PET Imaging of Individual Differences in Regional Mu-Opioid Activation in Motivational Brain Circuitry

by

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Chapter I

Introduction

Of the millions of people who try or even abuse drugs, why do only a small percentage of them go on to develop addictions? Why is it that most people who are injured or who endure surgery recover quickly and with minimal pain, but some endure pain long after the site of surgery or injury has healed? Even in healthy individuals, a wide range of normal variation is observed in clinical settings, for example, it is well known that patients exhibit wide ranges of sensitivity and tolerance to opiate analgesics. Humans exhibit a spectrum of responses to noxious events or exposure to drugs, ranging from resiliency to extreme susceptibility to chronic dysregulation. In order to be able to treat diseases of chronic pain and addiction or to better tailor treatment of post-surgical or post-injury pain, as well as understand etiology of and vulnerability to disease, it is essential to take into account the individual differences that have historically confounded treatments and studies involving pain and addiction.

Traditionally, scientific research has approached understanding the brain in terms of commonalities and averages: the brain mechanisms, structures, circuits, cellular pathways, molecules and genes that are conserved between species or that are common to

all humans. This approach is undeniably valuable and has been indispensible in advancing our knowledge of the brain. However, a recent movement in research is to advance beyond the commonalities and to focus on what makes us different; studying individual differences is a way to further advance our knowledge of the brain, a way to create a template for future research that takes individuality into account, and an essential step towards understanding and treating diseases of the brain.

Research in our lab has sought to tease apart some of the individual factors that predispose humans to the diseases of addiction and chronic pain and to develop research methods that most effectively highlight these various factors. Although they are seemingly disparate conditions, chronic pain and addiction often co-occur. Chronic pain is frequently comorbid with depression, anxiety, substance abuse disorders, somatization and personality disorders [1]. Similarly, addicts are commonly diagnosed with depression, anxiety, personality disorders. Studies have found that chronic pain patients have 15-28% rates of current substance use disorders and 23-54% lifetime prevalence of substance use disorders, compared to 16.7% lifetime prevalence in the general population [1]. Clearly the two conditions can have overlapping symptoms and accompanying disorders, and addiction risk increases with occurrence of chronic pain. These features point to the existence of common etiological mechanisms in subsets of susceptible individuals. Indeed, pain and addiction are mediated by at least one common pathway: the motivational circuitry of the brain.

It is important to apply methods that examine individual differences to both healthy and diseased populations in order to understand how these factors contribute to normal functioning, vulnerability to disease, and dysregulation in disease states. For

example, sex differences exist in the symptoms and incidence of a host of brain diseases, including mood disorders, schizophrenia, Alzheimer's, and autism, in addition to chronic pain and addiction, which are addressed in this dissertation. However, little is known about why these differences in symptoms and vulnerability exist or how to tailor treatments to best benefit men versus women. A timely news article in the March 26 issue of *Science* reports on the strong sex biases toward male research animals that exist in most areas of biomedical science, with Neuroscience as one of the most male-biased disciplines [2]. Knowledge about how individual differences such as a particular genotype influence brain states will enable clinicians to offer personalized treatments, or to identify vulnerable individuals and modify their treatments accordingly. Our research on individual differences in the motivational system has allowed us to study normal variations in its function, to learn about predispositional factors that influence motivational dysregulation, as well as, through placebo analgesia, to understand how the mind's interaction with the environment induces physiological responses in the motivational system.

In the pages that follow, I will first introduce the anatomy and functionality of the motivational circuitry in general, its involvement in natural reward and pain, as well as its dysregulation in addiction. I will then address one of the modulatory neurotransmitter systems within the motivational network, the μ-opioid system, and its regulation of pain and reward. The subsequent discussion will explain the relevance of using placebo analgesia as a tool for examining mind-body interactions and individual differences in motivational circuitry functioning. The literature on certain individual differences that

contribute to variations in motivational network function in addicted states will also be summarized.

Anatomy and Neurochemistry of the Brain Motivational Circuitry

The brain's motivational system is comprised of cortical, limbic, and motor structures. These include the basal ganglia, including the caudate, putamen, globus pallidus, and nucleus accumbens; the amygdala ventral tegmental area (VTA), the thalamus, insular cortex, anterior cingulate cortex, and the orbitofrontal cortex, all of which are interconnected. The structures at the heart of the motivational system, the basal ganglia, are situated at a convenient intersection between limbic and cortical circuits, so that they receive sensory information filtered through both affective and executive lenses. The basal ganglia receive and process this information about relevant or rewarding environmental stimuli and initiate appropriate corresponding motor behaviors[3]. The system evolved to enable organisms to adaptively perceive and evaluate the rewarding or aversive value of a stimulus and subsequently engage in either approach or avoidance behaviors, depending on the adaptive survival potential of the behavior.

