice found unavoidable social interaction aversive.

Mouse models have been developed to mimic genetics of ASDs and other related disorders. It is important to note that autism cannot be explained by one single gene and that only about 5-15% can be accounted for by a genetic defect of which does not even always result in an ASD diagnosis (Caglayan, 2010; Martin and Ledbetter, 2007). Children with Fragile X Syndrome (FXS) tend to show mild autism features specifically in social behaviour, eye contact along with additional anxiety-like behaviours(Moss and Howlin, 2009). Impaired social interaction, impaired social communication and presence of preservative/repetitive behaviours are three core features of autism of which at least one must be present to be a good model of autism (Spencer et al., 2011). Upon testing the Fmr1KO mice on multiple behavioural tests, Spencer and colleagues identified improved models for different behavioural symptoms present in autistic-like traits and that currently, the most optimal model is Fmr1KO Mice on the B6D2 F1 background, as it satisfies all three of the behavioural requirements of autism. Mouse models such as this have led to advances in understanding ASDs and are of great benefit in the future of studying autism molecularly (Schwartzer et al., 2013).

1.6 Use of Pain: Translatability from Rodent to Man
An evolutionary benefit of shared neural representations for self and others is that they can be used to learn, understand and potentially help one another. While positive experiences can have a reinforcing value like a dog seeing another dog getting a treat for sitting so it also sits. A
negative experience observed may cause individuals to avoid certain behaviors or situations that can cause harm without actually going through such experience for example a dog seeing another dog get in trouble with the owner for stealing treats so the first dog does not do the same. One is affected by another person’s state and feeling by the activation of ACC and AI corticies which are important regions of the affective pain system. Jeffrey Mogil says that the parallels between humans and rodents is evident and that translational research is very possible as existing data suggests that rodents are capable of simple forms of the same types of social interactions that affect both acute and chronic pain, that similar social sex differences are found in both species and that the probability of observing such phenomena depends on familiarity between social actors and also that reduction of social stress elicits a form of empathy in strangers (Martin et al., 2015; Mogil, 2012; Mogil et al., 2010). The social neuroscience of pain field has huge potential to one day lead to more successful insights into treatment of chronic pain in humans which is one of the leading causes of hospitalization (Fortner et al., 2002). Mice and other rodents are appropriate subjects for scientific study as they have underlying properties that are preserved throughout mammalian species.

Recent research repeatedly confirms that even rodents are capable of producing simplified versions of various social phenomena including empathy. There are many parallels that are now being discovered in rodents and humans. A recent study has found a direct translation between rodents and humans with effects of similar magnitude using similar sample sizes in both species (Martin et al., 2015). They had demonstrated that emotional contagion is readily translatable from mice to humans opening doors for further use of mouse genetics and known physiology to better understand the underlying mechanisms of the phenomena on which higher forms of empathy are dependent.
With the increasing evidence of empathy in rodents and knowledge of pain pathways, there is
great possibility of getting closer to bridging the gap between empathy behavior and its
underlying neural mechanisms.

1.7 Conclusions and the Power of Rodent Research

This literature review provides strong evidence that rodents show empathy-related behaviours
and have empathic capacities that were previously thought to be impossible. There is increasing
evidence of the social modulation of fear and pain in rodents however the detailed underlying
neural mechanisms still remain unclear. Further research in terms of the molecular mechanisms
underpinning empathy as well as its associated genes can help towards understanding Autism
Spectrum Disorders and its neural correlates in a way that was never thought possible. This will
shift social neuroscience from what it is today to reach its full potential in helping bridge the gap
between behaviour and biology, which without rodent models would prove difficult or even
impossible.
2 Introduction

