ELUCIDATING THE GENETIC INFLUENCES IN PAIN SENSITIVITY AND ANALGESIC EFFECT

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ELUCIDATING THE GENETIC INFLUENCES IN PAIN SENSITIVITY AND ANALGESIC EFFECT

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Pain impacts the quality of life for millions of people each year and is a significant drain on the country's health care resources. Pain is an innate response to a noxious stimulus indicating damage in a region of the body. This response elicits various reactions from the body including autonomic, inflammation, as well as stimulation of growth and repair. Under some conditions, often associated with plasticity in the nociceptive system, pain may become prolonged and lose its adaptive function. In this study, we are using data from human volunteers, which makes our findings directly clinically applicable compared to animal models. The clinical aspects of pain have been vastly studied but there is a deficiency of information regarding genetic influences on pain responses. There is new evidence supporting the notion that genetics contributes to pain sensitivity and response to analgesics. We have undertaken a scientific study to test the effects of candidate molecular receptor variants in this process. We have found positive associations of some single nucleotide polymorphisms with pain sensitivity and

analgesia, although no polymorphisms were implicated in case-control studies of chronic pain conditions with the current cohort of subjects. It is the dawning of a new era in pain research, in which our enhanced understanding of the molecular mechanisms contributing to pain may help elucidate the individual differences in pain responses. By improving our understanding of molecular contributions to pain, it will be possible to tailor treatment to individual patients, thus improving clinical outcomes.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Pain impacts the quality of life for millions of people each year and is a significant drain on the country's health care resources. It is the primary motivator for the utilization of health care (Knapp and Koch 1984), and approximately 1 in 5 Americans experiences chronic pain (Joranson and Lietman 1994). Pain medications are the second most prescribed medications (Schappert 1998) and the direct and indirect costs of treating pain is estimated to be over $125 billion annually (Turk et al. 1999). Nociception is the term used to describe the neural transmission of signals that may lead to the experience of pain. According to the International Association for the Study of Pain, pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (www.iasp-pain.org/terms-p.html#Pain). On this website is described the subjectivity of pain and that each person has unique parameters of experience to which pain is rated. Although pain is an emotional experience, this definition also acknowledges the role that biology plays in the pain process, as the noxious stimulus has an ability to cause tissue damage and thus incorporates physiological pathways.

The Afferent Nociceptive System

A pain response elicits various reactions from the body including vasodilation, inflammation, and the stimulation of growth and repair. Under some conditions, often associated with plasticity in the nociceptive system, pain may become prolonged and lose its adaptive function. Once the pain pathway has become sensitized, the nociceptive

pathway is more primed to sensation and this leads to a chronic pain state. Noxious stimuli are detected by specialized receptors in the nervous system called nociceptors. Nociceptors innervate all peripheral systems of the body to allow for the perception of a noxious stimulus. They make up part of the sensory system of the nervous system and allow the brain to analyze the nature, location, intensity and duration of the noxious stimulus (Riedel and Neeck 2001).

. There are two main types of nociceptors, Aδ fibers and C fibers\. Aδ fibers are myelinated and are thought to contribute to the experience of sharp shooting pain. This is usually the first sensation a person feels after the experience of a noxious stimulus. C-fibers are unmyelinated, and their activation can lead to the sensation of a dull throbbing pain, which tends to last for a longer duration of time. In a chronic pain condition, it is believed that prolonged C-fibers activation can cause second order spinal neurons to become sensitized and cause long-term pain. Tissue damage and peripheral nerve injury may cause an expansion of the dorsal horn receptor field, thereby increasing the input region from the periphery.

The cell body of the nociceptor is located in dorsal root ganglia and enter the central nervous system in the dorsal horn of the spinal cord. The C fibers synapse on spinal neurons in superficial region of the dorsal horn and are found in laminae I and II, while the Aδ fibers are located in lamina V (Rexed 1952). There is a high concentration of excitatory amino acids found in the superficial region of the dorsal horn, which include glutamate, aspartate, substance P (SP) and calcitonin gene related peptide (CGRP). These molecules represent the main nociceptive transmitters as they co-localize to the nociceptive neurons and have been found to be elevated, along with their receptors, in a

chronic pain situation (Riedel and Neeck 2001). The stimulus is routed through the cell body up the primary afferent axons to multiple regions of the neuraxis. The thalamus is a primary brain region involved in nociceptive processing. The thalamus is responsible for the propagation of the stimulus and routing of the impulse to the somatosensory cortex where the sensation is interpreted, as well as to other cortical regions for higher order processing.

Central Processing of the Noxious Stimulus

Exactly where pain is processed in the brain remains an enigma. Pain is regarded to be perceived in the subcortical structures of the brain. The first receiving unit is the thalamus and then the message is transmitted to the somatosensory cortex, as well as other cortical structures, and effector processes are initiated. This is supported by the fact that patients with cortical lesions do not lose the sensation of pain (Shibasaki 2004). A positron emission tomography-regional cerebral blood flow (PET-rCBF) activation study concluded that the primary and secondary somatosensory cortex (SI and SII respectively) are activated during a painful stimulus, as well as the cingulate cortex, contralateral to the side where the pain is being delivered (Talbot et al. 1991). Using functional magnetic resonance imaging (fMRI), researchers have shown that activation occurs in the SI not only during the actual stimulation, but also in anticipation of the noxious stimulus (Porro et al. 2002). A few groups demonstrated that central neuroplasticity and pain memory play a considerable role in clinical symptoms such as central neuropathic pain and phantom pain (Melzack et al. 2001; Garcia-Larrea et al. 2002). The SI seems to play a role in basic pain processing, while the SII and insula are involved in more intricate pain perception and sensitization. The emotional aspects of pain processing are controlled by

the anterior cingulate cortex and the posterior insula/parietal operculum structures (Shibasaki 2004).

Descending Anti-Nociception

Gamma-amino butyric acid (GABA) is a major inhibitory transmitter in the CNS. This compound has been implicated in the inhibition of acute and persistent pain (Malcangio and Bowery 1996; Schadrack and Zieglgansberger 1998). Anti-nociception has been attributed to cholernergic interneurons acting on opioidergic interneurons through the mu, delta and kappa opioid receptors, via enkephalins and dynorphins.

Along with pain relief via the efferent pain pathway, inflammation and redness may occur at the site of the stimulus, if there has been an immunological response to allow wound healing. During the inflammatory process, there is an actual disruption of the perineurial barrier around the primary afferent fibers, which allows endogenous and exogenous opioids released from immune cells to inhibit the nociceptive stimulus at the peripheral ending (Antonijevic et al. 1995). This is an additional system of pain relief.

Two Receptor Systems

Two important receptor systems known to be integral to the process of nociception and anti-nociception are the N-methyl-D-aspartic acid (NMDA) and the opioid receptor systems respectively. Opioid receptors are synthesized in the peripheral neurons and then are transported to the periphery and central endings of the nociceptive fibers. NMDA receptors can be found highly represented in the dorsal horn in lamina II along with the opioid receptors, which suggests that these receptors may be functionally related. Opioids have been shown to directly or indirectly modulate NMDA receptor mediated events within the CNS by either inhibition or potentiation of the electrophysiological message (Chapman et al. 1994; Sivilotti et al. 1995; Zhang et al. 1996; Vaughan and

Christie 1997). Research (Chen et al. 1995) has shown that the kappa opioids, such as dynorphin, are NMDA receptor antagonists and that the kappa opioid peptides have been found in the dorsal horn during inflammation, associated with a disruption of nociceptive transmission at the level of the spinal cord. Opioids, in fact, have been found to regulate NMDA receptors by inhibiting the calcium channel activity of these receptors (Basbaum and Fields 1984; Mao et al. 1995). One affecting the influence of spinal opioid receptors in anti-nociception (especially that of the mu- opioid receptor) is the amount of spinal cholecystokinin (CCK), whose action is inhibitory on spinal opioid efficacy (Stanfa and Dickenson 1995). Nitric oxide (NO) acts as a negative feedback regulator of NMDA receptors. This feedback loop is initiated by the release of calcitonin gene related peptide (CGRP) and substance P (SP), which is increased in the dorsal horn during hyperalgesia. A prolonged release of these factors from the primary afferent neurons activates the NMDA-NO cascade (McMahon et al. 1993). Hyperalgesia is defined as an increased response to a painful stimulus. NMDA activation, which is mediated by NO, has been implicated in the maintenance of hyperalgesia in chronic pain models (Meller and Gebhart 1993). This hyperexcitability of the spinal cord is known as central sensitization. This phenomenon is known as “windup” and is caused in part by the involvement of the C-fiber activity with the constant release of the neurotransmitters in the dorsal horn region, which affects post-synaptic transmission of the nociceptive signal (Mendell and Wall 1965; Basbaum and Fields 1984; Urban et al. 1994; Mao, Price et al. 1995). “Windup” is also known as temporal summation of pain and was first described by Mendell and Wall in 1965.

The clinical aspects of pain have been greatly studied but there is a deficiency of information regarding genetic influences on pain responses. There is direct evidence supporting the notion that genetics contributes to pain sensitivity and response to analgesics (Mogil et al. 2003; Zubieta et al. 2003). It is the dawning of a new era in pain research in which our enhanced understanding of the molecular mechanisms contributing to pain may help elucidate the well-documented and substantial individual differences in pain responses. By improving our understanding of molecular contributions to pain, it will be possible to tailor treatment to individual patients, improving clinical outcomes. A few genes stand out in the search for the genetic component of pain. These candidate genes are discussed below and are the major focus of my work.

Genetics In Pain

Two main influences in many life experiences are environment and genetics. The environmental influences in pain are discussed below. There is concrete evidence that genetics plays a role in pain susceptibility as well as analgesic effect. This is evident in twin studies of lower back pain and neck pain, examining dizygotic twins as well as monozygotic twins (MacGregor et al. 2004). In this extensive study, which included 181 monozygotic (MZ) and 351 dizygotic (DZ) twin pairs, the range of concordance found was 52%-68% for lower back pain and 35%-58% for neck pain. These numbers are considered strong indicators of genetic contribution. Association studies of candidate genes and pain have become common in the past five years, given that pain is a complex trait with genetic influences. Pain genetics studies were initially done using mouse models. Comprehensive mouse studies have been undertaken, and only in the past decade have human participants been the object of genetic candidate gene association

analysis of pain. We have based our human studies in part on data generated by murine QTL analysis.

Mouse Models of Pain

Human traits can often be mirrored and manipulated in the murine laboratory system, in which systems can be broken down into simpler events in inbred mice to control for genetic background. Classic tools include transgenic mice, which express an exogenous gene, and knock-out mice, in which an endogenous gene of interest is made non-functional by homologous recombination in embryonic stem cells. Genetically speaking, there are also differences between laboratory mouse strains. There are two main strains that have been bred to select for pain sensitivity in mice. One is the HA/LA mouse line, which displays high and low analgesia respectively (Panocka et al. 1986), which is induced during swimming in cold water. The second useful strain in the pain paradigm is the HAR/LAR mouse line, which was bred by Belknap and colleagues in 1983. These mice display high or low analgesia in response to the opioid analgesic levorphanol. These strains are theorized to have mutations which control response in a Mendelian fashion. Quantitative trait loci mapping has been undertaken in these and other strains, using microsatellites and candidate gene polymorphisms involved in pain sensitivity, in association with measured analgesic response (Mogil et al. 1997a, 1997b). In terms of mouse knock-out models, all the opioid receptors have been disrupted as well as all their identified endogenous ligands. From these studies, we have learned that there is great redundancy within the opioid system although the different knock-out mice have various behavioral deficits. The knock-out mice have been made by various laboratories including Zhu et al. 1999 and Filliol et al. 2000, who made a delta opioid receptor knock-out. The first few mu opioid receptor knock-outs were made in 1996-7 (Sora et al. 1997;

Tian et al. 1997; Matthes et al. 1998). The only kappa opioid receptor knock-out was made by Simionin et al. in 1998. In all these knock-out mice, homozygotes were viable and fertile. Absence of the receptor was shown by lack of binding of the selective agonist, but there was no major compensatory effect with respect to the anatomical expression of the other two receptors. There is a hypothesis that there may be an adaptation at the level of coupling efficiency instead of the difference being seen at the level of ligand binding (Matthes et al. 1998; Narita et al. 1999; Hosohata et al. 2000). Mice lacking the opioid peptide components have also been reported to be phenotypically normal for pain modalities. These mouse data have been extensively discussed in a comprehensive review by Kieffer and Gavériaux-Ruff (2002). In this review they also describe efforts to make double and triple knock-out mice through cross breeding.

Human Studies

One of the first studies to implicate genetics in pain was a study by Morris-Yates et al. in 1998, which showed that 56.9% of twins were concordant for irritable bowel syndrome symptoms. In 1999,Yunus et al. presented a genetic linkage analysis of families with fibromyalgia to the human leukocyte antigen (HLA) locus, which is a group of genes in the human histocompatiblity complex that encodes for cell surface antigen presenting proteins. In the early part of this decade, researchers examined candidate genes in relationship to pain sensitivity and the therapeutic effect of analgesics. In 2003, Zubieta et al showed that there were three different states (efficient enzyme, non-efficient enzyme and non-functional enzyme) of the protein involved in catecholamine metabolism depending on specific polymorphisms found in the catechol-O-methyltransferase gene (*COMT*) (Zubieta et al. 2003). In the same year Mogil et al found that a gene originally involved in skin pigmentation was in fact also involved in the mediation of analgesic

effect of a kappa opioid agonist in mice (Mogil et al. 2003). The human data from this paper will be discussed in chapter 4, as I have been involved in the human analysis of susceptibility genes for both acute pain as well as chronic pain as part of a large collaboration.

Gender Differences and Psychosocial Influences

The differences that have been found between men and women with regard to pain are now well documented (Maixner and Humphrey 1993; Fillingim et al. 1999). Women have a significantly lower pain threshold and tolerance than men, and also rate the same noxious stimulus more highly than men. There are also clinical data indicating that women suffer more from pain and chronic pain syndromes than men (Dao and LeResche 2000; Heitkemper and Jarrett 2001). There are many possible implications of this information. First, it is possible that men and women experience and respond to pain in a different manner. Also, a society stereotype mandates that men are supposed to be the stronger sex and not admit pain. Thus, there may be different nociceptive pathways in the different sexes, but psychosocial factors matter as well. Gender role expectations are, in fact, significant predictors of pain threshold, tolerance and unpleasantness (Rollman et al. 2004), though gender roles typically do not fully account for sex differences in pain perception. Animal studies have shown that there are differences in the pain response and analgesic effects of morphine between male and female mice of certain strains (Kest et al. 1999). Gene expression and hypophyseal portal artery (HPA) regulation in the hippocampus have also been found to be different between the sexes in a mouse chronic stress model (Karandrea et al. 2002). A review (Craft 2003) highlighted the numerous studies that have been conducted in rodents to indicate that mu opioid receptor agonists

(which often cross-react with kappa opioid receptors) have a more powerful effect on males compared to females, an effect which is reversed in humans.

Gonadal hormones (estrogens and androgens) also have a pronounced effect on pain thresholds in male and female rats (Liu and Gintzler 2000; Aloisi 2003). Women exhibit a significantly lower tolerance time compared to their male counterpart, as well as rate the predicted pain tolerance lower at the start of the study, suggesting a lower capacity to withstand pain than males (Rollman et al. 2004). It is hypothesized that due to the different biological demands on the male and female body, women experience pain earlier (e.g. at the onset of menses) and experience more pain more often due to the menstrual cycle. This cycling painful experience is the reason women are more vigilant about pain and seek healthcare more often than men (Stenberg and Wall 1995; Crombez et al. 1999; Aldrich et al. 2000). This theory extrapolates that because of this continuing cycle of pain, the nervous system becomes plastic and that more women undergo peripheral sensitization, which may alter the activity of the primary afferent neurons (Taddito et al. 1997, Craig and Andrew 2002, Woolf and Salter 2000). Many chronic pain conditions not involving the sex organs are more predominant in females, such as irritable bowel syndrome, biliary colic, oesophagitis, interstitial cystitis, fibromyalgia, rheumatiod arthritis, and temporomandibular disorder (Unruh 1996). Females rate an injection of intramuscular glutamate more painful than males (Cairns et al. 2001) and this may be attributed to the finding of differences in the descending inhibitory control pathways in the two sexes after experimental muscular pain induction (Ge et al. 2003). Men may be able to better inhibit the muscle pain compared to females.

An increase in pain sensitivity may also be attributed to psychological problems such as depression and panic disorders, as these patients have an increase of clinical pain complaints (Lautenbacher et al. 1999). In one study (Stephan et al. 2002), researchers using rats demonstrated that postnatal experiences such as maternal deprivation lead to differences in adult pain sensitivity, with there being an effect depending on the rat strain studied, as well as a sex difference. Female rats display an increase in pain sensitivity due to maternal deprivation across the strains, compared to their male counterparts. This increased sensitivity was shown to be reversible in adulthood with chronic antidepressant treatment or by additional stimulation directly after maternal deprivation as a neonate.

A positive family history of pain has been associated with increased pain complaints as well as greater experimental pain ratings in females, but not in males (Fillingim 2000). This study indicates a physiological difference between the sexes, which is related genetically to family pain history. The extent to which this association between pain sensitivity and family history is driven by genetic versus environmental factors is not known.

Candidate Genes

The Opioid Receptor Family

The opioid receptor family is paramount in analgesia. There are three classes of opioid receptors: μ, δ and κ. Each family has distinct but also interactive functions and each is a product of a single gene, with some alternative splicing resulting in several different isoforms (Pan et al. 1998; Pan et al. 2001). The receptors all have conserved transmembrane domains as well as intracellular loops, with class differences found in the extracellular loops, and the amino and the carboxy ends of the protein (Chaturvedi et al. 2000). Opioid receptors are found in non-neuronal cells as well as the CNS. The three

receptor types, although they are conserved in structure, have divergent expression patterns and regulatory mechanisms (Wei and Loh 2002). Hetero- as well as homodimers of the receptors are formed in the plasma membrane of peripheral neurons.

A gene on chromosome 6, containing four exons, encodes the μ (mu) opioid receptor. It is involved in the targeting and preferential binding of morphine. It has been previously reported that allelic variations in the μ opioid receptor lead to alterations in the endogenous system related to addiction susceptibility (Liu and Prather 2001). The two best characterized polymorphisms are in exon one of the μ opioid receptor gene (*OPRM1*), which encodes the extracellular domain of this G protein coupled transmembrane receptor. These polymorphisms are A118G (N40D at the protein level) and C17T (A6V). All the other known variants have a rare allele frequency of <5%, and no functional analysis has been attempted. Variations in the 5' regulatory region have also been found, but they do not appear to affect gene regulation (Mayer and Hollt 2001). The A118G SNP (single nucleotide polymorphism) has been functionally shown to specifically affect β endorphin binding (ligand of the endogenous opioid analgesic pathway) to the receptor, and eliminates the putative N-glycosylation site that the asparagine provided in the extracellular domain (Bond et al. 1998; Mayer and Hollt 2001). The ß endorphin binding affinity is increased three fold for protein encoded by the G allele. Binding of β endorphin to the μ opioid receptor activates the receptor, which leads to activation of potassium channels (Bond, LaForge et al. 1998).

