Social Modulation and Communication of Pain in the Laboratory Mouse

Dale J. Langford

Department of Psychology

McGill University, Montreal

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Abstract

Pain sensitivity is importantly affected by a number of factors, and may best be evaluated using a biopsychosocial framework. Although such a framework has been applied to the study and treatment of pain in humans, the concept of pain as a multidimensional experience in rodent models has been given considerably less attention. There is evidence, however, that pain is significantly modulated by a variety of psychosocial factors; therefore, identifying such factors is important, especially considering the extensive use of rodent subjects in pain research.

A previous study performed in our laboratory described a within-cage order-of-testing effect that suggested the social communication of pain amongst mice, a finding that inspired the research described in this dissertation. Using novel experimental paradigms, the research presented describes the effects of the immediate social environment on pain sensitivity in the laboratory mouse, as well as the converse, the effect of pain on social interactions among mice. Social conditions in pain experiments vary with respect to familiarity (i.e., cagemate versus stranger), sex, and pain state of the conspecific and such factors appear to significantly modulate both pain sensitivity and social behaviour.

Here I present social modulation of pain as evidence for empathy in mice, such that mice observing a cagemate in pain exhibit significant hypersensitivity to a noxious stimulus; such modulation is dependent on visual cues. I also show that varying perceived social threat by permitting or limiting full physical contact in stranger male dyads significantly modulates pain behaviour resulting in testosterone-dependent stress-induced analgesia or hyperalgesia, respectively.

Furthermore, I show that females display heightened social approach toward a cagemate displaying pain behaviour, that this social approach is also observed in mice lacking the oxytocin receptor gene, and that such behaviour may have analgesic properties. Finally, in light of our initial finding that pain is visually communicated, I present a novel coding system for facial expressions of pain in the mouse.

In summary, these findings suggest the importance of accounting for psychosocial factors affecting pain sensitivity in the laboratory mouse, and suggest potential animal models for such complex social processes as empathy and prosociality. Furthermore, facial expression is a novel dependent measure of pain that may provide a more complete description of an animal's pain experience.

Resumé

La sensibilité à la douleur est grandement influencée par un certain nombre de facteurs et pourrait être évaluée plus adéquatement selon un cadre bio-psycho-social. Bien qu'un tel cadre ait été appliqué à l'étude et au traitement de la douleur chez l'humain, le concept de douleur en tant qu'expérience multidimensionnelle chez le rongeur a bénéficié de beaucoup moins d'attention. Pourtant, il est démontré que la douleur est significativement modulée par un ensemble de facteurs psychosociaux; l'identification de ces facteurs demeure donc importante, particulièrement lorsqu'on considère l'usage considérable des rongeurs dans le domaine de la recherche sur la douleur.

D'après une étude réalisée précédemment par notre équipe, les souris qui cohabitent et qui sont témoins de leur souffrance mutuelle sont plus sensibles à la douleur que les souris soumises au test à la douleur de manière individuelle. Ce constat de l'influence de la cohabitation et de l'ordre selon lequel les souris sont testées, évoquant l'existence d'une communication sociale, a inspiré les recherches décrites dans cette thèse. Utilisant de nouveaux paradigmes expérimentaux, nos travaux décrivent les effets de l'environnement social immédiat sur la sensibilité à la douleur chez la souris de laboratoire et, inversement, les effets de cette douleur sur ses interactions sociales. Les conditions sociales varient en fonction du degré de familiarité (c.-à-d. souris consoeurs contre souris étrangères), du sexe et du niveau de douleur de la congénère, tous des facteurs qui semblent moduler de manière significative la sensibilité à la douleur et le comportement social.

J'expose ici que la modulation sociale de la douleur constitue une preuve

d’empathie chez la souris de sorte que des souris qui cohabitent et qui voient leurs consoeurs être en proie à la douleur affichent une hypersensibilité significative lors des tests de nociception; une telle modulation dépend de signaux visuels. Je démontre également que le changement de perception de menace sociale parmi une paire de souris mâles étrangères affecte leur sensibilité à la douleur, entraînant les réponses dépendantes à la testostérone et induites par le stress que sont l’analgésie, obtenue lorsqu’on permet un contact physique complet ou l’hyperalgésie, obtenue lorsqu’un tel contact est limité. Par ailleurs, je montre que les femelles manifestent une approche sociale plus marquée envers une consoeur en proie à la douleur, que cette approche se voit aussi chez des souris dépourvues du gène codant pour le récepteur oxytocine et qu’un tel comportement pourrait avoir des propriétés analgésiques. Enfin, à la lumière de notre découverte initiale révélant que la douleur est communiquée de façon visuelle, je présente un système de codage inédit des expressions faciales de douleur chez la souris.

En résumé, ces résultats suggèrent l’importance de tenir compte des facteurs psychosociaux dans l’analyse des facteurs pouvant influencer la sensibilité à la douleur chez la souris de laboratoire et proposent des modèles animaux potentiels pour l’étude de processus sociaux complexes comme l'empathie et la prosocialité. Par ailleurs, l'expression faciale constitue une mesure dépendante originale susceptible de décrire de manière plus complète l'expérience de douleur chez l’animal.

Acknowledgements

I am immensely grateful for the unwavering guidance, support, and encouragement of my supervisor, Dr. Jeffrey Mogil, from whom I have learned so much. I am also grateful for my labmates, with whom I have had helpful discussions and many laughs throughout my graduate studies, particularly Brandy Callahan, Dr. Michael LaCroix-Fralish, Melissa Farmer, Dr. Mona Chanda, Leigh MacIntyre, Ara Schorscher-Petcu, Sebastien Austin, Susana Sotocinal, Jennifer Ritchie, and my faithful undergraduate researchers. I am also thankful to Dr. Ken Craig for much helpful and inspiring advice regarding our work on mouse facial expressions. I could not have completed this thesis without the support and encouragement of my family: my amazing parents and brothers, Matt and Brad, who have unconditionally loved and believed in me, my wonderful in-laws and sister-in-law, Emily, for their constant support and interest in my work. Finally, I am undyingly grateful to my husband, Matt, who can always turn my day around with a few wise words, and with whom living life to the fullest is unavoidable. And to Kingston, my little man of one year, who keeps me balanced and amazes me every day.

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Abbreviations

AA	acetic acid abdominal constriction writhing test
AITC	allylisothiocyanate (mustard oil)
BW	both writhing
CAP	capsaicin
CAS	castrated
CCI	chronic constriction injury
CNS	central nervous system
CYP	cyclophosphamide cystitis
FACS	facial action coding system
F_{early}	formalin early phase
F_{late}	formalin late phase
fMRI	functional magnetic resonance imaging
HA	high autotomy
I	isolated
IP	intraperitoneal
LA	low autotomy
MGS	mouse grimace scale
$MgSO_4$	magnesium sulfate writhing test
NFCS	neonatal facial coding system
NOP	no observed pain
NW	none writhing
OP	observed pain
OT	oxytocin

OTR KO	oxytocin receptor knockout
OW	one writhing
PET	positron emission tomography
PFP	primal face of pain
Post-Op.	post-incisional model
rACC	rostral anterior cingulated cortex
SI	primary somatic area
SII	secondary somatic area
SC	subcutaneous
SIA	stress-induced analgesia
SIH	stress-induced hyperalgesia
SNI	spared nerve injury
TC	tail clip
TF	tail flick
TW	tail withdrawal
TP	testosterone propionate
VEH	vehicle
WT	wildtype
ZYM	zymosan

Contribution of Authors

This thesis is manuscript-based and includes portions of the text and figures from two published papers (Chapters 2 and 4), and two papers in preparation for submission (Chapters 3 and 5). In some cases I have reformatted or rewritten the accepted manuscript in order to better comply with doctoral thesis requirements and to better fit the style of the thesis as a whole. A complete reference list for all chapters is included at the end of the dissertation.

Chapter 2:

Langford DJ, Crager SE, Shehzad Z, Smith SB, Sotocinal SG, Chanda ML, Levenstadt JS, Levitin DJ, & Mogil JS (2006). Social modulation of pain as evidence for empathy in mice. *Science*, 312(5782): 1967-1970.
Most of the experiments were conducted by myself with a few exceptions. Sara Crager collected initial data comparing writhing behaviour in cagemate and stranger dyads (Fig 1A), which I completed. Zarrar Shehzad, an honours student in our lab at the time, spearheaded the formalin experiment (Fig. 4), for which I served a supervisory role. Shad Smith conducted the anosmia experiment (Fig. 3A). Susana Sotocinal provided assistance with blind testing in the writhing-hypersensitivity experiment (Fig. 5). Mona Chanda provided graphical support and Jeremy Levenstadt provided some help with video scoring. Daniel Levitin provided helpful discussion and assistance with some statistical analyses. Jeffrey Mogil served a supervisory role and provided support and inspiration for experimental design.

Chapter 3:

Langford DJ*, Tuttle AH*, Briscoe C, Harvey-Lewis C, Baran I, Sternberg WF & Mogil JS. Varying perceived social threat modulates pain sensitivity in male mice. (In preparation for submission; *co-first authors)
This project was based on my and Sara Crager's finding presented in Chapter 2. I supervised undergraduate honours students, Colin Harvey-Lewis and Inna Baran, who assisted with data collection presented in Fig. 4. I was also involved in the experimental design and statistical analysis of these data. I prepared the initial draft of the manuscript (with input from Wendy Sternberg and Jeffrey Mogil). Alex Tuttle collected the data presented in Fig. 2, and Ciara Briscoe collected the data presented in Fig. 3. Wendy Sternberg and Jeffrey Mogil served in an advisory capacity and assisted with statistical analyses.

Chapter 4:

Langford DJ*, Tuttle AH*, Brown K, Deschenes S, Fischer DB, Mutso A, Root KC, Sotocinal SG, Stern MA, Mogil JS, & Sternberg W. Social approach to pain and its consequences in laboratory mice. (In press at *Social Neuroscience*; *co-first authors).

Experiment 2 was based on a finding of mine that resulted from a review of video from Chapter 2. I also conducted statistical analyses for Experiment 2 and co-wrote the submitted article with Wendy Sternberg and Jeffrey Mogil (which I revised for the purposes of this thesis). In terms of data collection and scoring, I largely served a supervisory role. Sonya Deschenes and Susana Sotocinal assisted with data collection and scoring for Experiment 2. KC Root provided assistance with the design of experimental paradigms. Meanwhile, at Haverford College, under Wendy Sternberg's supervision, Tuttle, Fischer, Mutso and Stern ran and scored Experiment 1. Jeffrey Mogil provided guidance throughout the project.

Chapter 5:

Langford DJ, Drummond TE, Sotocinal SG, Klassen T, Echols S, Wong D, Craig KD, Mogil JS. The Mouse Grimace Scale: coding facial expressions of pain in the mouse. (In preparation for submission to *Nature Neuroscience*)

Most of these experiments were conducted by myself, with a few exceptions. Tanya Drummond assisted with data collection for the superficial pain assays and for the acetic-acid dose response experiment. Susana Sotocinal collected the zymosan dose response data. Klassen, Echols, Wong, and Dr. Ken Craig developed the Mouse Grimace Scale. Jeffrey Mogil and Kenneth Craig provided guidance and inspiration on the project.

Chapter 1

General Introduction

1. General Introduction

Pain is a complex, multidimensional, and ultimately subjective experience. The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey & Bogduk, 1994), contending that pain is not a purely physiological event. In fact, pain is a highly subjective and idiosyncratic experience importantly influenced by cognitive, affective and social factors. As a result, the biopsychosocial model (Engel, 1977) has been applied to the study and treatment of pain in humans (Andrasik, Flor, & Turk, 2005; Gatchel et al., 2007; Keefe et al., 2002; Turk & Okifuji, 2002). Considerable evidence suggests the need to apply this model to the study of pain in animal populations as well (reviewed in Section 1.2). The pain experience is therefore sensitive to modulation by a host of factors, allowing for tremendous individual variability in response to similar painful insults. Determining factors that explain this variability can provide valuable information, potentially leading to novel therapeutic approaches to pain prevention and management, as well as advocating for individually tailored pain treatment (Mogil et al., 2003).

The purpose of this dissertation is to identify social factors that influence variability in pain sensitivity, thereby providing support for the complexity of the pain experience in laboratory mice, which need to be accounted for in pain research. Furthermore, this dissertation describes a novel dependent measure that evaluates painful facial expression in the mouse (Mouse Grimace Scale; MGS), which may facilitate the accurate identification of pain in rodent models.

1.1 Brain-pain connection: evidence for pain as a multidimensional experience

Centuries ago, Rene Descartes proposed the concept of the "pain pathway" (Descartes, 1664), by which a painful signal is transmitted from the body to the mind. Although somewhat oversimplified and erroneous in its suggestion of a mind-body dichotomy, the concept of a pain pathway (or, more correctly, pathways) from the periphery to the central nervous system (CNS) is still accepted today.

Somewhat more recently, Melzack and Wall presented the gate control theory of pain, which posits that the dorsal horn of the spinal cord plays a significant modulatory role in either the inhibition or facilitation of pain transmission, and that this mechanism is not only affected by pain fibers from the periphery, but also by direct involvement of the brain (Melzack & Wall, 1962, 1965). This theory greatly impacted the field, as it helped to explain individual variability in pain sensitivity in response to identical stimuli, and conceptualized pain as a multidimensional experience involving more than simply a sensory message transmitted, one-way, to the brain. It also led to the conception of a "neuromatrix", a subset of brain regions involved in dynamic pain processing (Melzack, 1999). That the brain could significantly alter pain perception shed insight on such phenomena as stress-induced changes in pain sensitivity (Melzack, 1999), as well as phantom limb pain (Hill, 1999) and the placebo effect (Benedetti et al., 2005), all of which can be characterized by a disconnect between physiological events and perceptual experience.

The development of various neuroimaging techniques provides further evidence for the complexity of the pain experience. Across a wide range of positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies, the experience of pain has been shown to induce activation in a subset of brain regions (commonly known as the "pain matrix") (Price, 1999). The pain matrix includes not only sensory-discriminative areas of the brain (e.g., primary and secondary somatic [SI/SII], sensory-motor co-activations, lateral thalamus), but also regions associated with cognitive-attentional (e.g., posterior parietal and prefrontal cortices) and affective-emotional processes (rostral anterior cingulate cortex; rACC and insula) (Chen, 2007; Ingvar, 1999; Peyron, Laurent, & Garcia-Larrea, 2000). Furthermore, data from small animal fMRI studies suggest similar involvement of these structures in the rodent pain experience (Chang & Shyu, 2001; Lowe, Beech, & Williams, 2007; Malisza & Docherty, 2001; Tuor et al., 2000).

The brain therefore plays a major role in how nociceptive information is processed and thus how pain is perceived in both humans and non-human animals. This considerable plasticity and dynamic involvement of the brain also suggest that the pain experience may be vulnerable to modulation by a number of factors, both organismic and environmental.

1.1 The biopsychosocial model of pain

Since its inception, the biopsychosocial model (Engel, 1977) has been applied to the treatment and study of several conditions, including chronic pain

(Gatchel et al., 2007; Turk & Okifuji, 2002), head pain (Andrasik et al., 2005), and arthritis (Keefe et al., 2002). The model emphasizes the need to account for biological, psychological and social influences in order to fully understand complex disease processes. Indeed, the observations that physically identical injuries within and across individuals can result in drastically different pain experiences provide support for this concept.

As mentioned above, despite adherence to a biopsychosocial model of pain in humans, psychosocial factors are rarely taken into account in the study of pain in animals, even though considerable evidence suggests their importance in pain modulation. The field relies heavily on the use of rodent models to study the basic neural processes mediating the pain response (Mogil, Simmonds, & Simmonds, 2009), but basic researchers largely adopt a reductionist model, focusing on physiological processes irrespective of psychological or social influences. In fact, the term *nociception* – the purely physical effect of a painful stimulus on peripheral sensory cells – is commonly used in rodent pain research, even to describe the putative perceptual experience of the animal (Langford & Mogil, 2008). Clearly, the study of biological processes underlying the pain experience is necessary and meaningful; however, this failure to acknowledge and adequately capture the complexity of the pain experience in animal models of pain may at least partially account for the relative lack of successful translational findings from basic science laboratories to the clinic (Mogil, 2009).

In addition to a brief account of common biological factors influencing pain sensitivity, both psychological and social factors in humans and rodents are

reviewed below. It is important to be mindful that these factors can interact in a number of ways, and that it is difficult to partial out the relative contribution of any single factor to the pain experience. It should also be appreciated that a unidirectional relationship between psychosocial variables and pain sensitivity may not exist; that is, pain can also modulate psychosocial factors, such as mood (Fishbain et al., 1997), memory (Schnurr & MacDonald, 1995), anxiety (Narita et al., 2006), and sociability (Monassi, Bandler, & Keay, 2003).

1.1.2 Biological factors influencing variability in pain sensitivity

The study of genetic factors, and specifically the identification of genes involved in pain sensitivity, has become a topic of much study in the field, especially since the advent of powerful gene mapping techniques. In humans, family, twin, and association studies have resulted in the identification of various candidate genes involved in pain processing (Foulkes & Wood, 2008; LaCroix-Fralish & Mogil, 2009). In rodents, the creation of transgenic mice has resulted in hundreds of published papers reporting pain phenotypes associated with these mutants (Lacroix-Fralish, Ledoux, & Mogil, 2007).

Clearly genetics play an important role in pain sensitivity; however, heritability estimates (i.e., the degree of genetic influence on a phenotype) of such behavioural traits are typically less than 50% (Plomin, 1990). In fact, even in identically housed and experienced rodents, environmental or gene-environment interactions have been shown to account for 60% of the variance observed in the commonly used tail-withdrawal test of thermal sensitivity (Chesler et al., 2002).

It is therefore important to study and to account for the involvement of non-genetic factors.

Biological sex is also a major factor affecting pain sensitivity. Females represent the majority of clinical pain patients (Unruh, 1996), and are considerably more sensitive to a variety of experimental painful stimuli than their male counterparts (Fillingin et al., 2009). Substantial differences in pain sensitivity and response to analgesia (Craft, Mogil, & Aloisi, 2004; Dahan, Kest, Waxman, & Sarton, 2008) have been observed in both humans and non-human animals, and have also been shown to interact significantly with genotype (Mogil et al., 2005; Mogil et al., 2003).

Age also plays a role in modulating pain sensitivity, and is a topic of growing interest in the field. In humans, the impact of age on pain is complex and depends largely on the type of pain; for example, musculoskeletal pain peaks at middle-age, while neuropathic pain increases across the lifespan (Gagliese, 2009). Age-related changes in pain sensitivity in rodents are also quite complex and dependent on pain modality (Gagliese & Melzack, 2000). For instance, there is a similar age-related increase in the severity of neuropathic pain, but this is specific to mechanical hypersensitivity (Kim et al., 1995). Further complicating the field is a significant interaction between age and psychosocial variables, with middle aged rats (10 months) more susceptible to affective and cognitive deterioration associated with chronic pain (Leite-Almeida et al., 2009).

Body weight may also affect pain sensitivity. Obesity has been associated with increased prevalence of fibromyalgia (Neumann et al., 2008), arthritis

(Mehrotra et al., 2004) and low back pain (Kiess et al., 2001) as well as increased sensitivity to experimental pain (Zahorska-Markiewicz, Zych, & Kucio, 1988). At the opposite end of the spectrum anorexia and bulimia have been associated with increased pain thresholds (Lautenbacher et al., 1991b), although it is not clear whether this is due to body weight or to associated psychological or pathological factors (Lautenbacher et al., 1991a). There is a paucity of research describing the effects of body weight on pain sensitivity in rodents; however, two studies by the same group have observed an interactive effect of body weight and stress on pain sensitivity, whereby prenatally stressed rats with low body weight display increased sensitivity to inflammatory pain (Butkevich et al., 2008; Butkevich et al., 2009).

Several of these findings suggest the important entanglement between biological and psychosocial factors in modulating the pain experience. It is therefore important to understand and account for such variables when studying and treating pain.

1.2.2 Psychological factors influencing variability in pain sensitivity

Psychiatric comorbidity is prevalent among chronic pain sufferers (Compton, Darakjian, & Miotto, 1998), and contributes to the difficulty in studying and treating these patients. Indeed, several psychological disorders have been associated with abnormalities in pain perception, including affective, substance dependence, and anxiety disorders (Fishbain, 1999), as well as schizophrenia, borderline personality disorder, post-traumatic stress disorder, and

eating disorders (Klossika et al., 2006). For nearly all of these conditions, the exact mechanism by which this modulation of pain sensitivity occurs is unknown.

Empirical studies using priming techniques to induce changes in mood, such as the presentation of unpleasant olfactory or visual stimuli, have also documented a robust effect of mood on the pain experience, specifically modulating the affective component of pain; that is, pain unpleasantness (Loggia et al., 2008b; Villemure & Bushnell, 2009). Using an evaluative conditioning paradigm, one group showed that ratings of pain intensity also change with respect to unpleasant visual imagery (Wunsch, Philippot, & Plaghki, 2003). In a sample of chronic back pain patients, musically induced mood changes alter baseline pain ratings and pain threshold, with depressed mood enhancing and elated mood reducing the pain response (Tang et al., 2008).

Not surprisingly, there is considerably less research on the relationship between pain and emotion in animals; however, there is evidence to suggest a link. Firstly, a variety of antidepressants (with tricyclic antidepressants most efficacious) alleviate hypersensitivity to mechanical and thermal stimuli as well as anxiety related pain in mouse models of neuropathic pain (Matsuzawa-Yanagida et al. 2008). Secondly, surgically induced neuropathic pain (e.g., spinal nerve ligation) has been shown to produce both anxiety and depression in mice (Suzuki et al., 2007). Finally, genes whose functions have been associated with anxiety and depression have also been linked to abnormalities in pain processing, in a direction akin to those observed in human clinical populations (Konig et al., 1996; Bilkei-Gorzo et al., 2002).

Stress has also been shown to significantly affect pain sensitivity in both directions; that is, stress can either enhance or reduce pain sensitivity depending on the nature of the stimulus (Vidal & Jacob, 1982). In both children and adults, there is considerable evidence linking stress with elevated pain sensitivity (Ashkinazi & Vershinina, 1999; Cathcart & Pritchard, 2008; Dufton et al., 2008). However, research also suggests an analgesic effect of stress in humans, particularly when the stressor is acutely severe and physical in nature (Butler & Finn, 2009).

In rodents, there is a wealth of literature detailing stress-induced analgesia (SIA) using a variety of models including, but not limited to, forced swim (Terman, Lewis, & Liebeskind, 1986) and social defeat (McLaughlin et al., 2006). Interestingly, such stressors under slightly different conditions can also produce stress-induced hyperalgesia (SIH) (Quintero et al., 2000; Marcinkiewcz et al., 2009). That similar stressors may result in dichotomous responses to a painful stimulus is the topic of Chapter 3 of this thesis.

Distraction has also been shown to significantly affect pain sensitivity. In children, for example, it has been shown that such activities as using a kaleidoscope (Tufekci, Celebioglu, & Kucukoglu, 2009), watching television (Bellieni et al., 2006; Wang, Sun, & Chen, 2008), counting, listening to music or talking (Uman et al., 2006) result in reduced self-reported pain during needle procedures. Similarly for adults, playing action-related video games increases tolerance to experimentally induced pain (Raudenbush et al., 2009). Immersion in virtual reality has analgesic effects in both experimental (Rutter, Dahlquist, &

Weiss, 2009) and clinical settings (Sharar et al., 2008), and has been proposed as a treatment for procedural pain (Hoffman et al., 2006). Hypnosis, perhaps characterized by an intense distraction of the mind, has shown promise in reducing self-reported pain in chronic pain patients, perhaps due to a greater perceived control over their condition (Jensen et al., 2009).

There is very little research on the topic of distraction and pain in rodents; however, it has been documented that various strains of mice engaged in grooming behaviour display significant hypoalgesia in response to thermal and mechanical stimuli relative to other behavioural states, even in the face of neuropathic injury (Callahan et al., 2008).