Historical Context of Motivational Circuit

Historically, the nucleus accumbens and its interconnected regions were first mapped and characterized in relation to their integration of dopamine transmission and

adaptive motor behavior [4]. Early animal studies in the 1970s established the nucleus accumbens as a primary site of motor initiation and dopamine as a critical neurotransmitter in the process. Pharmacological enhancement of dopamine transmission in the nucleus accumbens resulted in exploratory motor behaviors, as opposed to the stereotyped motor behaviors caused by dopamine stimulation in other parts of the striatum [5]. Soon after, Mogenson *et al.* [4] characterized the motor circuit as comprising the nucleus accumbens as an integration site of telencephalic glutamatergic inputs from the amygdala and prefrontal cortex, and brainstem dopaminergic inputs from the ventral tegmental area. GABAergic outputs from the nucleus accumbens to the ventral pallidum then regulated motor initiation. Later, the role of the mediodorsal thalamus in this motor circuit was clarified when pharmacological manipulations in the mediodorsal thalamus initiated motor behavior [6].

While the nucleus accumbens and its circuitry were initially implicated in motor initiation and regulation, later studies of these structures revealed they were also involved in reward perception and responsivity. Specifically, initial studies revealed that animals would sustain electrical self-stimulation in the basolateral amygdala, prefrontal cortex, mediodorsal thalamus, nucleus accumbens, ventral pallidum, and VTA [7]. Subsequent experiments demonstrated that the same dopaminergic inputs to the nucleus accumbens that were critical for motor behavior also mediated reward responsivity to drugs, food and sex [8]. Thus, early research on the behavioral correlates of this circuitry resulted in the concept that the circuitry's connections and neurotransmission allowed for a system in which motor initiation and reward evaluation converged. This emerging idea of circuitry

that integrated reward and motor activity led to our current understanding of the functional connectivity of the motivational circuitry.

Current Understanding of Motivational Circuit

Dopamine is a central modulatory neurotransmitter in the motivational system, and it is crucial to eliciting reward responsivity and motor behavior. Dopamine-containing neurons arise in the midbrain and project to a diverse range of forebrain targets. The nigrostriatal dopamine system's neurons originate in the substantia nigra and project primarily to the dorsal striatum, where they are mostly associated with motor function. The mesolimbic and mesocortical dopamine projections both arise from the VTA. The mesolimbic neurons project to the ventral striatum (NAc), septum, amygdala and hippocampus, while mesocortical neurons innervate medial prefrontal cortex, cingulate cortex, and perirhinal cortex. Because the mesolimbic and mesocortical neurons have substantial overlap, the systems are often referred to collectively as the mesocorticolimbic dopamine system. The mesocorticolimbic system is primarily involved in motivational function [9].

Central to the motivational system is the VTA's dopaminergic projection to the nucleus accumbens. Reward and motor behavior are modulated both by excitatory input to VTA dopaminergic neurons and by disinhibition from GABAergic inhibition of these neurons. Electrophysiological experiments in active primates established that dopaminergic activity in the motivational circuit assigns appetitive and motivational value to external stimuli, not by directly signaling a rewarding event, but by encoding a

prediction error, which represents the difference in expected versus actual reward value [10]. Thus, dopamine activity signals novelty and salience of rewarding environmental stimuli rather than encoding reward receipt or the behavior elicited by reward.

In the nucleus accumbens, dopamine release encoding novelty and salience modulates excitatory glutamatergic input from orbitofrontal cortex, cingulate cortex, prefrontal cortex, hippocampus, amygdala and thalamus, in effect acting as an informational gatekeeper that either encourages or inhibits behavioral responses to potentially rewarding or non-rewarding environmental stimuli [5]. These glutamatergic neurons that project to the NAc from the aforementioned regions are also regulated by dopaminergic innervation from the VTA [11-14]. Thus, these regions also respond to reward encounter, delivery and intensity, and execution of appropriate behavioral responses [15-17].

The ventral pallidum receives GABAergic afferents from the NAc and dopaminergic inputs from the VTA, and is involved in initiating motor behavior by connecting with motor nuclei in the brainstem [18]. The mediodorsal thalamus, in turn, directs a unidirectional flow of information from ventral pallidum to prefrontal cortex, so this results in an indirect transfer of information from the more limbic to more motor portions of the NAc [19].