The genetic variants of the δ (delta) opioid receptor (gene *OPRD1*) have been linked to the heredity of pain sensitivity in mice (Mogil et al. 1997) and humans (Kim et al. 2004). It has been shown that activity of the δ receptor is μ receptor mediated and that

the δ receptor is found in the inactivated state intracellulary. Once the μ opioid receptor is activated, the δ opioid receptor is recruited to the membrane, and the μ and δ receptors have similar mechanisms of signaling (Cahill et al. 2001). The δ-opioid receptor can also heterodimerize with the μ and κ-opioid receptors (George et al. 2000; Gomes et al. 2000). The most frequent of the *OPRD1* gene variants is T80G (F27C), which is located in exon one and has a G allele frequency of 9% (Mayer and Hollt 2001).. The other predominant known variant is a silent polymorphism found in exon three, T921C. Although this is a silent mutation and the protein sequence is predicted to remain unaffected, this SNP has been linked to heroin abuse in the German population (Mayer and Hollt 2001). This suggests that either the silent variant might affect RNA splicing/stability, or it could be in linkage disequilibrium with another variant that carries a functional effect.

The κ (kappa) opioid receptor, whose gene (*OPRK1*) is found on the q arm of chromosome 8, has been linked to sex differences in analgesia (Gear et al. 1996). The kappa opioid receptor has been shown to heterodimerize with the delta opioid receptor, having a binding affinity different to that of each homodimer (Wessendorf and Dooyema 2001). The most common κ variants known appear to be silent polymorphisms, which are not predicted to affect the protein level. I am examining three of the most common SNP sites: *G36T* in exon one, and *A843G* and *C846T* in exon three (Mayer and Hollt 2001). These silent variants have proven negative in a few association studies of addictive disorders (Mayer and Hollt 2001), but there are no studies of these variants in pain or analgesic responses.

The Melanocortin Receptor Family

The melanocortin receptor family is very interesting because its members have diverse and distinct functions. They are G protein coupled receptors (GPCRs) and belong to the rhodopsin group of receptors (Fredriksson et al. 2003). Melanocortin 1 receptor (MC1R) has historically been known to be involved in coat color and pigmentation. This receptor is involved in the activation of eumelanin synthesis by the binding of this receptor to its endogenous ligand (∝melanocyte stimulating hormone, -∝MSH) and adrenocorticotropic hormone (ACTH) (Mountjoy et al. 1992). MC1R is spliced from a precursor gene called proopiomelanocortin (*POMC*). Upon activation of the receptor, activation of adenyl cyclase occurs and there is an elevation of cAMP levels in melanocytes, leading to increased melanin and pigmentation. Human mutations in the *MC1R* gene are associated with red hair and fair skin (type I and II in the Fitzpatrick Clinic Scale) (Valverde et al. 1995; Box et al. 1997). Rare individuals who are null at *POMC* have red hair, adrenal insufficiency and are obese due to lack of ∝MSH and also the lack of ability to stimulate the whole melanocortin receptor family (Krude et al. 1998). Loss of function of the *MC1R* gene results in a yellow coat color in mice (Robbins et al. 1993; Jackson 1997). In rodents, there are two loci that control pigment color-*extention* and *agouti*. *Extention* is also called *MC1R* and its endogenous antagonists are the agouti protein (ASIP) (Lu et al. 1994) and the agouti-related peptide (AGRP). A rescue study has been done in which the researchers expressed a *MC1R*-containing bacterial artificial chromosome (BAC) transgene in *Mc1r* knock out mice and observed a darkening of the coat color in a copy number dependent manner (Healy et al. 2001). *MC1R* is expressed in a number of peripheral tissues and cells including

leukocytes, where it mediates anti-inflamatory actions as an inhibitor of pro-inflamatory cytokines (Chhajlani 1996; Lipton and Catania 1998). MC1R has been shown to be expressed in the ventral periaqueductal grey (PAG) as well as in glial cells involved in the pain pathway (Xia et al. 1995; Wikberg 1999). Futhermore, our group and collaborators recently showed that *MC1R* variants affect analgesic efficacy and that this system is mediated by the kappa opioid receptor and influenced by cycling estrogen (Mogil et al. 2003). This work will be further discussed in chapter 4.

CALCA1/αCGRP Receptor

CALCA1/αCGRP receptors are synthesized in the thyroid by the parafollicular cells and can mediate a reduction in serum calcium levels. *CALCA1/αCGRP* is a polycystronic gene, which is alternatively spliced depending on the cell and tissue type into calcitonin (CALCA1), or the receptor for calcitonin (αCGRP). *αCGRP* is a 6 exon gene product, which is only neuronally expressed, and is an important regulator of vascular tone and blood flow. The *CALCA1* gene product includes exon 4 while the *CGRP* product instead includes exon 5. The first three exons are common in both gene products but exon 1 contains 5' untranslated sequence. There are two other genes that are similar: *CALCB* which produces a second CGRP without alternative splicing, and *CALCP*, which is a pseudogene. Elevated cerebrospinal fluid CGRP levels have been found in patients with depression (Mathe et al. 1994) and fibromyalgia (Vaeroy et al. 1989). Thus *CGRP* is a reasonable pain candidate gene as it is stimulated by the activated delta opioid receptor, inhibited by kappa and mu opioid receptor activation, and colocalizes in vesicles with Substance P (another neurotransmitter involved in the pain

pathway via the NMDA receptor) (Bao et al. 2003). CGRP causes vasodilation as a result of binding to CALCA1 receptors (Sato et al. 2000).

Chronic Pain Conditions

Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a common and often debilitating gastrointestinal disorder affecting up to 15% of the US population, predominantly adult females (Talley 1999). It is characterized by recurrent abdominal discomfort or pain associated with altered bowel habits with diarrhea/constipation, and is more common among women than men. There are specific criteria associated with the diagnosis of IBS and these are reviewed by Talley (Talley 1998). A hallmark of IBS is enhanced sensitivity to visceral stimulation, and some patients have reported enhanced pain sensitivity in remote anatomical regions (Verne et al. 2001). The pathophysiology of IBS remains an enigma. Heredity has been shown to play an important role in this disorder, through twin studies (Levy et al. 2001), and thus IBS is considered a multifactoral trait. A review of the literature indicated that there is a high comorbidity of irritable bowel syndrome with other functional gastrointestinal disorders and it is suggested that there may be a common pathophysiology. The review also mentions that IBS patients also suffer from other comorbid disorders such as depression, anxiety, fibromyalgia (49% of IBS patients), chronic fatigue syndrome (51%), temporomandibular disorder (TMD) (64%) and chronic pelvic pain (50%) (Whitehead et al. 2002). There is some evidence of generalized enhancement of pain sensitivity in IBS (Verne and Cerda 1997; Verne and Price 2002). Research has also examined the effects of sex hormones on visceral function and pain, there is a pronounced difference in pain sensitivity across the menstrual phases (Bajaj et al. 2002). Men display a shorter gastrointestinal (GI) transit time especially in the right

colon (Meier et al. 1995), and postprandial gastric relaxation is longer in females (Mearadji et al. 2001). It has been shown that bowel movements in females are altered during the menstrual cycle with prolonged GI transit times in the leuteal phase of the cycle (when progesterone is increased). Progesterone is a smooth muscle relaxant which might explain the gender differences in visceral pain (Wald et al. 1981; Waliszewski et al. 1997). It is interesting that IBS patients report an increase in symptoms during menses (Heitkemper et al. 1993). There is a rat model of the pathogenesis of this disease, which is achieved by the rectal injection of mustard oil which persists to cause a state of chronic visceral hypersensitivity (Al-Chaer et al. 2000). There is a mouse model of post infectious gut dysfunction, which leads to muscle hypercontractility and this study implicates a few genes in gut dysfunction. The investigators believe that post infectious irritable bowel syndrome may be a result of Th2 cytokine induced expression of TGF β1 and an up-regulation of the COX-2 and PGE2 in smooth muscle cells (Akiho et al. 2005).

Fibromyalgia

Fibromyalgia syndrome (FMS) is a rheumatological condition characterized by chronic widespread muscle pain, which affects women disproportionately (Staud 2002). FMS is associated with general soft tissue sensitivity in the body, lack of REM sleep, fatigue, parethesia, numbness, headache, swelling, and some patients may have other pain syndromes such as IBS. Ninety percent of patients are women and about half of them suffer with IBS in addition to their FMS symptoms (Wallace 1997). A specific diagnosis is usually made by excluding other diseases based on symptoms. FMS is characterized by a generalized heightened pain sensitivity to mechanical and non-mechanical stimulation, and its pathogenesis remains unclear. These patients display quantitative

abnormalities in pain perception under experimental conditions, in the form of allodynia (pain with innocuous stimuli) as well as hyperalgesia (increased sensitivity to a painful stimulus) (Staud and Smitherman 2002). Many FMS patients also meet Diagnostic and Statistical Manual of Mental Disorders VI (DSM VI) criteria for mood disorders such as depression. The precursor to serotonin, a molecule called 5-hydroxy-tryptophan (5HTP), has been the subject of heated debate as to its role in the pathology of this disease but the data remain inconclusive (Wolfe et al. 1997; Juhl 1998; Alnigenis and Barland 2001). This leaves the pathology of this debilitating syndrome open to other incriminating molecules, which we are investigating. In one study, 46% of affected patients reported a positive familial history of FMS (Offenbaecher et al. 1998), also implicating a genetic predisposition.

In the subsequent chapters, I describe my work as part of a collaborative group of human and mouse geneticists, pain researchers and physicians. Our goal has been to test candidate gene polymorphisms for a role in pain sensitivity, chronic pain, or response to pain medication. Furthermore, I undertook a laboratory investigation to study whether delta or kappa opioid receptor polymorphisms might have altered function.

CHAPTER 2
MATERIALS AND METHODS

Candidate Gene Selection

Literature Search

Pubmed (NCBI) was used to search current literature to identify genes involved in the pain pathway that might play a role in pain sensitivity and analgesia. The literature was examined and candidate genes were selected.

Single Nucleotide Polymorphism (SNP) Selection

Single nucleotide polymorphisms for candidate genes were identified using the NCBI websites Entrez SNP or Entrez Nucleotide databases. Only SNPs with a minor allele frequency of ≥ 5% were used, based on the probable final sample size we expected to have (200 people per patient population). This was to ensure we would have sufficient power to detect association.

Genotyping

Primer Design And Synthesis

PCR primers were designed using several different methods. The primer 3 program (Whitehead Institute, MIT http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used in selection of primers flanking some SNPs using nucleotide sequence obtained from the NCBI website. Primers reported in current literature were also used for some SNPs. Primers were synthesized by the Qiagen Operon company (Valencia, California). Primer sequences and PCR conditions are listed in table 2-1.

Polymerase Chain Reaction (PCR)

Primer annealing temperatures were optimized using a gradient PCR machine (MJ Research). Conditions varied for each specific primer set (see table 2-1). Either Hotmaster Taq (Eppendorf) or home-made Taq polymerase (made using Qiagen protocol) was used in polymerase chain reaction at a concentration of 0.62U per reaction (initial concentration of stock is 5U per microliter). For the Hotmaster reactions, the buffer provided was used at a concentration of 1X (2.5µL per reaction for a total reaction volume of 25µL). For the home-made Taq polymerase, Roche Applied Science (Indianapolis, Indiana) PCR buffer was used. dNTPs (Invitrogen) were used at a concentration of 10mM and the primer concentration was 10ng per reaction. Some PCR reactions required the addition of DMSO to abate the non- specific binding of the primers to the genomic DNA. The genomic DNA for each subject was added at a quantity of 50-100ng per reaction. The amplification was performed on either a MJ Research PCR machine (PTC 200) or a Hybaid (Thermo) PCR machine as follows: 95°C for an initial 5 minutes, then cycling (35X) at 95°C for 30 seconds, the specific annealing temperature for 30 seconds and then an extension at 72°C for 45 seconds. A final extension was performed for 10 minutes before the PCR was completed.

SNP Analysis

PCR products were visualized by electrophoresis and ethidium bromide staining on a 1.2% agarose gel (Bio-Rad). These products were either sequenced or digested to allow SNP detection, depending on the SNP as outlined in table 2-1. For restriction enzyme digests, 5µL of PCR product was used with 2µL of the appropriate restriction enzyme buffer, along with 0.5µL of enzyme in a final volume of 20µL (the reaction was spiked

with 0.5µL of enzyme after the first hour of digestion at the manufacturer's recommended temperature, to maximize the digestion efficiency). Digests were separated on a 1.6mm 8% native polyacrylamide gel (10mL 40% acrylamide, 5mL Tris buffer, 34.7mL dH_2O, 40 µL temed and 300µL ammonium persulfate) for two hours at 200V and were visualized by ethidium bromide staining. Cycle sequencing of PCR products utilized an ABI Prism R310 sequencer and Big Dye chemistry 2.0 at a dilution of ¼, using the PCR primers as sequencing primers. PCR products were purified using Millipore microcon filters (Fisher Scientific) and sequencing reactions were purified using Edge Biosystem sephadex columns (Gaitherburg, Maryland). Sequence data analysis was done using the Sequencher program (Gene Codes Corporation, Ann Arbor, Michigan). Some of the genotyping was done using the pyrosequencing core at the University of Florida, once the sample size became unmanageable for manual sequencing. The full list of genotyping is depicted in the appendix.

Cloning

Opioid Clones

The delta opioid cDNA clone was a generous gift from the lab of Dr. Brigitte Kieffer at the Louis Pasteur Institute in Paris, France. The kappa opioid receptor cDNA was a gift from Dr. Liu-Chen at Temple University in Philadelphia, USA.

Upon receiving the clones, primers with a Kozak sequence at the 5' end and restriction sites specific for the multiple cloning site of the pcDNA3.1v5/his (Invitrogen) were synthesized (figure 2-1) (Qiagen Operon) and PCR was performed using the high fidelity Taq polymerase Discoverase (Invitrogen). The PCR products were cloned into the TOPO4 vector (Invitrogen) and were transformed into TOP10 *E. coli* cells using the manufacturer's protocol. Cells were plated on LB agar plates containing 100µg/mL

ampicillin. Single colonies were selected after 16 hours at 37°C, and grown in 4mL of LB broth with ampicillin at a final concentration of 100µg/ml overnight. Plasmid mini preps were performed the next day using the Qiagen mini prep kit, following the manual provided in the kit. The clones were screened for an insert using single and double restriction digests, and positive clones were then sequenced with Big Dye 2.0 chemistry as described above except for that Big Dye was used at a ½ dilution. The sequences were generated by the Center for Mammalian Genetics (CMG) core. Once the correct sequence was identified, glycerol stocks of this clone were made using 1mL of the overnight culture mixed with 1mL of a 60% glycerol solution, and stored at -80°C.

Site Directed Mutagenesis

Four inconsistencies were found in our *OPRD1* cDNA sequence compared to the NCBI entry (NM_000911.2). We set out to correct our sequence and also create the more common T allele (the original construct from France had a G at this SNP location) by using the Stratagene QuikChange Multi Site-Directed Mutagenesis Kit. Four 5' phoshorylated primers were synthesized (Qiagen Operon) to incorporate the desired change into the plasmid sequence (see Table 2-2). For *OPRK1*, changes were introduced with site-directed mutagenesis. After the PCR and parental strand digestion with DpnI was performed, the single stranded plasmid was transformed into Stratagene XL- gold ultracompetent *E. coli* cells. Single colonies were selected and grown in liquid media under ampicillin selection as described above. Mini preparations were performed and the colonies were screened for the correct sequence by using the cloning primers. Once the desired clone was found, inserts were then digested using the specified restriction enzymes (method described above). The mammalian expression vector (pcDNA.1

v5/his) was digested with the same enzymes. In the case of the delta opioid receptor, the restriction sites were HindIII on the 5' site and BstB1 (isoschizomer of Sfu1) at the 3' end of the insert. For *OPRK1*, HindIII was designed into the 5' primer and AgeI on the 3' primer. The digested plasmids were run on a 1.2% low melt agarose gel and excised and extracted using the Qiagen gel extraction kit. The inserts were subsequently ligated into the expression vector using DNA T4 ligase (1µl emzyme, 2µl ligase buffer, 3µl insert, 1µl vector and 13µl d_2H_2O) and this was incubated at 4°C overnight. After incubation, 10µl of the ligation reaction was transformed into Stratagene XL1-Blue *E. coli* cells and incubated on LB agar with 100µg/ml ampicillin at 37°C overnight. Single colonies were selected and grown in liquid LB ampicillin media for 16 hours at 37°C. mini preps were done as described above and the plasmids were screened as above. Once positive clones were identified they were sequenced by using the forward flanking primer on the mammalian expression vector and the reverse cloning primer. Once sequence was confirmed, a maxi plasmid prep was performed using the Qiagen Maxi prep kit from a 500ml culture of a desired colony. The maxiprep plasmid DNA was quantified with a spectrophotometer (Bio-Rad).

Tissue Culture

Stable Transfections

HEK 293 cells (human embryonic kidney immortal cell line) were transfected with recombinant pcDNA3.1 vectors (OPRD1, OPRK1) as follows. In a 1ml transfection reaction, I added 20µg of plasmid DNA, 50µl 2.5M calcium chloride (CaCl), 450µl N-N-bis(2-hydroxylethly)-2-aminoethane-sulfonic acid(BES) buffered saline (BBS), and 500µl autoclaved deionized water. This reaction was incubated for 10 minutes at room

temperature and then was added to a 10cm tissue culture plate with HEK 293 cells at ~60% confluency. The plates were incubated for 24 hours at 33°C with 3% CO_2. The 293 media (Dilbecco Modified Eagle Medium-DMEM) with 10% neonatal calf serum (NCS), 1% penicillin, 1% streptomycin (Gibco-BRL) was changed after this time and the plates were then incubated for a further 24 hours at normal incubation parameters (37°C and 5% CO_2). After the next 24 hours the cells were split if they were at 90% confluency and then put under selection using 0.31g Geneticin (Sigma- RBI), in 500ml DMEM (10% Fetal Bovine Serum (FBS)) which was filter sterilized. The transformed cells were kept under selection conditions for three to five weeks and were then frozen in DMEM with 10% FBS and 10% DMSO. The cells were kept in liquid nitrogen.

Characterization and Verification of Stably Transfected Cell Lines

Gene expression levels

Transformed HEK 293 cells were defrosted from liquid nitrogen and grown in 293 media until confluent. The media was changed after 24 hours post defrost to remove the DMSO from the cells. These cells were no longer under selection conditions. Once confluent, the cells were counted and then plated out at concentrations that included 200 cells per 10cm dish as well as 1000 cells per dish, 5000 cells per dish and 10,000 cells per dish. These cells were monitored to detect single cell colonies. Cloning rings (Fisher) and silicone grease was used to isolate these single cell colonies by trypsinization (Gibco 0.25%) and moved into a 24-well plate to grow until confluent. Once these single cell colonies were confluent, the cells were split into two 6 well plates and allowed to grow to confluency once again. One of the plates was washed with 1mL phosphate buffered saline pH 7.4 (PBS, Gibco) followed by lysis with 750µl Trizol (Invitrogen) for RNA

extraction. The RNA was resuspended in 50µl of DEPC treated water (Ambion), and 1µl of the RNA was run on a 1.2% TAE agarose gel with ethidium bromide to observe general quality and quantity under UV illumination. Another microliter was used for spectrophotometric quantification.