Prior experience with pain may be considered a psychological factor influencing variability in pain sensitivity in that it may contribute to the expectation of future painful events, generally resulting in hypersensitivity. For example, individuals with chronic pain prior to amputation are more likely to experience phantom limb pain and associated changes in neural plasticity (Flor, 2008). Similarly, chronic pain experience in rodents has been associated with changes in sensitivity to other painful stimuli, namely formalin-induced inflammatory pain. Spinal nerve ligation (a surgical model of neuropathic pain) in rats has been associated with increased response to inflammatory pain (LaBuda, Donahue, & Fuchs, 2001); however, this finding was not replicated by other groups, who reported a reduction in inflammatory pain sensitivity (Vissers et al., 2003; Kaku et al., 2007). Among infants, experience with needle procedures is associated with remote hyperalgesia (Taddio et al., 2002) and

hyperalgesia to prolonged noxious heat in school-aged children (Hermann et al., 2006). Similarly, rat pups exposed to repeated painful stimulation in the first week of life exhibit increased sensitivity to pain even in adulthood (Johnston et al., 1993; Anand et al., 1999), a phenomenon likely due to neural plasticity (Torsney & Fitzgerald, 2003).

In line with the conception of prior pain experiences influencing expectations of future pain, it has been shown that the experimental modulation of expected pain intensity significantly alters both verbal pain ratings as well as activation in the pain matrix (Koyama et al., 2005). In complement, those who have "forgotten" past experiences with pain exhibit reduced pain sensitivity. For example, memory-impaired mice (via scopolamine or ketamine administration) have been shown to exhibit increased pain thresholds akin to those observed in Alzheimer's patients (Pickering et al., 2004).

1.2.3 Social factors influencing variability in pain sensitivity

Substantial evidence suggests the modulatory effect of social factors on pain sensitivity in humans. In fact, a social communication model of pain has been proposed in order to better characterize the pain experience and the robust social factors that modulate pain (Craig, In Press). The model amalgamates both intrapersonal and interpersonal influences on both the individual in pain as well as individuals in the immediate environment, suggesting not only the importance of social context on the experience of pain, but also stressing the effect of observing pain in others. The impact of social factors on pain is not straightforward.

Indeed, the evidence reviewed in this section suggests that social context can either enhance or diminish pain sensitivity.

Data from imaging studies using virtual social interactions indicate a shared neural basis of physical pain and social "pain" (i.e., social rejection) (Eisenberger, Lieberman, & Williams, 2003), and the experience of social distress under these circumstances enhances pain sensitivity (Eisenberger et al., 2006). On the other hand, social *support* has been shown to affect pain sensitivity in both patient and healthy populations. In fact, a lack of social support is a major factor in predicting the development of chronic pain (Turk, 1997). For example, fibromyalgia patients exhibit significantly reduced sensitivity to normally painful stimuli and increased thermal pain thresholds in the presence of their significant other (Montoya et al., 2004). However, social support can also result in *increased* sensitivity to pain, as has been observed in a subset of fibromyalgia patients who display increased pain behaviours in the presence of a solicitous spouse, but not when the spouse is absent (Thieme et al., 2005).

In healthy individuals, the mere presence of another person (stranger or friend) has been shown to reduce sensitivity to experimental pain (Brown et al., 2003). However, another study assessing social influence on experimental pain using the same noxious stimulus has shown that levels of social support as well as the presence of a same-sex friend are associated with increased pain sensitivity in women, but not men (McClelland & McCubbin, 2008), suggesting the contribution of both familiarity and sex in modulating effects of social support.

Studies of procedural pain in infants repeatedly indicate an attenuation of

pain responding when engaged in skin-to-skin contact with the mother during heel lance procedures (Johnston et al., 2003; Ludington-Hoe, Hosseini, & Torowicz, 2005; Freire, Garcia, & Lamy, 2008). In fact, the contact of a loved one has even been shown to attenuate activation in response to the threat of pain in adults, depending on the quality of the relationship (Coan, Shaefer, & Davidson, 2006).

Social modeling has also been shown to affect pain sensitivity. A number of studies have shown that the observation of another's verbal pain ratings significantly modulates, in a similar direction, the pain ratings of observing individuals; for example, those exposed to a "tolerant" model report less pain than those without a model (Craig, Best, & Ward, 1975; Craig & Prkachin, 1978; Crockett, Prkachin, Craig, & Greenstein, 1986). Evidence from studies using autonomic correlates of pain and psychophysical judgments indicate that such modeling effects are not reflective of a conscious change in verbal reporting, but actually reflect a change in the sensory-discriminative experience of pain (Craig, 1975).

The study of empathy for pain – in which researchers assess an individual's response to another's pain – has been a topic of much recent interest. Neuroimaging studies show that empathy for pain involves activation in the affective regions of the pain matrix (Singer et al., 2004), and that such a response is modulated by perceived fairness (Singer et al., 2006), as well as social (Akitsuki & Decety, 2009) and racial context (Xu et al., 2009). Furthermore, such activation of the observer's pain matrix appears to translate to enhanced pain

sensitivity that is dependent on the affiliative association among the individuals (Loggia, Mogil, & Bushnell, 2008a). Empathy for pain in rodents is the topic of Chapter 2 of this thesis.

The importance of social factors in modulating pain sensitivity in rodents has also been documented. A multitude of studies have examined the effect of prolonged social isolation on subsequent pain sensitivity (Panksepp, 1980; Puglisi-Allegra & Oliverio, 1983; Gentsch et al., 1988; Siegfried & Frischknecht, 1988; Coudereau et al., 1997), with the majority reporting a reduction in pain sensitivity compared to group-housed animals. Social isolation rearing has also been used as an animal model of schizophrenia, a disorder for which hypoalgesia has been repeatedly reported (Tuboly, Benedek, & Horvath, 2009). In line with these findings, mice housed in numbers fewer than three exhibit increased baseline pain thresholds relative to those in higher-density cages (Chesler et al., 2002).

In contrast, group housing has also been shown to have an ameliorative effect on pain sensitivity. For example, one study using a model of chronic pain revealed that autotomy behaviour after complete hind paw denervation in males was significantly reduced among those housed with a female compared to those housed alone (Berman & Rodin, 1982). Similarly, studies assessing the effect of housing conditions on wound healing consistently show that recovery is substantially quicker among pair-housed animals, and that this effect may be mediated by oxytocin, a neuropeptide implicated in social behaviours such as pair bonding and social recognition (Detillion et al., 2004; Lim & Young, 2006).

In fact, the pain state of a co-housed animal can overcome typically robust genetic factors. Using a rat model of autotomy, Raber and Devor (2002) demonstrated that caging rodents selectively bred to exhibit low autotomy (LA) behaviour with those bred to exhibit high autotomy (HA) behaviour resulted in a complete reversal of the typically stable phenotype. That is, LA rats housed with HA rats showed significantly increased autotomy; in fact, even LA rats exposed merely to the odour of HA rats showed an increase in autotomy, suggesting this is not a modeling or imitation effect. The study went on to show that altering such housing conditions eliminated the significant association of a quantitative trait locus on chromosome 15 previously observed to modulate this phenotype (Devor et al., 2007).

A much smaller literature documents that pain sensitivity can be modulated by brief social interactions. In fact, I am aware of only two relevant studies, in which sibling male mice, reunited after a period of separation, show increased huddling and an opioid-dependent increase in pain threshold (D'Amato & Pavone, 1996), as well as enhanced responsivity to morphine (D'Amato, 1998). The influence of social contact on pain sensitivity is the topic of Chapter 4 of thesis.

A study assessing the influence of various factors affecting pain variability on a commonly used pain assay (Chesler et al., 2002) provided evidence for the social communication of pain in the laboratory mouse, and constitutes the inspiration for the series of experiments presented in this thesis. Specifically, it was observed that the order in which mice were drawn (and subsequently tested)

from their home cage significantly influenced thermal pain thresholds, such that mice tested last exhibited significantly reduced thermal thresholds than those tested first. Mice placed in a holding cage rather than returned to their home cage did not exhibit this order-of-testing effect, thus suggesting a role of communication among mice in the home cage (Chesler et al., 2002). The potential involvement of pain communication via facial expressions in mouse is the topic of study in Chapter 5 of this thesis.

1.3 The mouse as a model system for studying pain

Rodents are most commonly used in pain research for both practical and ethical purposes. They are relatively inexpensive to purchase and maintain, can be group-housed, and generally breed well, with outbred strains (particularly CD-1®, the strain used most frequently in this dissertation) producing an average of 12 pups per litter (Giknis & Clifford, 2007). The use of rodents also curtails ethical problems associated with the study of pain in humans. Undoubtedly, researchers have considerably more control over experimental parameters (e.g., researchers can more readily minimize external variables and can randomly assign mice to conditions despite possible negative health outcomes). Furthermore, invasive techniques can be utilized in order to study the basic mechanisms of pain processing. One substantial obstacle in human studies of chronic pain is the inability to assess pain sensitivity prior to the onset of the condition, in contrast with rodent models, for which chronic pain states can be surgically or chemically induced. It is important to note, however, that a reliable and accurate dependent

measure of spontaneous chronic pain –the most important component of neuropathic pain (Backonja & Stacey, 2004; Scholz et al., 2009) – has not been identified (or does not exist) in rodent models (Mogil & Crager, 2004).

The ability to provide self-report in humans is both a benefit (e.g., spontaneous pain episodes can be indicated and described) and a hindrance to the study of pain, as it allows for tremendous subjectivity that may obscure the validity of a study. The use of rodents eliminates this subjectivity, and there are several algesiometric assays that elicit reliable and observable pain behaviours (described below).

The unique advantage of using mice as a model species in pain research is the ability to create transgenic or knockout mice (an advantage that may extend to rats as well) (Geurts et al., 2009), affording the opportunity for discovering specific genetic involvement in the modulation of nociceptive sensitivity. As discussed previously, several hundred transgenic mice have been shown to exhibit abnormalities in pain processing (Lacroix-Fralish et al., 2007). Because 99% of the mouse genome comprises analogues of human genes (Waterston et al., 2002), it is possible to conduct valuable translational research from mouse to human; in fact, one such study revealed that the same genetic mutation in the melanocortin-1 receptor gene was responsible for altered pain perception in both mice and humans (Mogil et al., 2005).

A standard battery of pain assays has been developed to study pain sensitivity in the laboratory mouse. These assays tap various pain modalities, which can be used to provide a thorough phenotypic characterization of a

particular strain of mouse (Mogil et al., 2006). The battery includes assays that measure reflexive responding to both thermal and mechanical stimuli (e.g., tail-withdrawal and tail clip tests), as well as more organized behavioural responses to chemical stimuli (e.g., acetic-acid abdominal constriction "writhing" and formalin tests). More detailed descriptions are included in Chapter 5.

The abdominal constriction ("writhing") test is most commonly used in the experiments described in this dissertation for a number of reasons. Firstly, the assay is relatively brief, with behaviours quantified for only 30 min post-injection. Secondly, it is mild in intensity and therefore sensitive to modulation by weak analgesics (Vander Wende & Margolin, 1956), making it more ethically desirable. Thirdly, perhaps due to its mild intensity, the writhing test is also sensitive to mild stress, which may account for its relatively high rate of non-responders (Mogil et al., 2001). This high sensitivity is not always desirable, but for the current purposes it is ideal, since it allows for the observation of effects of potentially subtle environmental factors, such as social context, on pain sensitivity.

1.4 The mouse as a model system for studying social behaviour

The mouse is appropriate for the study of social behaviour because it is a naturally social species. Indeed, competition for nursing, play, aggression, allogrooming, huddling, and social learning are reliably observed among rodent littermates (Wills et al., 1983). Moreover, group-housed mice establish stable social relationships, such as submissive-dominant relationships among males. Mice also display a preference for social contact, even over environmental

enrichment (Van Loo et al., 2004), and generally display a strong preference for social novelty (Moy et al., 2008). Upon meeting a novel conspecific, mice engage in stereotypic sequential set of investigatory behaviours, which can be used to assess social recognition, social discrimination, partner preference and pair bond formation (Winslow, 2002). Moreover, researchers can make use of established social behaviours in order to quantify the level of sociality among groups of mice that may vary with respect to genotypic and environmental factors, thereby potentially gaining insight into disorders characterized by social abnormalities, such as autism (Crawley, 2007) and schizophrenia (Labrie, Lipina, & Roder; O'Tuathaigh et al., 2008).

1.5 Thesis Overview

As a result of the study identifying within-cage order-of-testing as a significant factor affecting variability in pain threshold (Chesler et al., 2002), suggesting the social communication of pain in mice, our lab became interested in determining the effects of the immediate social environment on pain sensitivity. To this end, we tested mice in various social (dyadic) conditions, using the sensitive writhing test.

In Chapter 2, I show that similarly injected cagemates, but not strangers, exhibit significant hypersensitivity and co-occurrence of pain behaviour relative to isolated testing, and that this pain is visually communicated. Furthermore, pain behaviour can be bidirectionally modulated depending on the amount of pain behaviour displayed by a test partner. Finally, heightened sensitivity among mice

observing a cagemate in pain generalizes to a stimulus of entirely different modality. These findings are interpreted as empathy for pain, at least at the level of emotional contagion.

From the experiments described in Chapter 2, an interesting sex-specific pain inhibition amongst *unfamiliar male* dyads in which only one mouse was injected with acetic acid (i.e., OW condition) was observed. In Chapter 3, I describe the replication of this male stranger-specific inhibition of writhing behaviour in a different laboratory, explained as a form of social stress-induced analgesia, as well as the new finding that this inhibition is testosterone-dependent. Castration of the unaffected male abolishes this effect, and inhibition is reinstated with testosterone replacement. Reducing perceived social threat by limiting physical contact results in a complete reversal of the phenomenon, in which male mice display *hyperalgesia* rather than analgesia. This dichotomous response appears to be dependent on the nature and severity of the stressor.

In Chapter 4, we tested mice in a novel pain-related social approach paradigm, in which a test mouse was given access to two conspecifics placed at opposite ends of a Plexiglas runway. In this paradigm, females, but not males, displayed significantly increased approach to a cagemate displaying pain behaviour than to an unaffected or unfamiliar (but affected) conspecific. Surprisingly, female mice lacking the (affiliative) oxytocin receptor also display preferential approach toward a cagemate in pain. Using a slightly different paradigm, we assessed both approach behaviour of an unaffected mouse and pain behaviour of an affected mouse placed in a small end compartment of a Plexiglas

runway. In this paradigm, frequency of approach by the unaffected mouse is significantly associated with reduced pain behaviour in the writhing mouse.

Finally, the research presented in Chapter 5 stems from the earlier finding that the communication of pain is visually mediated. We wondered whether mice were attending only to the pain behaviour (i.e., the writhe) itself or also to facial expression. First, we needed to determine whether mice do indeed display reliable and observable facial expressions in response to noxious stimuli, and to that end we developed a facial coding system specific to mice in collaboration with human facial expression experts, which we call the Mouse Grimace Scale (MGS). The scale has high inter- and intra-rater reliability. Using the scale to code facial expressions on a variety of algesiometric assays resulted in the observation that painful facial expressions are specific to tests of deep pain. This painful facial expression is dependent on stimulus intensity and can be dose-dependently reversed by morphine administration, supporting the validity of the MGS.

Chapter 2

Social Modulation of Pain as Evidence for Empathy in Mice

2.1 Rationale

The significant order-of-testing effect observed in the Mogil laboratory, and described in Chesler et al. (2002), suggested the social communication of pain in mice. We wanted to follow up on this finding by studying the effects of the immediate social environment and online observation of pain in a conspecific on pain sensitivity.

2.2 Abstract

Empathy is thought to be an attribute unique to higher primates, and possibly to humans alone. However, empathy may only require basic sensorimotor processing, and therefore may be within reach of all mammals, including rodents. We have found that cagemate, but not stranger, mice tested together show significantly increased and co-occurring pain behaviour. The only manipulation that abolished these effects was a visual blockade, suggesting that this phenomenon is dependent on visual cues. Mice showed more *or* less pain behaviour depending on whether the dose of a noxious stimulus administered to their cagemate was higher or lower, respectively. Additionally, the observation of a cagemate in pain altered sensitivity to an entirely different painful stimulus, suggesting a generalized sensitization of the pain system upon observation of pain in a familiar. In light of recent attention to empathy in humans, an animal model of empathy will provide a powerful experimental tool for further understanding the phenomenon.

2.3 Introduction

Empathy is generally considered an attribute unique to humans, popularly thought to be a cognitively sophisticated process involving self-awareness and conscious effort. However, Darwin may have been the first to suggest that empathy is a trait very likely exhibited by all social species, claiming that "social affection," as he termed the phenomenon, is necessary for successful social functioning (Darwin, 1871/1982; Chartrand & Bargh, 1999). Indeed, the ability to attend and respond to the state of a conspecific, especially in one's social network, would be advantageous for all group-living species.

2.3.1 Empathy: a phylogenetically continuous phenomenon

Several studies provide evidence that empathy is phylogenetically continuous and thus very likely possessed by all mammals (Preston & de Waal, 2002). Preston and de Waal (2002) note that this evidence has been overlooked due to a lack of congruence between theoretical and empirical study of empathy, and claim that this discrepancy may be resolved by adopting a more cohesive and universal definition for the phenomenon. As such, they proposed the perception-action model of empathy, which posits that the observation of another's state automatically stimulates representations of a similar state in the observer, leading to the sensitization of associated physiological processes. Furthermore, the more similar the subject and object's state, the greater the activation of associated autonomic processes (Preston & de Waal, 2002).

This basic definition for empathy allows for such subclasses as emotional

contagion, described as a multiply determined phenomenon that can occur in its primitive form without conscious awareness. This contagion usually results in "attentional, emotional, and behavioural synchrony" amongst participants in a social interaction, termed physiological linkage (Kaplan & Bloom, 1960; Hatfield, Cacioppo, & Rapson, 1993). The consequential alignment of various physical and autonomic measures can often be used to reliably gauge relationship quality (Levenson & Gottman, 1983; Feldman, 2007), including dispositional empathy (Chartrand & Bargh, 1999). In humans, synchronous behaviours have been observed to co-occur within 50 ms of each other, further suggesting that this phenomenon happens automatically (Condon, 1982).

In human studies of empathy, fMRI data provide evidence that 'shared representation' of affective or behavioural states among individuals can modulate empathic responses (Lawrence et al., 2006). Furthermore, the accuracy of determining another's affective state is significantly correlated with the real-time alignment of autonomic measures (Levenson & Ruef, 1992). As such, it was suggested that physiological linkage may be the underlying substrate for empathic responding.

Online co-occurrence of behaviours has also been observed in non-human primates. For example, studies involving Japanese monkeys (Nakayama, 2004) and chimpanzees (Anderson, Miyowa-Yamakoshi, & Matsuzawa, 2004) have shown that the observation of a conspecific exhibiting grooming or yawning behaviour, respectively, resulted in the observing primate exhibiting the same behaviour. Because this response does not necessarily require higher-order

cognitive functioning (Condon, 1982; Hatfield et al., 1993; Preston & de Waal, 2002), it seems highly probable that empathy exists, at some level, among all social species.

There is a paucity of research involving adult-adult empathy in non-primate mammals; however, existing studies suggest that these species may be capable of recognizing another's state and may respond accordingly. For example, albino rats learned to press a lever to release a conspecific from a raised harness (Rice & Gainer, 1962), thus alleviating its distress. Also, in conditioning studies with rats, electric shock to a conspecific (CS) paired with a shock to the subject (US) resulted in distress upon observation of a shock to the conspecific (Church, 1959). These findings may be interpreted as arousal rather than empathy, yet despite this more parsimonious explanation, these results indicate that rodents may be capable of "understanding" a conspecific's state.

2.3.2 Empathy for Pain

More recently, researchers have explored the empathy phenomenon with respect to the experience of pain. For example, a study employing transcranial magnetic stimulation demonstrated that observation of pain in another results in motor responses similar to those associated with the experience of pain itself (Avenanti et al., 2005). Additionally, using fMRI techniques, Singer and colleagues (2004) showed that a mere auditory signal that a loved one was undergoing a painful stimulus was enough to activate the affective components of the brain's pain matrix in the "observing" subject. Furthermore, this shared

neural response was shown to be modulated by conditioned preferences, as participants showed greater empathy-related activation upon observation of confederates who they had learned to be fair players in a previously played game of trust (Singer et al., 2006).

In addition to providing evidence of empathy for pain and physiological linkage, these fMRI studies also demonstrate that the relationship between the subject and object is an important mediating factor, such that these phenomena were observed amongst loved ones and those manipulated to have a positive relationship. Preston and de Waal (2002) also stress the importance of familiarity in facilitating the empathic response. If an attended object is familiar, the ability to recognize emotional and physiological states and to respond accordingly should indeed be significantly easier and should provide advantages to inclusive fitness.

In a study intended to quantify the effects of laboratory environment on pain variability in a commonly used pain assay (Chesler et al., 2002), a serendipitous discovery was made that provided a stepping stone for testing for empathy in rodents. Specifically, an order-of-testing phenomenon was observed, such that the first mouse tested from a cage exhibited significantly reduced pain sensitivity, on average, relative to its subsequently tested cagemates. In fact, each mouse tested showed increased sensitivity relative to the preceding mouse. If subjects were placed in a holding cage rather than returned to their home cage, the order-of-testing effect was entirely abolished, suggesting the communication of pain (or pain-related stress) from one mouse to another.

We hypothesized that if empathy does indeed exist in mice, the real-time

observation of pain in one mouse might affect the responses of its conspecifics to painful stimuli in a bidirectional manner. Furthermore, because sensitization of the pain system has been noted amongst familiar humans, we expected any empathic responses to be specific to, or at least stronger amongst, familiar (i.e., cagemate) mice. We also conducted independent sensory deprivation experiments to determine the mode by which pain was communicated. Finally, we hypothesized that mere observation of a familiar mouse in pain would result in a general sensitization of the pain system, indicated by altered sensitivity to a stimulus of an entirely different pain modality. In order to address these issues, we modified commonly used behavioural assays of pain sensitivity such that mice were tested in dyads in addition to traditional isolated testing. Specifically, we used the 0.9% acetic acid abdominal constriction (writhing) assay, and tested mice in dyads where both mice were injected, such that each observed the other in pain, and in dyads where only one mouse was injected, such that a mouse in pain observed a naïve mouse. Thus, we used reliably quantifiable pain behaviours as a proxy for empathic responding.

2.4 Materials and Methods

2.4.1 Subjects

Subjects were naïve male and female outbred CD-1® mice (ICR:Crl), aged 6-13 weeks of age, purchased from a supplier (Charles River, Boucherville, QC), or bred onsite in our vivarium. In one experiment, inbred BALB/cJ mice obtained from a supplier (The Jackson Laboratory, Bar Harbor, ME) were used due to their

particular susceptibility to kanamycin-induced deafness (see Fig. 3A "Deaf" bars). Upon arrival or weaning, mice were housed in same sex groups of two or more, and allowed to habituate to our vivarium for at least one week prior to testing. Mice were kept under 12:12 h light/dark cycle with lights out at 19:00 h, and were provided with food (Harlan Teklad 8604) and tap water ad libitum. In all experiments, approximately equal numbers of males and females were tested, but when tested together, only same-sex pairings were used. 'Cagemates' refer to mice drawn from the same cage, and 'Strangers' to mice drawn from different cages and tested together.

2.4.2 Nociceptive Assays

(a) Acetic Acid Abdominal Constriction Test

The nociceptive assay used in most of the experiments was the acetic acid abdominal constriction test, also known as the "writhing" test. After 30 min of habituation to the testing apparatus, a clear Plexiglas cylinder (15 cm diameter; 22.5 cm high) on a glass floor, subjects were quickly removed and injected intraperitoneally (i.p.) with 0.9% glacial acetic acid using a 1-ml syringe with a 26-gauge needle in a volume of 10 ml/kg. Subjects were then returned to their respective cylinders and digitally recorded for 30 min post-injection. The digital video files were archived and subsequently scored, using behavioural analysis software (Noldus Observer^{TM}) by blind observers whenever possible. Videos were sampled every 20 sec, and the presence or absence of writhes within a 5-sec interval was recorded, yielding a measure of pain behaviour reported as "%

Samples Writhing" (i.e., total of 90 samples). A mouse was scored as "writhing" if there was a visible lengthwise stretch of the torso, or concave shape of the abdomen, usually followed by extension of the hindlimbs. This assay is commonly used in pain research, and typically involves testing subjects singly; however, because we were interested in the effect of the presence of a conspecific on pain sensitivity, we also tested mice in dyads (see Fig. 2.1 for depiction of conditions).