The ability to form and maintain relationships is undoubtedly essential to survival. Empathy behaviour, which most likely evolved to allow species to care for their young, has expanded from its root to be present across species. Empathy is a vital psychological construct, which involves the ability to recognize and share emotions with others. The term empathy has been defined in various ways with a large majority believing that this behavioural construct is exclusively higher-order and involves complex cognitive capacities. However, empathy relies on a perception–action mechanism that includes lower-order behaviours such as mimicry and emotional contagion (Preston and de Waal, 2002). The empathy literature consists of a great amount of human research and growing rodent research. Rodent models are vital in order to truly understand the neurobiological process of empathy-related behaviours. These findings can help bridge the gap between social behaviour of empathy and its underlying neural mechanisms. To study empathy, pain is often used as a stimulus because of its simplicity in measurement and its universal understanding. The current study used an empathy-pain paradigm to not only demonstrate that empathy is evident in lower mammals such as mice but also to further our understanding of the neuronal regulation of empathy behaviour in these rodents. This was achieved by exploring stress-related circuitry that regulates empathy-like behaviours in rodents with a specific focus on glucocorticoid receptors (GRs). The medial prefrontal cortex (mPFC) and ACC have a variety of important functions one of which is empathy related and thus were explored along with the paraventricular nucleus (PVN) and bed nucleus of the stria terminalis (BNST) which are important in stress regulation for this experiment.
2.1 Statement of the Problem

Empathy, which has often been attributed to higher-order species and believed to only be possible in humans and primates that have the capacity of complex cognition, is recently gaining increasing evidence to include lower-order animals such as mice and other rodents. Although there is extensive literature on human empathy and characteristics, the existing literature on the neural underpinnings of empathic behaviours has been quite limited. Rodent empathy research allows for a unique opportunity into bridging the gap between brain and behaviour through the manipulation of rodent models. Stress has recently been found to impair emotional contagion in familiar mice and stress reduction to promote empathy in stranger (STR) mice (Martin et al., 2015). However, the stress-related molecular mechanisms that blocks empathy-like behaviours in rodents remain unknown.

2.2 Purpose of the study

The purpose of this study was to explore whether GR activity in the various mentioned regions (mPFC, BNST, ACC and PVN) differs based on vicarious pain behaviours between different social conditions. The current study explored whether activation of GRs in empathy-or stress-related brain areas prevent empathy-like behaviours in mice through the use of behavioural protocols and western blot analysis. This is rarely done in social neuroscience. This study was conducted to increase the present understanding of the stress-related circuitry that regulates empathy-like behaviours in rodents with a specific focus on glucocorticoid receptors. Any insight into neural underpinnings of stress and the complex social behavior of empathy can help increase our molecular understanding of affective pain pathways and their relation to stress. Also neural findings such as this can really set the stage for work in Autism Spectrum Disorders (ASDs) which are characterized by a lack of empathy.
2.3 Research Question and Hypotheses

This thesis aims to answer the following three research questions.

1. **Social Condition Experiment: Can previous findings of pain responses being influenced by social condition (ie. experiencing pain with familiar, stranger or in isolated condition) be replicated?**

   Previous research demonstrated that manipulating social condition modulated pain behaviour (Langford et al., 2006). In their experiment, familiar mice showed greater emotional contagion (measured by greater contagious pain hypersensitivity) compared to stranger mice. Thus, as similar experimental methods are used in the current study; a similar effect of cagemates showing elevated pain responses compared to strangers was expected.

2. **Social Condition Experiment: Does glucocorticoid activity in empathy- or high-stress related regions differ between different social conditions?**

   Since the lack of emotional contagion in stranger mice had been correlated to increased social stress, it was expected that there would be higher GR activity in the regions such as mPFC, ACC, BNST or PVN for STR mice. A significant difference in the glucocorticoid activity between the different social conditions was expected. Specifically, the increased stress in the STR condition was predicted to result in greater glucocorticoid receptor phosphorylation detected in STR mice. If proves true, this would provide evidence for the molecular mechanisms by which stress prevents empathy behaviour in rodents and provide basis for exploring the modulation of empathy at a molecular level.
3. **Metyrapone Condition Experiment: Will blocking the endocrine stress response result in increased empathic-like responses in unfamiliar mice?**

Previous research demonstrated that stress played a role in the resulting pain exhibited by different social conditions (Martin et al., 2015). In their experiment stress was found to impair emotional contagion in familiar mice while stress reduction enabled emotional contagion in the stranger mice condition. Similar results were expected for this experiment.