Role of mu-opioid signaling in the motivational circuit

While dopamine is considered a primary neurotransmitter in the motivational circuit for eliciting reward-related behavior, endogenous opioids, particularly those acting

at mu receptors, also play an important role in reward and motivation processing in the circuit.

Opioid receptors and peptides are expressed widely throughout the brain. There are three main types of opioid receptors: mu, delta, and kappa, which all belong to the G protein-coupled receptor family. The opioid peptides β-endorphin and the enkephalins bind with highest affinity to mu and delta receptors, while dynorphins bind preferentially to kappa receptors [20]. Because mu-opioid signaling is most relevant to the topic of this dissertation, the discussion that follows will address only aspects of mu-opioid related transmission.

Mu-opioid receptors are expressed abundantly throughout the motivational circuitry. The mu-opioid receptors are the most densely expressed of all the opioid receptors in the amygdala, thalamus, midbrain (i.e. periacqueductal grey (PAG) and VTA), and some brain stem nuclei [20]. They are also expressed in cortex, caudate, putamen, nucleus accumbens, ventral pallidum, hypothalamus and pituitary. Protein precursors of opioid peptides that bind to mu receptors, preproenkephalin (Penk) and proopiomelanocortin (POMC), overlap largely with mu-opioid receptor sites [20].

One way mu-opioid activity mediates reward and motivation is by its engagement of the mesolimbic dopamine system in the VTA. Mu-opioid receptors lie on GABAergic interneurons that normally exert inhibitory control over VTA dopamine neurons. However, opiates or endogenously released opioids can act at the mu-receptors on GABAergic neurons, inhibiting their activity and thus disinhibiting dopamine cell firing and subsequent dopamine release in the nucleus accumbens and other VTA- dopamine-innervated structures [21, 22]. Mu-opioid receptor signaling is always inhibitory via

activation of intracellular G_i proteins, which leads to hyperpolarization of the cell. In many brain regions, mu-opioid receptors reside both presynaptically and postsynaptically on GABA neurons, inhibiting GABA release. Sometimes this can result in the release of nearby cells from GABA inhibition, enabling their activation.

Now that I have outlined the relevant structural and neurochemical components of the motive circuit, I will turn to discussing the role of the motive circuit in eliciting psychological states of motivation, reward pursuit, stress, emotion, and pain. This discussion of the normal functions of the motivational system will segue into a review of the consequences of aberrant reward processing as they regard susceptibility to addiction and chronic pain.

Role of Motivational Circuitry in Behavioral and Psychological States

Reward Responsivity

In 1954, Olds and Milner [23] uncovered the first neural correlates of reward when they observed that direct electrical stimulation in the septum of rats produced a conditioned place preference for the environment in which the stimulation was received. They subsequently demonstrated that rats learned to work (i.e. lever press) for septal brain stimulation, showing that stimulation could serve as an operant reinforcer. They hypothesized that this stimulation produced reward responses because it activated brain systems (the septum as well as other interconnected structures that we now refer to as the mesocorticolimbic dopamine system) that evolved to evoke behavioral approach responses toward natural rewards that were essential for survival [23]. Shortly after these discoveries, numerous studies showed that natural rewards like food, water and sex, as

well as intravenous drug rewards, can instill in animals very similar response patterns as those to intracranial self-stimulation [24], providing further evidence of a brain network that processes potentially relevant stimuli and translates their perception into appropriate behavior.

Since the initial studies of Olds and Milner laid the groundwork for the neural basis of reward, the reward/motivation field has exponentially expanded, resulting in an abundance of knowledge about the specialized roles of each of the structures in the motivational system, as well as the nuances of psychological and behavioral responses to motivational incentives.

Motivation/Positive Reinforcement

For decades, the dopaminergic mesolimbic circuitry has been theorized to mediate "reward". However, it has become increasingly realized that such a conceptualization is not only too simplistic, but perhaps even inaccurate. While there are many competing theories about what aspects of reinforcement and reward are and are not mediated by this circuitry, there is a general consensus that instead of mere "reward", it is more accurate to say that the mesolimbic dopamine system mediates motivation and responds to motivationally salient stimuli. "Reward" implies a positive affective or pleasurable quality of a salient stimulus, but it is now known that the system also mediates responses to salient stimuli with negative or aversive characteristics. Aversive motivation will be discussed in more detail in the next section.

As mentioned earlier, the first studies to connect dopamine and mesolimbic pathways with reward were ones that demonstrated animals would work to obtain direct electrical stimulation into most of the mesolimbic regions [23], as well as subsequent studies that showed similar behavioral responses to the prospect of obtaining food, water, sex and drugs. The role for this system in reward response was expanded to include the motivation to obtain rewards, as another series of studies showed.