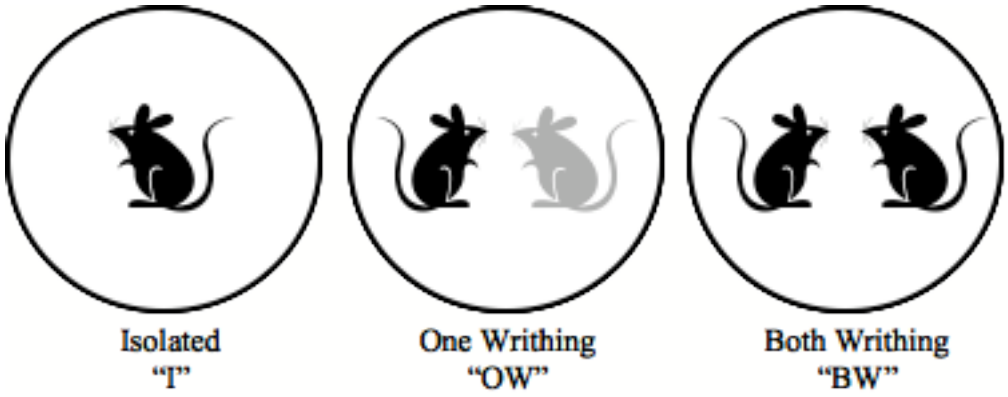

Fig. 2.1. Conditions for testing socially-mediated pain modulation. In the isolated condition, each mouse is tested singly. In the One Writhing ("OW") condition, one mouse is injected with 0.9% acetic acid and tested in the presence of a naïve mouse. In the Both Writhing ("BW") condition, both mice are injected with 0.9% acetic acid and tested in the presence of each other.

(b) Formalin Test

The formalin test is a commonly used measure of tonic inflammatory pain (Dubuisson & Dennis, 1977). Its injection into the hind paw results in a bi-phasic pattern of behaviour characterized by licking, biting, and/or shaking of the affected paw. For experiments using the formalin test, animals were acclimated to the observation cylinders for 30 min with their test partner, then removed and

injected with either 1% or 5% (20 μl) formalin into the plantar surface of the right hind paw using a 50-μl Hamilton microsyringe with a 30-gauge needle, and quickly placed back in their respective cylinders. For this assay, animals were also tested in dyads, where either both mice were injected with the same dose of formalin (1% or 5%; Fig. 2.4 'Same' condition), or where one mouse in the dyad was injected with 1% and the other with 5% formalin (Fig. 2.4 'Diff' condition). Subjects were digitally recorded for 60 min post-injection, and videos were later sampled every 1 min for 5 sec (i.e., 60 samples total) and scored for the presence or absence of right hind paw licking, to yield a measure of pain behaviour denoted as "% Samples Licking". Immediately after the 60-min observation period, animals were sacrificed and formalin-induced edema was confirmed by relative paw-body weight.

(c) Hargreaves' Radiant Heat Paw-Withdrawal Test

In the radiant heat paw-withdrawal test, animals were habituated to the Plexiglas cylinders on a glass surface and their test partners, for approximately two hours prior to testing. For this assay, inactivity is necessary to obtain accurate paw-withdrawal measures (i.e., paw-withdrawal must be due to thermal stimulus, not to natural movement) and thus requires relatively extensive habituation time. A light source was positioned 6 cm below the glass surface, and a high intensity (20%; ~45 W) light beam was directed at the plantar surface of the hind paw. The latency to purposeful paw withdrawal was recorded to the nearest 0.1 sec. Four baseline measures for each hind paw were obtained prior to

acetic acid injections, and two measures (one per hind paw) obtained every 5 min post-injection for 30 min. Subjects were simultaneously filmed from above in order to capture writhing behaviour.

2.4.3 Sensory Disruptions

(a) Anosmia

Mice were made anosmic through repeated intranasal zinc sulfate ($ZnSO_4$) injections (Fig. 2.3A "Anosmic" condition). This treatment has been shown to damage the olfactory epithelium, resulting in severe deafferentation of the main olfactory bulb and consequent profound anosmia within 1 week (Alberts & Galef, 1971; McBride, Slotnik, & Margolis, 2003). CD-1 mice were treated with 2-3 drops of the local anesthetic lidocaine, and 5 min later 50 μl of 5% $ZnSO_4$ (or saline) was injected into each nostril using a blunted, 4-mm-long, 26-gauge needle. Anosmia was confirmed behaviourally at 3-4 days post-treatment by assessing latency of food-deprived mice to detect a piece of mouse chow buried 0.5 cm under the bedding of a novel cage. Saline-treated mice retrieved the food in 30.9 ± 4.5 s; mice were considered anosmic if their latency exceeded 120 sec (two $ZnSO_4$-treated mice were discarded based on this criterion). Twenty-four to 48 h later, all mice were tested on the writhing test as described.

Mice were rendered deaf (Fig. 2.3A "Deaf" condition) using a chemotoxic strategy. Systemic injection of the aminoglycoside kanamycin produces ototoxicity and profound shifts in auditory thresholds across the frequency spectrum. This irreversible effect is strain-dependent in the mouse, with the

largest changes (up to 70 dB at 24 kHz) observed in BALB/cJ mice, with greater threshold shifts at higher frequencies (Wu et al., 2001), and so audition in the ultrasonic range is greatly impaired. BALB/cJ mice were given two weeks of twice-daily subcutaneous (s.c.) injections of 800 mg/kg (10 ml/kg volume) kanamycin base, and tested one week later on the writhing test as described. Subjects were subsequently assessed for startle response to high-frequency sounds, and found to be impaired compared to untreated BALB/cJ mice.

Tactile communication was prevented by placing a transparent Plexiglas barrier (20 cm high, 1/8-inch thick) between the two mice (Fig. 2.3A "Transparent" condition). The barrier was elevated 2 mm off the floor so as not to impede pheromonal communication.

Visual contact was prevented by placing an opaque Plexiglas barrier (20 cm high, 1/8-inch thick) between the two mice (Fig. 2.3A "Opaque" condition). This barrier was also elevated 2 mm off the glass floor to allow for pheromonal communication.

2.4.4 Statistical Analyses

After scoring digital video files, data was input into SYSTAT® (v. 10). For all statistical analyses, an alpha level of 0.05 was considered significant.

2.5 Results

2.5.1 Hyperalgesia and Co-occurrence in Cagemates, but not Strangers

Pain behaviours differed significantly depending on condition and social relationship (Fig. 2.2A). Based on a two-way Dunnett case-control comparison post-hoc test, cagemates (M = 56.90, SEM = 2.21) and siblings (M = 60.94, SEM = 3.63) tested in dyads showed significantly more pain behaviour relative to mice tested in isolation (M = 37.895, SEM = 3.20, $p < .05$). However, strangers tested in dyads (M = 42.27, SEM = 2.70) did not exhibit significantly increased pain behaviours relative to isolated testing. Additionally, in cagemate and sibling dyads where only one mouse was injected (M = 42.489, SEM = 3.73 and M = 45.80, SEM = 5.30 respectively), writhing levels did not differ from isolated subjects. Finally, in stranger dyads where only one mouse was injected, writhing levels were significantly lower (M = 25.00, SEM = 4.20) than isolated testing, an effect driven by a subpopulation of male mice who exhibited an almost complete inhibition of writhing behaviour when tested in the presence of a stranger male.

As depicted in Fig. 2.2B, both cagemate and sibling Both Writhing (BW) dyads exhibited significant co-occurrence of writhing behaviours (i.e., both mice writhing within same 5-sec interval) than would be expected by chance (i.e., zero); furthermore, cagemate dyads exhibited significantly greater co-occurrence (M = 5.00, SEM = 0.49) than stranger dyads (M = 2.60, SEM = 0.74; $t_{49} = 2.80$, $p < .01$).

Significant hyperalgesia, relative to isolated testing, was observed after 3 weeks of co-housing ($p < .05$, as assessed by a Dunnett one-way case-control

comparison test). Significant co-occurrence developed after 2 weeks of co-housing ($M = 3.71$, $SEM = 0.60$; $t_{15} = 6.15$, $p < .001$, by one-sample t-test compared to zero). Trend analyses revealed a significant linear trend for both hyperalgesia time course data ($F_{1,186} = 11.15$, $p = .001$) and synchrony time course data ($F_{1,73} = 8.90$, $p = .004$).

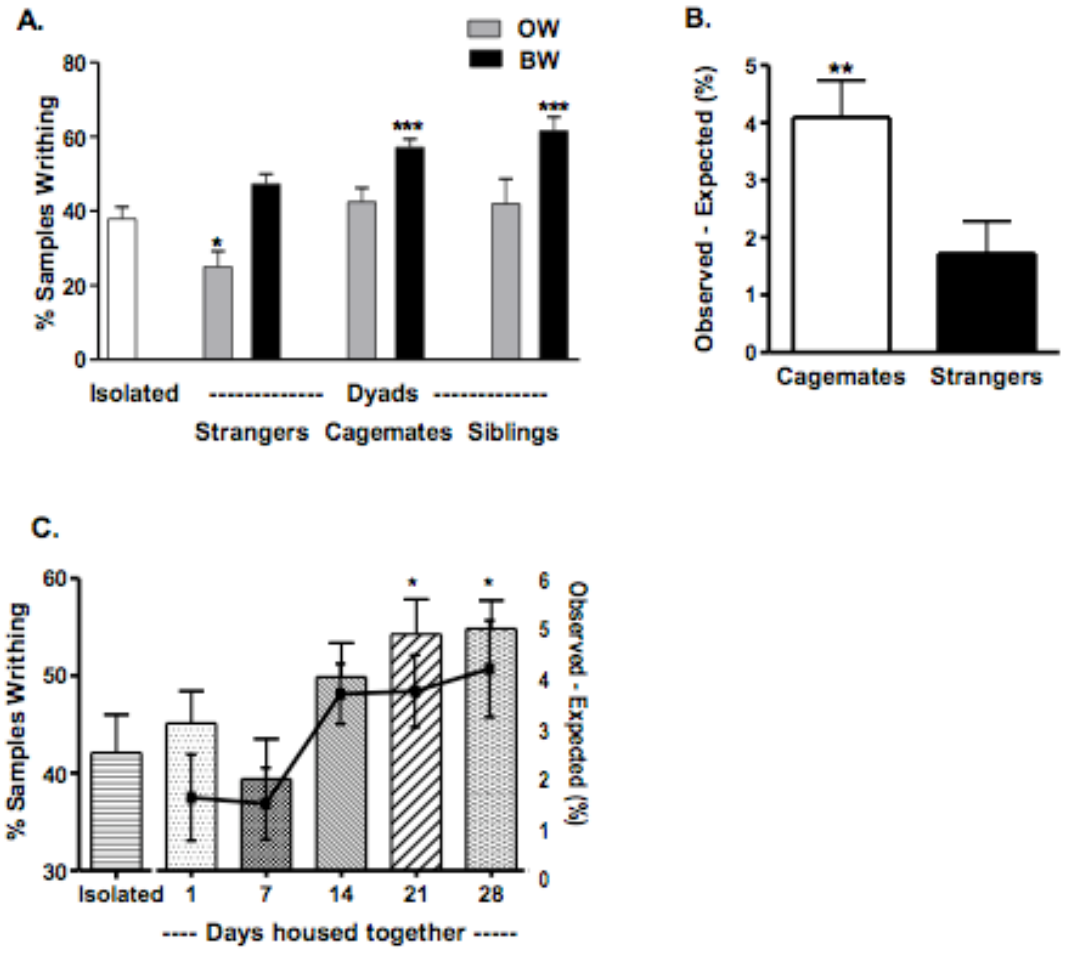

Fig. 2.2. Socially-mediated hyperalgesia and co-occurrence of pain behaviours in cagemate dyads where both mice are similarly injected. All graphs display means ± S.E.M . Graph A shows significantly increased pain behaviours in cagemate and sibling BW dyads relative to Isolated, $*p < 0.05$, $***p < 0.005$. Graph B shows a statistically significant co-occurrence in writhing behaviour in the Cagemates and Strangers conditions ($p<0.05$ in both cases); the co-occurrence was significantly higher in Cagemates, $**p < 0.01$ compared to Strangers. Graph C shows the time course for development of both hyperalgesia and co-ocurrence $*p < 0.05$ relative to Isolated.

2.5.2 Hyperalgesia and Co-occurrence Dependent on Visual Cues

Sensory disruptions of olfaction, audition, and tactility did not disturb the hyperalgesia previously reported, such that mice in BW dyads exhibited significantly greater writhing behaviour than those in dyads where only one mouse was injected (as assessed by Student's *t* test). However, placement of an opaque Plexiglas barrier between the two mice effectively abolished this difference (Fig. 2.3A). Blocking visual contact also reduced the co-occurrence of writhing behaviour in BW dyads to chance levels (Fig. 2.3B; $M = 0.05$, $SEM = 0.61$; $t_{40} = 5.90$, $p < .001$).

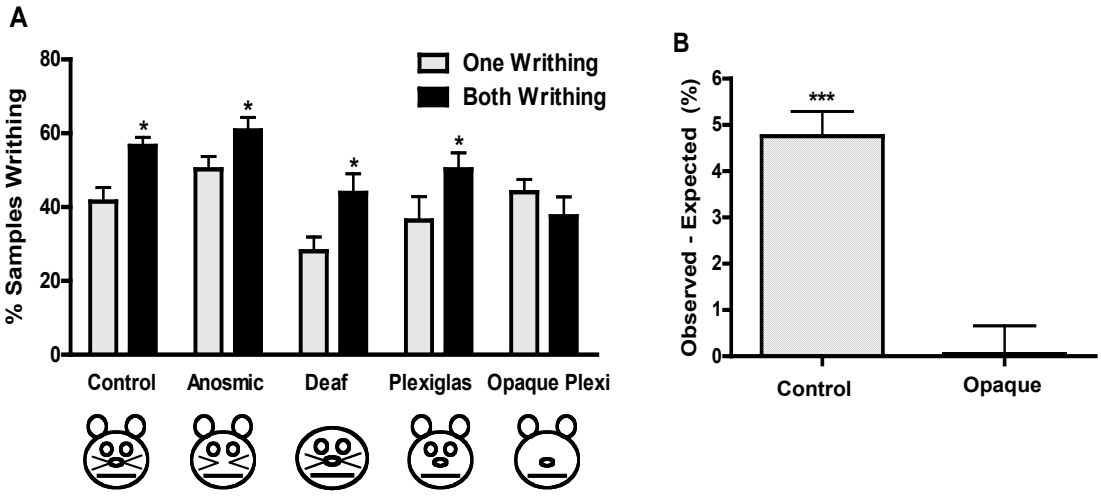

Fig. 2.3. Dependence of socially mediated pain hyperalgesia and co-occurrence on visual cues. Sample sizes are indicated in italics. "Control" data (intact mouse face cartoon) were taken from Cagemates condition in Fig. 2.1 for purposes of comparison. In graph A, bars represent mean ± S.E.M. percentage of sampled intervals showing writhing behaviour (% Samples Writhing). $*p < 0.05$ by Student's *t*-test compared to OW group. Graph B shows the elimination of co-occurrence in BW dyads in which a visual blockade was placed between the two mice (Opaque). Bars represent mean ± S.E.M. excess of observed samples with joint writhing above the expected value, as a percentage. $**p < 0.001$ compared to Control group (Student's *t*-test).

2.5.3 Bidirectional Modulation of Pain in the Formalin Test

Figs 2.4A and B depict the typical biphasic pattern of behaviour in response to formalin injections, as well as the effect of observing a cagemate administered the same or different dose of formalin on this pattern of responding. Because no significant differences were observed after 40 min post-injection, the following analyses were conducted on average licking percentages observed within the first 40 min.

A two-way (injected dose x observed dose) repeated measures ANOVA based on data from the formalin experiment (Fig. 2.4C), revealed a significant three-way interaction ($p < .05$), such that an effect of injected dose was only observed amongst dyads where both mice were injected with the same dose, that is either 1% ($M = 12.81$, SEM = 1.48) or 5% formalin ($M = 26.67$, $SEM = 2.14$; $t_{46} = -5.32$, $p < .001$), whereas dyads in which each mouse was injected with a different dose, where one mouse was administered 1% ($M = 17.39$, $SEM = 2.20$) and the other 5% formalin ($M = 19.32$, $SEM = 1.92$) displayed no significant difference in licking behaviour ($t_{42} = -0.66$, $p = 0.51$).

A significant two-way interaction ($F_{1,88} = 9.3$, $p < 0.005$) was also observed, such that mice injected with 1% formalin, observing a cagemate injected with 5% formalin tended to exhibit greater licking behaviour ($M = 17.39$, $SEM = 2.20$) than when tested with a cagemate similarly injected with 1% formalin ($M = 12.81$, $SEM = 1.48$; $t_{44} = 1.75$, $p = .09$), and alternatively, that mice injected with 5% formalin, observing a cagemate injected with 1% formalin showed significantly less licking behaviour ($M = 19.32$, $SEM = 1.92$) than

when tested with a cagemate similarly injected with 5% formalin (M = 26.67, SEM = 2.14; t_{44} = -2.54, $p < .05$).

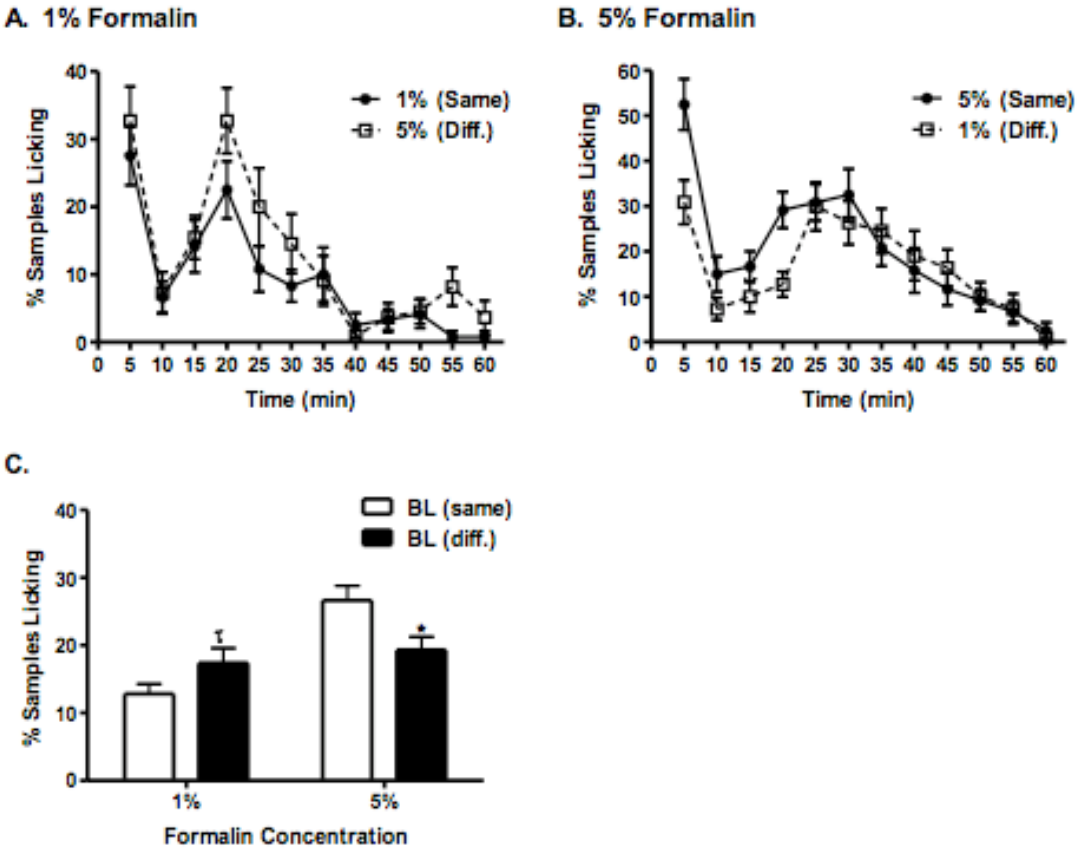

Fig. 2.4. Bidirectional modulation of pain behaviour in dyads where each mouse in the dyad was injected with a different dose of formalin. Graph A shows data from all mice receiving 1% formalin; the legend describes the status of the other mouse in the dyad. Graph B shows data from all mice receiving 5% formalin; the legend describes the status of the other mouse in the dyad. Graph C shows means for all conditions from 0-40 min post-injection, after which there was no longer significantly different licking behaviour between 1% and 5% groups, $^{*}p < 0.05$ compared to analogous "same" condition, $^{\dagger}p < 0.10$ compared to analogous "same" condition.

2.5.4 Observation of Cagemate in Pain Leads to Hypersensitivity to Pain of Different Modality

A two-way (dose x observation) ANOVA revealed a significant main effect of dose ($F_{1, 178} = 11.78$, $p < .01$), such that mice injected with acetic acid (Fig. 2.5A, "OW (Inj.)" and "BW" conditions) exhibited significant reductions in paw-withdrawal latencies ($M = 5.06$, $SEM = 1.75$, and $M = 9.85$, $SEM = 1.52$, respectively) relative to baseline ($t_{27} = 2.89$, $p < .01$ and $t_{27} = 6.47$, $p < .001$, respectively). This analysis also revealed a significant main effect of observation, such that mice observing a cagemate in pain ("OW (Uninj.)" and "BW") exhibited significant reductions in paw-withdrawal latencies ($M = 4.35$, $SEM = 1.44$, and "BW" reported above) relative to baseline ($t_{27} = 3.03$, $p < .01$).

Furthermore, mice tested in BW dyads exhibited significantly greater reductions in paw-withdrawal latencies than OW dyads ($t_{56} = 2.07$, $p < .05$). Finally, dyads in which neither mouse was injected with acetic acid showed no change in paw-withdrawal latencies relative to baseline ($M = -0.18$, $SEM = 1.35$; $t_{27} = -0.13$, $p < 0.89$).

In this paradigm, BW dyads displayed significantly more writhing behaviour ($M = 23.74$, $SEM = 4.30$) relative to OW dyads ($M = 11.70$, $SEM = 3.00$; $t_{34} = 2.29$, $p < .05$), replicating the core phenomenon of hypersensitivity in familiar BW dyads (data not graphed). Finally, there was a significant negative correlation between writhing levels of the injected cagemate (OW and BW dyads only) and average (post-injection) paw-withdrawal latencies of the observing cagemate (Fig. 2.5C; $r = -0.40$, $p < 0.05$).

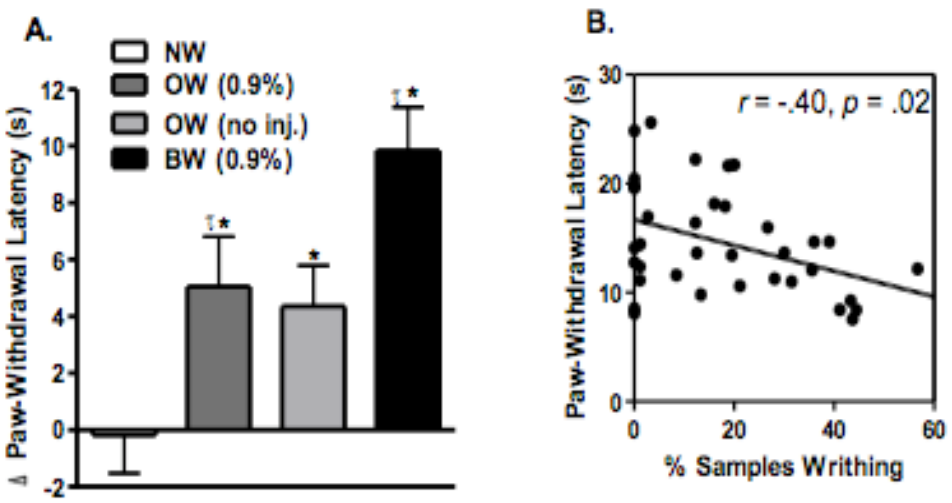

Fig. 2.5. Thermal hyperalgesia produced by injection of acetic acid, by mere observation of a cagemate injected with acetic acid, or both. Bars in graph A represent mean ± S.E.M. average change in paw-withdrawal latencies from the baseline latency, *p<0.05 compared to NW group and zero; $^{\dagger}p$<0.05 compared to the group immediately to the left. Graph B illustrates a significant correlation (r = -0.40; p<0.05) between the writhing behaviour of one mouse in a dyad (ordinate; BW and OW-Inj. only) and the average (post-injection) paw-withdrawal latency of its dyadic counterpart (abscissa; BW and OW-Uninj. only).