### 2.4 Theoretical Framework

The development of the current project was based on findings throughout decades of pain and empathy research. The knowledge that empathic behaviours have been demonstrated in rodents through the social modulation of pain in mice by familiar observers (Langford et al., 2006) and that social stress could block this empathy (Martin et al., 2015) has led to the development of the current research questions. However, the molecular explanations behind these effects are unknown at this time. This is essentially what the present study aimed to begin to uncover. Based on previous work, it was hypothesized that glucocorticoid activity in empathy and stress related regions would be increased in stranger mice as a result of social stress (based on social observational pairings) and that this is what reduces empathic responding (measured through pain responses specifically writhing behaviour).
3 Materials and Methods

3.1 Subjects

The subjects for the social condition experiments were naïve male CD-1 ® mice (at least 6-10 weeks). All mice were housed with the same-sex in groups of 4 mice per cage. Each plastic cage consisted of a grate top containing wood chips as bedding and one plastic dome. This was maintained in a temperature-controlled (20 ± 1° C) environment with 14-/10-h light/dark cycle with access to food (Harlan Teklad 8604) and water ad libitum. Experiments were conducted only during the light period. Before testing, all subjects were acclimated to the laboratory for at least one week.

3.2 Habituation and Setup

Prior to the actual testing, all mice were taken from the animal holding room and given one hour to habituate to the testing room in their respective cages. At the start of the experiment, mice were weighed and separately marked based on conditions. Mice were tested either in isolation or dyads. Dyads consisted of either stranger pairs (mice taken from two different cages) or cagemate pairs (both mice taken from same cage). Mice were also given 30 minutes to habituate in their respective cylinders prior to acetic acid injections. The experimental apparatus was set up as displayed in Figure 3.1.
Figure 3.1. Empathy Paradigm Setup. The behavioural experiments were set up as shown in the figure above. There were 8 transparent Plexiglas cylinders (15 cm diameter, 22.5 cm high) set up on a clear glass shelf mounted on the wall. Under each Plexiglas Cylinder there were cameras set up in order to record the behaviour.

3.3 Protocol

3.3.1 Social Condition Behavioural Experiment

The experimental timeline consists of a half an hour habituation period starting when the mice are put in their respective cylinders followed by a 0.9% acetic acid injection to each mouse (10 ml/kg, intraperitoneal). Behaviour was recorded for an additional 30 minutes post-injection. Recording began at the start of the 30 minute habituation period and was stopped after the 30 minute post-injection period. The experimental timeline for the Social Condition Behavioural Paradigm is shown in Figure 3.2. The order of the social conditions assigned to each Plexiglas Cylinder were randomly distributed for every experimental run.
Figure 3.2. Social Condition Behavioural Experiment Timeline. The experimental timeline for the Social Condition Behavioural Experiment is shown in the figure above. The mice are given 60 minutes to habituate to testing room after which cameras are turned on and mice are placed in their respective cylinders and given 30 minutes to habituate to the new cylinder environment. After the cylinder habituation of 30 minutes, video is recorded for another 30 minutes of actual pain response after which brains are immediately removed.

3.3.2 Metyrapone Behavioural Experiment

Metyrapone is a glucocorticoid synthesis inhibitor that was dissolved in saline at a dose of (50mg/kg). The Metyrapone Behavioural experiment was conducted with the use of the same paradigm as the Social Condition Behavioural Experiment. The only difference was that injections of either saline or Metyrapone (2-methyl-1,2-di-pyridyl-1-propanone) were intraperitoneally administered at a volume of 10ml/kg prior to the initial 30 minute cylinder habituation. Similar to the Social Condition Behavioural Experiment, acetic acid injections were given after the 30 minute habituation. The experimental timeline for the Metyrapone Behavioural Experiment is shown in Figure 3.3. This experiment had four conditions consisting of an isolated mouse pretreated with Metyrapone (ISO-MET), isolated mouse pretreated with saline (ISO-SAL), stranger dyad pretreated with Metyrapone (STR-MET) and stranger dyad pretreated with saline (STR-SAL).
3.3.3 Microdissection

After the behaviour portion of the experiment, the brain of each mouse was removed immediately following decapitation. Using a microscope and a mouse brain matrix, the brain was microdissected for the removal and isolation of the mPFC, ACC, BNST, and PVN. The microdissected tissue was then stored at -80°C until protein extraction.