For northern blot, the RNA was subjected to electrophoresis on a 1% agarose gel made with 1X MOPS buffer and 6% formaldehyde (Fisher Scientific). The appropriate amount of RNA was added to an individually wrapped RNAse free eppendorf tube along with 16 µl loading buffer (300µl formamide, 105µl filter sterilized 37% formaldehyde solution, 60µl tracking dye, 60µl 10X filter sterilized MOPS buffer and 3µl ethidium bromide). The samples were incubated at 65°C for 10 minutes and then electroporated on the formaldehyde gel at 4°C in 1xMOPS at 105V for one hour and fifteen minutes. An RNA ladder was run with the samples (Invitrogen). The gel was then photographed using the Eagle Eye photo documentation system. The 28S, 18S and 5S ribosomal subunits were marked on the gel using India ink, and the molecular weight markers from the ladder were measured using a ruler relative to the wells of the gel. The gel was then blotted overnight using two pieces of Whatmann 3MM paper as a wick to allow the 20x SSC to be absorbed through the gel. The Gel was placed upside down, with the Hybond N+ (Amersham Biosciences/GE Healthcare, Piscataway, New Jersey) nylon membrane placed on top of the gel. Two pieces of Whatmann 3MM paper and a stack of paper towels were placed on top of the membrane. A plate was applied to the top and then it was all weighted down for an overnight transfer. The next day, the blot was dismantled, and the wells were marked on the membrane using a pencil and writing directly through the gel. The 28S, 18S and 5S subunits were also marked on the gel at this time and the

lanes were numbered. The membrane was then placed in the vacuum dryer for 2 hours to allow cross-linking of the RNA to the membrane.

The full-length cDNA PCR products were used as a probe for the Northern blot. A β actin PCR product was used as a probe in a separate hybridization to control for loading. Approximately 20ng of the products were used for radiolabeling. Five microliters of random primers were added to the product in 11.5µl, boiled for 5 minutes and then put on ice. To the cooled tube, 5µl of dCTP buffer was added as well as 2.5µl [P^{32}]dCTP (25µCi) (Amersham Biosciences/GE healthcare, Piscataway, New Jersey) and 0.8µl Klenow polymerase fragment (Stratagene PrimeIt II labeling kit). This was gently mixed and incubated at 37°C for at least 15 minutes. The labeling reaction was purified using a Quiagen kit. The manufacturer's protocol was followed and then the probe was boiled for 5 minutes. The blot was briefly soaked in 2xSSC and then allowed to pre-hybridize in 20ml Church and Gilbert hybridization solution (500mM sodium phosphate pH7.2, 7% SDS) for 30 minutes in a glass hybridization tube in a 65°C rotator. After the pre-hybridization step, the solution was removed and another 5ml of the hybridization solution was added to the blot as well as the 200µl of the probe. This was returned to the 65°C rotator to hybridize overnight. The next day, the hybridization solution was removed and the blot was washed twice with 65°C 1xSSC, 0.1%SDS for 15 minutes each. The blot was then washed with 0.1xSSC, 0.1%SDS at 65°C for 20 minutes. The blot was then allowed to dry slightly, wrapped in saran wrap, and placed between two screens in an x-ray cassette. An x-ray film (Kodak XAR)was exposed to the blot at -80°C for 2-7 days, and the film then developed in an automated film developer. The blot

was then stripped in 0.1xSSC, 0.1%SDS by microwaving until the solution boiled, and re-probed using the β-actin probe in the same manner as described above.

Protein expression levels

A western blot was performed to see the relative expression from the plasmids at the protein level. Stably transfected cells were grown on a plate to confluency. The cells were trypsinized, 5ml of PBS was added and the cells were transferred to a 15ml conical tube. They were then spun down at 500g for 5 minutes and the supernatant was removed by aspiration. 5ml of PBS was added and the cells were resuspended and then re-centrifuged for a further 5 minutes. After the PBS was decanted, 1ml of RIPA protein lysis solution (250mM NaCl, 50mM Tris-HCl pH7.4, 1% Nonident NP-40, 0.25% Na deoxycholate in H_20 with 1x complete protease inhibitor cocktail (Boehringer-Mann) added right before use) was added to the cells. The lysate was transferred to a 1.5mL microfuge tube kept on ice. The cells were further mechanically disrupted by sonication 2x for 5 second bursts from the probe sonicator set at 30. The lysate was centrifuged at 10,000g for 5 minutes at 4°C, and the supernatant was then transferred to a new tube. Samples were then mixed with sodium dodecyl sulfate- containing electrophoresis loading buffer containing 2mol/L urea and 5% 2-ME to denature the proteins, and then the samples were boiled for 10 minutes. 50μg of each cell lysate were loaded onto a precast 12% mini SDS-acrylamide gel and run at 4°C overnight at 45mA run in a MOPS-SDS running buffer. The next day, the samples were electroblotted to nitrocellulose sheets in transfer buffer containing 0.1%SDS. The blot was rinsed in 1X Tris buffered saline (TBS) 3x for 5 minutes each and then the blot was blocked in 1% BSA/5% milk for one hour. After blocking, the blot was washed once again in TBS 3x for 5 minutes

and then I added a 1:2000 dilution of Alkaline Phosphatase conjugated anti-his antibody (Invitrogen, Calsbad, California) along with 0.1% Tween-20 and 1% non-fat dry milk. The primary antibody was left on the blot overnight. The next day, the primary solution was removed and the blot was washed. Next, a chemiluminescent developing solution was added, which consisted of NBT, BCIP, in NTMT (5M NaCl, Tris-HCl pH 9.2, 1M $MgCl_2$, 10% Triton-X). After the blot had developed sufficiently, the solution was removed and the blot was analyzed by exposure to x-ray film.

Functional Analysis

Immunocytochemistry

Stably transfected HEK 293 cells were grown on an 8 well chamber slide (Fisher) until about 50% confluent. The media was then removed and the cells were washed with 200µl of PBS per well. This was removed 5 minutes later, and 200µl of 100% methanol was added for 5 minutes to allow the fixing of the cells. After this incubation the cells were washed twice for 5 minutes each, with PBS. The cells were then blocked by the addition of 200µl of PBS with 10% FBS and were incubated for 20 minutes at room temperature. After the blocking solution was removed, a PBS/10% FBS solution with either a 1:250, 1:500 or a 1:1000 dilution of the FITC conjugated anti-his antibody was added. Analysis of the slides was done using fluorescent microscopy.

cAMP Activation Assays

The Stable cell lines were transfected as described above with a pCRE plasmid containing the β-galactosidase gene. This transfection was transient, so the cells were left to grow for 48 hours and then stimulated by the addition of a sequential dilution of agonists (β-endorphin, DPDPE for OPRD1 and Dynorphin A, U69593 and Tan 67 for OPRK1-Sigma Aldrich, St. Louis, Missouri). These serial dilutions of agonists were

made starting at 10^{-4} or 10^{-6} depending on the stock concentration. The dilutions were made in media containing 1X 3 isobutyl-1-methylxanthine (IBMX), which inhibits intracellular phosphodiesterase and allows for the accumulation of cAMP. Included also in half of the dilutions was forskolin, which activates the receptor in the absence of ligand as the opioid receptors decrease the cAMP levels in the cell. The dilutions were added to the wells in duplicate (150µl/well), in a dose dependent manner and were incubated at 37°C with 5% CO_2 for 6 hours and then lysed using 50µl/well cell lysis buffer (250mM Tris-HCl pH=8.0, 0.1% Triton X-100 in H_20). A protein assay was performed on 10-20% of the lysate by Bradford analysis using a commercial protein dye (Bio-Rad), while the rest of the lysate was mixed with an ortho-nitro-phenyl-β-D-galactopyranoside (ONPG) solution (for 100mL: 84.05mL dH_20, 15mL 0.4M Na_2HPO_4, 100µl 1M $MgCl_2$, 500µl 2M KCl, 200mg ONPG). After addition of this solution, the plates were incubated at 37°C to aid in developing, and samples were measured at a wavelength of 405nM at various intervals.

Table 2-1 Primer sequences and PCR conditions as well as genotyping detection strategy.

Gene (SNP)	**PCR Primers**	**Strategy**	**Annealing temp.**
MC1R (V60L, K65N, D84E, V92M)	5'-cct ggc agc acc atg aac ta 3'- aga ggc tgg aca gca tgg	Sequence (494 bp)	62˚C
MC1R (R151C, R160W, R163Q)	5'-tgc agc agc tgg aca aat g 3'- atg tgg acg tac agc acg g	Sequence (292 bp)	62˚C
MC1R (D294H)	5'-tgc atc tca cac tca tcg tcc 3'-ata tca cca cct ccc tct gcc	Digest 228 bp PCR product with αTaqI restriction enzyme	62˚C
OPRM1 (C17T, A118G)	5'-gaa aag tct cgg tgc tcc tg 3'-gca cac gat gga gta gag gg	Sequence (302 bp)	61˚C
OPRD1 (T80G)	3'-cgc cgg ccc gca gcg gac tca 5'-gcg gcg gag ccg gcc ggc agc c	Sequence (272 bp)	75˚C
OPRK1 (G36T)	3'-gag tag acc gcc gtg atg at 5'-atc ccc gat tca gat ctt cc	Digest 203 bp product with PspOM1	60˚C
OPRK1 (A843G, C846T)	3'-ggc gta gag aat ggg att ca 5'-tga cta ctc ctg gtg gga cc	Sequence (358 bp)	62˚C
CALCA1 (G-855A, T-624C, C-590G)	3'-ctc gtg gga aac aag aga cg 5'-agt aga gga ctg aag tgc ggg	Digest 547 bp with BsmAI and AciI	65˚C
CALCA1 (Leu66Pro)	3'-cct tcc tgt gta tga tgc tgc g 5'-gcc ctg tcc cct agg act c	Digest 332 bp with AluI	65˚C

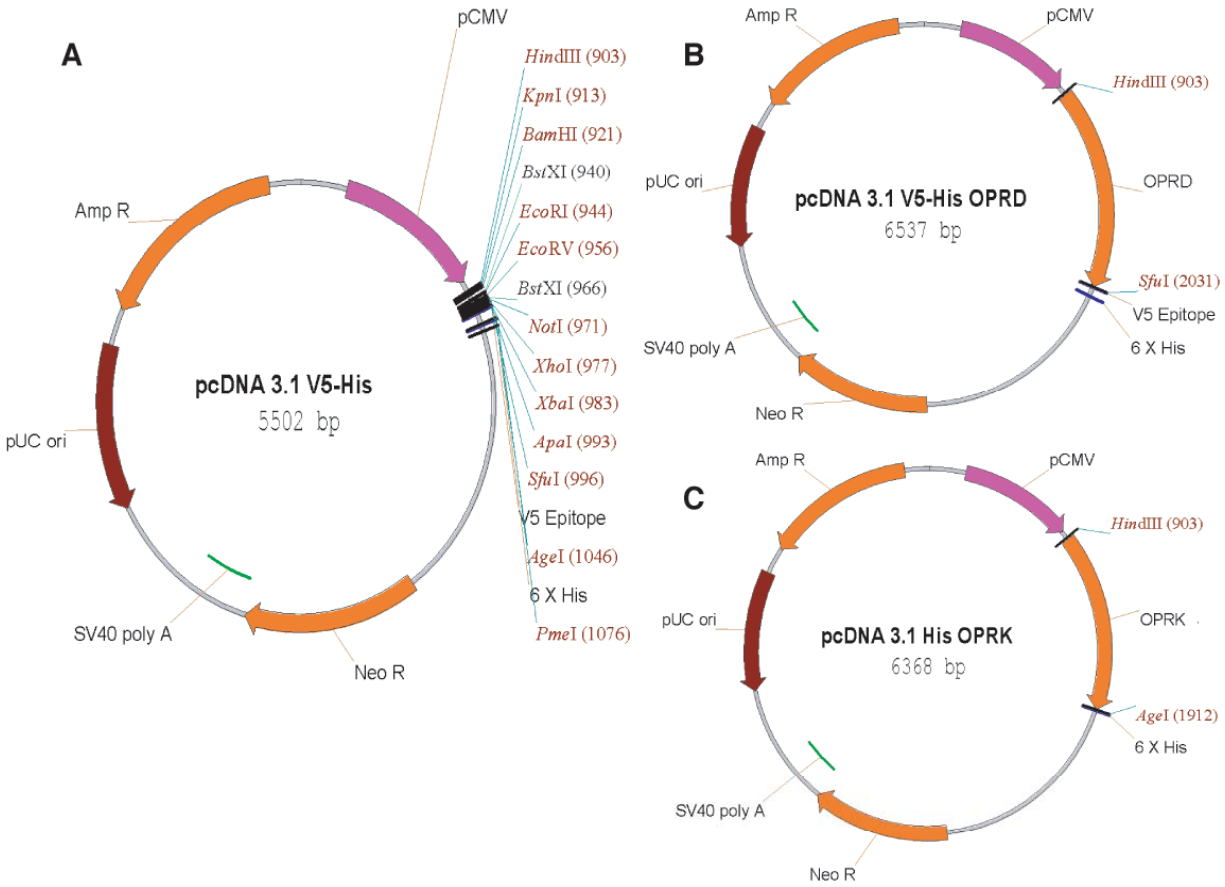


Figure 2-1 Vector maps made using the Vector NTI software. A) illustrates the empty vector highlighting the features of the vector including the multiple cloning site. B) represents the *OPRD* cDNA inserted into the HindIII and SfuI sites. There were two vectors made for this gene, one representing each allele. C) depicts the *OPRK1* gene in the HindIII and AgeI sites, which removed the V5 epitope tag from these constructs as there were also two different constructs made in order to represent the two alleles of the OPRK G36T polymorphism

Name of Primer	Primer Sequence (5'-3')
hDOR sense G80T	[phos]ccctagcgccttccccagcgctgg
hDOR sense T462C	[phos]ctgccaccctgtcaaggccctggact
hDOR sense A892G	[phos]cgctggtggtggctgcgctgcacc
hDOR sense G1108C/C1109G	[phos]ccggcggtggccgtgccgccttcg
hKOR G36T-T	ttccgcggggagcctggccctacctgcgccccgagc
hKOR G36T-T rev	gctcggggcgcaggtagggccaggctccccgcggaa

Figure 2-2 List of primers used for the site directed mutagenesis. The hDOR primers were 5' phosphorylated as a multi change kit was used and all these primers were used in one reaction. For the T allele, the first primer on the list was left out as the original cDNA had a T allele in base pair position 80. For the hKOR vector, there were no other sequencing errors so a single change kit was used.

CHAPTER 3
INTRODUCTION TO ASSOCIATION STUDIES

Candidate Gene Approach

Association studies have become the most popular method used today in the search for genes involved in complex genetic disease. The most common cause of Mendelian diseases is a single mutation resulting in a non-synonomous change in a codon, leading to an amino acid substitution or stop codon. Complex traits are more involved as both genetics and environment play a role in the pathogenic mechanism.

Some consider association studies to be a "fishing expedition," without justification or a testable hypothesis (Eisenach 2004). Clinical pain genetics studies in humans are now underway, with justification based on recent findings of differences in thermal and chemical stimulation to induce acute and chronic pain as well as nerve injury models in various inbred mouse strains (Mogil et al. 1999; Seltzer et al. 2001; Lariviere et al. 2002). These results suggest common genetic entities involved in pain processing are conserved throughout the mammalian species. The understanding of genetic factors involved in pain has been successfully approached from two different angles: 1) genetic screening of individuals with and without pain in order to identify novel proteins involved in the development of pain or pain processing, and 2) the identification of high risk populations who may develop chronic pain (Eisenach 2004).

Many association studies may lack power if the sample size is too small to elucidate a significant result, depending on the allele frequencies of the polymorphisms

examined. The greater the heterozygosity, the smaller the sample size needs to be in order to observe a true association. One test of association is to compare allele frequencies between cases and controls to allow the ascertainment of any change in the relative risk based on genotype (Belfer et al. 2004). In addition to this important predictive benefit, a goal of association studies is to ultimately lead to identification of the actual genetic changes that effect susceptibility, therefore providing improved prediction and targets for therapy. Two recent successes in this area involve identifying culprit genetic changes in Type 1 Diabetes (Onengut-Gumuscu et al. 2004) and macular degeneration (Esfandiary et al. 2005). Because association studies lack a long-distance power like linkage analysis, it is important to test multiple SNPs in a gene (when possible) since an effect may not be revealed unless a genotyped SNP is very close to (or is) the actual pathogenic change.

As technology is still relatively expensive in performing association studies, we had to restrict the number of candidate genes to be studied, and these were chosen with the following criteria in mind: evidence of the gene being involved in pain processing, allele frequencies, and the likelihood that the SNP may have a functional effect (Belfer et al. 2004). There are several different methods to predict the possible functional impact of a polymorphism based on whether it is located in the promotor region, the coding region, intronic regions or untranslated regions of the gene. If the polymorphism is located in the coding region, one can predict if the change is synonymous (silent) or non-synonymous, and how a non-synonymous change would affect the local environment of the protein. Protein prediction programs can ascertain if the change in the amino acid at the polymorphic position will have an effect on the protein structure or function, but the

caveat is that there has to be a structurally similar protein already in the database, which can act as a backbone for the assembly of the protein structure. In addition, even if an amino acid change does not affect structure significantly, it could be a key residue for post-translational modifications such as phosphorylation or glycosylation. Polymorphisms located within promoters may affect the structure of the promoter region, which may have an effect on how accessible this region is to transcription factors. They may directly affect binding affinity of these transcription factors or RNA polymerase to the promoter. Intronic polymorphisms may also confer regulatory alterations to the RNA, as splicing motifs may be altered as a result (Mendell and Dietz 2001). SNPs in the untranslated region of the gene may affect RNA stability. Because finding genes involved in pain is still in its relative infancy, association studies are paramount to the progress in the pain field, to be followed by functional analysis.

In the association studies done to date, Quantitative Trait Locus (QTL) mapping has suggested to the researchers that most SNPs involved in complex trait disease will be in the regulatory regions of the gene (King and Wilson 1975; Mackay 2001) even though most reported SNPs are in gene introns (Glazier et al. 2002). The Catechol-O-Methyltransferase (*COMT*) gene, which has some SNPs in the promotor region of the gene, has been comprehensively researched within the past few years and it has shed light on pain sensitivity as it relates to genetics (Zubieta et al. 2003). Recently, this gene's effect has been examined using different potential haplotypes that are based on five common polymorphisms within the gene. Researchers identified three different haplotypes that encompass 96% of the human population, and these haplotypes were able to predict pain sensitivity as well as predict individuals at risk for developing a chronic

pain condition called myogenous temporomandibular disorder (Diatchenko et al. 2005). Haplotypes can be more powerful than single SNPs for some analyses.

Silent coding-region SNPs have also been implicated in the pathogenesis of a disease state, which may reflect a linkage to a nearby functional SNP, or a haplotype effect, or an effect on RNA. For example, significant association was reported between a silent mutation in the FAS coding region and papillary thyroid carcinoma (Basolo et al. 2004). Our laboratory examined the most plausible candidates involved in human pain sensitivity and analgesic effect as determined from mouse data, and inferences from literature. We assessed three different populations of participants: a healthy subset of individuals, and two chronic pain groups who have either been diagnosed with fibromyalgia (FMS) or irritable bowel syndrome (IBS) but not both.

As discussed in greater detail in chapters 4 and 5, we measured a number of phenotypic variables in our subjects. This included obvious items such as gender, age, ethnic background, and diagnosis. However we also attempted to gather data particularly helpful relative to certain candidate genes (skin tone/hair color for *MC1R*), other clinical parameters and experimental pain test results. Thus we had the possibility of finding significant associations of certain SNPs with only some variables, which could help shed some light on actual biochemical mechanisms.