2.6 Discussion

We have shown that familiar mice (co-housed for 2 to 3 weeks) when tested together, show significant hyperalgesia and co-occurring pain behaviours (Fig. 2.2). The online observation of pain in a cagemate appears to have a sensitizing effect on the observer and implies the communication of pain from one mouse to another. In fact, the co-occurrence of pain behaviours in familiar individuals may itself be evidence of empathy, as it represents a compelling analogue to the demonstrations of physiological linkage in empathizing humans (Levenson & Reuf, 1992).

Hyperalgesia was not observed in stranger dyads where both mice were

injected, and although these dyads exhibited co-occurring pain behaviours, they did so to a significantly lesser degree than cagemate dyads (Fig. 2.2B). This specificity to familiar dyads is congruent with findings in the human literature showing that relationship quality is a significant factor mediating the empathic response (Singer et al., 2004; 2006). Empathizing with individuals in one's social circle might present an advantage for group-living animals, as it may provide the observer with important social information not consciously accessible, thus guiding behavioural responses. Moreover, the subject inspiring the empathic response serves to benefit from any ameliorative behaviour offered by the empathizing observer.

By blocking each sensory modality independently, we showed that the phenomenon persisted in the absence of olfactory, auditory, and tactile cues, but was effectively abolished by the blockade of visual contact (Fig. 2.3A). The visual blockade also eliminated above-chance co-occurrence of pain behaviours (Fig. 2.3B). This finding of visual-dependence seems surprising considering the general conception of rodents' poor vision and typical reliance on olfactory communication; however, the albino CD-1 mice used in these experiments possess reasonable visual acuity, passing perceptual tasks such as the visual cliff (Adams et al., 2002). Although the identification of a familiar versus unfamiliar mouse was most likely established via pheromonal contact, it is evident that the communication of the pain itself appears to be visually mediated.

We have also shown that the communication of pain sensitivity can be modulated bidirectionally. Pain behaviour was influenced by that of its

neighbour, such that formalin-induced licking was marginally increased in mice receiving a low dose while observing a high-dose-injected cagemate, and significantly *reduced* in mice receiving a high dose while observing a low-dose-injected cagemate (Fig. 2.4). Typical dose-dependent licking behaviour was observed in dyads where mice were similarly injected; however, the alignment of pain behaviours in dyads where mice were differentially injected effectively abolished any effect of dose. The ability of social context to completely eliminate established dose-dependent actions of a noxious stimulus is remarkable, indicating the robust effect of social factors on pain perception. No significant effects were observed among strangers (data not shown), again suggestive of the importance of relationship in modulating this response.

Finally, we showed that observation of a cagemate in pain results in a general sensitization of the pain system, as observing mice showed significant hypersensitivity to a stimulus of an entirely different pain modality, also ruling out imitation (and potentially mirror neurons) as a potential explanation for the observed phenomena (Fig. 2.5A). Mechanisms underlying these phenomena are thus more likely to be found in the sensory/perceptual system than in the motor system. The observation and direct experience of writhing appeared to have a summative effect on thermal hyperalgesia, as BW dyads showed approximately double the reduction in paw-withdrawal latency than OW dyads. We also found a significant relationship between the writhing behaviour of one mouse in the dyad and the thermal sensitivity of the observing mouse, such that higher writhing levels were correlated with lower thermal thresholds in the observer (Fig. 2.5B).

Again, these findings were not observed in stranger dyads (data not shown).

Collectively, these experiments provide evidence for empathy in mice. Attending to a familiar individual in an altered physiological or behavioural state resulted in the modulation of the observer's state to better match the attended subject, thus implying the existence of physiological linkage in mice. As expected, these results were specific to familiar individuals.

2.6.1 Future Directions and Implications

Stranger BW dyads did not show significant hyperalgesia relative to isolated testing; however, stranger dyads where only one mouse was injected exhibited significant analgesia relative to isolated testing. As aforementioned, this finding was specific to male dyads, such that approximately half the males, when tested in front of an uninjected stranger male, exhibited significantly reduced writhing behaviour, presumably in light of a perceived need for hypervigilance in a potentially threatening situation. Factors modulating this inhibition of pain behaviour as well as the influence of proximity of a conspecific are assessed in the proceeding chapters.

There is a possibility that the communication of pain involves a combination of modalities (i.e., not solely vision). The olfactory manipulation (Fig. 2.3) did not produce damage to the vomeronasal organ; therefore, communication was intact across all sensory disruptions, and cannot be ruled out. Manipulations that effectively block the vomeronasal pathway would be beneficial in order to officially rule in (or out) the involvement of pheromonal

communication.

Despite potential involvement of pheromones (at least in the recognition of familiar versus unfamiliar test partner), the finding that vision is a key factor in the communication of pain opens up the possibility that mice may be communicating via facial expressions, and perhaps, that subtle differences in facial activity could be detected under different social conditions. Whether or not this is true, the ability of a human researcher to detect the presence of a painful state via facial expression would offer a unique and powerful advantage for the field of pain research. As a result, we have undertaken a project (see Chapter 5) assessing the existence and pervasiveness of facial expressions of pain in the mouse.

Future research should determine the mechanism through which this empathy phenomenon occurs. The potential contribution of affiliative hormones, such as oxytocin and vasopressin, endogenous opioid and/or dopaminergic activity may be potential candidates for the key mediating factor of the observed empathy effect.

Our findings are entirely consistent with the perception-action model of empathy proposed by Preston and de Waal (2002), both in the automatic priming of somatic responses in a state similar to that of the attended object, and in the modulating effects of familiarity and similarity of experience between subject and object. Our observations cannot be easily explained by stress, imitation, or conditioning, and neither depend on nor necessarily indicate the presence of sympathy, conscious (cognitive) representations or altruism.

Empathy for pain is currently a topic of much study in humans (Jackson, Rainville, & Decety, 2006; Singer et al. 2004; Avenanti et al. 2005), and "mirror neurons" responding to another's pain may have been identified in human anterior cingulate cortex (Hutchison et al., 1999). A large human literature documents the effects on pain report of observation of pain in others (Craig & Weiss, 1971); the present data suggest that these effects may be mediated precognitively; that is, without conscious awareness.

As mentioned earlier, these findings have implications for the design of basic research in pain. Many behavioural pain assays are conducted using multiple mice per run. In order to obtain the most valid and unbiased results, communication between mice must be controlled (e.g., visual blockade between test subjects, etc.), and the influence of subtle social factors should not be overlooked.

Additionally, presuming that the same sort of pain empathy exists in humans, these results may have implications for patients housed together, and for familial chronic pain sufferers. Observation of another in pain may cause a general sensitization of the pain system such that, regardless of the type of pain, one may perceive his or her own condition as significantly more severe. Our model may thus allow us to study the robust social factors that modulate chronic pain in humans.

Importantly, these findings provide a viable animal model of empathy that may provide a unique and powerful tool for investigating the biological mechanisms of the phenomenon. The importance of empathic responding is

evident when considering the consequences of its absence; for example, empathy deficiency has been associated with mental disorders including psychopathy (Ellis, 1982), autism (Charman et al., 1997), and schizophrenia (Langdon, Coltheart, & Ward, 2006). This model may afford the opportunity for better understanding these empathy-deficit disorders. We now have the resources necessary to conduct important genetic and pharmacological studies that may lead to unique and effective treatment for these disorders.

Chapter 3

Varying perceived social threat modulates pain behaviour in male mice

3.1 Rationale

In Chapter 2, we found a sex-specific inhibition of pain behaviours in male stranger dyads in which only one mouse was injected (i.e., OW condition), perhaps a form of social stress-induced analgesia. We wanted to determine whether we could modulate this inhibition through hormonal and testing parameter modulations.

3.1 Abstract

We previously demonstrated that male mice displayed significantly reduced pain behaviour on the acetic acid abdominal constriction test when confined in close proximity to a stranger male mouse (Langford et al., 2006). We show here the testosterone-dependence (via castration and testosterone propionate replacement) of this phenomenon, likely a form of (social) stress-induced analgesia. However, when similar male dyads are separated by vertical metal bars, allowing only partial physical contact, we find that the mice exhibit hyperalgesia, not analgesia, in response to both acetic acid injection and noxious radiant heat, relative to testing in isolation. This finding was specific to same-sex male dyads, and no change in nociceptive sensitivity was observed when males were tested in the presence of a female conspecific. We propose that pain sensitivity varies with respect to the severity of the social stressor: mild social stress produces hyperalgesia and more severe social stress produces analgesia.

3.3 Introduction

In an effort to determine the effects of the immediate social environment on pain sensitivity, we previously tested mice — using the sensitive acetic acid abdominal constriction ("writhing") test — in various dyadic (social) conditions, comparing pain behaviour to that of mice tested in isolation. We observed significant modulation of pain behaviour (hypersensitivity and temporal synchronization) in familiar dyads in which both mice received the acetic acid injection, interpreting these findings as evidence for empathy for pain in mice (Langford et al., 2006). We also observed an interesting sex-specific phenomenon amongst stranger dyads in which only one mouse in the dyad was injected. Specifically, we found that a subset of male mice, when tested in the presence of a naïve stranger male, exhibited greatly reduced pain behaviour relative to testing in isolation (Langford et al., 2006).

We speculated that this inhibition observed amongst stranger male dyads was a form of social stress-induced analgesia. The impact of stress on pain sensitivity is well established; stress has been observed to inhibit or exacerbate pain perception depending on the nature and/or parameters of the stressor (Imbe, Iwai-Liao, & Senba, 2006; Kelly, 1982). Indeed, it would be advantageous to inhibit pain behaviour in a potentially dangerous situation in order to facilitate escape, whereas under other circumstances vigilance to painful stimuli may be more beneficial. We became interested in determining whether this social modulation of pain behaviour could be reversed by altering the perceived threat, either by manipulating hormonal status through gonadectomy, or by manipulating the

testing environment to ensure physical safety from conspecific aggression.

Castration has been shown to reduce social conflict and attack in rats (Barfield, Busch, & Wallen, 1972) and mice (Luttge, 1972). In non-human primates, gonadectomy during adolescence has been shown to significantly impair social behaviour evidenced by reduced displays of dominance and relative disinterest in unfamiliar conspecifics (Richards et al., 2009). Conversely, testosterone administration has been shown to facilitate inter-male aggression in rodents (Gandelman, 1980; Giammanco et al., 2005). If the inhibition of pain behaviour previously observed was truly due to social stress related to the possibility of inter-male aggression, the phenomenon should be attenuated using a gonadectomized partner and reinstated if that partner received testosterone replacement. It is also conceivable that gonadectomy of the test mouse would abolish the effect, by signaling the submissive role of the test mouse, similarly defusing the possibility of aggression.

Using similar logic, we also predicted that we might block the phenomenon by limiting the opportunity for physical aggression, presumably thereby reducing the inherent social stress. In our original paradigm (Langford et al., 2006), mice were tested in a Plexiglas cylinder (15 cm diameter; 22.5 cm high), with no barriers between the mice. In the present study, we placed a barrier (vertical metal bars) between the two mice, eliminating the possibility of effective attack, but still allowing social interaction. We also predicted that female dyads as well as male-female dyads tested in this paradigm would not evince any stress-related pain modulation, since female mice presumably pose no physical threat to larger

males (≈20 g versus ≈35 g, respectively) and female-female aggression rarely occurs in laboratory mice (Hrapkiewicz, Medina, & Holmes, 1998; More, 2008).

3.4. Materials and Methods

These studies were conducted at Haverford College and McGill University. All procedures were approved by local Institutional Animal Care and Use Committees, subject to national (U.S. and Canadian) guidelines.

3.4.1 Subjects

Mice used in this study were of the outbred CD-1® (ICR) strain (Harlan, Indianapolis, IN or Charles River, Boucherville, QC), aged 6-12 weeks. Animals were housed in a light- (12:12 h light:dark cycle, lights on at 07:30 h), and temperature (20°C)-controlled facilities, housed in standard shoebox cages in same-sex groups of 3-6 mice, with food (Harlan Teklad 8604) and tap water available *ad lib*. Mice were habituated to the vivarium for at least one week before testing. All experiments were conducted during the animal's light cycle.

3.2.2 Nociceptive Assays

(a) Acetic acid abdominal constriction "writhing" test

We used the acetic acid abdominal constriction test, in which 0.9% acetic acid is injected intraperitoneally (10 ml/kg). This test of tonic inflammatory nociception involves a clear quantifiable pain behaviour (stretching of the abdominal musculature), and is relatively mild in intensity, allowing the detection

of subtle modulatory factors (Jordan & Mogil, 2006). After a 30-min habituation period, mice were injected with acetic acid and immediately returned to the testing apparatus (see below). Animals were digitally videotaped for 30 min post-injection, and pain behaviour was quantified, by an observer blinded to experimental condition, using a time-sampling method in which the presence/absence of writhing was scored for the first 5 sec of every 20-sec interval (Langford et al., 2006).

(b) Radiant heat paw-withdrawal test

In this assay (Hargreaves et al., 1989), mice are confined atop a ¼-inch-thick glass floor located 6 cm above a projector lamp bulb (IITC Model 336 Plantar Analgesia Meter). A 2-3 h-long habituation time is necessary to reduce activity levels sufficiently to allow testing (Callahan et al., 2007). After habituation, a noxious radiant heat stimulus (20% maximal intensity; ≈45 W) was applied to the plantar surface of the hind paw, and the latency to purposeful paw withdrawal was recorded to the nearest 0.1 sec. Five measurements per hind paw (separated by at least 20 sec) were recorded and averaged for each subject.

3.2.3. Testing Apparatus

In some experiments, mice were habituated and tested (in dyads) in transparent Plexiglas observation cylinders (15 cm diameter; 22.5 cm high), allowing completely unimpeded physical contact between them (Fig. 3.1A). Overt physical aggression was rare, but did occur in less than 4% of intact male

dyads. No physical aggression was seen in dyads containing a castrated male or female mouse. In other experiments, mice were habituated and tested in adjacent Plexiglas observation cubicles (9 x 5 x 5 cm high), separated by thin vertical metal bars (Fig. 3.1B). Social interactions could and did occur between the two mice, but physical aggression was not possible, since the attacked mouse could withdraw beyond the biting range of the attacker. We have determined in pilot studies (not shown) that the different dimensions of the testing apparatuses do not affect writhing behaviour.

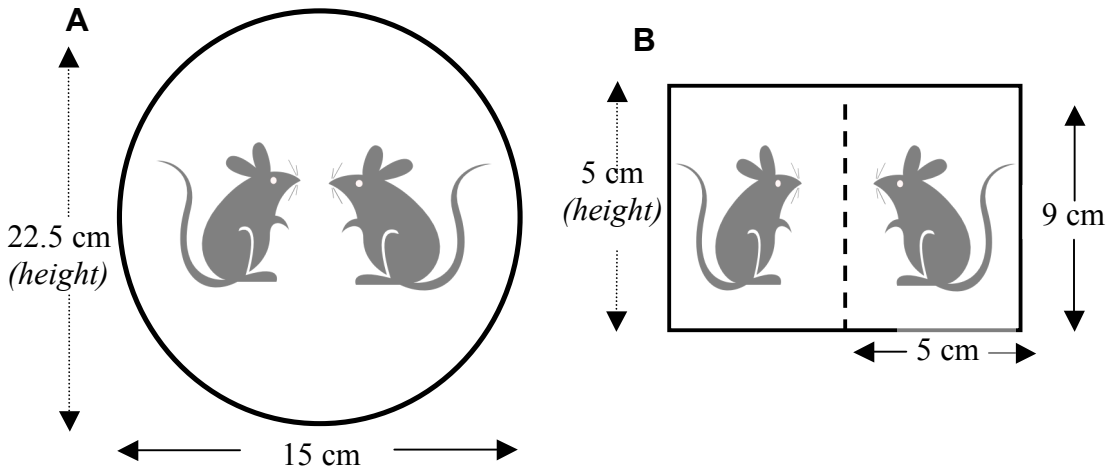

Fig. 3.1. A graphical depiction of the test apparatuses. In both paradigms, one mouse was subjected to the painful stimulus while the other mouse was not. Mice were either tested (A) in a Plexiglas cylinder affording opportunity for unlimited contact, or (B) in a Plexiglas cubicle separated from their test partner by vertical metal bars, allowing for partial contact.

3.2.4 Gonadectomy and Hormone Replacement

(a) Gonadectomy

Castration surgery was performed under isoflurane/oxygen anesthesia. Bilateral incisions were made in the scrotum, and testes were isolated and

exposed. A hemostat was used to clamp the vas deferens, and the testis and testicular fat was removed from each side. Sutures were placed using 3-0 silk as necessary to close the incision. Sham gonadectomy was performed under similar conditions, except that no testicular tissue was removed. Behavioural testing commenced no less than two weeks following surgery.

(b) Hormone Replacement

Approximately one week following gonadectomy, Silastic tubing (0.062" i.d.) was cut to a length of 15 mm, and packed with crystalline testosterone propionate to a length of 10 mm (approximately 10 mg). The ends of the capsule were sealed with Silastic adhesive. Pellets were cured overnight in PBS and rinsed with 70% ethanol followed by sterile saline just prior to implantation. These procedures are adapted from Lindzey et al. (1998), who found capsules of these dimensions to restore 80% of seminal vesicle weight and reverse the plasma testosterone reduction resulting from castration in male mice. Empty pellets were used as a control. Pellets were implanted subcutaneously at the shoulder, under isoflurane/oxygen anesthesia. The incision was closed with 3-0 silk. Behavioural testing commenced no less than two weeks following pellet implantation.

3.2.5. Fecal Boli

Fecal boli deposits were determined by counting boli immediately post-injection, and subtracting this amount from the number of boli counted at the end of the post-injection period. Initial boli counts did not differ between groups.

3.4.6. Statistical analyses

All data was input into SYSTAT v.10 for statistical analysis and graphically displayed using Prism 5.0 (GraphPad Software). A criterion alpha = 0.05 was set for all statistical analyses.

3.5 Results

3.5.1 Pain inhibition produced by social interaction with unaffected, androgenically intact, stranger male mice.

One-way ANOVA performed on writhing behaviour indicated a significant main effect of condition ($F_{3,57} = 4.73$, $p < 0.01$). Posthoc tests revealed that mice tested in the presence of an (uninjected) intact or castrated but hormonally replaced male mouse exhibited significantly less writhing behaviour than those tested either in isolation or in the presence of an (uninjected) castrated male mouse (Fig. 3.2).

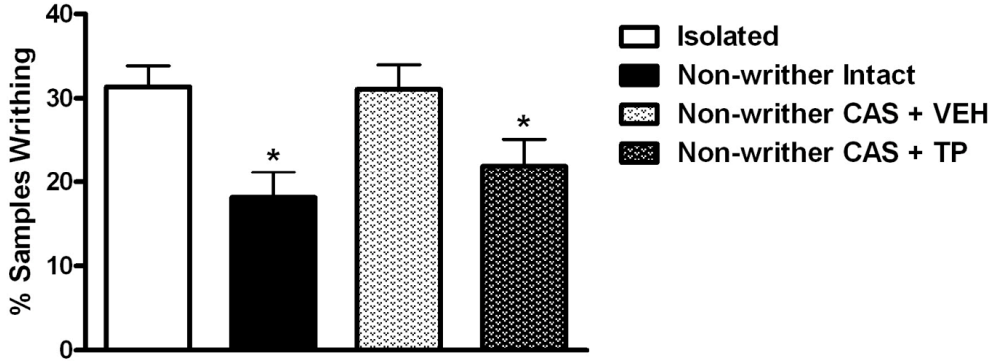

Fig. 3.2. Reduction of pain behaviour in stranger male dyads in which the non-writher is hormonally intact or testosterone replaced and full contact is permitted. Bars represent mean ± S.E.M. samples featuring writhing behaviour. Male mice injected with acetic acid ("writher") tested in the presence of a hormonally intact, unaffected, stranger male mouse ("non-writher") significantly inhibit their writhing behaviour. Castration of the non-writhing mouse abolishes the effect, and testosterone propionate (TP) replacement reinstates the inhibition. VEH = vehicle treatment (sesame oil). *p<0.05 compared to Isolated condition.

3.5.2 Pain hypersensitivity produced by limited social interaction with unaffected stranger male mice.

A two-way (sex x testing condition) ANOVA performed on writhing behaviour revealed a significant interaction ($F_{1,92} = 7.2$, $p < 0.01$). Subsequent *t*-tests comparing to the relevant isolated condition revealed significant differences in writhing in one case only, between two males tested beside each other (separated by jail bars), who each displayed significantly more writhes ($p < 0.05$; Fig. 3.3A). A trend towards *decreased* writhing in female-female dyads was also observed ($p = 0.12$). In contrast, no significant changes were observed in mixed-sex dyads under similar conditions (Fig. 3.3B). Male mice in same-sex dyads deposited significantly more fecal boli than isolated males ($F_{1,90} = 4.8$, $p < 0.05$; $t_{46} = 2.5$, $p < 0.05$) (Fig. 3.3C). This effect of testing condition was not observed amongst same-sex female dyads or amongst different sex dyads (Fig. 3.3C,D).

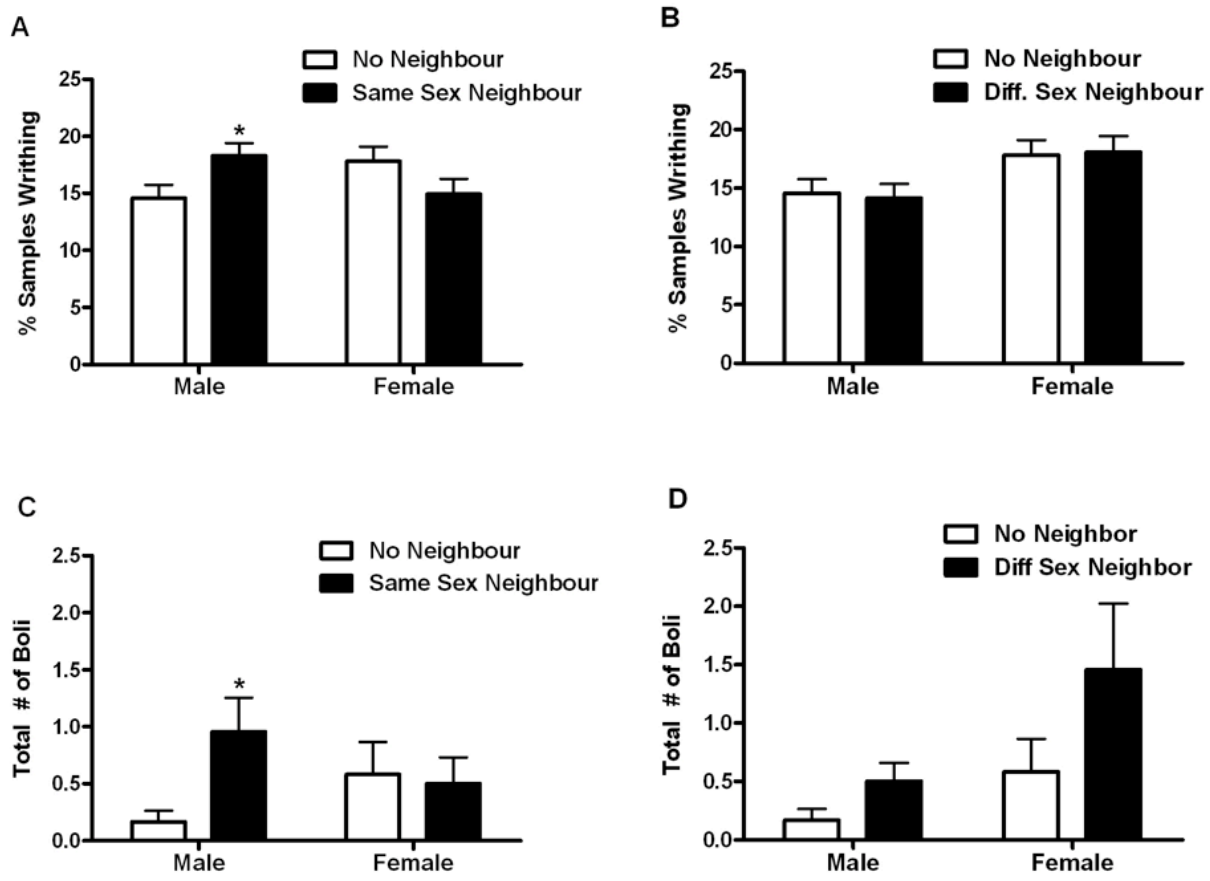

Fig. 3.3. Hyperalgesia in same-sex male dyads in which limited contact is permitted. Bars in A and B represent mean ± S.E.M. samples featuring writhing behaviour. (A) Male mice display significantly increased writhing behaviour in the presence of an unfamiliar neighboring male non-writher ("Same Sex Neighbor") separated by vertical metal bars. *p<0.05 compared to relevant Isolated condition. Female mice display a trend (p=0.12) in the opposite direction. (B) Male writhers tested in the presence of female non-writhers and female writhers tested in the presence of male writhers ("Diff. Sex Neighbor") do not exhibit altered writhing behaviour. Bars in C and D represent mean ± S.E.M. number of fecal boli deposited during the writhing test. (C) Male writhers deposit significantly more fecal boli when tested in the presence of an unfamiliar male non-writher, whereas females and mice tested in the presence of the opposite sex (D) deposit similar numbers of fecal boli compared to those tested in isolation. *p<0.05 compared to relevant Isolated condition.