3.3.4 Western Blot

The respective tissues were lysed and prepared for protein determination followed by separation on SDS-polyacrylamide gel transferred to a nitrocellulose membrane with the use of the Trans-Blot Turbo Machine (Bio-Rad). The membrane was blocked and incubated with Phospho-Glucocorticoid Receptor (Ser211) Antibody (Cell Signaling Technology #4161) for GR-phosphorylated overnight at 4°C. The secondary antibody, Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology, #7074), was used at room temperature followed by chemiluminescence detection (ImageQuant LAS 500, GE Healthcare Life Sciences). The
membranes were then stripped and re-probed for Glucocorticoid Receptor (D6H2L) XP ® Rabbit mAb (Cell Signaling Technology #12041) to detect GR total and β-Actin (D6A8) Rabbit mAb (HRP Conjugate) (Cell Signaling Technology #12620) as a loading control in order to determine whether there was a true difference in GR activity between conditions.

3.4 Data Collection and Analysis Procedures

3.4.1 Behaviour Scoring

The total number of lengthwise constrictions of the abdominal musculature was counted in the 30 minute experimental period using VLC media player. Within the 30 minute video recording, specifically the first 5 seconds of every 20 seconds was watched and counted for writhing. The behavioural coders were tested for inter-rater reliability and coded the videos while being blind to the conditions. The time period of 30 minutes was chosen as writhes after this point tend to occur at low levels eventually becoming completely absent. The average percentage of writhing of all mice in each condition was pooled in order to look for significant differences.

3.4.2 Western Blot Analysis

Membranes from chemiluminescence detection were transferred as tiff files from Image Quant Las 500 apparatus to a computer where the protein bands were quantified using the densitometric analysis program ImageJ from NIH. Protein expression levels were determined by western blot analyses whereby expression of phosphorylated glucocorticoid receptor antibody (P-gluc) was normalized to the loading control Beta actin and total glucocorticoid antibody (T-gluc). After the bands were quantified using ImageJ, the values for P-gluc and T-gluc intensity were then divided by β -Actin intensity after which the ratio of the two resulting values were calculated to give a final intensity value. This value was then used to make comparisons between the condition
groups of the experiments using one-way ANOVA. The equations used to calculate the intensity values are shown in Figure 3.4.

\[
\text{Ratio 1: } \frac{P - gluc}{B - actin}
\]

\[
\text{Ratio 2: } \frac{T - gluc}{B - actin}
\]

Final Ratio of Intensity Value: \( \frac{ratio1}{ratio2} \)

Figure 3.4 Western Quantification Calculations. A ratio was calculated for the P-gluc and T-gluc to the loading control β-Actin. These two ratios were used to calculate the final ratio which was then used to calculate statistical difference between social conditions.

3.4.3 Statistics

The statistical analyses were conducted using SPSS statistical program and GraphPad Prism 6. One-way ANOVA with three comparison groups (representing the three social conditions) followed by post-hoc Tukey’s test was used for behavioural analyses to determine differences between groups. p <0.05 was the criterion for statistically significance. A simple one-way ANOVA followed by post-hoc Tukey’s multiple comparison test was also used with the three comparison groups to find differences in each of the brain regions of glucocorticoid phosphorylation for molecular data. One-way ANOVA was used for the Metyrapone Behaviour experiments with four comparison groups.
3.4.4 Animal Ethics

All experimental procedures adhered to federal and institutional guidelines and were approved by the University of Toronto Mississauga Animal Care Committee.

4 Results

4.1 Contagious Pain Hypersensitivity in Cagemates but not Strangers

Consistent with the Martin et al. study, empathic-behaviour or in this particular case pain responses were higher between familiars. There was a significantly greater percentage of writhes found in the cagemate (CM) condition (46.00 ± 2.9) when compared to both the STR (35.48±2.86) and ISO (31.29±3.16) conditions. There was a significant effect of condition on writhing response at the p<0.05 level for the three social conditions [F(2,72)=6.65, p=0.0022]. Post hoc comparisons using the Tukey HSD test indicated that the average writhing percentage for the CM condition (46.00± 2.9) was significantly different when compared to both the STR (35.48, SD±2.86) and ISO (31.29±3.16) condition. However, the STR and ISO condition did not significantly differ from one another.
Figure 4.1 Contagious pain hypersensitivity in cagemates but not strangers. The results found significant difference between CM group compared to ISO and STR but no difference between the ISO and STR.