Statistical Analysis

Two different types of analyses are commonly used in the interpretation of an association study such as ours. These two statistical methodologies are quantitative trait locus analysis (QTL) and analysis of variance (ANOVA). Although it is a characteristic of statistics to make certain assumptions with different algorithms, these have been

considered and taken into account in our analyses. ANOVA and QTL are being used to screen for relationships between the SNPs and the phenotypic data.

QTL

In order to perform QTL linkage of a marker to a disease state, one must have a large experimental population. A QTL is the inferred location of a gene that affects a trait measured on a linear scale. These traits may be complex traits, being affected by more than one gene. This type of mapping has become commonplace due to the commercialization of molecular markers, which are highly polymorphic and easily genotyped, as well as the sequencing of the human and mouse genomes. QTL analysis is actually just a specific model of regression and likelihood analysis (Henshall and Goddard 1999). This allows one to compare marker genotype classes for different phenotypes instead of having to look at the phenotype first. The regression model of statistics makes the assumption that there is a linear relationship between the two variables of interest.

ANOVA

The analysis of variance is designed to be used when one is comparing two or more different groups and there are multiple variables being examined. This statistical method is used to determine if the observed differences can be attributed to something other than just chance variation in the population. When necessary, potential confounding variables (e.g. age ethnicity) can be controlled for using analysis of co-variance (ANCOVA). Usually from these statistics, a main effect is analyzed as well specific interactions in the population under analysis. ANOVA is more intricate t-test and therefore has similar assumptions in its usage, which are that the standard deviations in the different populations are equal. This statistic also assumes that samples are randomly selected

from the population (http://www.ccnmtl.columbia.edu/projects/qmss/anova_about.html). If we accept that each statistic has its own short-coming, we can proceed with analysis and lay a foundation for the unraveling of the complexity of the role of genetics in pain. ANOVA can help determine which variables (such as genotype) account for what percentage of the variation in each phenotype measured.

Negative Association Studies

We examined a total of six different genes encompassing fifteen different SNPs. These 15 SNPs were genotyped in 347 healthy controls, 74 IBS patients and 97 FMS patients. The positive associations found are described in the following two chapters but here we will impart some of the negative results we have found, which can be almost as important. The raw data of our three different groups have been compared to each other. The *OPRM1* SNPs do not have significantly different allele or genotype frequencies between the IBS patients, the FMS patients and the healthy subjects. The p values for C17T and A118G are 0.55 and 0.155 respectively, indicating that there is no significant difference between these groups of participants. For OPRD1, the p value with respect to the T80G polymorphism between the different groups is 0.64, again indicating no significant difference between the three populations. 0.38 and 0.98 are the p values for the *OPRK1* polymorphisms G36T and A843G, however, the C846T polymorphism appears to be significantly different between the three populations, but upon examining the raw data, this significance seems to likely due to rare allele effects, which may violate assumptions of our statistical test. The different genotypes are divided up into three different groups; the homozygotes for the major allele (CC), the heterozygotes (CT), and the homozygotes for the minor allele (TT). In the healthy population, as well as the FMS patients, there are no individuals who are homozygous for the minor allele (TT), whereas,

3.3% of the IBS patients have this genotype. This actual number of patients is 2. This is the only field where there is more than a 1% change in the allele frequency between genotypes and between groups, so this significant p value, in fact is not likely clinically significant and may be attributed still to chance.

The *CALCA* gene also showed in no convincingly positive results, as we found a p value of 0.42 for P4 and again we had a false positive for P2 (which includes two linked SNPs located 3 base pairs apart from each other) at 0.016. In this SNP analysis, we found that 2 people had the TTCC genotype in the normal population (constituting 0.58% of the group), and 3 individuals with IBS had this genotype (making up 4.1% of this group). However, given the implication of calcium metabolism in gut function, we hope additional subjects may strengthen this possible association. There were a total of 514 individuals genotyped in this analysis. MC1R was not analyzed in this case-control manner as we have shown that the minor alleles in this gene are associated with fair skinned people and this was not reported in the medical files of the chronic pain patients. Our studies are ongoing, and due to the low number of chronic pain patients we have at the moment, it may explain the lack of statistical significance we have found in our populations. As our patient numbers grow, our results may change. In addition, further phenotypic data are being gathered, such that we can perform association analyses within each group to see if any clinical parameters relate to genotype. Such data could be very helpful to understanding variability in phenotype (e.g. why some IBS patients have diarrhea and others constipation). A novel QTL algorithm is being developed for us by Dr. Rongling Wu (Dept. of Statistics) to help analyze our data using this method, which

will also screen for haplotype effects. Thus, the future may reveal additional discoveries from our work, based on new statistical analysis and additional clinical data.

CHAPTER 4
THE MELANOCORTIN-1 RECEPTOR GENE MEDIATES SEX-SPECIFIC MECHANISMS OF ANALGESIA IN HUMANS

MC1R

We decided to investigate the melanocortin-1 receptor gene because it has been implicated in pain sensitivity by QTL mapping in mice, which localized to the region where the *MC1R* gene is located (distal mouse chromosome 8). This was found to be linked to stress induced analgesia in female mice but not male mice (Mogil et al. 1997). Although this gene has classically only been related to the formation of pigment (Sturm et al. 1998), this QTL explained 17%-26% of the overall pain variance in stress-induced analgesia-treated female mice. By embarking on a murine study, it was found that the gene in this QTL responsible was, in fact, the *Mc1r* gene. It was found that the mouse strain with a recessive yellow mutation ($Mc1r^{e/e}$) (Cone et al. 1996; Tatro 1996), carries a frameshift in the region encoding the second extracellular loop of the protein. This frameshift leads to a completely non-functional receptor. When both sexes of these mutant and heterozygous mice were tested for pain sensitivity by a 49°C hot water tail withdraw assay, both before and after the administration of U50,488, (a kappa opioid receptor selective agonist) latencies were found to be significantly longer in males and were blocked in males by the NMDA antagonist MK-801 (Mogil et al. 1993). This MK-801 blocking action was obliterated in female mice with the $Mc1r^{e/e}$ genotype. This difference is related to cycling estrogen, as it was reversed by performing an ovariectomy and reinstated by subsequent hormone replacement therapy. These data suggest that

Mc1r functions in mediation of U50,488 analgesia. This was the basis for studying effects of *MC1R* variants in human pain. This latter mouse research was done by our collaborator Dr. Jeffrey S. Mogil, and published along with our subsequent human data, led by Dr. Roger Fillingim and described below (Mogil, Wilson et al. 2003).

Results

Pentazocine Studies

Redhead humans are analagous to the *Mc1r*$^{e/e}$ mice since most redheads are compound heterozygotes or homozygotes for *MC1R* rare alleles, which in this context will be called mutant alleles. Three major *MC1R* variants have been identified in association with red-headedness, which include the amino acid substitutions R151C, R160W and D294H (Rees et al. 1999). Our lab examined additional polymorphisms in the gene, which included V60L, V92M, and R163W. These variants have been shown to cause a loss of the function of the protein (Schièoth et al. 1999; Scott et al. 2002). The *MC1R* gene coding region was sequenced for these three mutations and other variants as described in chapter 2. We tested 18 females and 24 males (all healthy) who had different natural skin types as well as hair color (see figure 4-1). The people with type 1 or 2 skin (those who always burn when in the sun and who are usually red headed) were found to have two *MC1R* variant alleles (5 females and 9 males), while participants with a darker skin type (those less likely to burn upon exposure to sunlight) (13 females, 15 males) had one or no variant alleles. These participants were tested for thermal and ischemic pain sensitivity and tolerance, both at baseline and after the intravenous bolus administration of either 0.5mg/kg pentazocine or saline in a double blind randomized fashion. The thermal pain testing consisted of the administration of short, repetitive suprathreshold thermal stimuli to the right volar forearm, to assess temporal summation

of pain (Price et al. 1977). These pulses of heat were at 52°C and each pulse lasted for <1 second with a 2.5s interval between pulses, when the thermode returned to the baseline temperature of 40°C. The participants were asked to rate the pain on a scale of 0-100 and the assay was terminated when they rated the pain at 100 or asked that the assay be stopped. Only the first five pulses were used in the analysis, since 28% of the participants terminated the assay before all 10 intended pulses were administered. The ischemic pain assay was conducted by the submaximal effort tourniquet procedure (Moore et al. 1979). The left arm was exsanguinated by elevating the arm above the heart for 30s. A standard blood pressure cuff was then inflated to 240mm of mercury. Participants were then asked to perform 20 handgrip exercises, which were performed at 50% of their maximum grip strength. A pain threshold measurement was recorded (when the subject first reported pain) as well as a pain tolerance measure (when the participant asked for the assay to be terminated). The maximum assay length was 15 minutes and subjects were asked also to rate the pain intensity and unpleasantness every minute. This clinical pain testing was conducted by our clinical psychology collaborators under the guidance of Dr. Roger B. Fillingim at the UF/Shands Clinical Research Center. At these sessions, a vial of blood was collected for our genetic research.

As expected, ischemic pain thresholds and tolerances were found to be increased after pentazocine administration, along with decreases in ratings of pain intensity and unpleasantness at the same time. There was also a decrease in thermal pain intensity ratings after the drug bolus, which was not seen after the control saline dispensation (all p values <0.05 in relation to saline administration). These results suggest a strong analgesic effect of this drug. For ischemic pain, ANOVA showed there was a significant

sex x genotype effect ($P < 0.05$) and this interaction approached significance in thermal pain ratings ($P = 0.056$). In all the measures, the significant effects of genotype were found in females but not males. In fact, males reported only modest analgesia at this dose, while females with two of the MC1R mutant alleles (type I or II skin type and who tended to be redheads) displayed robust analgesia against ischemic pain, and was the only group to show any noteworthy analgesia against thermal pain (figure 4-2). It is clear from our analyses that *MC1R* genotype was more reliable when considering skin type than hair color. We found that lighter skinned people were much more likely have two or more rarer alleles compared to the darker skinned subjects and this did not hold quite as true for hair color. These results were mirrored in the mouse studies where female mice with the D6/D6 genotype (which during the linkage studies had two copies of the non-functional *Mc1r* gene), had a higher analgesia rating as tested by tail withdrawal latency from a 49°C water bath at different time points post U50,488 injection. The mice also demonstrated that this analgesic effect was mediated by the kappa opioid receptor as the non-functional strain of *Mc1r* mice was used for subsequent experiments on a B6 background. These $Mc1r^{e/e}$ mice were given U50,488, then they were given either a saline injection or MK-801, which is an NMDA receptor antagonist. The analgesic effect was lost in male mice after antagonist injection irrespective of the genotype of the mouse, but the wildtype females had no effect after MK-801 injection, which was lost in the $Mc1r^{e/e}$ mice. This experiment illustrated that the analgesic effect of the *Mc1r* gene is mediated by kappa opioid receptor activation. These data were published in 2003 (Mogil et al. 2003).

M6G Data

Pentazocine is not used as commonly as it once was in the clinic. Thus, along with another group of collaborators who did the mouse work, we decided to look at the effect of M6G, a metabolite of morphine which acts mainly on the μ opioid receptor, and the *MC1R* gene. This agonist was administered subcutaneously at a dose of 0.3mg/kg. The pain testing was done slightly differently in this study at the laboratory of Dr. Albert Dahan in the Netherlands. Here acute pain was induced by the application of electrical current via two surface electrodes placed on the skin over the tibial bone (the shin) on the left leg. Ten pulses of a 10Hz each were administered for a duration of 0.1ms. The intensity of the electrical current was increased in a stepwise fashion of 0.5mA/s from 0mA to a cut off current of 128mA. The participants (47 in all: 29 redheads and 18 non redheads) were instructed to press a button when they could no longer handle an increase in current and this measure became their pain tolerance measure and this also indicated the end of the stimulus. Genotyping showed that all the redheads had two or more *MC1R* mutant alleles, but none of the non-redheads. One of the redheads had an A insertion at base 27 which caused a frameshift, instead of the previously described alleles.

Baseline pain tolerance differed significantly between genotypes, with greater currents being tolerated by the participants with two *MC1R* mutant alleles (-20.9 (1.7 SEM) mA) compared to those subjects with zero or one mutant allele (-15.8 (1.2) mA) ($p= 0.018$, see figure 4-3). There was no significant sex by genotype effect. The effect of the M6G analgesic was significantly higher in participants with two mutant alleles compared to those with 0-1 mutations. The area under the time effects curves (pain tolerance relative to baseline) was 1.49 (0.09) mA and 1.18 (0.04) mA respectively with $p=0.003$ (figure 4-6). These numbers suggest that there is an increase in tolerance due to

M6G administration of 18% (4mA) in 0-1 mutant allele people and 49% (10mA) in two mutant allele people (figure 4-5). Again, this effect was not based on the sex of the participant, only the genotype, unlike pentazocine. The genotypic differences seen in the patients can be attributed to the pharmacodynamics of the M6G ligand acting on the μ-opioid receptor, as the plasma M6G concentrations remained the same at different time points post injection between the two genotypic groups (figure 4-7). Again, this human study was a translational research project, and these results were mirrored in Dr. Mogil's study of the *Mc1r*$^{e/e}$ mouse model. In these mouse studies, wildtype B6 mice were used as controls and compared to their *Mc1r*$^{e/e}$ littermates. These mice underwent a battery of pain tests such as withdrawal latencies from water either at 47°C or 49°C, a hotplate test, hot lamp test, binding clip test and a writhing test post 0.09% inter peritoneal acetic acid injection. We published these data in 2005 (Mogil et al. 2005)

Discussion

Pentazocine Studies

After murine QTL mapping and functional studies tested a candidate gene hypothesis, support for a female-specific role of Mc1r in pain sensitivity was evident. *MC1R* was tested and found to be the gene for this phenotypic effect as it is expressed in the peripheral neurons as well as brain glial cells (Wikberg 1999) and neurons of the ventral periacqueductal grey (Xia, Wikberg et al. 1995), a region of the brain which is critical for the modulation of pain. The exact relevant endogenous ligand of MC1R is unknown but one of the *POMC* gene splicing products is α-MSH (melanocyte stimulating hormone), which is an endogenous ligand. It has been shown that α-MSH acts as an antagonist in thermal nociception (Walker et al. 1980; Ohkubo et al. 1985) and

this ligand has also been revealed to have an anti-opioid role wherein it reduces tolerance of opioid ligands (Gispen et al. 1976). The regulation of α-MSH release by κ-opioid receptors seems to be sexually dimorphic in humans (Manzanares et al. 1993), however our group has not been able to reproduce this result in mice. A possible ligand for the role of MC1R in this paradigm is the dynorphrin class, which are classically selective κ opioid receptor ligands, that bind the melanocortin receptors with nanomolar affinity (Quillan and Sadee 1997). Our research set up the hypothesis that MC1R activation would cause an anti-opioid effect in females, which red heads (fair skinned females) would lack. Pentazocine, which has activity at the κ-opioid receptor site, also has an affinity for the μ receptor as well. Our next study using M6G actually addresses this point. These results suggest that there are qualitative sex differences in processing of pain inhibition. From these data, we can conclude that females with two mutant alleles need a lower dose of pentazocine for the same analgesic effect that females feel who have zero or one mutations, and men. This was a ground breaking study, discovering that MC1R accounted for kappa opioid mediated pain sensitivity in females. The NMDA receptor has long been known to mediate pain response in males, but not females, and we were able to fill this gap in knowledge.

M6G study

A problem with pain management is the variability between individuals in baseline pain sensitivity and the effects of analgesics (Aubrun et al. 2003). In this study, we have found a positive association between the *MC1R* gene and pain tolerance as well as a link to the efficacy of M6G. We have determined that there is a greater M6G induced analgesic responses in people who have two or more mutant *MC1R* alleles. We did not

observe a sex dependent result with this drug as we saw in the pentazocine study. However, the decreased pain sensitivity in these people with non-functional *MC1R* as well as the *Mc1r*$^{e/e}$ mutant mice (compared to the wild type people and mice) implies that endogenous activation of MC1R may have an anti-analgesic effect. The reason we may not see any differences in baseline pain sensitivity may be due to the different pain modality that was used in this study (electrical pain vs. ischemic and thermal pain used in our pentazocine study). Here, we have again demonstrated the power of direct mouse to human translation in genetic studies of a complex trait.

		Hair color*		Skin type†		*MC1R* genotype‡	
	Sex	RH	NonRH	I & II	III & IV	Two variant alleles	0/1 variant alleles
Number of subjects	Females	9	9	11	7	5	13
	Males	12	12	15	9	9	15
Δ Ischemic pain threshold§	Females	85.2	6.6	97.4	−35.0	116.6	18.7
		(145.7)	(139.2)	(148.4)	(97.8)	(181.7)	(124.5)
	Males	17.4	98.3	20.2	120.6	8.8	87.3
		(106.7)	(164.3)	(95.5)	(186.1)	(119.7)	(149.3)
Δ Ischemic pain tolerance¶	Females	237.0	62.0	233.3	24.8	408.0††	37.1
		(268.3)	(58.8)	(236.4)	(61.1)	(284.1)	(82.8)
	Males	62.3	84.6	84.6	59.6	46.4	84.1
		(217.8)	(137.4)	(190.6)	(155.1)	(149.3)	(182.8)
Δ Sum ischemic pain intensity‖	Females	55.0	16.6	58.4††	0.3	84.8††	16.9
		(58.1)	(42.1)	(54.0)	(26.6)	(50.3)	(41.9)
	Males	20.9	40.3	25.4	39.3	18.7	37.8
		(37.2)	(37.6)	(35.1)	(42.8)	(36.2)	(38.3)
Δ Sum ischemic pain unpleasantness**	Females	46.1	11.1	54.4††	−11.9	79.6††	9.0
		(60.0)	(51.8)	(55.6)	(31.9)	(45.7)	(49.6)
	Males	26.4	33.0	26.9	34.3	24.2	33.0
		(48.1)	(44.4)	(43.3)	(51.1)	(41.0)	(49.0)
Δ Sum thermal pain intensity††	Females	−1.4	−15.9	6.1	−31.9	28.0††	−22.8
		(56.1)	(38.9)	(46.9)	(41.1)	(48.9)	(40.1)
	Males	17.7	22.4	13.9	29.8	9.2	26.2
		(48.8)	(61.9)	(46.4)	(66.6)	(51.0)	(56.5)

Values presented are means (SD appears in parentheses). For all measures, greater values indicate more robust analgesia.

*Redhead (RH) includes auburn (two) and strawberry (three) hair colors; NonRH includes blonde (five), brown (fourteen), and black (two). As seen by others previously (27), all subjects with two variant alleles were RH, but only 12 of 21 (57%) RH subjects had two variant alleles.

†Based on Fitzpatrick skin type classifications: I: burn, never tan; II: burn, then tan; III: tan, sometimes burn; IV: tan, never burn. All subjects with two variant alleles had type I or type II skin.

‡Of the 14 subjects (29%) with two variant alleles, three were homozygous for R151C, one was homozygous for D294H, six were R151C/R160W compound heterozygotes, two were R151C/D294H compound heterozygotes, and one was a V92M/R160W compound heterozygote. Other than V92M, R151C, R160W, and D294H, the only nonconsensus allele observed was R163Q in one non-RH female. Observed allelic frequencies were very similar to published data (27).

§Calculated as: (postdrug pain threshold − predrug pain threshold).

¶Calculated as: (postdrug pain tolerance − predrug pain tolerance).

‖Calculated as: (sum of all predrug ischemic pain intensity ratings − sum of all postdrug ischemic pain intensity ratings).