A two-tailed Student's *t*-test performed on paw-withdrawal latencies also indicated a significant effect of condition, such that male mice, tested in the presence of a same-sex unfamiliar, unaffected, physically barred conspecific displayed significantly reduced paw-withdrawal latencies relative to isolated testing ($t_{46} = 2.03$, $p < 0.05$; Fig. 3.4A). No difference relative to the isolated condition was observed amongst different sex dyads ($t_{42} = 0.64$, *ns*; Fig. 3.4B).

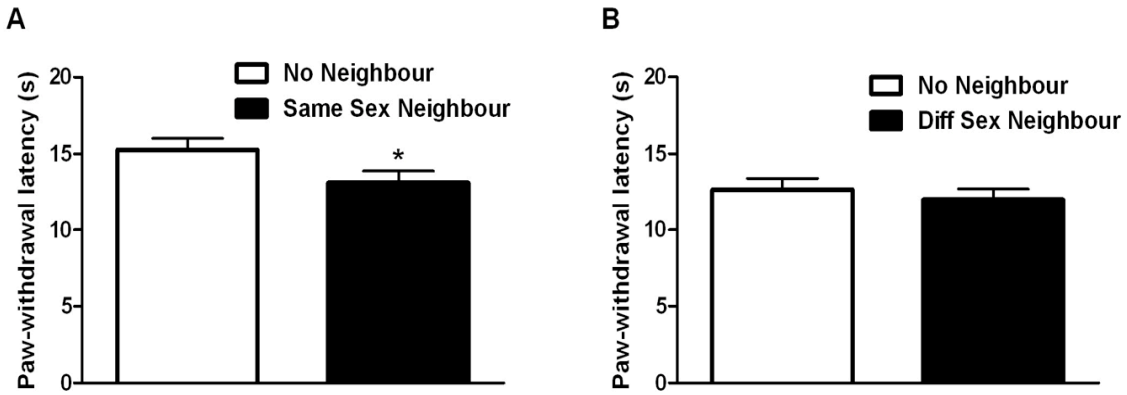

Fig. 3.4. Thermal hypersensitivity among same-sex male dyads in which limited contact is permitted. Bars in A and B represent mean ± S.E.M. latency to withdraw from a noxious thermal stimulus applied to the hind paws. (A) Male mice display significantly reduced paw-withdrawal latencies (i.e., hyperalgesia) when tested in the presence of an unfamiliar male neighbor. Male mice in different-sex dyads (B) show no alterations in noxious thermal sensitivity.

3.5.3 Laboratory Effects

Baseline (i.e., isolated condition) writhing levels differ considerably between the experiments reported here (compare Isolated conditions in Fig. 3.2A,B vs. Isolated conditions in Fig. 3.3A,B). This is likely due to the different

testing environments, as the cylinder experiments were conducted at Haverford College, and the cubicle experiments conducted at McGill University. This is not particularly surprising considering the substantial effect of varying laboratory environments on behaviour (Crabbe, Wahlsten, & Dudek; Chesler, 2002). In both experiments, however, pain behaviour in the isolated condition is at intermediate levels, allowing for the observation of hyperalgesia or analgesia (i.e., no floor or ceiling effects).

3.6. Discussion

These results replicate, in a different laboratory, our previous finding of decreased pain behaviour of male mice tested in the presence of an unaffected stranger male (Langford et al., 2006). We find that this inhibition is dependent on gonadal hormone status, such that when the non-writhing member of the dyad has undergone castration, the effect is abolished, and is reinstated by testosterone replacement. This phenomenon is wholly specific to males, in that females do not display reduced pain behaviour in the presence of an unfamiliar female (Langford et al., 2006), and the effect cannot be induced by administration of testosterone to females in adulthood (unpublished observations).

Furthermore, we find that by limiting physical contact between two stranger male mice, not only is analgesia in the test mouse abolished, but pain is modulated in the entirely opposite direction (i.e., hyperalgesia). This effect is similarly specific to same-sex male dyads, not being observed in female-female or male-female pairings, and was replicated using a noxious stimulus of an entirely

different modality.

The relevant mechanism of social communication between the mice is currently unknown. Previous studies have shown that urinary odors, perhaps through hormonally derived pheromones, are powerful signals for behaviour in rodents (Chamero et al., 2007). Therefore, the most obvious route for the observed threat communication is olfactory. However, the social communication of pain status producing the empathy effect we described previously was *visual*, and that modality may also subserve the modulations seen here. In the writhing test, there are two potential sources of visual information: the abdominal constrictions themselves, and facial expressions of pain that we have shown to reliably accompany those constrictions (unpublished data). It is much more difficult to imagine, however, a visual transmission of information in the radiant heat paw-withdrawal test, in which the pain behaviour (a simple withdrawal from the stimulus) occurs within fractions of a second and is *not* associated with a facial expression recognizable to the experimenter (see Chapter 5).

3.6.1. Social Stress-Induced Analgesia

We have suggested that the unimpeded proximity of an unfamiliar male may result in a form of social stress-induced analgesia (SIA) in the test mouse (Langford et al., 2006). Because the mice are drawn from separate cages and placed together in a novel environment, dominance hierarchies are not set, and the 30-min acclimation period prior to injection gives little time to settle this issue. Note that the testing apparatus is a neutral environment, novel to both members of

the dyad, and thus there is no implied dominance like that found in a resident-intruder paradigm (Thurmond, 1975). As a result, the presence of an unfamiliar and potentially dangerous conspecific likely initiates a stress response that may in turn affect pain sensitivity in the injected test mouse. Indeed, social stimuli have long been known to induce SIA in rodents. The most well-studied example is SIA from social defeat (the result of an aggressive encounter by a conspecific) in male rodents, which involves both opioid and nonopioid mechanisms (McLaughlin et al., 2006; Thompson & Shuster, 1982; Randall & Rodgers, 1988). Cross-species threat stimuli involving predators (or their odors) also produce SIA in rodents (Kavaliers & Colwell, 1994; Lester & Fanselow, 1985; Vendruscolo et al., 2006) and the underlying neurochemistry is known to vary by sex (Kavaliers & Choleris, 1997; Kavaliers & Colwell, 1991). We demonstrate here that the presence of a gonadally intact stranger male — who in the natural environment, would represent a rival for territory, resources, and females — may also serve to activate the same descending antinociceptive circuitry that produces defeat and predator SIA, even prior to an adversarial encounter. That the presence of an unaffected stranger male mouse is stressful to the injected test mouse has been previously demonstrated by the increased number of fecal boli emitted compared to similar testing in isolation, with familiar males, or when *both* mice are injected with acetic acid (Langford et al., 2006, Fig. S4B). Normal social interactions between unfamiliar mice are, of course, common and even preferred by even adult males over non-social options (Van Loo, Van de Weerd, Van Zutphen, & Baumans, 2004; Winslow, 2003). What appears to make the difference in this

case is the fact that one of the mice in the dyad is in pain, and thus especially vulnerable. Indeed, we have shown that stress levels (as estimated by fecal boli) in the habituation period (i.e., before acetic acid injection) are equivalent across different social testing conditions (Langford et al., 2006, Fig. S4A).

That castration of the non-writher eliminates the observed pain inhibition suggests that removal of gonadal hormones abrogates the social threat. Pheromones contained in urine have been shown to specifically promote inter-male aggression in hormonally intact mice (Chamero et al., 2007; Novotny et al., 1985). The absence of such aggression-promoting pheromones may therefore result in reduced social threat perception and therefore normal pain responding.

3.6.2. SIA or Avoidance of Pain Behaviour Display?

It is important to note that we cannot here distinguish whether the social stimulus reduces pain sensitivity in the observed mouse (i.e., produces true SIA), or simply reduces the *display* of pain behaviour (consciously or otherwise) without any reduction in perceived pain. The inhibition of writhing behaviour was not reversed by naloxone (10 mg/kg, i.p.) in our hands (data not shown), likely ruling out an opioid-mediated SIA, but non-opioid forms of SIA would be unaffected. If pain behaviour is interpreted by conspecifics as indicating vulnerability it could invite aggressive attacks. Therefore, males may have evolved the tendency to suppress the overt display of pain when in the presence of a potentially threatening conspecific. It is also obviously adaptive to hide evidence of vulnerability from predators, but it is not at all clear why only males

would choose to do so. Note that the SIA and display avoidance explanations of the current phenomenon are not mutually exclusive, since an ideal means of inhibiting pain display would be to activate endogenous analgesia circuitry such that there was less pain *to* display.

3.6.3 Social Stress-Induced Hyperalgesia

Although SIA has been far more extensively studied, stress has also been shown to modulate pain sensitivity in the opposite direction, and there is growing interest in the phenomenon of stress-induced hyperalgesia (SIH) (Imbe et al., 2006). A number of models have also been established to assess SIH, such as inescapable holding (by the nape of neck) and novelty exposure (Vidal & Jacob, 1982), exposure to low-frequency vibration (Dufton et al., 2008), repeated cold (Ohara et al., 1991), restraint (Torres et al., 2001), swim (Quintero et al., 2000), and social defeat (Andre et al., 2005; Marcinkiewcz et al., 2009) stress. It should be noted that many of these models are also used to establish SIA; the determining factor appears to be the repetitiveness of the stressor or time lapsed post-stress. Generally, models that induce SIH involve chronic exposure to the stressor or only evoke SIH days after the stressor (King et al., 2003), thus implying that SIH may be the result of more psychological stress (versus more acute physical stress in SIA models). Indeed, this observation is in line with evidence from human literature detailing strong comorbidity between mood disorders and chronic pain (Bair et al., 2008). By limiting physical contact in our paradigm, we have eliminated this acute stress evoked by the potential for

physical aggression, perhaps triggering psychological stress in the mere presence of an unfamiliar stranger male, who still represents competition and potential aggression. However, it should be noted that incidences of fighting were almost never observed in our cylinder (i.e., physical contact) condition.

It has also been proposed that the severity of the stressor differentially modulates pain sensitivity, such that more severe stressors evoke SIA, while less severe stressors evoke SIH (Vidal & Jacob, 1982). This hypothesis is most clearly corroborated by human accounts of a complete lack of pain perception despite major injuries in sporting events, major accidents, or battle versus enhanced pain perception amongst those with anxiety disorders (Gureje, 2008). In humans, it has also been shown that fear (induced by electric shock) produces analgesia, whereas anxiety (induced by the threat of electric shock) induces hyperalgesia (Rhudy & Meagher, 2000). Our results appear to support these hypotheses, such that the immediate physical threat in the cylinders may have induced fear and therefore SIA, whereas reducing this threat by limiting contact or appraisal of the potential danger may have induced only anxiety (by the mere presence of a potential foe), thereby producing SIH.

That these phenomena are specific to male mice is not surprising. First, physical aggression is largely specific to males, denoted by the considerable involvement of testosterone in mediating aggressive behaviour (Giammanco et al., 2005); therefore, these paradigms may serve as sex-specific stressors. Second, crowding has been shown to be stressful in males, but not females (Brown et al., 2003); the close proximity imposed by the testing apparatus may have also thus

exerted sex-specific effects. Finally, there appears to be a basic sex difference in the behavioural response to stress, which evokes the canonical "fight-or-flight" response in both sexes, but females may secondarily activate a "tend-or-befriend" response (Taylor et al., 2000) that in the current paradigm would mitigate against pain-related vulnerability in front of a conspecific being interpreted as a stressor.

3.6.4 Conclusions

Studying the effects of such social stressors may be important to all social species, especially considering the robust social factors affecting pain sensitivity in humans (Chambers, Craig, & Bennett, 2002; Flor, Turk, & Rudy, 1989), as well as recent evidence suggesting the impact of social factors in rodent pain models (Giosa et al., 2009; Langford et al., 2006; Raber & Devor, 2002). Furthermore, the observation that a similar social stressor may modulate pain in either direction suggests the involvement of different pathways in each case, and their study may lead to a better understanding of the underlying neural mechanisms of stress-induced changes in pain sensitivity. The present findings obviously have direct implications as well for the design of rodent pain experiments, in which social effects on pain behaviour are unappreciated modulatory factors.

Chapter 4

Social Approach to Pain in Laboratory Mice

4.1 Rationale

A review of video taken from our original empathy phenomenon, specifically of OW dyads, led us to hypothesize that social contact, initiated by the non-writhing mouse, might be associated with reductions in pain behaviour exhibited by the writhing mouse. We therefore used novel paradigms to determine patterns of social approach in dyads where only one mouse was injected as well as the relationship between social approach and pain behaviour. We also wanted to determine whether the absence of oxytocin signaling affected pain-related social approach.

4.2 Abstract

It has been recently demonstrated that an animal's pain behaviour can be modulated by the presence of a conspecific, but what remains unclear is whether such pain behaviour can serve the function of soliciting social approach. Using a novel social approach paradigm, we tested mice in various dyadic or triadic conditions, including "jailed" mice — some in pain via intraperitoneal injection of 0.9% acetic acid — and test mice free to approach or avoid the jailed mice. We observed a sex-specific effect whereby female, but not male, mice approached a familiar same-sex conspecific in pain more frequently than an unaffected familiar or unfamiliar (but affected) conspecific. Despite a substantial literature emphasizing oxytocin's role in affiliative and pair-bonding behaviour, this effect was also observed in female mice lacking the oxytocin receptor, suggesting that pain-related social approach may not be mediated by oxytocin. Furthermore, we found that the frequency of contact by the test mouse negatively correlated with

the pain behaviour of the jailed mouse, suggesting that proximity of a familiar unaffected conspecific may have analgesic properties.

4.3 Introduction

The experience of pain clearly serves adaptive functions. It acts as a warning signal leading to adaptive avoidant behaviour, as a healing promotor by inhibiting potentially damaging movement, and as positive punishment protecting against similar future painful encounters. In humans at least, behavioural responses associated with the pain experience also serve adaptive functions, such as the solicitation of aid. The cry of an infant in pain, for example, is a salient cue for appropriate caregiving responses (Johnston & Strada, 1986). Similarly, verbal pain complaints effectively solicit aid from family, friends, and health practitioners. Also, more subtle behavioural responses, such as facial expressions, can be accurately detected and responded to in order to diminish pain, especially in clinical populations where communication is limited or absent (Grunau et al., 1998; LaChapelle, Hadjistravopoulos, & Craig, 1999; Manfredi et al., 2003; Nader et al., 2004). It is unclear, however, whether pain behaviours exhibited by rodents serve such an adaptive function in a social environment. The present study aims to determine whether the display of overt pain behaviours can influence the behaviour of an observing mouse, and whether such ensuing behaviour impacts pain sensitivity.

Recent evidence suggests that mice can recognize and respond to the painful state of a conspecific. Raber and Devor (2002) showed that rats bred to exhibit low levels of autotomy after neuropathic injury housed with rats bred to

exhibit high levels of autotomy displayed significantly increased pain behaviours that effectively masked the typically robust genotype. Similarly, we have shown that co-housed (familiar) dyads exhibit significant hypersensitivity to a noxious stimulus when both mice were injected simultaneously; that is, when observing a cagemate in pain. Moreover, we showed that the mere observation of a cagemate in pain was enough to sensitize an animal to a noxious stimulus of an entirely different modality (Langford et al., 2006), suggesting effective communication of pain — interpreted as empathy — amongst familiar dyads. In a very recent study, Gioisa and colleagues (Gioiosa et al., 2009), also described social communication of pain amongst familiar male mice that displayed *reduced* pain behaviour in the presence of a similarly affected cagemate, despite differential social status. Collectively, these studies provide support for the effective social communication of pain, and stress the importance of familiarity in mediating the phenomenon.

The present experiments assess whether mice can indeed distinguish the presence or absence of pain in another, and also whether such pain communication facilitates adaptive behaviour in an unaffected observing mouse. If the purpose of communicating a pain state is to solicit aid, observation of another's pain behaviour should produce a response in the observer that is consistent with providing such aid, such as social approach. In this case, social approach may be described as "prosocial" in that providing such aid incurs no benefit to the observer. That it also imposes no obvious cost on the observer suggests it should not, however, be characterized as altruism (Trivers, 1971).

Given the extensive literature documenting the role of the neuropeptide, oxytocin (OT), in social behaviour (Winslow & Insel, 2002), we hypothesized

that this ability to distinguish and/or respond to the pain states of familiar others would be reduced in mice lacking the gene encoding the OT receptor.

Finally, if approach toward a mouse in pain is to be considered adaptive, it may be associated with lower levels of pain behaviour. In light of findings implicating familiarity as a mediator of pain communication, we also predicted that such effects be specific to familiar mice. We have addressed these questions by observing the pain and positional behaviour of mice in a set of dyadic or triadic social interactions.

4.4 Materials and Methods

These studies were conducted at Haverford College and McGill University. All procedures were approved by local Institutional Animal Care and Use Committees, subject to national (U.S. and Canadian) guidelines.

4.4.1 Subjects

Subjects were naïve male and female outbred CD-1® mice (ICR:Crl), aged 7-12 weeks old, purchased from a supplier (Charles River, Boucherville, QC or Harlan, Indianapolis, IN), or bred onsite in our vivarium (McGill University). For the oxytocin (OT) experiment, subjects were oxytocin receptor null mutant (*Oxtr*$^{-/-}$; OTR KO) or their wildtype (WT) counterparts. Heterozygous male and female mutant OTR KO mice were obtained from Dr. Larry J. Young (see Takayanagi et al., 2005), and interbred in our vivarium at McGill University to produce new WT and KO mice of both sexes (heterozygotes were not tested, but rather used for further breeding), confirmed by polymerase chain reaction (PCR).

Mutant mice are on a C57BL/6J fully congenic background. Mice were housed in same-sex groups of 4-6 mice under a 12:12 h light/dark cycle with lights out at 19:00 h, and were provided with food (Harlan Teklad 8604) and tap water *ad libitum*. All mice were tested only once.

4.4.2 Nociceptive Assay

The nociceptive assay used in these experiments was the acetic acid abdominal constriction ("writhing") test, a model of visceral inflammatory pain that involves an intraperitoneal injection (10 ml/kg) of dilute 0.9% glacial acetic acid. The writhing test was chosen for its obvious behavioural display consisting of abdominal constriction accompanied by stretching of the body and hind paw extension. Furthermore, the writhing test is sensitive to mild analgesics and subtle modulatory effects, lending itself well to the study of social modulation of pain (Jordan & Mogil, 2006).

4.4.3 Testing Apparatus

For both social approach paradigms, we used a covered Plexiglas alley (77 cm long x 5 cm wide; 15 cm wall height), with one or both ends separated from the central region by wire mesh screens (Experiment 1) or vertical metal bars (Experiment 2) to create a separate "jailed" compartment (Fig. 4.1). A piece of white tape was placed on the alley 10 cm (approximately 1 body length) from the wire mesh to denote the "proximal" region in Experiment 1.

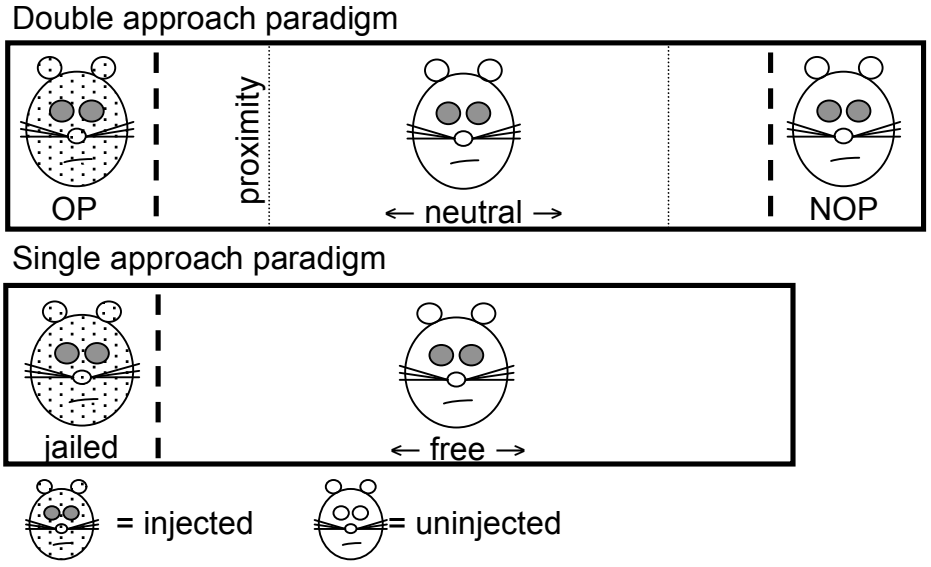

Fig. 4.1. Schematic diagram of social approach apparatus used in Experiment 1 (double approach paradigm; top) and Experiment 2 (single approach paradigm; bottom). OP: Observed pain; NOP: No Observed Pain.

4.4.4 Experimental Paradigms

Experiment 1: Double approach paradigm

For each freely moving "observer" mouse, two "demonstrator" mice were placed in the apparatus, one in each end compartment. In each trial, one of the demonstrator mice was injected with acetic acid prior to being placed in its compartment ("Observed Pain" or "OP" condition); at the other end (counterbalanced between runs) was a similarly handled but uninjected demonstrator mouse ("No Observed Pain" or "NOP" condition). Location scoring of the observer mouse was conducted via time-sampling (every 20 sec) by an observer blind to condition; at each sample, location was scored as either proximal to (within 10 cm of) the Observed Pain demonstrator mouse, proximal

to the No-Observed Pain demonstrator mouse, or in the neutral territory between them. Four sub-experiments, varied with respect to sex and genotype, were conducted as described below.

(a) Male (n=13) and female (n=15) CD-1 mice were tested in the double approach paradigm (all same-sex cagemates), as described above.

(b) Female mice (n=13) were tested in the double approach paradigm, with stranger female mice (as opposed to same-sex cagemates) serving as demonstrators.

(c) Female mice (n=9) were tested in the double approach paradigm, with cagemate demonstrators, except that instead of the wire mesh screen, clear Plexiglas barriers separated observer from demonstrator mice in the apparatus.

(d) Female OTR-KO mice (n=15) and their wildtype (n=8) counterparts were tested in the double approach paradigm, with same-sex/genotype cagemates serving as demonstrators.

Experiment 2: Single approach paradigm

Male (n = 25 dyads) and female (n = 30 dyads) CD-1 mice were tested using the single approach paradigm described below. The jailed mouse was either a cagemate of the free mouse or a stranger, in a between-subjects design. Thirty min prior to the start of the observation period, the "free" mouse was placed in the central region of the apparatus, and a "jailed" mouse was placed into the end compartment. After habituation, the jailed mouse was briefly removed and injected with acetic acid. In this experiment, instances of physical contact of the free mouse with the jail bars and/or the jailed mouse were recorded via time-

sampling (every 20 s). Both pain behaviour (of the "jailed" injected mouse) and position (of the "free" uninjected mouse) were scored.