First were the discoveries that ablation of dopamine fibers near the hypothalamus as well as nigrostriatal dopamine fibers markedly reduced feeding and drinking behaviors [25, 26]. Additionally, damage to mesolimbic dopamine fibers resulted in decreased forward locomotion characteristic of reward-seeking [27]. Later studies using neuroleptics, drugs that selectively block the actions of dopamine at dopamine receptors, found that blocking dopamine function interferes with instrumental responding for food, rewarding effects of lateral hypothalamic electrical stimulation, intravenous amphetamine or cocaine injections, and water seeking [9]. Low doses of dopamine antagonists reduce lever pressing for food and water, but do not interfere with general appetite or thirst when food and water are readily available without work requirements. This evidence indicates a role for dopamine circuitry in motivation and expending effort to obtain natural "rewards", but not in appetite for them [28].

An important series of studies established separate neurochemical correlates of wanting and liking of rewards. Injection of μ-opioid receptor agonists into certain regions within the nucleus accumbens and ventral pallidum of rats enhances hedonic "liking" expressions in response to sweet tastes [29]. However, lesions to dopamine

projecting neurons to the striatum and resulting dopamine depletion do not impair hedonic "liking" responses to sucrose. Pharmacological blockade of dopamine activity produces similar results. Brain manipulations to enhance dopamine also do not alter hedonic impact of natural rewards. Amphetamine microinjections into nucleus accumbens do not increase "liking" reactions to sucrose, but they do result in increased "wanting" behaviors for sucrose reward. Similar results were found in mutant mice whose dopamine transporter gene was knocked down, resulting in hyperdopaminergic brains. Taken together, these studies strongly suggest that dopamine is not responsible for the hedonic impact of rewards, but that it is involved in mediating the wanting of rewards [29].

Despite competing theories that attempt to explain exactly what role the mesolimbic dopamine system plays in reward and motivation, a consensus has emerged on the broad roles of many of the network's structures.

Parts of the amygdala are responsive to motivationally and emotionally relevant stimuli; basolateral amygdala receives sensory information from cortex and thalamus and shares glutamatergic connections with the centromedial amygdala, which in turn sends efferents to autonomic brainstem nuclei, somato- and visceromotor control sites, and the striatopallidum [30]. Thus, basolateral amygdala is thought to evaluate the emotional value of environmental stimuli, while the central amygdala integrates their motivational elements and then sends this information to downstream regions that elicit appropriate bodily and behavioral responses. Evidence of this comes partly from animal lesion studies, in which basolateral lesions alter sensitivity to changes in reward value of a

stimulus. Lesions to the centromedial amygdala disrupt the motivational aspects of rewarding stimuli [30].

One structure to which the amygdala sends its stimulus valence information via glutamatergic projections is the ventral pallidum. The ventral pallidum may act on this information and elicit appropriate approach or withdrawal behaviors depending on whether its input was positive or negative in valence [30].

Basolateral amygdala and the anterior cingulate cortex (ACC) have a reciprocal glutamatergic connection through which information is transferred relating to decision making based on stimulus valence information from the amygdala [28], while nucleus accumbens dopamine is thought to mediate overlapping aspects of motor activation, motivation, and affect.

Aversive Motivation

One of the biggest challenges to the original notion that mesolimbic dopamine mediates only the pleasurable or positive aspects of reward is an accumulation of evidence from numerous studies showing that aversive and unpleasant stimuli activate the system in the same ways rewards do. In animal studies, microdialysis measurements of increased accumbens dopamine transmission are caused by a wide variety of unpleasant stimuli, including footshock, tailshock, tail pinch, restraint stress, social stress, conditioned aversive stimuli, and anxiogenic drugs [28]. In electrophysiology studies, conditioned aversive stimuli and restraint stress enhance activity of VTA dopamine neurons [28]. Conditioned behavioral responses to aversive stimuli are enhanced after

amphetamine administration [31]. Nucleus accumbens lesions interfere with responses to conditioned aversive cues [31]. Injection into rat nucleus accumbens of either a dopamine antagonist or an opioid antagonist blocks the antinociceptive effects of anesthesia on pain-reactive reflexes [32]. The responsiveness of the mesolimbic dopamine system to aversive stimuli and their predictors indicates that it may serve a role in mediating incentive motivation for behaviors that promote survival and safety via avoidance, in addition to mediating the production of approach behavior.

The animal studies are consistent with imaging studies of aversive stimuli in humans. Patients diagnosed with post-traumatic stress disorder, when presented with unpleasant stimuli like combat sounds, demonstrate increased blood flow to the accumbens [33]. PET measurements of [^{11}C] raclopride indicated dopamine release in ventral striatum in response to social stress, which was also correlated with cortisol release [34].