**Calculated as: (sum of all predrug ischemic pain unpleasantness ratings − sum of all postdrug ischemic pain unpleasantness ratings).

††Calculated as: (sum of predrug thermal pain intensity ratings trials 1–5 − sum of postdrug pain thermal intensity ratings trials 1–5).

††Significantly higher than corresponding within-sex group, $P < 0.05$ (see text).

Figure 4-1 The measures of pentazocine analgesia in humans by sex, hair and skin phenotypes, as well as *MC1R* genotypes. This is a comprehensive figure of all experimental pain testing compared to people grouped with regard to their hair color, skin type and *MC1R* genotype (Mogil et al. 2003).

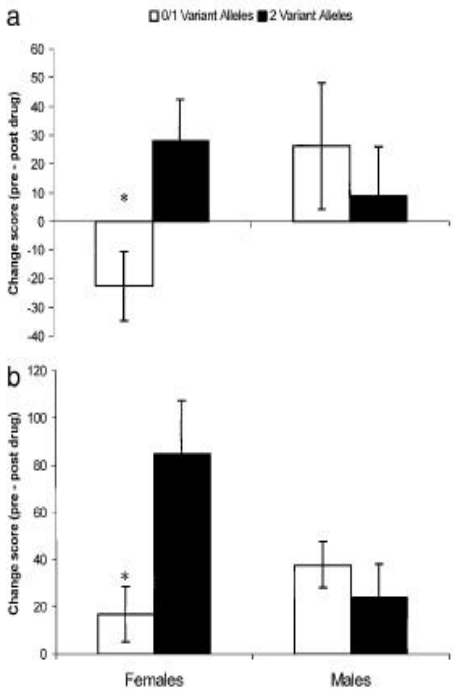


Figure 4-2 Change in pain ratings after pentazocine analgesia separated by sex and genotype. a) is the results of the thermal pain testing while b) is the result of ischemic pain testing. There was no difference between the groups in baseline pain measurements or response to saline administration across sex or genotype. The * indicates a significant difference found in the sex between the two different genotypic groups ($p<0.05$) (Mogil et al. 2003).

Genotype	No. of variants	Genotype*	n (%)†	Phenotype‡
+/+ (consensus sequence)	0	0/1	10 (21%)	All non-redhead
V60L/+	1	0/1	2 (4%)	Both non-redhead
V92M/+	1	0/1	1 (2%)	Non-redhead
R151C/+	1	0/1	3 (6%)	1 Non-redhead; 2 redheads
R160W/+	1	0/1	3 (6%)	2 Non-redheads; 1 redhead
V60L/V60L	2	0/1	1 (2%)	Non-redhead
V60L/V92M	2	0/1	1 (2%)	Non-redhead
V60L/D294H	2	0/1	1 (2%)	Redhead
V92M/D294H	2	0/1	1 (2%)	Redhead
R151C/R163Q	2	0/1	1 (2%)	Redhead
R160W/R163Q	2	0/1	1 (2%)	Redhead
ins29/ins29	2	2+	1 (2%)	Redhead
R151C/R151C	2	2+	3 (6%)	All redheads
R151C/R160W	2	2+	12 (26%)	All redheads
R151C/D294H	2	2+	1 (2%)	Redhead
R160W/R160W	2	2+	4 (9%)	All redheads
R151C/R151C *and* R160W/+	3	2+	1 (2%)	Redhead
Totals		0/1: 25 2+: 22	47	Non-redheads: 18 Redheads: 29

*Experiments in transfected cell lines have determined that the following *MC1R* variants are unable to induce cyclic AMP production when stimulated by α-melanocyte stimulating hormone (α-MSH) or a long lasting analogue, NDP(Nle,[4] D-Phe[7])-MSH (see Schaffer and Bolognia[8]): R142H (Arg142His), R151C (Arg151Cys), R160W (Arg160Trp), D294H (Asp294His), and the insertion mutations ins29 and ins179. The R142H and insertion mutations are very rare (<1% allele frequency), and were not seen in this study except for one subject homozygous for ins29. Subjects with two (or in one case, three) total variants at ins29, R151C, R160W, and/or D294H (all redheads) were thus classified in the non-functional MC1R genotype group (''2+''). The V60L (Val60Leu) and V92M (Val92Met) are somewhat common mutations, but do not lead to MC1R loss of function, and thus subjects with these *MC1R* variants were classed with the functional MC1R genotype group (''0/1''). R163Q (Arg163Gln) has not yet been tested for cAMP stimulation; we conservatively classified this variant as not affecting MC1R function. It should be noted that the genotypic effect on both baseline sensitivity and M6G analgesia is significant at the $p<0.05$ level regardless of the classification of R163Q containing subjects. We also assayed for the K65N (Lys65Asn) and D84E (Asp84Glu) variants, but found none.

†Percentages do not add up to 100% due to rounding. Allele frequencies in these subjects were: +/+ (wildtype): 31%; ins29: 2%; V60L: 6%; V92M: 3%; R151C: 27%; R160W: 26%; R163Q: 2%; D294H: 3%. These values agree well with those previously reported in the literature.

‡Subjects classified as ''redheads'' had red hair ranging from orange to auburn, fair (type I/II) skin, and blue or green eyes. Twenty two of the 29 redheads (76%) possessed two or more MC1R inactivating variants, also in excellent agreement with the existing literature.

Figure 4-3 The grouping of subjects by the location of the SNP in the *MC1R* gene and a description of their phenotypic characteristics (Mogil et al. 2005).

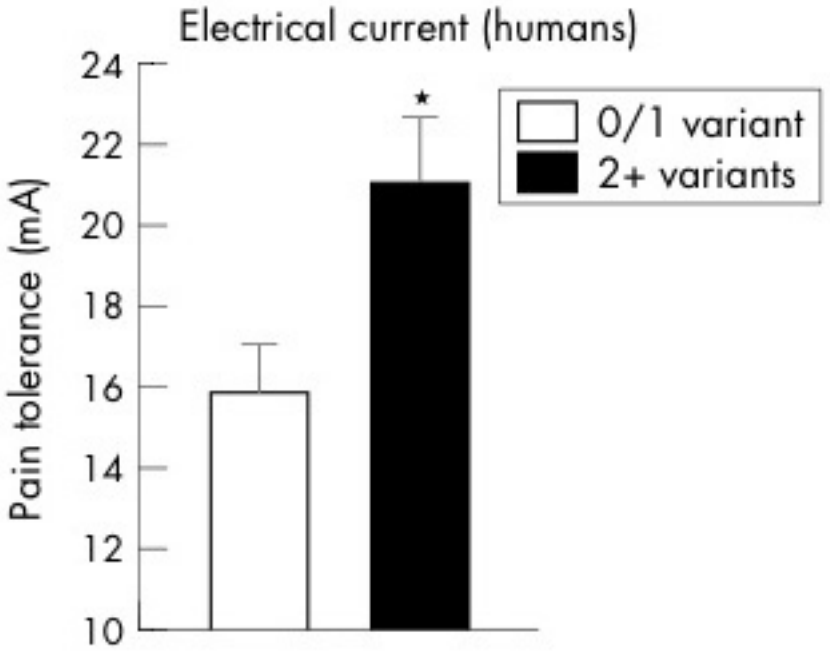


Figure 4-4 Effects of MC1R functionality on baseline nociceptive and pain sensitivity in humans. These data are separated by genotype only as significant effects of genotypes were observed in both sexes (* $p<0.05$) (Mogil et al. 2005).

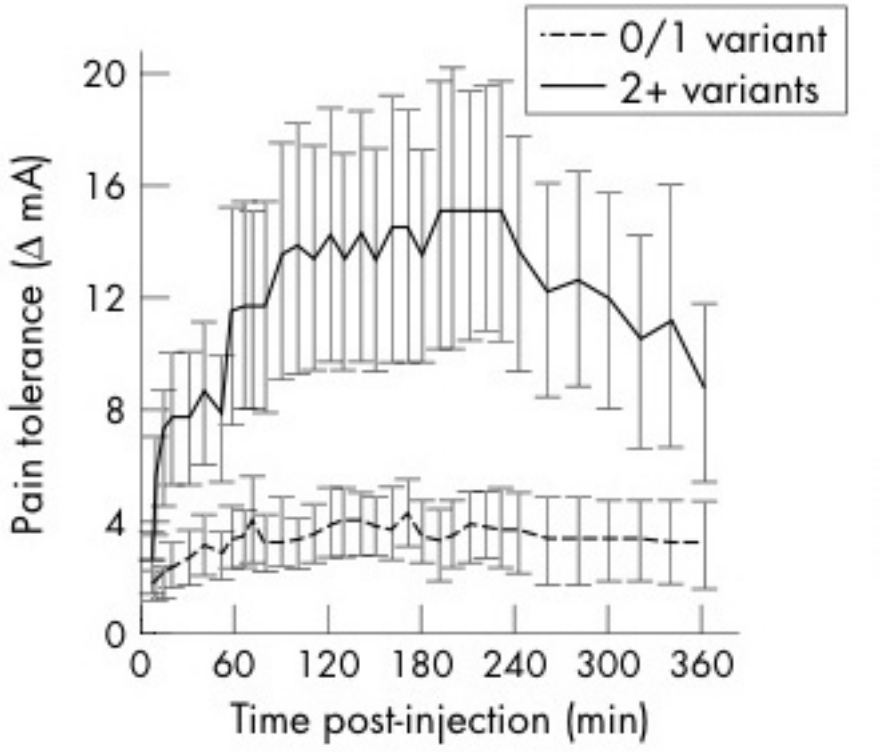


Figure 4-5 The change in pain tolerance over time after the administration of M6G at a dose of 0.3mg/kg. Here we have charted the different genotypes of our participants and see a functional difference in tolerance over time depending on the grouping (Mogil et al. 2005).

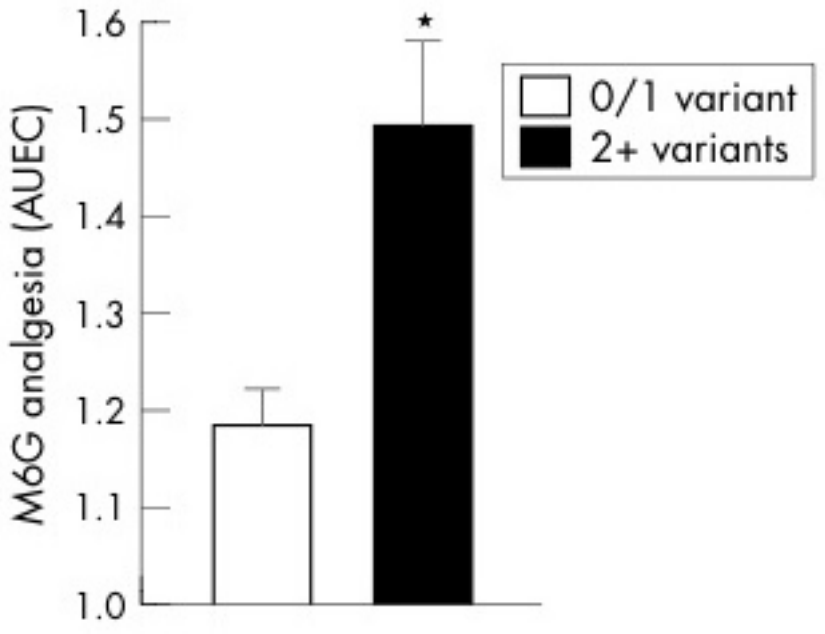


Figure 4-6 M6G analgesia expressed as the area under the time effect curve. This is a significant measure ($p<0.05$) (Mogil et al. 2005).

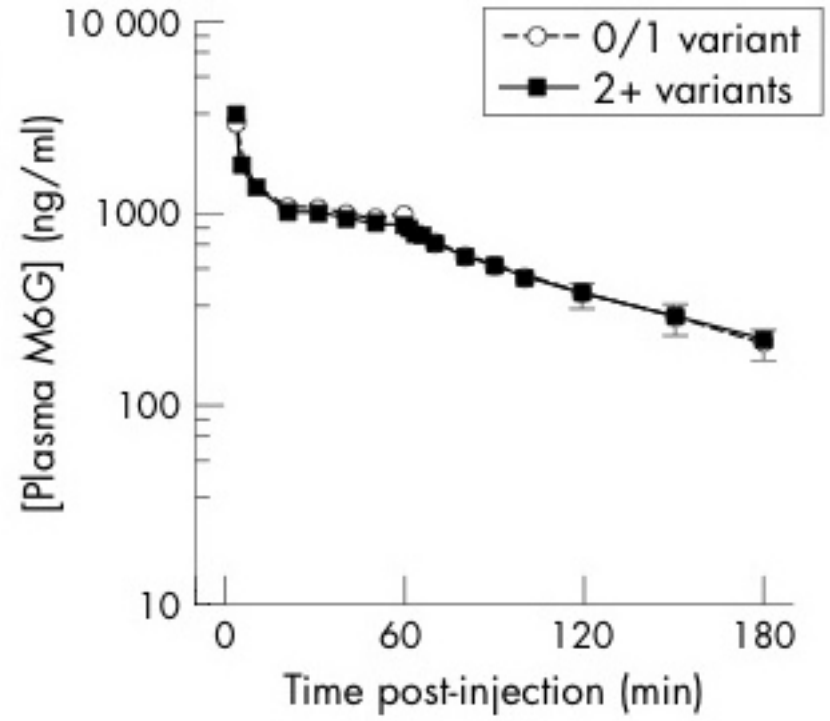


Figure 4-7 Concentrations of M6G plasma levels in participants at time points after M6G administration at a dose of 0.03mg/kg (Mogil et al. 2005).

CHAPTER 5
THE A118G SINGLE NUCLEOTIDE POLYMORPHISM IN THE μ-OPIOID RECEPTOR GENE IS ASSOCIATED WITH PRESSURE PAIN SENSITIVITY

Introduction

In 1999, Uhl et al. suggested that the μ-opioid receptor gene (*OPRM1*) may be a likely candidate to be involved in pain sensitivity in humans. It is located on chromosome 6 at band q24-q25. There is a SNP located at nucleotide number 118 which changes the sequence from an A nucleotide (major allele) to a G (minor allele). When this change occurs, it causes an amino acid change from a polar amino acid asparagine to the acidic amino acid aspartate (N40D). The G allele occurs in the general population at a frequency of 20%-30% (Bond et al. 1998; Grosch et al. 2001; Szeto et al. 2001). Until our study, no reports of associations with the *OPRM1* gene and baseline pain sensitivity in humans had been published. In the past, pressure pain threshold had been assessed in monozygotic and dizygotic twins and the results suggested a 10% heritability, but these data may be skewed since the twins were in the same room at the time of testing (MacGregor et al. 1997). Another group of investigators (Kim et al. 2004) found heritability of 22%-46% across three pain modalities in healthy individuals. Several genetic association studies have been done in analgesic response. The μ-opioid agonist M6G has been demonstrated to reduce pupil constriction in subjects with a rarer *OPRM1* allele (Lotsch et al. 2002), and the rarer G allele was associated with lower M6G potencies (Romberg et al. 2003). *OPRM1* polymorphisms have been associated with opioid addiction and abuse in various studies in a case-control experimental design (Bond

et al. 1998; Hoehe et al. 2000; Szeto et al. 2001; Tan et al. 2003), but there are almost as many studies that have failed to replicate these results (Compton et al. 2003; Crowley et al. 2003; Franke et al. 2003). On the evidence of previous findings that aspartate in position 40 of the protein increased the binding affinity of this receptor's endogenous ligand (Bond et al. 1998), we hypothesized that people with one or more of the G alleles would have diminished sensitivity to experimental pain. This was tested on a set of healthy individuals from Dr. Fillingim (n= 167).

Results

Genotyping disclosed that 24% of females and 17% of males had one or two G alleles (24 and 12 individuals respectively). In all, 96 females and 71 males were genotyped and their demographic information recorded (figure 5-1). Because AG/GG individuals were older that those with the AA genotype ($p < 0.05$) and women were slightly younger than the male participants ($p = 0.07$), age was controlled for in all of our analyses. Our participants underwent the same ischemic and thermal testing as described in the previous chapter. These data are represented in figure 5-1. Women had significantly lower heat pain thresholds (HPTh $p<0.05$) and heat pain tolerances (HPTo $p<0.001$) compared to men, but there was no effect due to genotype ($P>0.05$). Individuals in this study additionally underwent pressure pain testing in which an algometer was used to apply pressure with a $1cm^2$ size probe at a rate of 1kg/sec. Pressure was applied to the masseter (approximately halfway between the ear opening and the corner of the mouth), the center of the right upper trapezius (posterior to the clavicle) as well as the right ulna (on the dorsal forearm, about 8cm distal to the elbow), with this measure taken at three different times. For this measure, subjects were asked to report when the pressure first became painful (pressure pain threshold (PPT)). The

results from the pressure pain testing are presented in figure 5-3 and show that there was a significant main effect of genotype that emerged for pressure pain threshold (PPT) at all three measured sites. (trapezuis $p = 0.002$; masseter $p = 0.023$; ulna $p = 0.049$). At all three sites, individuals with at least one minor allele at this locus displayed higher PPTs than those with two of the more major alleles. Women reported lower PPTs at all three sites compared to their male counterparts ($p < 0.001$). Women also described significantly higher heat pain ratings during temporal summation of pain at both temperatures (49°C and 52°C, $p< 0.001$), but there was no overall genetic effect ($p > 0.10$). There was, however, a sex by genotype effect for pain ratings at 49°C ($p <0.05$). No significant associations between the A118G SNP and ischemic pain threshold (IPTh) or ischemic pain tolerance (IPTo) emerged from these data ($p > 0.10$).

Discussion

In this study, we examined a large group of young adults and we found an A118G SNP allele frequency similar to those reported previously (Bond et al. 1998; Grosch et al. 2001; Szeto et al. 2001). The results indicate that having one or more *OPRM1* G allele is associated with a lower sensitivity to pressure pain than having the AA genotype. A sex by genotype interaction was observed for heat pain ratings at 49°C, suggesting that the G allele was associated with lower pain ratings among men and but a higher rating among women with the same genotype. A similar trend was seen in heat pain tolerance but it was not statistically significant ($p = 0.08$). As previously reported (Fillingim and Maixner 1995; Berkley 1997; Riley et al. 1998), women communicated lower heat pain tolerance, higher heat pain ratings and lower pressure pain threshold compared to the men in our study. A possible explanation of the association between the *OPRM1*

polymorphism and mechanical pain sensitivity is the observation that there is a greater binding affinity for β-endorphin to the aspartate at amino acid 40 (Bond et al. 1998), which may allow for a more robust effect of endogenous opioid analgesia. This SNP may also be in linkage disequilibrium with other *OPRM1* polymorphisms that contribute to this effect (Hoehe et al. 2000). There was a varying pattern of associations across different pain assays, between genotype and pain perception. This may be explained by the fact that previous findings have only shown low to moderate associations between genotype and responses to different pain assays, which suggests distinct factors may be the culprit for the variability observed in the different pain measures (Janal et al. 1994; Fillingim et al. 1999). Parallel studies conducted in mice supported the genetic association found in our study (Lariviere et al. 2002). The mechanism underlying our association findings may be modulation of mechanical pain, either exclusively or preferentially. There is evidence suggesting that descending opioid systems inhibit deep pain more efficiently than cutaneous pain (Yu et al. 1991), which would explain the association we found to mechanical pain and the lack of association to thermal pain. There may be underlying associations with pain modalities other than mechanical pain, but due to the relatively low frequency of the G allele and the size of our sample group, we may not have enough power to reach significance. This could explain why we found a marginal significance in heat pain tolerance ($p = 0.08$) and the significant association found in heat pain ratings at 49°C ($p < 0.05$) when examining the sex by genotype interaction of this SNP. These results reflect a marginally higher heat pain sensitivity among the G allele female group compared with the AA genotype females. Also, by analyzing the different means and effect sizes, the association of the G allele and

mechanical pain appears to be stronger in men than women. These data advocate the continuing research of genetic contributions of candidate genes in pain sensitivity with special emphasis to be given to sex differences and the relative strengths of associations as it pertains to gender. Since our study has the potential to have been underpowered, we cannot rule out the possibility of an association of *OPRM1* variants with heat or ischemic pain. As there are large variations in allele frequencies in different ethnic groups, this may affect studies conducted with mixed groups (Crowley et al. 2003), although race was controlled in our study. Our study is the first to find such A118G associations in these measures. If these data are reproduced in another group of participants, it would be worthy to investigate possible underlying mechanisms.