4.4.5 Statistical Analyses

After scoring digital video files, data was input into SYSTAT® (v. 10). Data were analyzed by ANOVA or Pearson's correlations, as appropriate, followed by posthoc testing using Tukey's test or simple main effects analyses. For all analyses, an alpha level of 0.05 was considered significant.

4.5 Results

4.5.1 Experiment 1: Approach behaviour is dependent on pain state of conspecific in outbred, wildtype and OTR KO females.

Data for Experiment 1 were analyzed according to procedures used by Nadler et al. (2004). Where a significant overall effect of location in the apparatus was observed, the number of bins in which the animal was located in the "neutral" territory was subtracted out, and a binomial probability was calculated to test the null hypothesis that the number of bins located in proximity to the OP mouse was equal to the bins in proximity to the NOP mouse.

(a) *Female-specific approach behaviour towards a cagemate in pain*

A 2 (sex) x 3 (location: proximal to OP, proximal to NOP, neutral) mixed factorial ANOVA was conducted. There was a significant sex by location interaction ($F_{2,52} = 4.0$, $p < 0.05$), such that female mice ($F_{2,28} = 4.2$, $p < 0.05$) exhibited increased frequency of proximity to the Observed Pain demonstrator

relative to the No Observed Pain demonstrator; (44/73 bins, binomial probability < 0.05). In contrast, males were found in each of the three regions with equal frequency ($F_{2,24} = 0.1$, *ns*) (Fig. 4.2A; left).

(b) *Female proximity to mouse in pain not observed in stranger dyads*

One-way repeated measures ANOVA conducted on female mice tested for their behavioural response to the presence of strangers yielded no significant main effect of position ($F_{2,24} = 0.5$, *ns*). There was no difference among the mean percentage of time spent in the three locations (Fig. 4.2A).

(c) *Female proximity to cagemate in pain not observed when tactile contact is blocked*

A one-way repeated measures ANOVA conducted on female mice tested for their behavioural response to the presence of cagemates separated by Plexiglas barriers yielded no significant main effect ($F_{2,22} = 0.02$, *ns*). As with the behavioural response to stranger mice, there was no difference among the mean percentage of time spent in the three locations in the apparatus (Fig. 4.2A; right).

(d) *Both WT and OTR KO females display similar approach to cagemate in pain*

A mixed factorial ANOVA conducted on female OTR KO mice and their wildtype counterparts, tested in the presence of cagemates separated by wire mesh barriers, yielded a significant main effect of location ($F_{2,42} = 5.0$, $p = 0.01$), that was not dependent on genotype (genotype x location interaction: $F_{2,42} = 0.03$, *ns*). As in the female outbred mice, both OTR KO and WT females spent more time

proximal to the Observed Pain demonstrator than the No Observed Pain demonstrator (48/79 bins; binomial probability = 0.01; Fig. 4.2B).

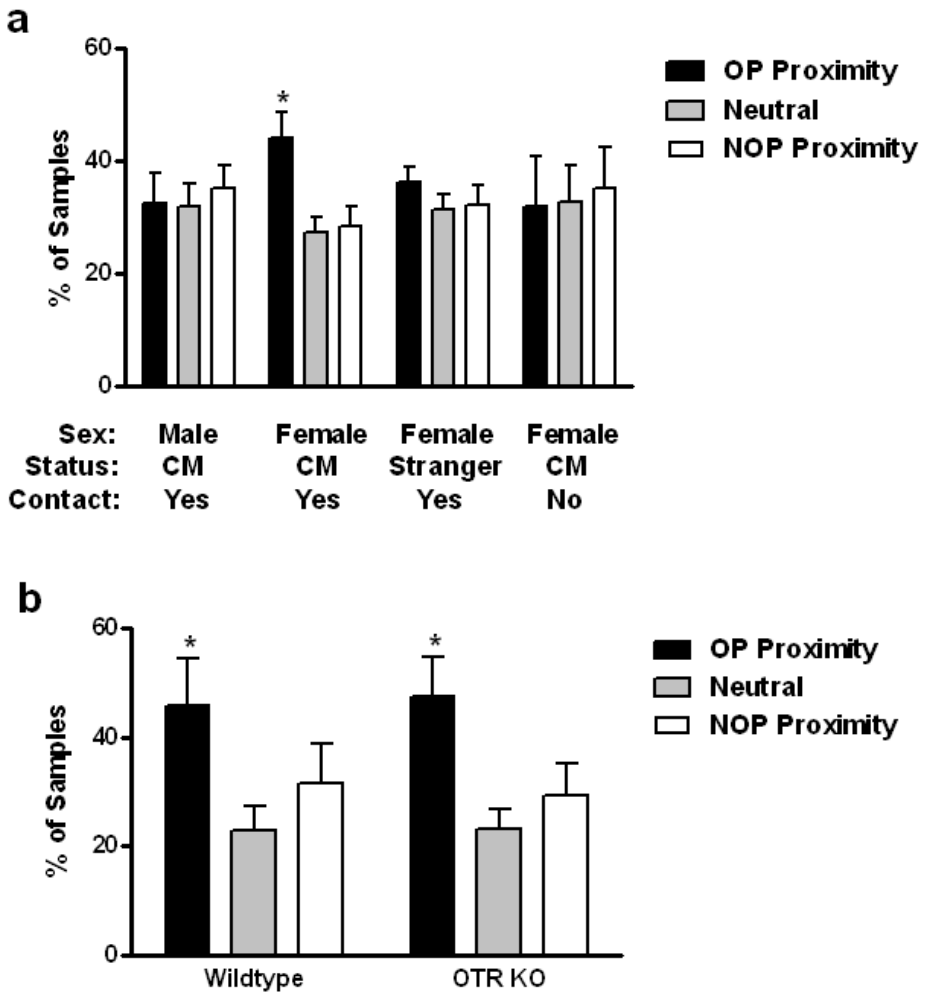

Fig. 4.2. Approach behaviour in male and female CD-1 (**a**) and (**b**) WT and OTR KO observer mice in the double approach paradigm. CM: cagemate. Bars denote mean ± S.E.M percentage of samples in which observer mice were found to be located in either proximity region (OP or NOP) or in the central neutral region. *Significantly different from other regions within-condition, $p < 0.05$.

4.5.2 Experiment 2: Cagemate location is correlated with pain behaviour

Frequency of contact (by the free mouse) with the ”jail” was significantly negatively correlated with writhing behaviour of the jailed mouse in cagemate (r = -0.41, $p < 0.05$, df = 28), but not stranger (r = -0.22, *ns*, df = 23) dyads (Fig. 4.3). Analysis by sex revealed a stronger relationship in female cagemate dyads (r = -0.47, p = 0.06, df = 16) than male cagemate dyads (r = -0.32, p = 0.29, df = 10).

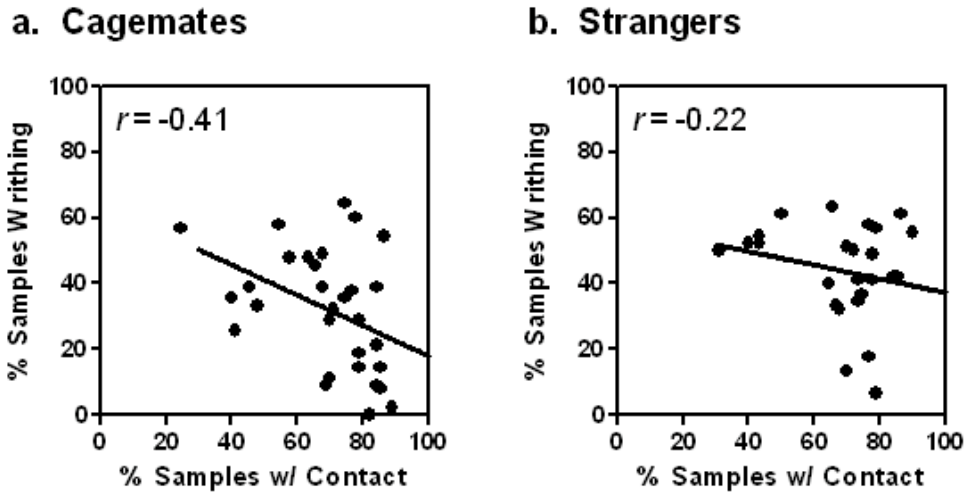

Fig. 4.3. Scatterplots indicating the relationship between physical proximity (x-axis; percentage of samples featuring contact between the free mouse and the “jail”) and observed pain behaviour (y-axis; percentage of samples featuring writhing behaviour) in male and female cagemate (**a**) and stranger (**b**) mice tested in the single approach paradigm.

4.6 Discussion

Using a novel experimental paradigm, we have demonstrated that female mice are more likely to approach a cagemate that is displaying pain behaviour than an unaffected, but equally familiar, mouse. This pattern of approach was not

observed amongst familiar males, nor was it observed amongst unfamiliar females. Prevention of physical contact by a Plexiglas barrier abolished this preferential approach behaviour toward a familiar female in pain. Despite its established role in mediating a variety of social behaviours, OT was not required for mediating preferential social approach toward a familiar female in pain. This pattern of approach was also observed in females lacking the gene encoding the OT receptor, suggesting a distinction between affiliation and social approach. Finally, a significant negative correlation was present between the proximity of a cagemate to an affected mouse and the pain behaviour displayed by that mouse. No correlation between location and pain behaviour was observed in strangers. These findings are consistent with the hypothesis that overt pain behaviours serve to solicit aid from conspecifics in the immediate social environment.

4.6.1 Sex Differences in Social Approach

Experiment 1 shows that preferential social approach to a familiar mouse displaying pain behaviour was an effect specific to female mice. Approach behaviour amongst familiar male triads did not differ with respect to the demonstrators' pain state. This increased approach among females cannot be explained by the novelty associated with the behavioural display as this pattern was not observed amongst female strangers, nor is there any reason to expect that males should not show a similar frequency of approach to a novel stimulus. In fact, in light of a rodent's natural preference for social novelty (Moy et al., 2008), it is interesting that females choose to approach a cagemate more frequently than a stranger. Therefore, the pain display of a conspecific appears to be a more

salient cue for social approach.

Sex differences in social behaviour have been well documented in the rodent literature, and an extensive investigation of social behaviour specifically in the CD-1 strain was recently reported (Malloy et al., 2005). This pattern of approach observed may also be associated with differential coping mechanisms in response to stress (Taylor et al., 2000). In our paradigm, it is possible that the observation of pain acted as a mild stressor, resulting in a "tend-and-befriend" response comprising increased parental care and affiliative behaviour amongst females (versus a "fight-or-flight" response in males). Literature on rodent prosocial behaviour generally concerns parental care, which is highly sexually dimorphic in mammals, with females displaying greater levels of pup care behaviours than males in 95% of mammalian species (de Jong et al., 2009). In the rare rodent species that exhibit biparental care, such as the monogamous male prairie vole (*Microtus ochrogaster*), deviations in estrogen receptor-α distribution appear to mediate prosociality (Cushing & Wynne-Edwards, 2006). Therefore, robust sex differences in prosocial behaviour, as displayed in the current study, could be expected in species (such as *Mus musculus*) with sexually dimorphic parental care.

4.6.2 Oxytocin and Social Approach

There is a large literature documenting oxytocin's role in modulating social behaviours in both humans (Kosfeld, et al., 2005) and rodents (Insel, 1992; Ferguson et al., 2000). It was therefore surprising to find that female mice lacking the OT receptor did not show deviation in social approach behaviour from

that of their WT or outbred counterparts, especially considering the significant role OT plays in sexually dimorphic traits (Taylor et al., 2000).

This finding suggests that observed social approach behaviour toward a cagemate in pain is distinct from other affiliative behaviours such as social recognition and pair bonding. In fact, OT knockouts have been shown to exhibit similar levels of social approach to their WT counterparts as well as the gregarious C56BL/6J strain (Crawley et al., 2007). Moreover, in humans, oxytocin has been shown to facilitate trust (Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005), but not altruistic behaviour (Zak, Stanton, & Ahmadi, 2007) in games of monetary exchange.

Oxytocin has been reported to be analgesic in a variety of pain tests when administered into the brain (e.g., Ge, Lundeberg, & Yu, 2002) or systemically (Lundeberg et al., 1994); however, unpublished data from our laboratory suggests that this analgesia is actually mediated via the vasopressin-1A receptor. Furthermore, we have run OTR KOs on a battery of pain tests (including the writhing test), and found no significant genotype differences in any assay (unpublished data).

4.6.3 Social Proximity and Pain Display

Experiment 2 indicates that the proximity of a cagemate is inversely related to the pain behaviour expressed by the demonstrator exposed to the noxious stimulus. We have noted previously a very similar inverse correlation between contact initiated by an unaffected mouse and pain behaviour in the affected mouse in cagemate, but not stranger, dyads completely free to interact

socially within a cylindrical enclosure (data not shown). Although we cannot draw causal conclusions regarding a putative analgesic effect of a "friendly" social stimulus, there are several potential interpretations. It is possible that the proximity of a familiar other provides a kind of social buffer with analgesic properties. Social buffering is a well-established phenomenon found in a variety of species, including rodents, whereby the presence of a conspecific reduces distress responses (Kikusui, Winslow, & Mori, 2006). We show that contact of the unaffected free mouse appears to be associated with lower pain behaviour in the affected mouse, but only if the animals are cagemates. That is, in cagemate dyads, the amount of time the observer spent in proximity to the jail is negatively correlated with the amount of pain behaviour in the demonstrator. Socially induced analgesia among familiar others has also been observed among male sibling mice (D'Amato, 1998) that, when reunited after a period of separation, exhibit reduced sensitivity to a noxious thermal stimulus, as well as increased morphine analgesic sensitivity.

An alternative explanation is that mice that are experiencing less pain "solicit" less social approach behaviour from the familiar conspecific. This hypothesis might be tested by varying concentrations of the noxious stimulus to induce different levels of behavioural response, to determine if the intensity of the noxious stimulus produces reliable variations in the frequency of social approach. It is also possible that the display of pain behaviour is itself an aversive stimulus, causing the free mouse to avoid the afflicted animal. However, it is not clear why this behavioural response would depend on the social relationship among the animals, since the positional behaviour of strangers was not correlated with pain

behaviour in the demonstrator.

We conclude that some form of social communication is taking place among the mice in these interactions, although we cannot yet be sure which specific sensory mechanism communicates pain/distress between the animals. In Langford et al. (2006), pain communication was visually mediated; here, however, we find that the enhanced social approach displayed by female subjects toward a cagemate in pain is completely abolished when the subjects are separated by a clear Plexiglas barrier, suggesting that vision alone is not sufficient, and that physical contact may play an important role in this form of communication.

4.6.4 Conclusion

This investigation permits a broader consideration of the *significance* of social modulation of pain in mice (Langford, et al., 2006). Hypersensitivity amongst similarly affected cagemate, but not stranger dyads, was interpreted as evidence for "empathy" (at the level of emotional contagion) in rodents, which reflects a "self-oriented" or inward experience of pain empathy. The current observation of enhanced social approach to a cagemate in pain reflects an "other-oriented" or outward experience of pain empathy. We believe our findings are potentially consistent with the presence of other social emotions like "prosociality" in these animals, at least in females. If indeed we have successfully modeled a form of prosocial behaviour among adult unrelated dyads (social approach towards a cagemate exhibiting pain behaviour), this paradigm may serve as a useful methodological tool for understanding the neural basis of

prosocial behaviours.

Chapter 5

The Mouse Grimace Scale: coding facial expressions of pain in the laboratory mouse

5.1 Rationale

In light of the finding that social communication of pain is visually mediated (See Ch. 2, Fig. 3) we were interested in determining whether mice communicate their pain via facial expression. In an effort to establish whether mice do indeed display observable and reliable facial expressions in response to painful stimuli we collaborated with human facial coding experts at the University of British Columbia to create a facial coding system specific for pain in the mouse.

5.2 Abstract

Here we present the development and assessment of the Mouse Grimace Scale (MGS), a standardized coding system used to calculate an animal's facial pain score and to make pain-no pain judgments. The MGS comprises five codable features, with three similar to those involved in human facial expressions of pain, scored in terms of intensity relative to the animal's own baseline. We demonstrate high intra-rater and inter-rater reliability of the scale, as well as good accuracy in classifying a painful state in the mouse. We tested the MGS on a variety of commonly used pain assays and found that the scale revealed significant changes in intensity of facial expression in response to stimuli invoking deep pain. In contrast, superficial and neuropathic pain models failed to demonstrate evidence of facial pain. Mice administered cyclophosphamide (100, 200, 400 mg/kg) causing bladder pain or zymosan causing inflammatory pain (0.25, 0.5, 2, 10 mg/ml) demonstrated dose-dependent increases in pain face intensity that was, in turn, dose-dependently reversed by morphine administration (5 and 10 mg/kg). In animal models, where information gleaned from overt

behaviours is limited, other indicators, such as facial expressions, may provide meaningful insight into the animal's pain experience.

5.3 Introduction

Darwin famously asserted that non-human animals are capable of expressing emotion (including pain) through facial expression (Darwin, 1872), using similar movements as do humans, and that this ability may be both innate and adaptive. The ability of infants as well as the congenitally blind to display similar facial expressions of pain as children and adults lends credence the notion of a Primal Face of Pain (PFP; Schiavenato et al., 2007). In humans, of course, the ability to communicate one's mental state presents an advantage to both the sender and receiver, such that help may be offered when the signal is one of distress, or a warning signal may be heeded that ensures the receiver's survival (Williams, 2002).

Facial expressions have been well characterized in humans, and can be reliably coded using the anatomically based Facial Action Coding System (FACS; (Ekman & Friesen, 1978). The FACS has been useful for the evaluation of virtually every emotional expression, including facial expressions of pain. Similar scales, such as the Neonatal Facial Coding System (NFCS), have been adopted specifically to assess pain in particular populations (Grunau & Craig, 1987), and have become a tremendously useful tool in clinical settings for assessing pain and analgesia in clinical populations in which verbal communication is limited or non-existent, such as young children (Grunau et al., 1998), individuals with intellectual disabilities (LaChapelle et al., 1999), autists

(Nader et al., 2004), and those with dementia (Manfredi et al., 2003).

Aside from overt behavioural responses that may lack specificity, there is relatively little we can observe in non-human mammals that would reliably indicate their internal state. Facial expressions have been fairly well characterized in chimpanzees, for which a modified version of the FACS has been applied in order to make direct comparisons of facial expression structure between chimp and human (Parr et al., 2007). Facial expressions have also been studied in rats in response to consummatory stimuli (Grill & Norgren, 1978), where researchers noted a stereotypic and differential display in response to pleasant and unpleasant taste stimuli. Yet, despite evidence that non-human mammals exhibit facial expressions, no systematic attention has been given to facial expressions of pain in any non-human species. Considering the pain field's heavy and continuing reliance on rodent models (Mogil et al., 2009), the ability to reliably and accurately detect pain using facial expression might offer a unique and potentially powerful scientific tool.

A collaboration between a human facial expression of pain laboratory and a mouse pain behaviour laboratory was set up to achieve this goal. The result is a coding system specific to the mouse (but potentially usable across a wide range of mammalian species): the *Mouse Grimace Scale (MGS)*. We report here that for a subset of algesiometric assays—tests of deep pain—the MGS displays admirable accuracy and reliability as a novel dependent measure of pain in the mouse. In addition, experiments reported herein suggest that pain perception in many common assays might be more paroxysmal than previously thought.

5.4 Materials and Methods

5.4.1 Animals

All subjects were CD-1® (ICR:CrI) mice, aged 6-18 weeks, bred in our vivarium with mice obtained from Charles River Laboratories (Boucherville, QC). Mice were housed in groups of 2 or more, under a 14:10-h light cycle (lights on at 07:00 h) in a temperature controlled environment (20 ± 1ºC) with *ad lib* access to food (Harlan Teklad 8604; Madison, WI) and tap water. Each assay utilized a new cohort of mice, such that no subject participated in more than one pain assay. Each cohort consisted of 8-20 mice, with approximately equal numbers of each sex. We found no evidence sex differences in facial expression of pain using the MGS.

5.4.2 Noxious Chemicals

All compounds were obtained from Sigma-Aldrich, and dissolved in physiological saline, except where otherwise noted. See specific assays below for precise doses.

5.4.3 Initial Frame Capture and Scale Development

Mice were individually placed in cubicles (9 x 5 x 5 cm high) with two walls of Plexiglas and two walls of removable stainless steel. A digital video camera was placed immediately outside both Plexiglas walls in order to maximize the opportunity for clear headshots. Mice were acclimated and filmed for 30 min prior to injection (*baseline/"no pain"*) and for 30 min post- intraperitoneal (i.p.) injection of 0.9% acetic acid (*post-injection/"pain"*). Using Windows Media

Player©, individual frames of the WMV files were "grabbed" whenever a clear, unobstructed head shot was observed. In the post-injection period, frames were grabbed specifically during the exhibition of the writhing/stretching behaviour—lengthwise constrictions of the abdominal musculature—normally used as the dependent measure in this assay. The resultant JPEG files were cropped (so that body position was no longer visible) and auto-adjusted for contrast and brightness in Adobe Photoshop® CS 8.0.

Multiple collages of "pain" and "no pain" photographs were compiled and sent to collaborators at University of British Columbia, who used these collages—along with individual baseline and post-injection photos for each subject tested—in order to devise a coding system consisting of facial features they perceived as potentially reliable indices of pain. This coding system was named the Mouse Grimace Scale (MGS; Fig. 5.1a).

Following a formal training session based on the MGS, a randomized set of photos was presented to seven blinded coders at McGill University in order to assess accuracy, reliability and validity of the MGS.

5.4.4 Nociceptive Assays

The following assays were used. All stimuli are thought to be noxious in mice since mice either: 1) reflexively withdraw from them, 2) exhibit presumably recuperative behaviours such as licking/biting/shaking in response to them, or 3) display hypersensitivity (i.e., hyperalgesia and allodynia) to evoking thermal and/or mechanical stimuli. In many cases identical or highly similar insults are known to be associated with pain in humans. Note, however, that there is no

direct evidence for the existence of spontaneous pain in many of these assays. In all cases mice were habituated for 30 min before testing began.

a. Acetic acid abdominal constriction ("writhing") test (AA)

Diluted (0.9%) acetic acid was injected intraperitoneally in a volume of 10 ml/kg. Mice were returned to cubicles and filmed for 30 min post-injection.

b. Allyl isothiocyanate (AITC; mustard oil)

AITC (5%) was injected subcutaneously in a volume of 20 μl into the plantar surface of the right hind paw. Mice were returned to cubicles and filmed for 30 min post-injection.

c. Capsaicin (CAP)

Capsaicin (125 μg/ml; dissolved in 80% saline, 10% Tween and 10% ethanol) was injected subcutaneously in a volume of 20 μl to the plantar surface of the right hind paw. Mice were immediately returned to their cubicles and filmed for 20 min post-injection.

d. Chronic constriction injury (CCI)

CCI surgery was performed under general anesthesia essentially as described by Bennett & Xie (1988). An incision was made at the level of the sciatic nerve, on the right side. Four loose ligatures were tied around all three branches of the sciatic nerve, and the wound closed. Mice were returned to their home cages until testing. On test days, mice were habituated for 30 min before being videoed.

e. Cyclophosphamide cystitis (CYP)

Cyclophosphamide (100, 200, or 400 mg/kg) was injected intraperitoneally in a volume of 10 ml/kg. Mice remained in their home cages for 3.5 h, and were then returned to observation cubicles for 60 min.

f. Formalin test (F_{early}, F_{late})

Formalin (5%) was injected subcutaneously in a volume of 20 μl to the plantar surface of the right hind paw. Mice were returned to cubicles and filmed for 60 min post-injection. The early (acute; F_{early}) phase of the formalin test was denoted as the first 5 min post-injection, and the late (tonic; F_{late}) phase as 15-60 min post-injection.

g. Magnesium sulfate abdominal constriction test ($MgSO_4$)

Magnesium sulfate (125 mg/kg) was injected intraperitoneally in a volume of 10 ml/kg. Mice were returned to cubicles and filmed for 20 min post-injection.

h. Post-incisional model (Post-op.)