4.2 Phosphorylation Differences of the Glucocorticoid Receptor

GR phosphorylation at Ser(211) is known to be a biomarker for GR activation in vivo (Rogatsky et al., 1998). GR activity was looked at by quantifying bands from the western blots run on tissue of various regions in the mice. The P-gluc intensities were normalized to T-gluc and β-actin after which the mean, SD, and SEM were calculated for each condition based on the brain region. One-way ANOVA tests followed by post-hoc Tukey’s multiple comparisons test was run on the data. The results for each of the brain regions analyzed are presented in the following subsections.
4.2.1 Increased Phosphorylation of Glucocorticoid Receptors in the mPFC and ACC (Empathy-Related Brain Regions) between Social Conditions

There was a significant increase in the phosphorylation of the glucocorticoid receptor levels in the medial prefrontal cortex of stranger mice (67.77±9.98) compared with the isolated condition (32.79±5.75). However the cagemate condition (41.71±9.77) did not significantly differ from the STR and ISO conditions. The mPFC showed a significant effect of condition on glucocorticoid phosphorylation level at the p<0.05 level for the STR and ISO condition [F(2,14)=4.017, p=0.0418]. Post hoc comparisons using the Tukey HSD test indicated that the average score for the STR condition in phosphorylation level in the PFC(67.77±9.98) was significantly different when compared to the ISO (32.79 ±5.75) condition (Figure 4.2). Taken together, these results suggest that there was increased glucocorticoid activity seen in unfamiliar mice when compared to when tested alone. However this difference was not seen when tested with a familiar. A representative western blot is shown for the mPFC of all three social conditions in Figure 4.3.
Figure 4.2 mPFC GR Phosphorylation graph. There was a significant increase in the phosphorylation of the GR in the mPFC of STR mice compared to ISO condition.

Figure 4.3 A representative western blot image for the medial prefrontal cortex showing bands for P-GR, t-GR and β-actin.
There was also a significant increase in the phosphorylation of the glucocorticoid receptor in the ACC of stranger mice (103.00±38.00) compared with the isolated (57.11±17.15). However the familiar condition (68.53±20.86) did not significantly differ from either the STR or ISO conditions. The ACC showed a significant effect of condition on glucocorticoid phosphorylation level at the p<0.05 level for the STR and ISO conditions [F(2,15)=4.727, p=0.0256]. Post hoc comparisons using the Tukey HSD test indicated that the mean score for the STR condition in phosphorylation level in the ACC(103.00±38.00) was significantly different when compared to the ISO (57.11±17.15) condition (Figure 4.4). Taken together, these results are similar to the previous mPFC data in that there was increased glucocorticoid activity seen in unfamiliar mice when compared to when tested alone while this effect was not seen with familiairs.
Figure 4.3 ACC GR Phosphorylation graph. There was a significant increase in the phosphorylation of the GR in the mPFC of STR mice compared to ISO.

4.2.2 No significant difference in Phosphorylation of Glucocorticoid Receptors in the PVN and BNST (Stress Response Regulating Regions) between Social Conditions

There was no significant difference in the phosphorylation of the GR in the PVN and BNST of STR mice compared with familiars and isolated. Specifically in the PVN there was no significant difference \( F(2,15)=1.584, p=0.2375 \). found at the \( p<0.05 \) between STR (63.32 ± 12.52), CM (84.51±7.52) and ISO (84.72±8.54) conditions (Figure 4.5). In the BNST brain region, there was also no significance found \( F(2,15)=1.584, p=0.2375 \). found at the \( p<0.05 \) between STR (21.50 ± 7.40), CM (47.84±12.80) and ISO (47.13±12.60) conditions (Figure 4.6).
Figure 4.5 PVN GR Phosphorylation Graph. There was no significant difference in the phosphorylation of the GR in the PVN of the three social conditions.
Figure 4.6 BNST GR Phosphorylation Graph. There was no significant difference in the phosphorylation of the GR in the BNST of the three social conditions.