A	Male		Female	
	AA	AG/GG	AA	AG/GG
No	59	12	74	24
Age (y [SD])*	25.0 (5.6)	27.2 (7.3)	22.8 (4.3)	25.3 (6.6)
White (%)	75	83	75	63
African-American (%)	7	0	6	5
Hispanic (%)	12	8	14	13
Other ethnicity (%)	7	8	6	21
Oral contraceptive (%)	NA	NA	50	46

Abbreviation: NA, not applicable.
*Genotype difference, $P < .05$.

B	Male			Female		
	AA (n = 59)	AG or GG (n = 12)	Effect Size*	AA (n = 72)	AG or GG (n = 24)	Effect Size
Heat pain threshold†	40.6 (2.7)	41.2 (2.8)	0.23	40.2 (2.8)	39.9 (3.2)	–0.10
Heat pain tolerance†	46.8 (2.4)	48.0 (1.8)	0.52	45.7 (2.5)	45.4 (2.8)	–0.12
Pain ratings at 49°C (0-100)†	41.1 (21.5)	34.0 (13.4)	0.35	53.3 (24.9)	65.3 (27.6)	–0.47
Pain ratings at 52° C (0-100)†	68.8 (23.5)	61.5 (22.7)	0.31	82.9 (18.1)	84.0 (22.3)	–0.06
Ischemic pain threshold	206.9 (196.1)	214.3 (151)	0.04	167.9 (151)	201.8 (162.4)	0.24
Ischemic pain tolerance	620.1 (245.4)	674.6 (185.8)	0.23	583.6 (281.3)	609.4 (277.1)	0.09

*Effect size is Cohen D representing the magnitude of the difference between the AA and AG groups, separately for men and women. Positive values indicate greater pain sensitivity in the AA group, whereas negative values represent greater sensitivity in the AG group.
†Main effect of sex, $P < .05$.

Figure 5-1 This figure illustrated the details of the subjects in this study. A) Represents the demographic information by genotype and sex. B) Shows heat pain and ischemic pain measures for male and female participants divided into *OPRM1* A118G genotype (Fillingim et al. 2005).

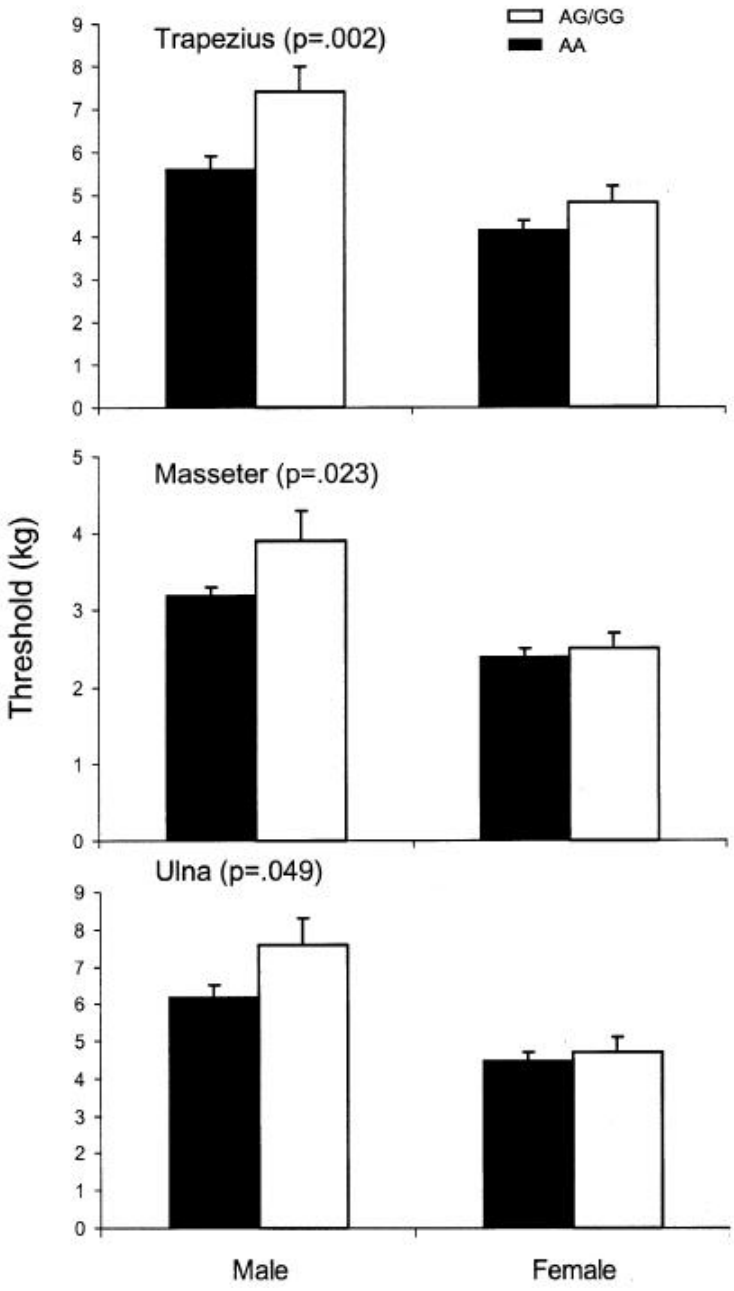


Figure 5-2 Pressure pain threshold at all three sites tested (trapezius at the top, masseter in the middle and ulna at the bottom). Men are depicted on the left and women on the right. Effect sizes (Cohen D) for the genotype effects are: trapezius, men=0.89, women=0.38, masseter, men=0.65, women=0.14 and ulna, men=0.61, women=0.11. Significance is presented on figure represents overall genotype effect for each site (Fillingim et al. 2005).

CHAPTER 6
FUNCTIONAL ANALYSIS OF SINGLE NUCLEOTIDE POLYMORPHISMS

Introduction

While our association studies have correlated pain phenotypes with polymorphisms, it would be clinically useful to elucidate the mechanisms underlying these associations. Functional studies are a logical method to assess the biochemical manner in which the polymorphism may affect the phenotype. Comprehensive studies have been performed on mouse models of Mc1r receptors rendered non-functional by the minor alleles (Cone et al. 1996; Tatro 1996). The mu opioid receptor has already been the subject of these kinds of studies, which showed that the minor allele at position 118 reduces receptor function (Bond et al. 1998). Thus, we decided to examine the effects of the different alleles of the delta and kappa opioid receptor. Discussed below is the rationale behind the studies, and our results.

The Delta Opioid Receptor and T80G (F27C)

The T allele at base number 80 is the more common allele at this position, while the G allele has been observed in this position at a frequency of 0.09 (Gelernter and Kranzler 2000) to 0.12 (Kim et al. 2004). This allele frequency implies that one in ten alleles will be a G, and as we each have two alleles, this means about one in five people will have one or two G alleles. The T to G change in the nucleotide sequence converts the amino acid residue from a phenylalanine to a cysteine (F27C). Phenylalanine is an aromatic amino acid that is hydrophobic, while cysteine is polar and, because of its sulfhydryl side chain, has the ability to form disulphide bonds and make a sulphur bridge. This is

involved in proteins forming their tertiary and quaternary structure, which confers functionality to the protein. With Dr. Mavis Agbandje-McKenna, we have modeled this non-conservative change in the amino acid sequence using another G-protein coupled receptor (GPCR) as a basis. The GPCR used in the computer modeling of the delta opioid receptor is the bovine rhodopsin receptor. There is a 19% identity in the amino acid sequence and a further 33% similarity (ClustalW alignment). The phenylalanine in position 27 is conserved between the two GPCRs (see figure 6-1) (and in fact this residue is conserved in *OPRD1* of rodents as well). In the computer model, we changed residue 27 to a cysteine (figure 6-2) and have illustrated the potential difference in the local environment between the phenylalanine and cysteine in this position. In the past, research has focused on cloning this gene and functional analysis independent of naturally occurring SNPs (Evans et al. 1992). A mutation study was performed on the delta opioid receptor where all the cysteines were replaced one by one with either a serine or an alanine, and testing of the expressed mutant protein suggested that the replacement of either extracellular cysteine resulted in a receptor lacking delta agonist or antagonist binding activity (Ehrlich et al. 1998). Thus the fact that the F27C SNP adds a cysteine in the extracellular region of the protein is an interesting aspect of our study. This natural allele variant has the potential for an alternate disulphide bridge formation.

Recent studies have found positive associations of the delta opioid receptor F27C SNP and alterations in pain sensitivity (heat pain intensity) in a sex-dependent manner (Kim et al. 2004). These findings were consistent with mouse studies, which suggested a sex-specific QTL on chromosome 4 (where murine *Oprd1* is located), that mediates thermal nociception measured with a hot plate (Mogil et al. 1997). Hot plate sensitivity

differences have been noted between knock out mice and their wildtype counterparts (Zhu et al. 1999). These data suggest that genetic variants do affect the function of the protein. It has been our undertaking to elucidate possible functional differences of the delta opioid receptor protein variant F27C.

The Kappa Opioid Receptor and G36T

The delta opioid receptor is known to heterodimerize with the kappa opioid receptor and thus, modulate the function of each other (Jordan and Devi 1999). The heterodimer has a distinct function, with its own set of selective agonists. For this reason, we decided to examine the effects of the synonymous G36T polymorphism in the kappa opioid receptor, the most common SNP, since there are no known non-synonymous SNPs. Silent polymorphisms have been implicated in functional consequences in other systems. For example, a silent polymorphism in the delta opioid receptor (T307C), has been associated with biobehavioral phenotypes in heat pain intensities in humans (Kim, et al. 2004). This effect may be mediated through epigenetic mechanisms related to the nucleotide substitution (Dennis 2003). There is also evidence for synonymous SNPs having functional consequences as part of a haplotype, where compound heterozygotes have a different functional consequence from each isolated polymorphism effect (Duan et al. 2003). The kappa opioid receptor has been implicated in visceral pain sensitivity (Simonin et al. 1998). That study found that kappa opioid receptor knock out mice displayed increased visceral writhing in response to an agent, compared to their wildtype littermates. Thus OPRK1 is a very interesting receptor to study in chronic pain conditions such as irritable bowel syndrome, and to study functionally with respect to natural variants. Thus, while the prior expectation is that there will be nonfunctional

differences between the two alleles, there is justification to test this scientifically especially given the heterodimerization system with *OPRD1*.

Results

After unsuccessful attempts to clone the delta opioid receptor myself by RT-PCR (probably due to multiple amplicons from delta opioid receptor type 2) and low level of expression in leukocytes, we received the full-length receptor *OPRD1* cDNA in pcDNA1 (Invitrogen) from the laboratory of Dr. Brigitte Kieffer at the Louis Pasteur Institute in Paris, France. At the same time we requested the cloned kappa opioid receptor gene from Temple University in Philadelphia from the laboratory of Dr Liu-Chen. This cloned cDNA was in the pcDNA3 vector (Invitrogen). After I performed site directed mutagenesis and sequencing to attain the desired alleles, the vectors (cDNA inserts in pcDNA3.1 with C-terminal tags) were transfected into HEK 293 cells (which have no endogenous opioid receptors) and underwent stable selection using Geneticin (Gibco). Positive pooled colonies were used initially in functional analysis. However, these showed a negative western blot analysis using a tagged antibody to detect the His tag at the carboxy terminus and were negative for immunocytochemistry. Thus, we proceeded with making clonal populations of transfected cells. Activation assays were also performed on the pooled stable cell lines, which were frozen down in different aliquots due to having to split the cells before initial selection. These data suggested that the protein was not being expressed (figure6-3), as there was no dose response curve. After clonal selection, the clones were frozen and protein and RNA extracted. The clones were screened for expression by northern blot (figure 6-4), and 1-3 clones were chosen from each allele: a low expressing cell line, a medium expressing cell line and a high expressing cell line. Three clones were not always available for each allele, as high

expressing clones tended to die before we could freeze down the colony. The surviving clones underwent western blotting to test for protein expression. Two different antibodies were used for this purpose: an alkaline phosphatase (AP) conjugated anti-histidine antibody (Invitrogen), and a polyclonal anti-histidine antibody (Covance), with an AP secondary antibody. Two different methods were used for detection as well: AP development using NBT and BCIP as well as an chemiluminescent (ECL) method (Pierce). A positive control protein consisted of a his-tagged protein, which was 40.8kDa in size. The ECL method produced the clearest results, which were negative for opioid receptors but positive for the control protein (figure 6-5). Immunofluorescent analysis of the colonies (immunocytochemistry) revealed only background fluorescence in the cells, and binding assays showed no dose response curve. Together these data suggest that there is little or no expressed recombinant protein in the clones.

As a secondary test, the vectors were transfected into COS-7 cells, using the Fugene reagent (Roche) in a 3:2 ratio. A total of four $10cm^2$ plates were transfected with each vector, and a β-galactosidase reporter gene was cotransfected into two of the four plates. These two plates were subsequently used in binding assays two days later, while in the same time frame, one of the two remaining plates was harvested in Laemli buffer (Bio-Rad) with 5% beta-mercaptoethanol (BME, Sigma). The last plate was split 24 hours post transfection onto 4-well chamber slides, grown overnight and then fixed, permeabalized and immunostained with the anti-his antibodies. This immunocytochemistry revealed no signal above background, while there was a total lack of a dose response curve in the binding assays and yet another negative western blot (figure 6-6). In a separate experiment to analyze possible quantitative or splicing effects

of *OPRK1* G36T by itself, RT-PCR of a portion of the cDNA (and *GAPDH* as a control) was done using leukocyte RNA from individuals who had genotypes GG and GT. There was no difference in expression levels by RT in the GG sample compared to the heterozygote in the kappa RNA, nor any aberrant bands (data not shown). This indicates that the minor allele does not obviously affect expression of the gene.

Discussion

The vector sequence is normal and in frame, including the his tag, prior to the stop codon. Planning of the construct included removing as much of the multiple cloning site (MCS) as possible as to remove the most extraneous material out of the finished product. In fact, the whole MCS was removed (see figure 2-1 chapter 2), from the first restriction site (HindIII) to the SfuI site in the case of the delta opioid receptor alleles, and the AgeI site in the kappa opioid receptor vectors. A Kozak sequence was added 5' the translation start site to aid in expression of the gene.

The mRNA was about 1.2kb, which is the expected size, and this was confirmed via both northern blot analysis and RT PCR. We could even visualize the difference in expression levels of each of the single clones via RT PCR (figure 6-7). There were three different bands in the northern blot analysis of the receptor RNAs, using a full-length cDNA probe. The three-band pattern has been reported before, which was explained to be the hybridization of the probe to related receptor mRNA, but may represent residual non-specific hybridization in our case as well (Evans et al. 1992).

The lack of stable expressed protein in our clones could be due to multiple reasons. The vector might not be as conducive to mammalian expression as we had hoped in these cells. Also, protein expression may be below detectable levels, however the binding assays indicate absolutely no expression, and ECL is a sensitive system. The binding

assays should have shown a dose response curve in response to the addition of receptor agonist during stimulation. These experiments have been performed before, with a slightly different experimental design (Evans et al. 1992; Jordan and Devi 1999; Decaillot et al. 2003), by the addition of forskolin to the agonist. We also added a varying amount of forskolin to the agonist and planned to observe the change in inhibition depending on the amount of agonist added to the well. We did not observe this difference as others had, which may be explained by lack of protein. The background seen in the immunocytochemistry showed a faint signal around the nucleus, which suggests that the recombinant protein may be expressed but retained in the endoplasmic reticulum (ER). There is evidence from other groups that the majority of an expressed delta opioid receptor does not make it out of the ER and is subsequently degraded due to protein misfolding or problems in post-translational modifications (Petaja-Repo et al. 2000; Petaja-Repo et al. 2001). There may also be a confounding effect of a low level of expression of the vector and post-translational problems that may account for the lack of functional protein. It is interesting that the high expressing (RNA) clones tended to die quickly, suggesting that expression of the protein is occurring but causing cell death in those clones, possibly due to a cytotoxic effect in the maturation of the protein. These experiments proved to be a starting point for our functional analyses and will need further troubleshooting in the future.

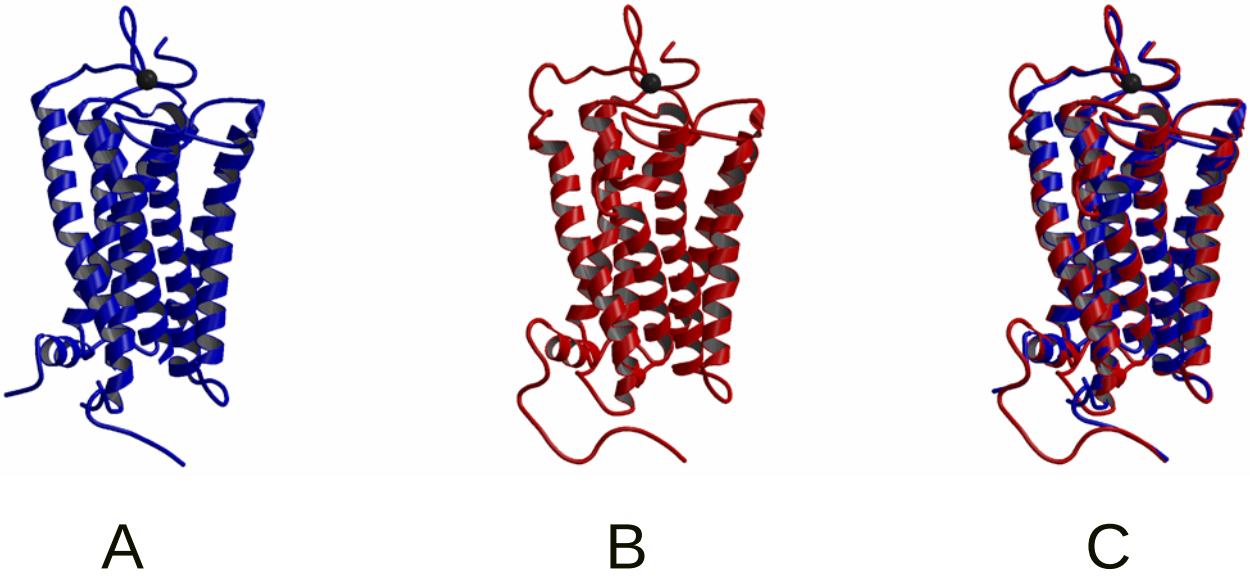


Figure 6-1 Model of the delta opioid receptor (B), based on the bovine rhodopsin receptor as a homology template (A) with the ball representing the conserved phenylalanine residue. C is both receptor models superimposed on top of each other to illustrate the similarities between these models.