Incision was performed under general anesthesia as described by Brennan et al. (1996). A 1-cm longitudinal incision (skin, fascia and muscle) was made on the plantar surface of the hind paw.

i. Spared nerve injury (SNI)

SNI surgery was performed under general anesthesia as described by Shields et al. (2003). An incision was made at the level of the sciatic nerve, exposing its three branches. The tibial and common peroneal nerves were tightly ligated, while the sural nerve was left intact. The wound was closed, and mice returned to their home cages until testing. On test days, mice were habituated for 30 min before being videoed.

j. Tail-clip test (TC)

An alligator clip applying 700 g of force was applied ~1 cm from the base of the tail. The nocifensive endpoint was a purposeful attempt to remove the clip (i.e., head movement toward the tail). Because this assay is sensitive to repeated testing, only one trial was conducted per subject.

k. Tail-flick test (TF)

A radiant heat source was applied to the animal's tail (~3 cm from base). The nocifensive endpoint was reflexive withdrawal of the tail. The intensity of the commercial device (IITC Model 33) was set to 5% of maximum output, resulting in mean latencies of approximately 4 s (data not shown).

l. Tail-withdrawal test (TW)

The distal half of the mouse's tail was immersed in a temperature-controlled hot water bath (45 ºC or 49 ºC). The nocifensive endpoint was reflexive withdrawal of the tail.

m. Zymosan test (ZYM)

Zymosan (0.25, 0.5, 2, or 10 mg/ml) was injected subcutaneously in a volume of 20 μl to the plantar surface of the right hind paw. Mice were returned to cubicles and filmed at 2 h post-injection.

For assays involving a reflexive response (e.g., TC, TF, TW), we grabbed frames during the exhibition of the response itself (*pain*) and compared these to frames grabbed after the onset of the stimulus but well before the nocifensive

response (*no pain*). For assays involving a spontaneously emitted behaviour (e.g., AA, AITC, CAP, FORM, $MgS0_4$), we grabbed frames during the exhibition of such behaviour, or immediately preceding the behaviour (in the case of licking) (*pain*); we compared these frames to those grabbed from baseline video (pre-injection; *no pain*). For the ZYM and CYP assays, we grabbed frames beginning 2 or 3.5 h, respectively, post-injection (*pain*) and compared these to frames grabbed from baseline video (pre-injection; *no pain*). For the Post-op. assay, we grabbed frames 1-2 h post-surgery (*pain*) and compared these to frames grabbed 1 day prior to surgery (*no pain*). For the CCI and SNI neuropathic assays, we grabbed frames from video recorded 1, 7, and 14 days post-surgery (*pain*) and compared these to frames grabbed from baseline video (collected one week prior to surgery).

For all nociceptive assays described above, frames were collected from digital video files, saved as JPEG files, and cropped and edited as described previously. Edited JPEGs were then copied and pasted in a randomized order into a Microsoft Word or PowerPoint file. Photo identifications were removed in order to ensure that coding was performed blind.

5.4.5 Morphine Analgesia

Morphine analgesia was assessed in the cyclophosphamide cystitis model using the highest dose of cyclophosphamide (400 mg/kg). Cyclophosphamide was administered i.p. in a volume of 10 ml/kg after baseline filming. Saline or morphine (5 and 10 mg/kg) was administered subcutaneously (s.c.) 3.25 h post-cyclophosphamide injection, and began filming at 3.5 h post-injection.

5.4.6 Statistical Analyses

All statistical tests were conducted using SYSTAT v.10, and displayed in GraphPad Prism v.5. For all tests, an alpha level of 0.05 indicated significance.

5.5 Results

5.5.1 MGS Features and Reported Statistics

Analysis of the breakdown of changes in individual facial features revealed the absence of any one feature or cluster of features driving overall scores. Therefore, considering the average of these feature changes (See MGS; Fig. 5.1A) appears to represent the most appropriate method of calculating grimace intensity.

In human literature, the FACS is typically used to assess facial expressions of pain, and is often reported by observation in terms of intensity (equivalent to our mean grimace score), frequency and duration. We chose to evaluate intensity compared to baseline intensity in order to obtain a difference score for each subject. Assessing pain by frequency of observable pain features did not yield any difference in results (data not shown), and we believe that comparing post-injection scores to baseline constitutes the most efficient and reliable way to assess pain by facial expression.

5.5.2 Reliability and Accuracy

Inter-rater reliability was assessed by comparing the average scores for

each initial observation to the average scores of these coders (Fig. 5.1B). This analysis revealed a significant positive correlation ($r_{avg} = 0.80$, $p < .001$). Inter-rater reliability was also assessed using Chronbach's alpha, yielding a value of 0.89. Intra-rater reliability was assessed for each coder across all 10 randomized orders and averaged across coders, yielding an average Chronbach's alpha value of 0.81. Trained coders consistently assigned higher pain scores to photographs of mice in pain than to photographs of mice before the painful stimulus was applied (data not shown). When using an arbitrary cut-off score to discriminate pain from no pain, coders tended to be somewhat conservative, a finding denoted by a substantially greater proportion of misses than false alarms (that is, classifying a photo of a mouse in pain as not in pain). In terms of scale development we believe that false alarms (analogous to Type I errors) are the graver of the two errors and are thus optimistic that the scale may be successfully applied to make dichotomous pain-no pain classifications in the mouse. When making "pain-no pain" classifications, coders were accurate 72% of the time, and only 10% of observations were false alarms, and this accuracy level increased to 82% in a coder with 1 year of experience (Fig. 5.1C).

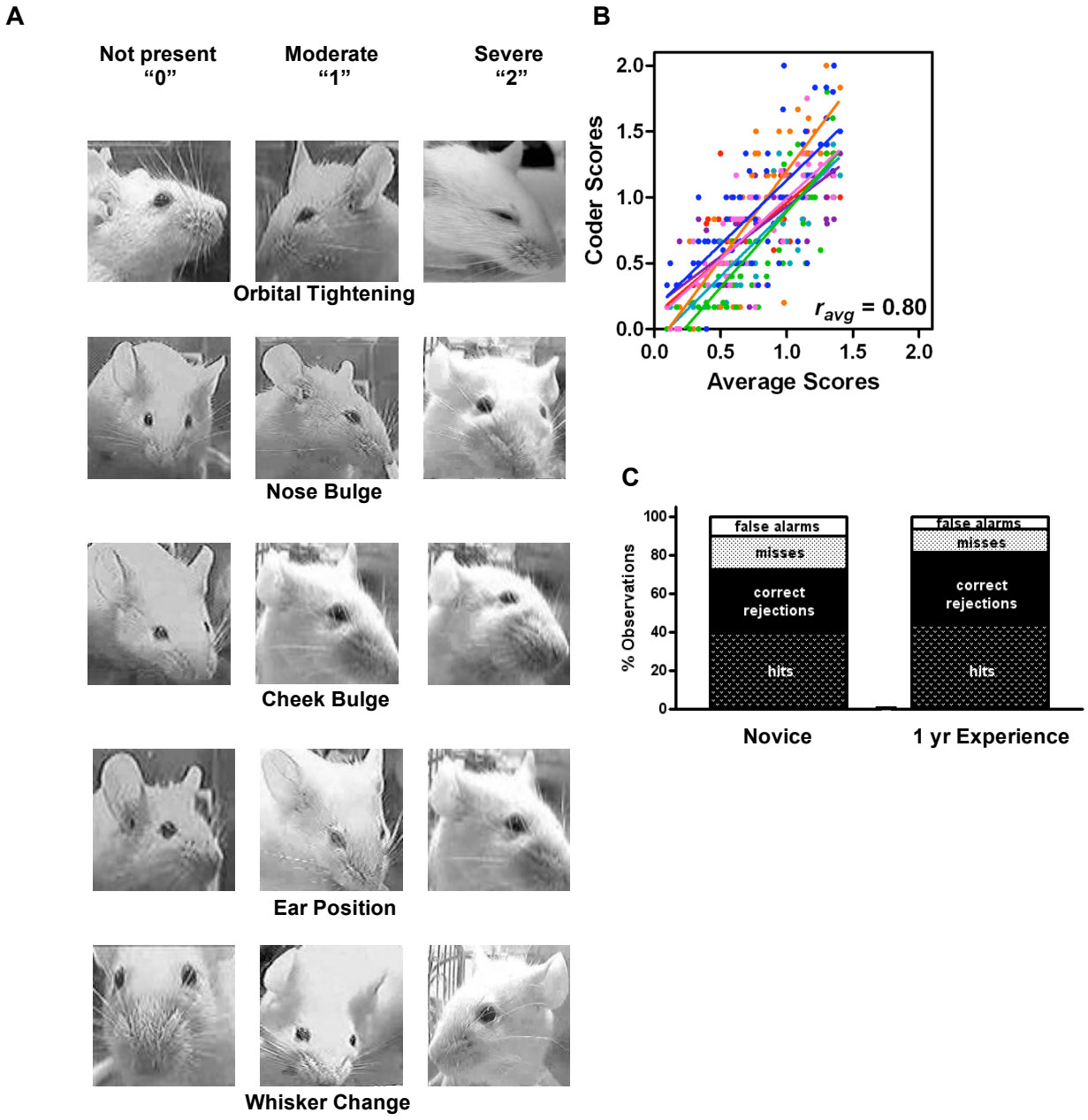

Fig. 5.1. The MGS, and its interrater reliability and accuracy. (**A**) Intensity of each feature is coded on a 3-point scale ("0": not present; "1": moderately visible; and, "2": severe). Scores are averaged across all five features to determine a mean MGS score. Features are defined as follows. *Orbital tightening*: narrowing of the orbital area, with a tightly closed eyelid or an eye squeeze (denoted by wrinkle around eye). *Nose bulge*: rounded extension of skin visible on the bridge of the nose. *Cheek bulge*: convex appearance of the cheek muscle (between eye and whiskers) from its baseline position. *Ears:* pulled back from their baseline position, or vertical ridges from ear to ear may be drawn together. *Whisker change:* movement of whiskers either backwards, against the face, or forwards, as if standing on end. Whisker change can also be noted by darkening of the whisker pad. (**B**) Interrater reliability of the MGS scale on the abdominal constriction test, using a selection of 64 randomized (*pain* and *no pain*) photographs. The mean MGS scores of each of six individual coders were compared to the average of all coders. (**C**) Signal detection of novice and experienced coders on the data set described in (**B**). See p. *xi* for list of abbreviations.

5.5.3. Evaluating MGS Amongst Common Algesiometric Assays

The ability of the MGS to detect pain among commonly used pain assays was assessed by comparing faces at the time of the response (e.g., during tail flick, writhe, etc.) to the appropriate baseline (Fig. 5.2). As mentioned above, for assays in which the response involved an action of the head, frames were grabbed ~2-5 s prior to the behaviour. Mean difference scores were compared to zero by one-sample *t*-tests and false discovery rate was controlled (Benjamini & Hochberg, 1995). Superficial and neuropathic pain assays failed to show significant changes in pain face. However, all stimuli inducing deep pain — resulting from stimulation of the muscle, fascia, or viscera — resulted in significant changes in facial expression from baseline (See Fig. 5.2 for significance values).

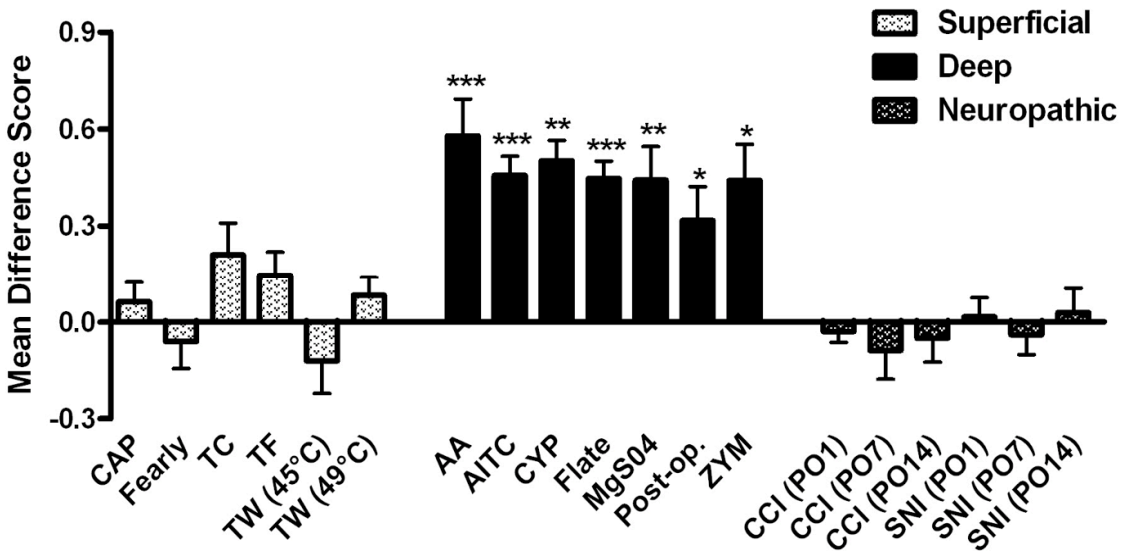

Fig. 5.2. MGS specificity to assays of deep pain. Bars represent difference scores (±S.E.M.) calculated by subtracting average of MGS scores for *baseline* photographs from MGS scores for *pain* photographs for each subject. Difference scores were compared to zero by one-way Student's *t*-tests. *$p<0.05$, **$p < 0.01$, ***$p<0.001$.

5.5.4 Stimulus-Response and Morphine Analgesia

Although we observed dose-dependent increases in frequency of pain behaviour (main effect of AA concentration: $F_{3,36} = 35.4$, $p < .0001$; Fig. 5.3A), we found no evidence of a dose-dependent increase in pain face intensity; that is, the pain face *during* the writhe remained constant (Fig. 5.3B). When we randomly sampled writhing videos, however, we obtained more frames depicting the paroxysm for mice injected with higher concentrations of AA; therefore, we did observe dose-dependency of the painful facial expression (main effect of AA concentration: $F_{3,36} = 5.5$, $p < .01$; Fig. 5.3C).

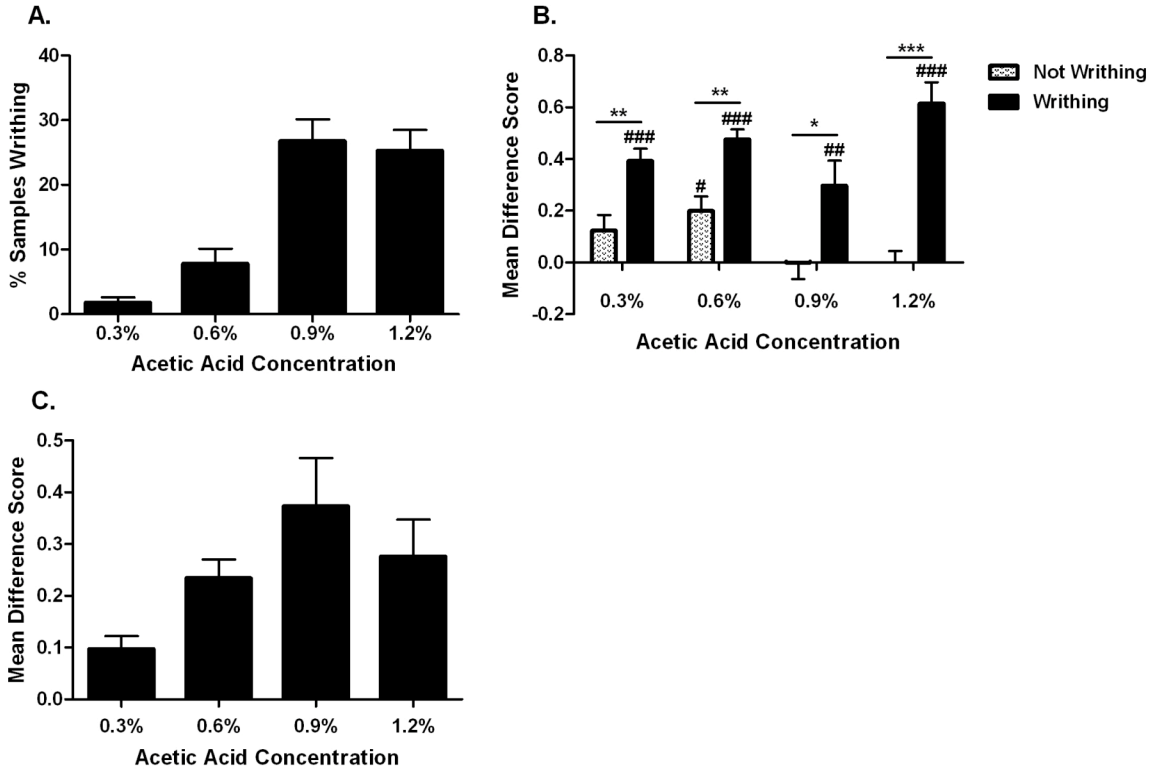

Fig. 5.3. Stimulus dependency of writhing behaviour (**A**). No dose-dependency when frames grabbed during exhibition of writhe, and no painful facial expression observed in inter-writhe interval (**B**). Dose-dependency of painful facial expression with random sampling of writhing video (**C**). # Significantly different from zero by one-sample Student's *t*-test (## $p < .01$, ### $p < .001$). * Significantly different from "Not Writhing" by paired Student's *t*-test (**$p < .01$, ** $p < .001$).

We therefore chose to use algesiometric assays that lack observable pain behaviours. We found that increasing doses of ZYM correspond to increasing MGS difference scores (main effect of ZYM dose: $F_{3,31} = 3.0$, $p < 0.05$; Fig. 5.4A). Also, increasing doses of CYP correspond to increasing MGS difference scores (main effect of CYP dose: $F_{2,17} = 8.9$, $p < 0.005$; Fig. 5.4B). We also observed dose-dependent morphine reversal of 400 mg/kg CYP-induced MGS difference scores (main effect of morphine dose: $F_{2,12} = 26.6$, $p < 0.001$; Fig. 5.4C). Note that 10 mg/kg morphine by itself (No CYP+10) produces no changes from baseline.

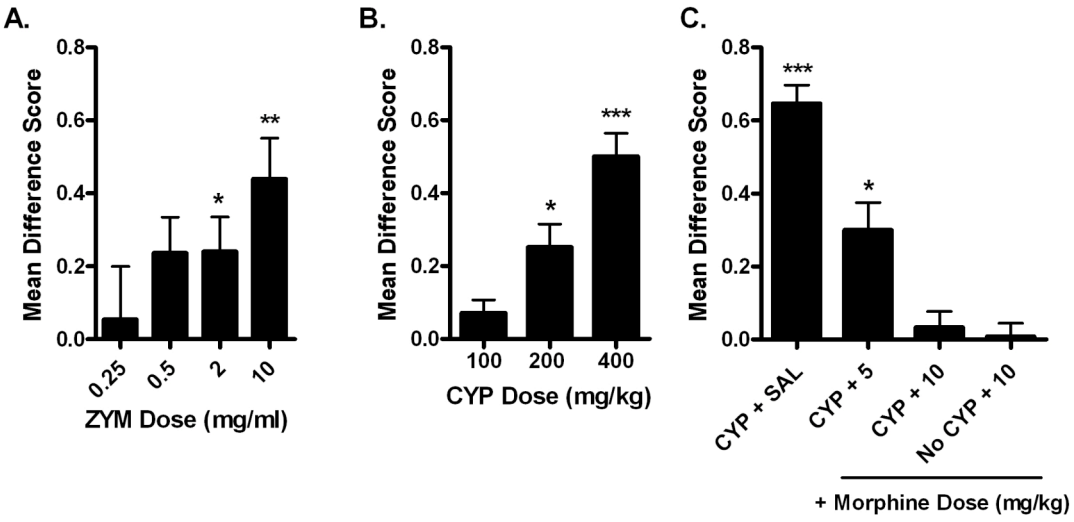

Fig. 5.4. Dose dependency of ZYM-induced (**A**) and CYP-induced (**B**) painful facial expression. Dose-dependent reversal of CYP-induced painful facial expression, with no effect of morphine administration alone (**C**). No dose-dependency when frames grabbed during exhibition of writhe, and no painful facial expression * Significantly different from zero by one-sample Student's *t*-test (* $p < .05$, **$p < .01$, ** $p < .001$).

5.5.5 Specificity of MGS Features

To determine the specificity of this set of facial features to the pain experience, we also assessed changes in facial expression from baseline to other conditions and behavioural states (Fig. 5.5). We assessed facial expressions post-injection with lithium chloride, a drug that reliably induces conditioned taste aversion in rodents (O'Donnell & Gould, 2007), and found that these mice displayed some, but not all features coded by the MGS. Sleeping and grooming mice also display some overlapping features, and we suggest that such photos not be scored in order to reduce the risk of false alarms.

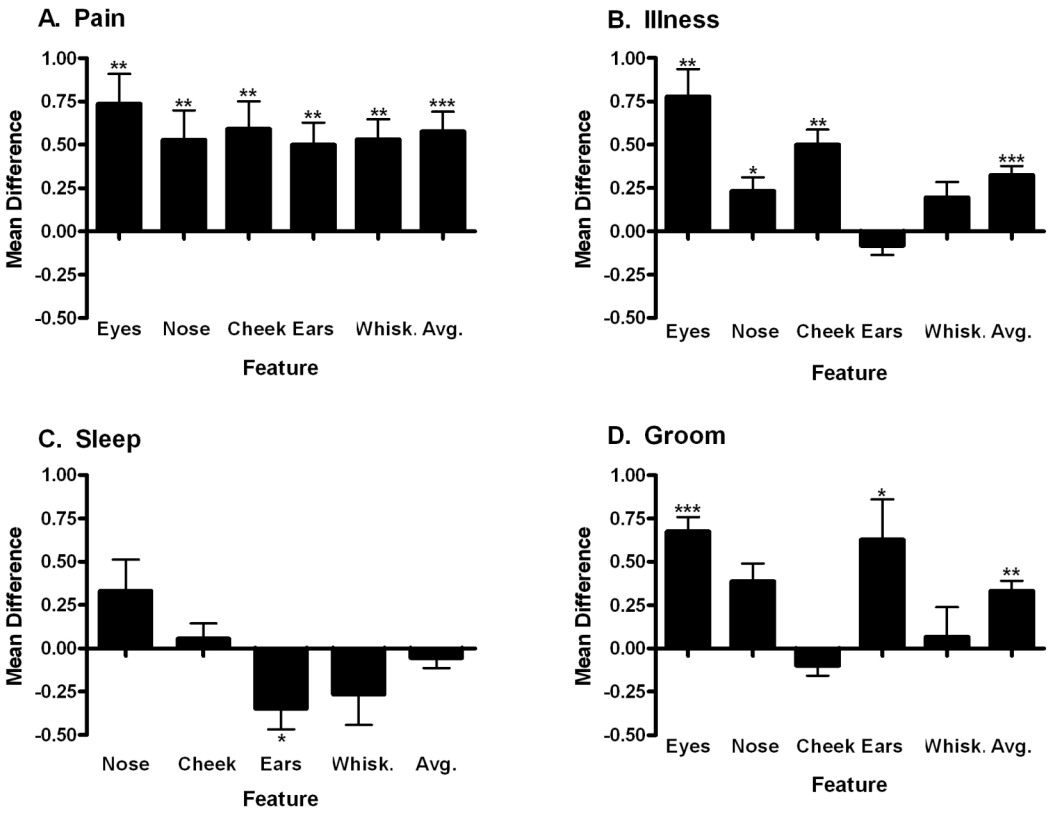

Fig. 5.5. Mean difference scores by feature for mice injected with 0.9% AA (**A**) 25 mg/ml lithium chloride (**B**), sleeping mice (**C**), and grooming mice (**D**).

Additionally, a separate between-groups analysis of baseline scores revealed that stress (produced by mild restraint) results in inflated baseline scores (data not shown), and should be minimized where possible (see Discussion).