4.3 Pharmacological Blocking of the Endocrine System Response on Contagious Pain Hypersensitivity in Strangers

Mice were pretreated with the glucocorticoid synthesis inhibitor Metyrapone then run through the same empathy paradigm. The behaviour was then coded for the 30 minute block after the acetic acid injection. The behavioural analyses of the four conditions were run through a one-way ANOVA.
All conditions were compared to one another and one statistical significance was found in writhing response between the STR-MET (55.95±1.60) compared with the ISO-MET(40.06±5.09). However, all other condition comparisons were found to be not significant at the p<0.05 level. Specifically, there was no statistical significance found between STR-MET (55.95±1.60) compared with the STR-SAL(49.33±2.99). There was no statistical difference between ISO-SAL (46.00±0.94) and ISO-MET (40.06±5.09) groups. Also no statistical significance found between ISO-SAL (46.00±0.94) and STR-SAL (49.33±2.99) either. Figure 4.7 shows these results in a graph. Taken together, the only significant difference found after comparison of all the conditions to each other happened to be STR-MET to ISO-MET.
Figure 4.7 Metyrapone Empathy Behaviour Graph. A significant difference between STR-MET and ISO-MET was found for this experiment. No other significant differences were found between conditions for this experiment.
5 Discussion and Future Directions

5.1 Summary of Results

This study used a behavioural empathy paradigm to provide unprecedented insight into the neuronal regulation of empathy. The presence of a cagemate as opposed to a stranger or being tested alone while in pain resulted in higher pain responses measured, consistent with previous findings using the same paradigm (Langford et al., 2006; Langford et al., 2011; Martin et al., 2015). This study went a step further in to looking at the neuronal implications behind the behaviour that manifests from the paradigm. The brains of the mice subjects were microdissected immediately after the study and the brain areas known to be involved with empathy such as the medial prefrontal cortex and the anterior cingulate cortex as well as important regulators of the stress response including the bed nucleus of the stria terminalis and paraventricular nucleus of the hypothalamus were isolated. Western blot analyses on the microdissected brain regions demonstrated that the glucocorticoid receptor activity was altered in the stranger mice (the mice that did not engage in empathy behaviour by our definitions). Specifically it was found that there was increased phosphorylation of glucocorticoid receptors in the empathy involved regions of the mPFC and ACC of stranger mice compared with familiar mice and isolated controls. However there was no significant difference found in glucocorticoid receptor changes of the PVN or BNST (stress regulating regions).

Using these neuronal findings in terms of the stress response and with the knowledge that stress could be a potential blocker of the empathy behaviour of strangers, an experiment was conducted using Metyrapone, the endocrine stress response inhibitor. This experiment consisted of four conditions where mice were either pretreated with saline or Metyrapone and run in stranger
dyads or tested alone. This experiment found only a statistical significance between ISO-MET and STR-MET conditions.

5.1.1 Contagious Pain Hypersensitivity in Cagemates but not Strangers

Our results demonstrated empathy-like responding was present in rodents as only familiars showed greater pain responses when tested together. Specifically, there was a significantly greater percentage of writhes found in the CM condition when compared to both the STR and ISO conditions. Taken together, these results suggest that the presence of familiars resulted in increased pain responses exhibited. Specifically, the results suggest that it is the actual presence of a familiar that causes this increase in empathy response as being tested alone still resulted in the same pain response as being tested with a stranger. These results are important in that they show a replication of findings in the literature, which strengthens the use of this specific empathy paradigm for the future.