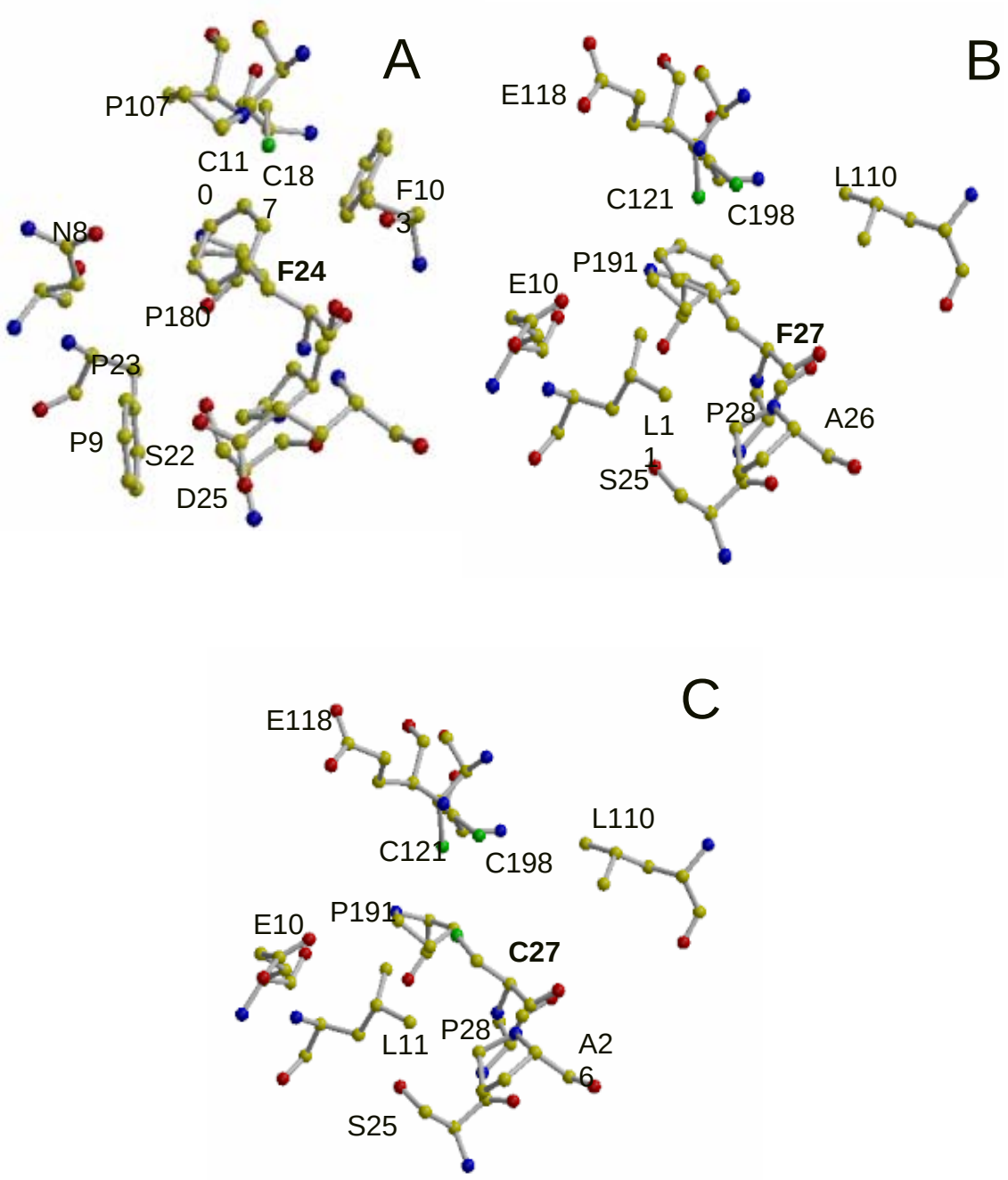


Figure 6-2 The local environment of the hydrophobic pocket where the phenylalanine is located. (A) is the conserved region in the bovine rhodopsin receptor while (B) is the region in the delta opioid receptor. (C) illustrates the possible change in the region when replaced by a cysteine residue.

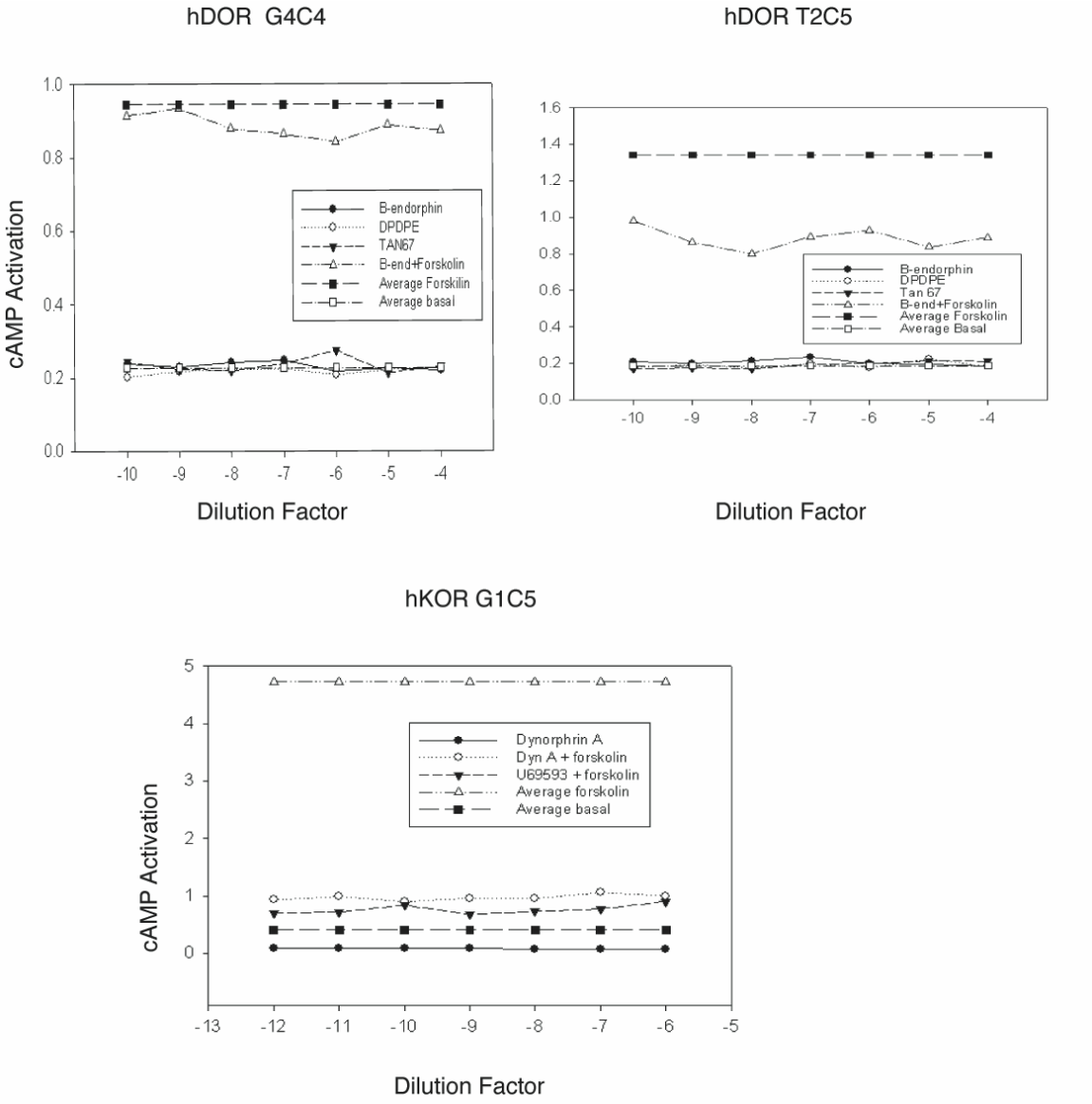


Figure 6-3 Activation assay results show that there was no dose response in the activation of the stably transfected cell lines. The forskolin (10mM concentration) is the maximum activation possible as this allows for the cAMP accumulation (or in our case, decrease) without having to signal through the receptor. In theory, we should see a dose response curve with a decrease in activation as the amount of agonist added increases.

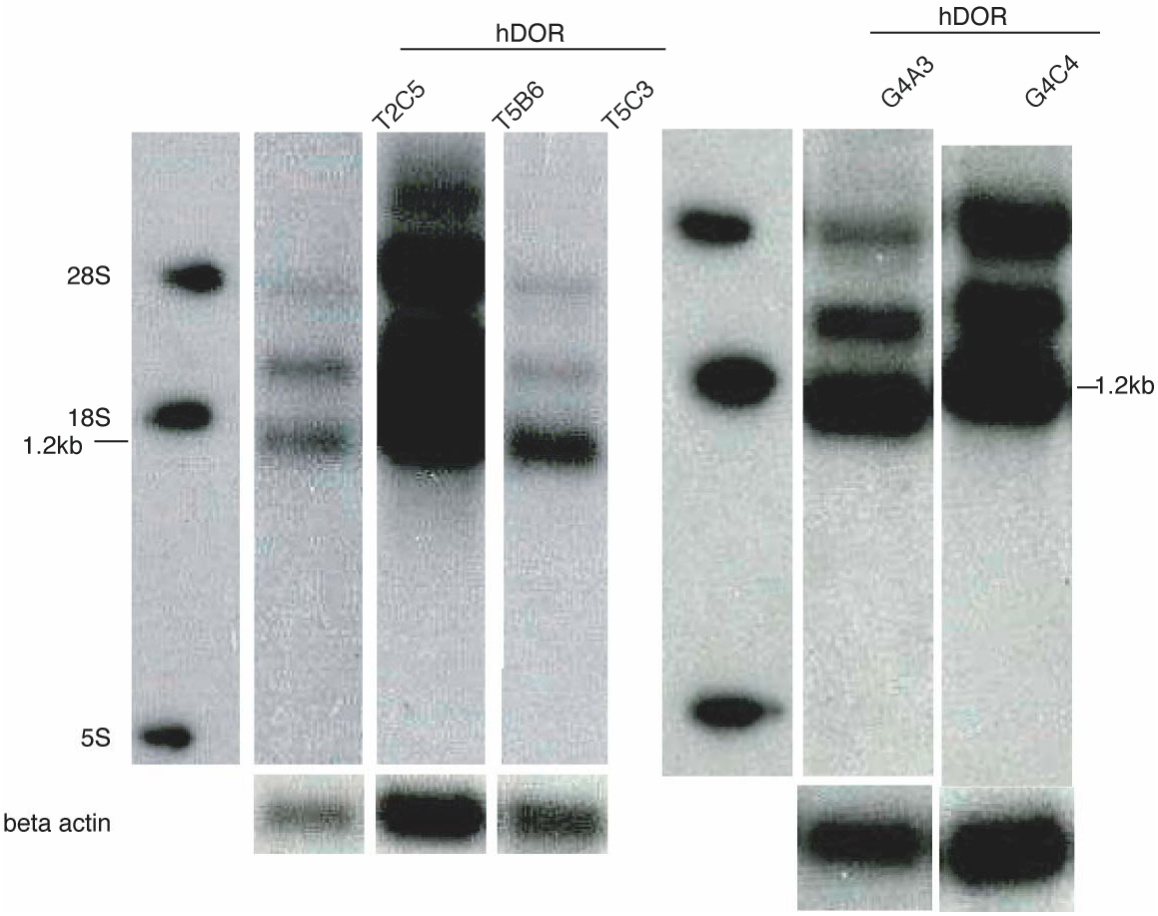
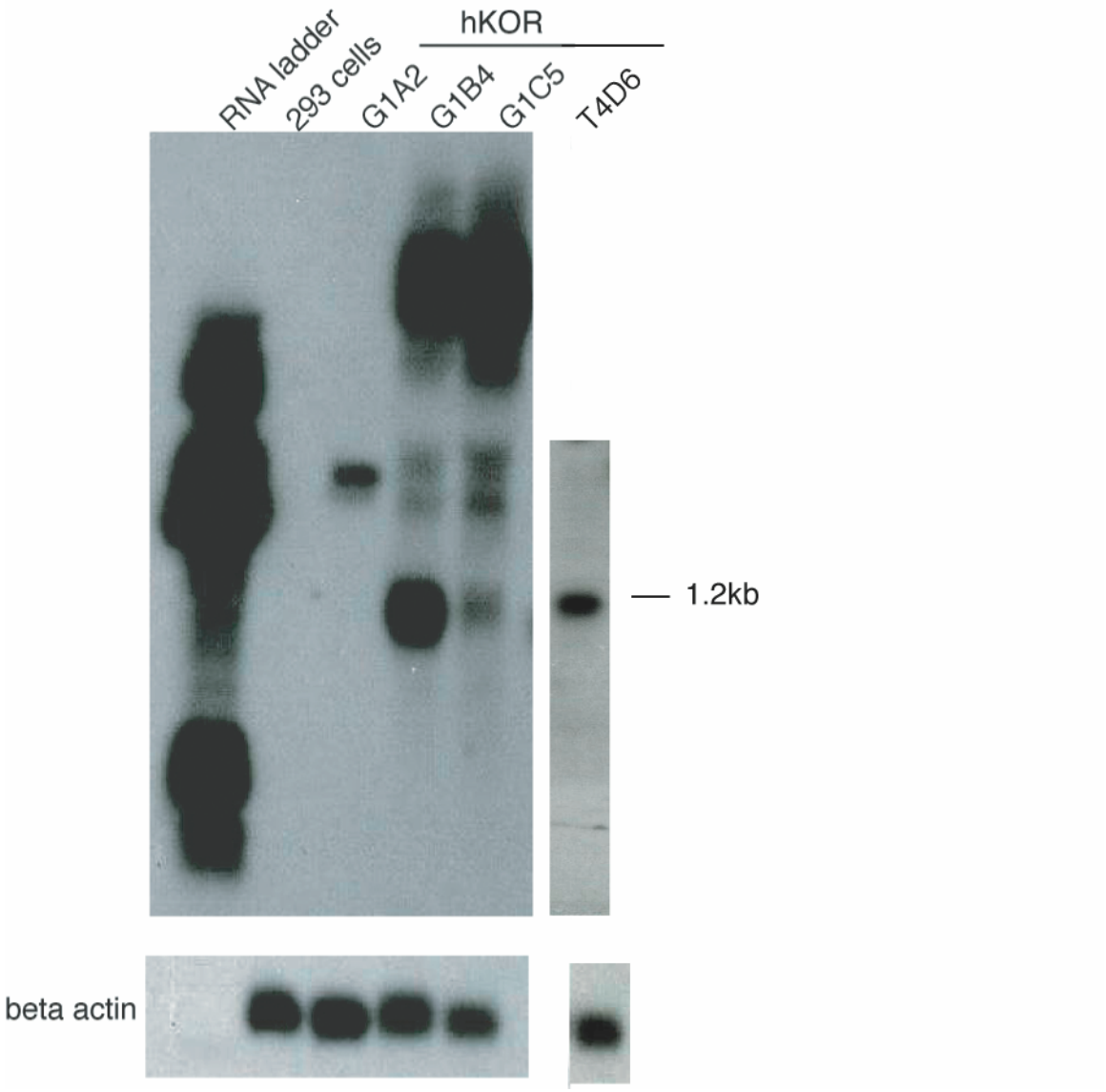


Figure 6-4 Northern blot analysis of the human delta- and kappa opioid receptor stably transfected cell lines. The 293 cells are untransfected and since the hKOR G1A4 cell colony produced no mRNA, it was used as a mock transfected cell line. There seemed to be substantial non-specific hybridization of the full-length cDNA probe, although the 1.2kb mRNA can be observed clearly.


RNA ladder
293 cells
hKOR
G1A2
G1B4
G1C5
T4D6
1.2kb
beta actin

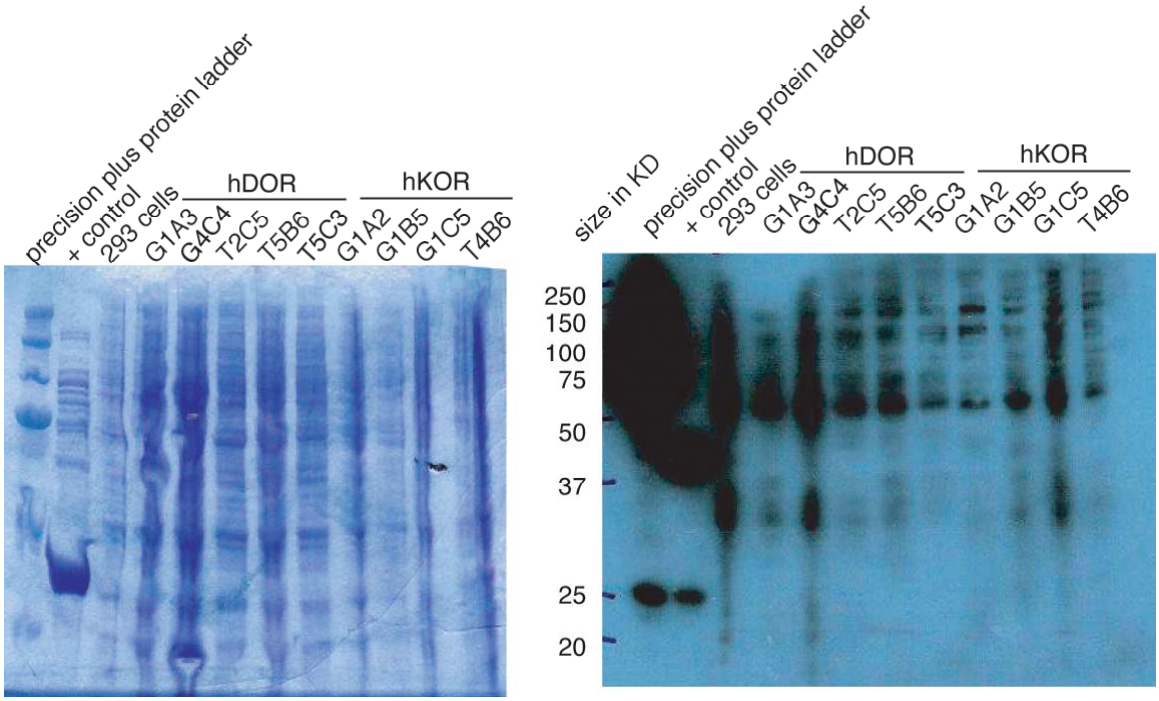


Figure 6-5 The western blot comassie stained is on the left and the ECL blot is on the right. The positive control protein is 40.8KD, which is about the expected size of our protein. There is no difference in the banding pattern between the untransfected 293 cells and the stable colonies. 2µg of the positive control was loaded on the gel along with 15µl of total cell lysate of each of the cell lines.