5.6 Discussion

Based on several photos of mice in pain and not in pain, we have created an objective, standardized coding system, *The Mouse Grimace Scale (MGS)*, which consists of 5 observable facial features coded on an intensity scale of 0-2 (absent-severe), and can be used to determine a mean pain score which can be compared to baseline, or to determine a dichotomous "pain-no pain" classification. Data from trained coders indicate high inter-rater and intra-rater reliability as well as consistently good accuracy (i.e., well above chance). Of the assays evaluated, the MGS revealed a significant increase in painful facial expression only for stimuli invoking deep pain – specifically pain arising from the viscera (acetic acid, magnesium sulfate, and cyclophosphamide cystitis), as well as from the muscle or fascia (formalin, mustard oil, zymosan, and post-operative pain). The discrepancy between early and late phase formalin is interesting but not surprising since early phase formalin is thought to be the result of direct stimulation of sensory nociceptors, and late phase the result of inflammation that is associated with the long lasting tissue damage and secondary hyperalgesia (Dubuisson & Dennis, 1977; Veiga et al., 2004), both of which comprise characteristics of deep pain (Gebhart & Ness, 1991). Moreover, although not observed in the acetic acid abdominal constriction test, dose-dependency of the pain face was observed in deep pain assays involving no other observable painful

paroxysms, namely the cyclophosphamide cystitis and zymosan inflammatory models. The pain face observed with the highest dose of cyclophosphamide administered was also dose-dependently reversed by morphine administration.

5.6.1 Evolutionary significance: communicative versus sensory function

It is interesting, and perhaps not surprising, that the majority of the MGS features are also present in human facial expressions of pain (Prkachin, 1992), particularly orbital tightening, which is reported as the most consistent indicator of pain in human literature (Craig, Prkachin, & Grunau, 2001). This finding supports Darwin's century-old prediction that facial expressions are evolutionarily conserved and universal. Facial expressions, including painful facial expressions, have largely been described as a means of social communication among humans. In the case of non-human animals, a painful facial expression may communicate danger to its conspecifics, warning them to avoid a threatening situation. Alternatively, the expression may communicate distress in an effort to solicit helping behaviour from its conspecific. Regardless of these potential advantages, it is perhaps surprising that mice exhibit observable facial expressions in response to painful stimuli. Such a response may serve as a signal of vulnerability to potential prey, and it may best be suppressed in order to improve survival likelihood. Our finding that some male mice inhibit their pain behaviour in front of an uninjected stranger male support this notion, although in these experiments no stranger (or familiar) mice were present during testing.

Despite its potential social significance, whether or not this expression is recognized and interpreted by an observing conspecific is unclear, and is the topic

of ongoing research in our laboratory. It is of note, however, that many mice have relatively poor visual acuity and thus, these facial changes may not be obvious enough to be detected.

It is also possible that the observed painful facial expression do not serve a communicative function, but rather a sensory function. Darwin (1872) and more recently, Susskind et al. (2008), suggested such facial expression might serve a sensory function, by modifying sensory input in a beneficial direction; for example, fearful facial expressions involve feature changes that facilitate sensory acquisition (e.g., orbital widening) in potentially dangerous situations improving the likelihood of successful fight or flight (Susskind et al., 2008). Alternatively, facial expressions of disgust involve facial changes that limit sensory input thereby reducing the effects of an unpleasant stimulus (Rozin & Fallon, 1987). Therefore, it is plausible that these painful facial expressions evolved as a means of limiting sensory (e.g., eye closure, whisker retraction) input thereby potentially reducing (deep) pain perception.

5.6.2 Specificity to deep pain

Considering the human literature on facial expressions of superficial (or cutaneous) pain, it is interesting that we find no evidence of changes in facial expression with the application of such painful stimuli in mice. Several potential explanations may account for this finding. Firstly, it may be evolutionarily adaptive to suppress a painful facial expression in order to reduce vulnerability to potential prey. Because these assays involved relatively short-acting stimulation, mice may have been able to inhibit their facial expressions at the time of

response. Secondly, by necessity, many of these superficial assays involved more restraint (tube) than deep pain tests (cubicle), resulting in inflated baseline scores. However, a selection of superficial assays were modified to use less restraint and still no differences in facial expression were observed (data not shown). The finding that stressful facial expressions share similar features to painful facial expressions (though lesser intensity) has been noted in preterm infants (Holsti et al., 2005). Thirdly, because the scale was developed based on photos of mice during the acetic acid writhing test, it is possible that the scale is specific (or at least biased) to this type of pain. However, because the MGS covers virtually every feature of the mouse face and after observation of several hundred head shots, it is difficult to imagine what other features might be involved in superficial pain assays. Furthermore, from the coder's subjective perspective no overall differences could be detected between baseline and pain faces in these assays. Finally, evidence suggests that such expressions are less consistent and universal than they appear in the literature, and that they may be better described as "microexpressions," observed in a proportion of, but not all, subjects (Badali, 2008, Doctoral dissertation, University of British Columbia).

Differences between visceral and cutaneous pain have been described in the human literature both in terms of psychophysical (Strigo et al., 2002) and cortical (Aziz et al., 2000; Strigo et al. 2003; 2005) processing, with experimentally induced visceral pain described as more unpleasant than both phasic and tonic cutaneous pain. Moreover, this pain is described using more affective terms (Strigo et al., 2002). It is therefore possible that the MGS captured the affective component of the pain experience and not the response to

the nociceptive stimulus *per se*.

We did not find any evidence of painful facial expression in chronic pain assays. Typically, nociceptive sensitivity in these models is assessed by measuring mechanical allodynia (von Frey) or thermal hyperalgesia (Hargreaves test), but in our model we did not apply any stimulus at the time of post-surgical filming in hopes of observing a spontaneous painful facial expression, the most consistently troubling component in the human experience of chronic pain. We did not observe any painful facial expressions in these post-surgical photographs; however, this may have been due to the fact that animals were tested for relatively short periods of time (60 min videos sampled every 2 min) in an environment outside their home cage. The most powerful assay would most likely involve 24 hour/day filming of the animal in its home cage, perhaps combined with some novel mouse facial recognition software. It is noteworthy however, that spontaneous chronic pain has never been reliably detected in animal models, either reflecting a failure to establish an adequate dependent measure (Mogil & Crager, 2004) or simply a fundamental difference between rodent and human; that is, perhaps these surgical techniques do not induce spontaneous pain in the rodent.

5.6.3 Stimulus-response and morphine reversal

In order to validate the MGS it was necessary to determine whether the scale could detect changes in facial expression that correlated with the intensity of the stimulus, as the relationship between facial expression and stimulus intensity has been reported in humans (Williams, 2002). Because we purposefully selected frames during the exhibition of the paroxysm, this stimulus-dependency was

unobservable in the writhing test. This observation is quite interesting in that it suggests that the pain experience *during* the pain behaviour is not affected by stimulus intensity. We also did not find any evidence of a painful facial expression *between* writhes in this data set, which may reflect an absence of pain perception during the inter-writhe interval (i.e., pain is experienced only *during* the writhe). Such information could not be gleaned by other means.

Because we did not observe stimulus-dependency with the writhing test, we selected painful assays that involved no observable spontaneous pain behaviour, including cyclophosphamide cystitis and zymosan, and altered stimulus intensity by varying dose or concentration. Here we found a positive relationship between the dose of cyclophosphamide and pain face intensity, thus providing support for the efficacy of the MGS in determining not only the presence of pain, but also its intensity, at least for assays involving no spontaneous behaviours. Dose-dependent reversal of the pain face with morphine provides further support for the MGS as a valid dependent measure of pain in the mouse.

5.6.4 Conclusions and future directions

The *Mouse Grimace Scale (MGS*) is an adequately reliable and accurate standardized scale that can be used to determine the existence and intensity of facial expressions of (deep) pain in the mouse. Its specificity to algesiometric assays involving deep pain stimuli may reflect the affective component of the pain experience in these animals, as this type of pain is associated with more unpleasantness in humans. The scale may be useful in assessing pain in assays

that lack other observable behaviours, such as the cyclophosphamide cystitis and zymosan inflammatory models. As we have previously shown that mice communicate pain visually (Langford et al., 2006), we can now determine whether observing mice are attending specifically to facial expression, as well as whether painful facial expressions can be modulated by the social context. Such experiments are underway.

Chapter 6

General Discussion

6. General Discussion

Collectively, the projects described in this thesis provide support for a complex pain experience in mice, not unlike that observed in humans, and therefore stress the importance of accounting for such complexity in basic pain research. The data also suggest that certain traits previously thought to be unique to humans, such as empathy and prosociality, may be phylogenetically continuous, manifest in all mammals in some form.

Specifically, these experiments show that pain sensitivity in the mouse is influenced by psychosocial factors such as social context, social stress, and social approach. We have observed that pain sensitivity can be modulated merely by the real-time observation of a familiar mouse in pain (Chapter 2), that the stress-inducing proximity of an intact unfamiliar mouse can facilitate or reduce pain sensitivity in males (Chapter 3), and that frequency of contact is increased and can have a buffering effect on pain in females (Chapter 4). Finally, the experiments described in Chapter 5 demonstrate that mice display reliably observable facial expressions in response to deep pain stimuli (including the stimulus used in the preceding chapters to assess social modulation of pain), which may provide a more complete picture of the pain experience in these animals and may even mediate the social influence on pain sensitivity observed herein, considering the finding in Chapter 2 that pain communication is dependent on access to visual cues (see Section 6.2).

6.1 Key factors modulating the effect of social context on pain

Two major features impacting the effect of social context on pain sensitivity have emerged from this series of projects (particularly Chapters 2-4): familiarity and sex. In many cases, these factors determine whether or not social modulation occurs, and both can generally be explained in light of their adaptive significance.

6.1.1 Familiarity

Familiarity has proven to be a vastly important feature of the social modulation of pain observed in our laboratory. In virtually all experiments, familiarity amongst mice in a dyad (i.e., mice co-housed for 21 days or sibling mice) was a requirement for social modulation of pain to occur. First, the empathy phenomenon — characterized by hypersensitivity and co-occurrence of pain behaviours among BW dyads — was specific to familiar mice. In fact, the dyadic testing of either sibling or non-sibling cagemates revealed similar changes in pain sensitivity, suggesting the importance of affiliation rather than kinship in mediating these effects. Second, both socially mediated bidirectional modulation of pain and general sensitization of pain were specifically observed in familiar dyads. Finally, the social approach and buffering effect of contact initiation observed amongst female dyads described in Chapter 4 were only observed in familiar mice. None of these phenomena were observed amongst strangers. Familiarity also proved important in the perception of social threat in male mice, such that pain inhibition in the presence of an intact, unaffected mouse was only observed amongst unfamiliar dyads.

A number of studies suggest the importance of familiarity in mediating socially induced changes in pain sensitivity (Coan et al., 2006; Montoya et al., 2004; Thieme et al., 2005), including empathic responding in humans (Loggia, Mogil, & Bushnell, 2008; Singer et al., 2004) and animals (Preston & de Waal, 2002). Indeed, it would be an unwise use of energy to physiologically respond to the behaviour of every observed conspecific (de Vignemont & Singer, 2006), and responding selectively to familiar individuals would be most adaptive, in that transmitted signals may be more reliable (and therefore *worth* responding to) and that the resulting behaviour may incur some benefit to the observer itself (i.e., reciprocal altruism) or to the group as a whole (i.e., inclusive fitness). Familiarity implies experience (built from repeated social interactions), through which subjects may more easily detect and attend to a change in behaviour, resulting in automatic state-matching (i.e., emotional contagion), and guiding behavioural responses (i.e., social approach). Familiar animals would also have established stable social relationships — dominance hierarchies in the case of male mice — in which behaviour was predictable and (most likely) non-aggressive. In fact, established familiarity among female dyads can inhibit aggression under normally competitive circumstances (Palanza et al., 2005).

6.1.2 Sex

Sex was also an important mediator of these social phenomena, particularly of the effects of social stress and social approach on pain sensitivity. First, although we found no sex differences in empathic behaviour, we did observe sex-specific pain inhibition in unfamiliar OW male, but not female,

dyads. Moreover, this sex-specific effect was testosterone dependent, as it was only observed amongst gonadally intact male dyads. Second, females, but not males, chose to approach a familiar mouse in pain more frequently than an unfamiliar or an unaffected familiar mouse. Although contact initiation with the affected mouse was associated with a reduction in pain behaviour in both sexes, the effect was significantly stronger amongst females.

These sex differences are perhaps not surprising in light of the fact that outbred CD-1 mice have been shown to exhibit significant sex differences in a number of dyadic social behaviours (Malloy, et al., 2005). Furthermore, coping mechanisms in response to stress have also been shown to differ with respect to sex (Taylor et al., 2000). Taylor and colleagues contend that males adopt a "fight-or-flight", while females adopt a "tend-and-befriend" behavioural response to stress. This notion is based on differential drives, such as offspring protection and attachment in females versus territorial establishment and status in males. Moreover, the "tend-and-befriend" response reduces distress, potentially alleviating the pain of a conspecific and potential ally, while the "fight-or-flight" response reduces the likelihood of physical danger, perhaps in some cases resulting in stress-induced analgesia in order to maintain heightened vigilance.

In the wild, males would be in competition for food, mates, and other resources. Aggression would be involved in resolving disputes over such matters, and therefore the proximity of a potentially aggressive competitor would likely result in a stress response. The close proximity (i.e., crowding) of males has in fact been associated with increased corticosterone levels in male rodents (Brown & Grunberg, 1995). In contrast, group-housed females are rarely aggressive

(More, 2008) and are less likely to form dominance-submissive relationships; as such, the proximity of another female mouse would pose no threat and therefore result in no stress response. In fact, females exhibit no increase in corticosterone levels in crowded conditions, but do exhibit a reduced stress response if housed in groups rather than in isolation (Brown & Grunberg, 1995).

6.2 Proposed relationship between facial expression, emotional contagion and social approach

In Chapter 2, we observed a change in the sensory perception of an unaffected mouse merely by observing a familiar mouse in pain, a finding suggestive of a shared physiological response (i.e., emotional contagion). Given the finding that such emotional contagion is visually mediated, it is suggested in Chapter 5 that facial expressions may also play an important role in the social communication of pain. This ability to identify and to automatically "feel" another's pain may impact behaviours unrelated to pain perception *per se.* Under the perception-action model (Preston & de Waal, 2002), empathy is phylogenetically continuous and comprised of five subtypes ranging from the most primitive (emotional contagion) to the most advanced (prosocial behaviours). Therefore, visual communication of pain and consequential emotional contagion may provide the basis for adaptively guiding behavioural responses, such as social approach. For example, it has been anecdotally noted in rhesus macaques that the distress of a young monkey spreads to the group, resulting in such behaviours as social approach, huddling and mounting, thereby relieving the distress of the entire group (de Waal, 1996). Also, fearful facial

expressions and body postures in monkeys have been shown to elicit fear in observing conspecifics, resulting in adaptive avoidance behaviour (Mirsky, Miller, & Murphy, 1958). Moreover, a variety of infant pain assessment tools use facial expressions and body posture as measures of pain perception; of course, such measures evoke appropriate caregiving responses from observers (Batton, Barrington, & Wallman, 2006).

It is therefore plausible that mice communicate their pain via visual displays, which can result in emotional contagion and altered social behaviour under specific circumstances of familiarity and sex (Fig. 6.1).

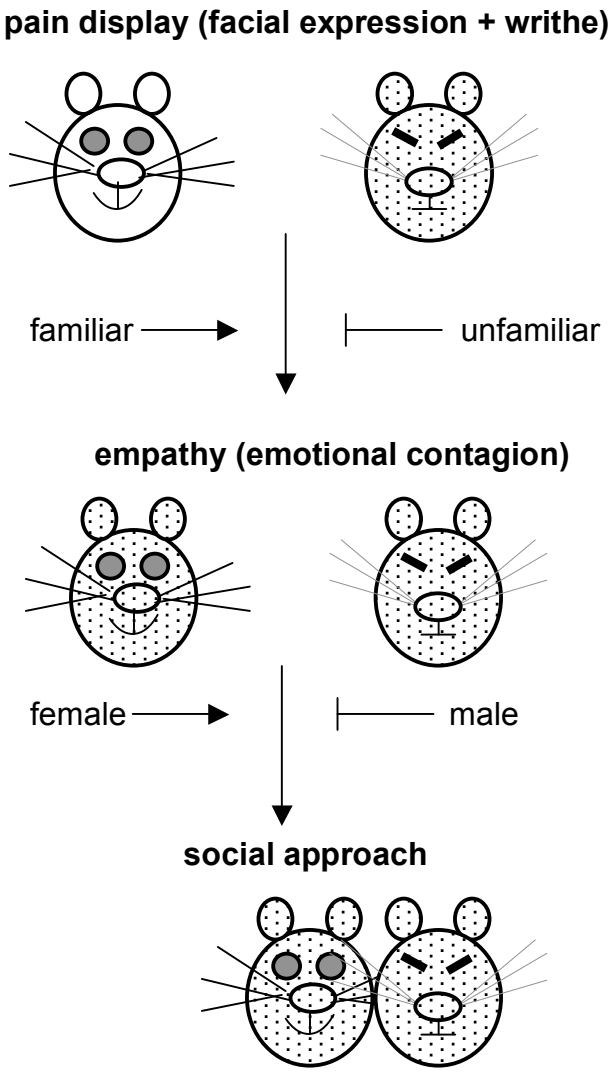

Fig. 6.1. Theoretical pathway whereby pain is communicated visually through pain behaviour and/or associated painful facial expression, subsequently shared by familiar conspecifics, and finally adaptively responded to in a sex-specific manner. Arrows indicate facilitation, and closed lines indicate inhibition of such responses.

6.3 Implications for basic pain research

The findings presented in this dissertation indicate modifications that may be applied to basic behavioural pain research. Moreover, these findings suggest opportunities for the study of pain and pain-related social behaviour in rodents.

6.3.1 Animal models of pain: the need for a biopsychosocial model

Hundreds of millions of dollars are invested in funding basic and clinical pain research a year by National Institutes of Health alone (Bradshaw et al., 2008); however, a major hurdle in the field is a scarcity of translational studies that identify novel therapeutic targets from basic science laboratories and successfully apply them to clinical populations. One particularly notorious example is the neurokinin-1 (NK1) receptor antagonist, which despite promising preclinical data, showed little analgesic efficacy amongst clinical populations (Hill, 2000). Perhaps one explanation for this incongruence is the failure to apply the biopsychosocial framework to animal models of pain. Indeed, Hill (2000) suggests that the additional function of NK1 in mediating behavioural responses to stress in rodents that may not necessarily translate to the clinic, highlighting the complicated involvement of psychosocial factors. Given evidence of substantial psychosocial influence described in the general introduction and detailed throughout this dissertation, as well as the increasing prevalence of animal subjects in pain research (Mogil et al., 2009) it is imperative to both understand and account for such variables in animal models.

6.3.2 Practical changes to pain behaviour protocols

Some specific changes to basic pain research protocols should be made in order to obtain the most accurate results. Clearly, animals (although group-housed) should be tested in isolation, and an opaque partition should be placed between test subjects during habituation and testing in order to eliminate opportunity for visual communication and physical contact. Similarly, the

induction of pain, by surgical or pharmacological techniques, should be done in the absence of conspecifics to minimize the transmission of pain information. It may also be necessary to avoid testing males from separate cages simultaneously, since even limited interaction evokes stress-induced changes in pain sensitivity.

6.3.3 The Mouse Grimace Scale as a complementary pain assessment tool

Chapter 5 introduces a novel dependent measure that may be utilized by pain researchers to better describe an animal's pain experience, particularly in response to deep pain stimuli that do not evoke other quantifiable behaviours. Furthermore, the identification of a reliably observable pain face associated with spontaneous (non-evoked) pain in the mouse is of utmost importance because of its relevance to the clinical experience of chronic spontaneous pain. As previously mentioned, spontaneous pain is the most troubling component of neuropathic pain and most reliably reflects overall pain ratings (Backonja & Stacey, 2004); yet there exists no reliable measure of such type of pain in neuropathic rodent models (Mogil & Crager, 2004). In fact, behavioural assays generally rely on dependent measures of hypersensitivity to mechanical and thermal stimuli, considerably less prevalent components of the clinical neuropathic pain experience (Backonja & Stacey, 2004). This failure to adequately capture the clinical pain experience may also contribute to the lack of successful translational findings in the field. Therefore, the ability to reliably identify non-evoked painful facial expressions in the mouse offers a unique, and potentially very powerful tool, for the study of spontaneous pain. If models of neuropathic pain do indeed elicit spontaneous pain in rodents, and this pain is

associated with observable facial expression changes, then around-the-clock home cage surveillance combined with mouse facial recognition software may offer an effective way of monitoring for facial expressions associated with the experience of spontaneous pain in response to neuropathic injury as well as the efficacy of pharmacological agents. This technology does not yet exist, but is clearly feasible.

In general, given the limited information one can gain from the analysis of pain behaviour, this additional measure may act as a complementary dependent measure to other objective and quantifiable pain behaviours. Animal care technicians can also use the MGS in order to objectively and reliably assess pain in laboratory animals, thereby ensuring the health and ethical treatment of test subjects. Moreover, the MGS provides a unique tool for assessing whether pain is communicated via facial expressions, and whether expressions change with respect to varying social contexts.

6.3.4 Pain as a tool for studying social behaviour

The findings presented in this dissertation suggest a need for continued research and understanding of social modulatory factors, not only to better facilitate pain research, but also to better understand social behaviours associated with the experience of pain. Pain is a powerful motivator that can result in substantial physiological and behavioural changes in order to avoid, escape, or cope with the aversive experience. It is evident from this dissertation that social factors can significantly modulate the pain experience, but it is also clear that the experience and observation of pain can significantly impact social behaviours, as

well as shed light on the dynamics of complex social processes, such as empathy and prosociality. In fact, the aversiveness of the pain experience may provide a more powerful means of studying these social phenomena, as human literature suggests that negative states are more accurately identified and result in greater physiological state-matching than positive ones (Levenson & Ruef, 1992).

In particular, these models lend themselves well to the study of genetic factors potentially involved in mediating such behaviours. With the use of methodologies such as quantitative trait locus mapping (QTL), it is possible to identify regions of a chromosome responsible for a phenotypic trait (Broman, 2001). Also, the availability of transgenic knockout mice affords the opportunity to verify involvement of particular genes. As the social behaviours described in this dissertation have relevance to a variety of social disorders, including schizophrenia, psychopathy and autism, insights into specific genetic involvement may potentially suggest novel therapeutic targets. At the very least, such information would afford the opportunity for early detection, and therefore early intervention.

With the advent of small animal fMRI, researchers may also be able to observe the anatomical bases of such behaviours. Although technically challenging, this technique has been performed on awake rodents with more than one animal in the scanner (Febo, Numan, & Ferris, 2005); therefore, it may be possible to adapt our paradigms in order to make use of this revolutionary technique. We would then gain the opportunity for an in depth look at the neural correlates of such social phenomena and compare them to relevant phenomena in humans.

6.3.5 Non-primate mammals: more than meets the eye

The research described in this dissertation suggests that mice possess attributes commonly thought to be uniquely human, such as empathy and prosociality. Some research on non-human primates suggests that skills such as imitation and theory of mind may not be present in this "higher" species (Heyes, 1998). It has been suggested, however, that these null findings may in fact be due to testing procedures, in that they lack a species-relevant dependent measure (Call & Tomasello, 2008). In fact, experiments involving more ethologically relevant paradigms support the notion that such traits are well within reach of non-human primates (Miklosi, 1999). This dissertation also provides support for this contention, in that appropriate experimental paradigms may be implemented in order to assess sophisticated social behaviours in mice. The observation that mice show evidence of empathy and prosociality indicates that these attributes may be possessed, at least at some level, by all group-living mammals. Moreover, non-primates may be capable of other abilities previously considered to be unique to humans and other primates, such as theory of mind and altruism. In fact, altruism has even been observed in single-celled organisms, where an amoeba will "commit suicide" for the sake of a larger organism (Strassmann, Zhu, & Queller, 2000), lending further support for the notion that such phenomena are phylogenetically continuous and evolutionarily conserved. Perhaps, we need only formulate appropriate experimental paradigms in order to observe these behaviours in new species.

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Original Contributions to Knowledge

1. First evidence of empathy in mice, at least in the form of emotional contagion.

2. Novel mouse model of social stress induced changes in pain sensitivity, either SIA or SIH depending on nature of the stressor.

3. Evidence of prosociality in the mouse and that social contact may have analgesic properties.

4. Novel reliable measure for assessing (deep) pain in the mouse.

Appendices