5.1.2 Phosphorylation Differences of the Glucocorticoid Receptor

Cortisol exerts its biological activity through glucocorticoid receptors. GR phosphorylation at Ser(211) is reported to be a biomarker for GR activation in vivo (Rogatsky et al., 1998). The results from this experiment showed a difference in phosphorylation for the different social conditions in specific brain regions. Both stress regulating regions and regions known for being involved in empathy were explored. Interestingly, it was only the empathy involved regions of the mPFC and ACC that showed a significant difference in terms of glucocorticoid activity. It was originally expected that all 4 brain regions that were explored would show greater
glucocorticoid activity for stranger mice however it was only the mPFC and ACC that did. The stress regulating regions of the PVN and BNST were found to be insignificant but it seemed almost as if the opposite effect of what was found in the mPFC and ACC was what the data was trending towards. If this were to be the case, an explanation could be perhaps some sort of negative feedback within these two sets of regions such that the increased glucocorticoid activity in the empathy involved regions as a result of the social stress is counteracted with the stress regulating regions lowering or downregulating their activity. This is only a speculation and further data and research would be needed to support this.

5.1.3 Pharmacological Blocking of the Endocrine System Response on Contagious Pain Hypersensitivity in Strangers

Previous studies showed that stress levels in stranger dyads are higher than in cagemate or isolated mice. The current study found only one statistical significance in empathy behaviour when stress response was blocked by Metyrapone and this was between STR-MET and ISO-MET. It is interesting to find such an effect as this was not present between STR and ISO mice in the Social Condition experiment or in the STR-SAL and ISO-SAL conditions of the Metyrapone Behaviour experiments. The only difference between the STR-MET and ISO- MET comparison with respect to the other two comparisons mentioned is the injection of Metyrapone. The Social Condition experiments involved no pre-treatment injection while the STR-SAL and ISO-SAL involved saline injections. There was no statistical significance found between the ISO-SAL and ISO-MET which was expected and shows that Metyrapone did not have any external effect on the pain behaviour of a mouse when tested alone. Also no statistical significance was found between ISO-SAL and STR-SAL which was also expected as saline was just control and there
should be no difference between these two conditions as this is what was indicated by the Social Condition Behaviour experiments.

It was expected that because stress was most likely the factor that prevented empathy in the STR mice that the simple blocking of the stress response would result in empathy behaviours similar to cagemates. However, there was no statistical significance found between STR-MET and STR-SAL. There are a number of reasons as to why the results yielded no effect in this case. One possible reason was that because the STR-SAL were always run after the STR-MET, the STR-SAL had a bit more time to habituate to the room and environment which may have reduced stress resulting in greater pain responses measured. Another potential factor that could have influenced the outcome was that the control saline that was used was actually found to be acidic in nature at a pH of 5.5 when measured. Also the fact that the first pretreatment injections were given intraperitoneally and the acetic acid injections were also given by the same route may have caused irritation. Future studies should randomize the order of the conditions that are run, use physiological pH saline and the pretreatment injections should be given in another route such as subcutaneously. With the proposed changes results may be different and I have already found different results from one preliminary experiment after making the following changes. However, a greater number of experiments will need to be conducted before any conclusions are reached.

### 5.2 Limitations and Future Directions

One limitation to this study is that it was not tested whether stress or specifically corticosterone levels were higher in STR mice. In the future blood should be collected and assayed via enzyme-linked immunosorbent assay (ELISA) to determine whether blood corticosterone levels correlate with expected empathy-like behaviours. A limitation to this study is that the found effects alone
do not establish a direct neural link between stress and social behaviour. Many manipulations of these social experiments will be needed in order to establish any concrete conclusion about the neural correlates of the complex behaviour of empathy. There are various experiments that can be done as a follow up to the current study.

Various stress condition alteration experiments can be done to holistically measure the effects of stress on empathy behaviour. The present experiment looked at blocking the endocrine stress response of STR mice however another variation similar to Martin et al. is introducing stress to the CM condition and then measuring glucocorticoid activity in different brain regions. Also perhaps corticosterone levels itself could be increased in mice and then the resulting behaviour and expression could be measured. Also to explore whether increased GR activity in the mPFC and ACC block empathy, viral genetic techniques can be used in order to determine whether an upregulation of GRs in the mPFC and ACC precludes empathy. Mice can be microinjected with a virus to specifically knockdown GRs in the mPFC and ACC, then be examined for resulting behaviours and changes in GR expression using both molecular and immunohistochemical techniques. Together, these experiments will allow us to start to understand the extent to which the stress response influences the sharing of emotional states.