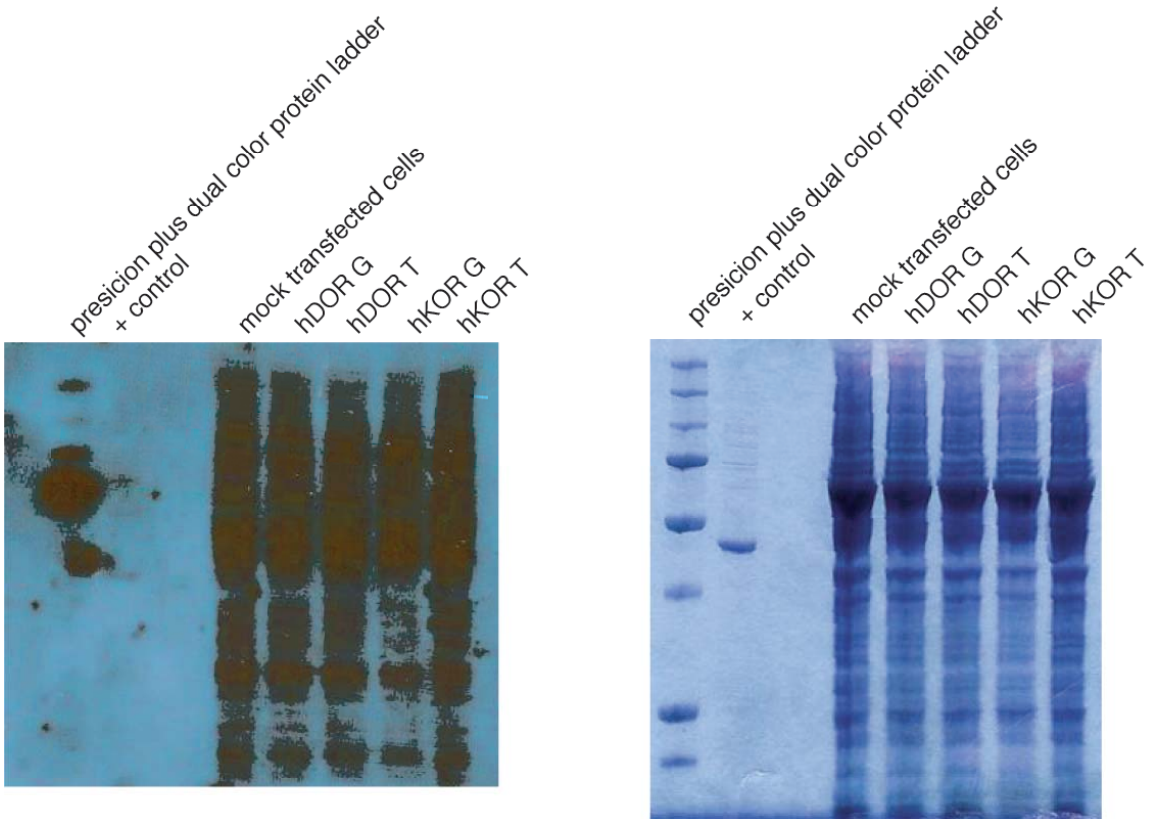


Figure 6-6 Western blot of COS-7 transiently transfected cells, 48 hours after transfection using the Fugene transfection kit. There is no difference in protein expression between the mock transfected cells, which received empty vector, and the vectors containing the cDNA of the four different alleles. The left picture is of the ECL autograph of the different cell lines. There is no difference in the protein expression profiles between the transfected cell lines and the mock transfected cell line indicating that there is no OPR protein being expressed. On the right is a duplicate comassie stained blot.

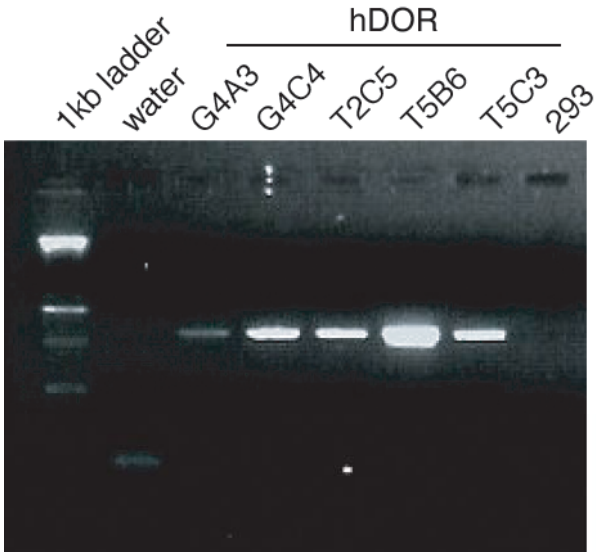


Figure 6-7 RT-PCR of the OPRD1 cDNA from stably transfected colonies after 25 extension cycles. GAPDH expression was equivilant in all the samples. These data illustrate the differences of expression levels between the different single colony cell lines used for our analyses, and that the clones are producing RNA from the plasmid, consistent with the northern analysis.

CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

MC1R

Although our functional studies proved inconclusive due to the lack of protein expression, we have found some associations between candidate genes in pain sensitivity and pain responses at baseline and after the administration of analgesics. We were the first group to elucidate a genetic link between fair skinned females and the response to pentazocine analgesia, where fairer skinned females have a greater analgesic response after the intravenous administration of a kappa opioid selective analgesic. This finding has far reaching implications as it had been anecdotally reported by the anesthesiology field that red head females need less anesthetic than the general population. Our findings have proposed a mechanism for their observations. This association is mediated through the *MC1R* gene, which is widely known in its role in the formation of skin pigment. Because pentazocine is not widely prescribed clinically anymore, we are currently conducting a study with morphine and examining baseline pain sensitivity and analgesic effect in a similar population. We have proposed that there is, in fact, a difference between fair-skinned individuals compared to darker skinned individuals when examining effects of morphine-6-glucoronide (M6G), a morphine metabolite in which we found that fairer skinned people had a higher analgesic effect via electrical stimulation compared to their darker skinned counterparts. M6G, although slower in its analgesic action, has less of an effect on respiration depression and it seems to have a smaller

sedative effect (http://www.medicalnewstoday.com/medicalnews.php?newsid=22657) than morphine, and may possibly be a better option for clinical use in the future.

OPRM1

One of the other candidate genes thus far found to have a positive association with baseline pain sensitivity, is the mu opioid receptor (OPRM1). It is the classic pain receptor in the nociceptive response and most analgesics are either directed either selectively or indirectly for this receptor. Our study into the baseline pressure pain sensitivity in different *OPRM1* genotypes was the first of its kind. We have linked the mu opioid receptor with mechanical pain sensitivity and the A118G polymorphism, which has been previously associated with other phenotypes such as the feeling of intoxication and family history of alcohol abuse (Ray and Hutchison 2004). The A118G polymorphism has also been associated with heroin dependence in Asian populations (Tan et al. 2003) and the minor allele in this polymorphism has also been shown to elicit an enhanced cortisol response to naloxone and reduced agonist effect of M6G (Hernandez-Avila et al. 2003).

The mu opioid receptor A118G polymorphism is also interesting, because it has been observed that the major allele is more highly expressed in post-mortem human brains compared to the G allele. In order to explain this finding, transfections of each allele were made in Chinese Hamster Ovary (CHO) cells and it was found that the A allele expressed more protein than the G allele. This research suggests an allelic consequence, which implicates a defect in transcription or mRNA maturation/stability of the minor allele (Zhang et al. 2005). There is also evidence that the A118G polymorphism is in linkage disequilibrium with the silent C17T polymorphism in the part of the gene encoding the extracellular region (Tan et al. 2003).

OPRD1

Although ANOVA analysis failed to find an association with pain sensitivity in our population of participants with the delta opioid receptor and pain sensitivity, another group recently found an association (Kim et al. 2004). Due to the gross amino acid change in the protein sequence and the environmental difference within the protein that is caused by this change, there is still a possibility of a functional implication of this polymorphism. Our lab has only performed three different pain modalities on our subjects, and we may be missing a pain modality associated with pain sensitivity or analgesic effect of this allelic variation. We are also pursuing QTL analysis of our data, which may be more sensitive to an underlying association, and enrolling more subjects.

Clinical Testing

Different clinical pain testing modalities were used in the different populations in the clinic. This makes it somewhat difficult to compare results obtained from these distinct populations. Our consortium of researchers and clinicians have started to use the same tests and facilities, which will make comprehensive conclusions more plausible in the continuing studies. For example, the IBS patients are now undergoing the same experimental pain testing as our healthy subjects. This could be a very interesting comparison.

Future Directions

Association Studies

Our research can be continued in the future by expanding our profile of candidate genes to analyze. We are currently starting to examine the adenosine receptors in our fibromyalgia patients in collaboration with a group in New York. After genotyping the polymorphism in this receptor, we have found that there is a higher frequency of the

minor allele in the FM patients (17%) compared to 1.5% in normal controls. This frequency difference warrants further validation and research into the exact mechanism of the adenosine receptor in the perception of pain and its role in the pathogenesis of a chronic pain syndrome. Also, we should examine the IBS chronic pain population and see if there is a difference of allele frequencies in this population compared to the FM patients and the normal controls. The β-2 adrenergic receptor is on our list of future candidate genes as it has been implicated in nociception in the mouse (Bastia et al. 2002). This genotyping is currently being undertaken in collaboration with the Belfer group at the NIH as well as our own facilities. In our collaboration, we are also examining the interleukins (IL), which are a large group of cytokines and are involved in the inflammatory response. The interleukins under examination are IL1α, IL1β, IL2, IL10 and IL13. As calcium levels have been found to be elevated in chronic pain patients (Ai et al. 1998), it is interesting to learn that IL1 is implicated also in the modulation of extracellular fluid calcium homeostasis (Sabatini et al. 1988). Both ILα and ILβ are released as a result of cell injury independent of the insult (Hogquist et al. 1991). IL1β is the major molecule responsible for the induction of cyclooxygenase 2 (COX2), which leads to the release of prostanoids. Prostanoids then invoke peripheral sensitization of nociceptors and causes localized pain hypersensitivity (hyperalgesia) (Samad et al. 2001). IL2, formerly known as T-cell growth factor, is an immunoregulatory molecule produced by lectin- or antigen- activated T cells. IL10 is also known as cytokine synthesis inhibitory factor, and is suggested to possibly arrest and reverse the chronic inflammatory response in atherosclerosis (Terkeltaub 1999). It has also been shown that IL10 works synergistically with glucocorticoids (Franchimont et al. 1999). Mice homozygous for a

disrupted IL10 gene seem to have an altered regulation of an immune response to enteric flora are more prone to inflammatory bowel disease (Kuhn et al. 1993). IL10 has also been connected to cytokine deficiency-induced colitis by QTL analysis (Farmer et al. 2001). IL13 is involved in the inhibition of inflammatory cytokine production, induced by lipopolysaccharides in blood monocytes (Minty et al. 1993). In fact, the c-terminal tail of IL13 (which dimerizes with IL4) interacts with the tyrosine kinases of the Janus kinase family (JAK), which interact in turn with STAT6 and regulate gene expression by binding to promoters of genes that are regulated by IL13 (and IL4). Differences in the gene sequence due to polymorphisms have been linked to differences in IL signaling (Kelly-Welch et al. 2003). Over-expression of IL13 in the lung of the mouse has been shown to cause inflammation and an accumulation of adenosine, as well as a decrease of adenosine deaminase activity with the simultaneous increase of various adenosine receptors (Blackburn et al. 2003).

We are also in the process of collaboratively genotyping polymorphisms in the cannabinoid receptor 2, which are involved in response to tetrahydrocannabinol (THC), which is used in some states as a pain reliever and an anti anxiety medication. Another potential candidate gene is the α-1-antitrypsin gene, which is known to modulate inflammation and has been shown to control fibromyalgia symptoms upon infusion (Blanco et al. 2005). Once these receptors have been genotyped, we can test their potential role in pain sensitivity and analgesic responsiveness. We could also use our samples that we have collected and replicate results found in other studies examining the vallanoid receptor subtype 1 gene (TRPV1) and its association with its increase in cold withdrawal times (Kim et al. 2004). I think that we should also focus our attention on the

NMDA receptor as this receptor is more involved in anti-nociception in males. We have also genotyped all our samples for all the COMT SNPs and are awaiting analyses, as we are also working on this aspect of the project in a collaborative fashion. Ultimately, strong reproducible associations can lead to improved pain management based on genetics, and shed light on biochemical systems involved in pain for future research.

Functional Analysis

If we do not find a positive association in our on-going studies of pain sensitivity with the delta and kappa opioid receptors, it may be difficult to justify future functional analyses of these polymorphisms. Our strategy needs troubleshooting if it is repeated in the future. For example, it may be helpful to first test this vector expressing a lacZ gene in HEK 293 cells before the effort is made to redo the stable cell lines. Also, others have used HEK cells that grow in suspension instead of our subpopulation of the HEK-S cells that grow adherently (Decaillot et al. 2003). The 5'UTR may be needed for proper translation of the protein, so next attempt, this region of the gene should maybe included in the vector. I think that the first experiment that should be attempted is the expression of the original vectors that were sent to us in a COS 7 cell system to see if we were actually sent cDNA that is translatable. I performed a quick transient co-transfection of the original vectors with β-galactosidase (figure 7-1) and it seems as though the kappa opioid vctor produces a functional protein. This experiment no only needs to be repeated, but mRNA and western blot analysis should be performed. The vectors can be expressed transiently, but we would have to then co-transfect a reporter vector to establish transfection efficiency, and we should be careful to make sure how different transient cell transfections express our receptor. It would also be important once we have protein, to

conduct binding assays to examine whether there is a difference in ligand affinity for the receptor based on the polymorphism located near the start of the gene, which translates into the extracellular region of the protein. This can be done using radiolabeled ligand and unlabelled ligand and analyze the competitive binding of the receptor. It would be worthwhile to look at the affects of the heterodimerization states of the opioid proteins. The polymorphisms in the genes of *OPRD1* and *OPRM1* confer amino acid substitutions in the encoded protein. The mu opioid receptor A118G polymorphism in the gene has already displayed altered function in the protein, but this has not been studied in a heterodimer state.

General

Our collaborations and studies are ongoing in this long-term project. We are currently looking at how differences in ethnic backgrounds affect pain sensitivity. We have started to standardize the testing amongst our three different populations and we plan to examine the evolution of the genetics of pain in the future. Our lab has expanded its collaborations since the inception of this described project and we are now studying patients with rotator cuff pain as well. This project was just the start of our lab's entry into the pain genetics field and, after four years, we are still one of the few laboratories in the country studying human pain sensitivity in the paradigm of genetics.

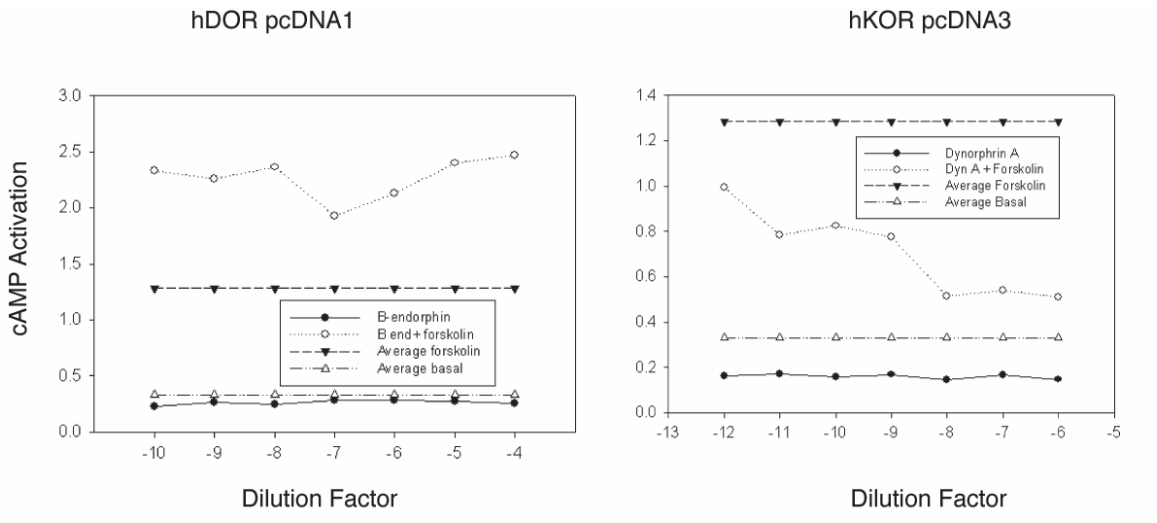


Figure 7-1 A transient transfecion with the original vectors received from collaborators. The hOPRD1 protein had no receptor activity in the activation assay, while the hOPRK1 protein seem to be expressed as we have a dose response in the cells where forskolin and agonist were added.

APPENDIX A
GENOTYPING RESULTS

Object A-1. Excel spreadsheet containing the list of genotype results in all polymorphisms studied. (object1.xls, 118KB)

Object A-2. Comma separated variable (CSV) version of the list of genotype results in all polymorphisms studied. (object2.csv, 28 KB)

APPENDIX B
GENOTYPE FREQUENCIES

Genotype frequencies of the different gene polymorphisms between three different pain populations. The actual headcount is followed by the represented genotype frequency in the population in parentheses. The between group value ids represented under the polymorphism name.

population	OPRM1 C17T (n=511) p=0.55			OPRM1 A118G (n=506) p=0.155			OPRD1 T80G (n=507) p=0.64			OPRK1 G36T (n=482) p=0.376		
	CC	CT	TT	AA	AG	GG	TT	TG	GG	GG	GT	TT
Healthy Subjects	320 (94.12)	18 (5.29)	2 (0.59)	263 (77.81)	67 (19.82)	8 (2.37)	265 (78.4)	64 (18.94)	9 (2.66)	257 (81.07)	51 (16.09)	9 (2.84)
IBS patients	68 (95.77)	2 (2.82)	1 (1.0)	54 (76.06)	13 (18.31)	4 (5.63)	57 (82.61)	11 (15.94)	1 (1.45)	61 (85.92)	10 (14.08)	0 (0)
FMS patients	97 (97.0)	2 (2.0)	1 (1.41)	73 (75.26)	24 (24.74)	0 (0)	78 (78.0)	20 (20.0)	2 (2.0)	82 (87.23)	11 (11.70)	1 (1.06)

population	OPRK1 A843G (n=410) p=0.98			OPRK1 C846T (n=405) p=0.021			CALCA P1/P2 TC/CG (n=514) p=0.0167			CALCA P4 TC (n=460) p=0.422		
	AA	AG	GG	CC	CT	TT	TTCC	TCCG	CCGG	TT	CT	CC
Healthy Subjects	172 (66.41)	78 (30.1)	9 (3.47)	231 (89.53)	27 (10.47)	0 (0)	169 (49.4)	131 (38.3)	42 (12.3)	280 (96.5)	9 (3.1)	1 (0.34)
IBS patients	41 (66.13)	18 (29.03)	3 (4.84)	55 (88.71)	5 (8.06)	2 (3.2)	38 (52.8)	30 (41.7)	4 (5.56)	67 (93.1)	5 (6.9)	0 (0)
FMS patients	61 (68.54)	25 (28.09)	3 (3.37)	75 (88.24)	10 (11.76)	0 (0)	42 (42.0)	48 (48.0)	10 (10)	96 (97.9)	2 (2.04)	0 (0)

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BIOGRAPHICAL SKETCH

Lee Kaplan was born in Cape Town South Africa on July 23rd 1976. After taking a few years off after high school, she attended the University of Stellenbosch in Stellenbosch South Africa and majored in genetics and psychology. Wanting to pursue a career in human genetics, Lee decided to head to the United States of America and more specifically, to Florida to attain her PhD in human genetics. After meeting Dr. Wallace, Lee knew that she would be able to meet her goals in this lab. Lee plans on going on to a fellowship in clinical cytogenetics and molecular genetics, which will allow her to direct laboratories key to diagnosing human genetic disease. Although clinical work will be her main focus, Lee plans also to run a basic science lab researching the mechanism of human disease.