In the current experiment, the stress regulating regions and high empathy involved regions were chosen. Other candidate brain regions such as the hippocampus and amygdala can also be explored, as being able to identify a familiar requires social memory and empathy behaviours are likely to require the limbic system. It is interesting that the current results demonstrated almost what looked like an opposing effect between the mPFC and ACC with the PVN and BNST so it would be interesting to see how the limbic system plays a role in this and perhaps other stress
regions as well. This could be an important step in uncovering some possible neural mechanisms behind stress pathways and empathy behaviour.

Oxytocin- the social bonding hormone- that also diametrically opposes stress can be explored in order to determine whether its activity varies in empathy-related brain regions of the different conditions(Burkett et al., 2016; Rodrigues et al., 2009; Taylor et al., 2000). Similar to the way the current study conducted experiments, it would be interesting to conduct similar studies but look at oxytocin receptor (OXTR) expression and how this correlates with rodent empathy expression. It would be even more interesting then to explore whether GRs and OXTRs interact to regulate rodent empathy. Oxytocin may enhance social bonding and empathy by decreasing the stress response, but the neural mechanisms underlying oxytocin’s effect on stress remain unknown. Exploring whether GRs in the mPFC and ACC block empathy by decreasing the activity of oxytocin receptors would be an interesting avenue for future research along this line. Co-immunoprecipitation techniques could be used to determine whether GRs and OXTRs physically interact and whether their levels are negatively correlated. Further, intranasal oxytocin could be administered to mice in order to determine whether this promotes empathy-like behaviours in rodents by decreasing GR expression/activity in the mPFC and ACC. The stress-oxytocin relationship is one that has not greatly been studies and could be of great significance as stress and social bonding could have many implications in social disorders and other social dysfunctioning.

Empathy is increasingly being studied by scientists because of its known role in psychological disorders, such as Autism Spectrum Disorder and psychopathy. There is also limited understanding in the neurobiological differences between mouse strains of varying sociability.
The present study and other studies that will be conducted related to this will help understand the 
roots of ASDs and underlying stress-related mechanisms. In the future, experiments could be 
conducted using the exact paradigm used in the current study with endless variations such as for 
instance exploring the differences between social and less social strains of mice and then looking 
at the differences in glucocorticoid activity. An enhanced cortisol response to social stress has 
been found in children with autism compared to control groups (Apratt et al., 2012). However 
molecular mechanisms underlying these increased stress responses to social contact are yet to be 
found. In rodents, Chen et al. found that BALB/cJ (BALB) mice displayed less social motivation 
and reduced social fear learning compared to the more social C57BL6/J (B6) mouse (2009). 
Meyza et al., (2015) found that BTBR T +lpr3t/J (BTBR) mice displayed antisocial behaviour 
compared to the d c57BL/6J (B6) through the use of two behavioural paradigm tests. It is 
expected that with the use of either the less social strains such as BALB or BTBR, it will result 
in findings of increased glucocorticoid activity similar to that of stranger mice. This could lead to 
interesting insight in to the molecular mechanisms that underlie social deficits and ASDs. Also 
eventually, genes can be identified that may play a vital role in empathy and/or social 
communication.

5.3 Conclusion

Rodent models of empathy such as the paradigm used in the current study will set a strong 
foundation for providing insight into the central mechanisms that engage empathy-related neural 
circuits at a detailed cellular and molecular level. The current study has shed light on some of the 
functional changes at the molecular level by which stress prevents empathy-like behaviors in 
rodents by examining neuromolecular mechanisms, something that is rarely if ever done in social
neuroscience. ASDs being characterized by a lack of empathy can thus be proficiently studied with this exact model to provide a novel understanding of the neural underpinnings of sociability and differences in glucocorticoid activity in the brain. Future studies conducted using this paradigm and with the pharmacological and molecular techniques following it will result in providing insight into the neuronal regulation of empathy behaviour. This is a transformative step towards bridging the gap between social behaviour and its underlying mechanisms, which will allow us to embark on a new stage of integrative studies in the domains of social behaviour and rodent empathy.
References