Most relevant to the topic of this dissertation is the imaging literature on human responses to pain, which provides clear evidence of both mesolimbic circuitry activation and dopamine and opioid activation within mesolimbic structures. An early fMRI study found that structures in reward and classic pain circuitry were activated during application of noxious thermal stimuli in humans. The activated structures included extended amygdala, VTA, PAG, ventral striatum and nucleus accumbens [35]. A PET study in our lab demonstrated dopamine activation within the basal ganglia in response to deep sustained somatic pain. Specifically, dopamine D_2 neurotransmission in dorsal striatum was positively associated with ratings of sensory and affective aspects of pain. Ventral striatum dopamine activation, presumably involving D_2 and D_3 receptors, was

associated with increases in negative and fear-related internal affective state [36]. A subsequent PET study replicated these findings in its comparison of dopamine activation in response to pain in fibromyalgia patients and healthy subjects. While healthy subjects showed dopamine activation in the basal ganglia in response to pain, fibromyalgia patients did not [37]. These studies established a clear role for the dopaminergic motivational system in modulating responses to pain and aversive stimuli, clarifying the fact that dopamine's role is to encode novelty and salience of environmental stimuli instead of just their rewarding aspects.

Expectation/Anticipation

Another aspect of the updated emerging view of the role of the reward and motivation system is its involvement in expectation, anticipation, and prediction of salient stimuli. Schultz and colleagues, who have become well known for their electrophysiological studies on reward prediction, have found that in monkeys, midbrain dopamine neurons code reward prediction errors [38]. This interpretation is based on experiments that show that dopamine neurons initially fire upon receipt of unexpected rewards. As these events become predictable over time, the cells reduce (and eventually stop) responding to predictable reward receipt and increase responding to cues that precede and predict reward. Thus, mesolimbic dopamine neurons are more responsive to reward predictors and the anticipation of the reward than to the actual reward itself [38].

A similar pattern of mesolimbic activity has been observed using brain imaging in humans. One PET study using [^{11}C] raclopride found that dopamine release was

enhanced in the striatum during both rewarding and anticipatory portions of a video game involving monetary reward [39]. fMRI studies have shown that the mesocorticolimbic circuitry is differentially activated by reward anticipation versus reward outcome; ventral striatum responds preferentially to anticipation while the medial prefrontal cortex responds to outcome [40].

Motivational Circuitry and Addiction

Drugs of abuse are chemically and structurally diverse and their primary sites of action in the brain vary widely. However, they all share the characteristics of being acutely rewarding and engaging the mesolimbic system by enhancing dopamine release. Their immediate rewarding effects promote repeated use, which leads to common brain adaptations, and, in susceptible individuals, eventually addiction.

The VTA-nucleus accumbens connection has been studied the most and seems most essential for mediating the acute rewarding effects of drugs. All drugs, through varying pathways, activate VTA dopamine neurons, which release dopamine into the nucleus accumbens [41]. For example, stimulants such as cocaine and amphetamines act directly on VTA dopamine terminals in the nucleus accumbens at dopamine transporters to enhance dopamine release. Alcohol acts on $GABA_A$ receptors at GABA neuron terminals in the VTA, inhibiting GABA release onto dopamine cell bodies, causing disinhibition of dopamine neurons. Nicotine, whose actions are most relevant to the dissertation topic, directly activates VTA neurons by stimulating cholinergic receptors on dopamine cell bodies and indirectly activates VTA cells by stimulating cholinergic

receptors on glutamatergic nerve terminals to enhance glutamatergic stimulation of VTA neuron activity. Importantly, nicotine and alcohol promote endogenous opioid release, which is thought to disinhibit VTA dopamine neurons by endogenous opioid action at opioid receptors on VTA GABA interneurons [41].

Drugs of abuse also elicit common adaptations after chronic exposure. Tonic dopamine levels are reduced, and natural rewards fail to induce as large of a phasic dopamine increase as before. Conversely, drugs of abuse sensitize the dopamine system, so that dopamine is released at higher levels in response to drugs or drug-related cues [41]. Imaging studies have shown that prefrontal cortex, anterior cingulate cortex and orbitofrontal cortex show reduced baseline activity after chronic exposure to drugs. Their involvement in executive functions like working memory, attention, behavioral inhibition and decision making is therefore disrupted, and this dampened function is thought to contribute in part to the severe impulsivity and compulsivity characteristic of addicts [41].

Individual Differences in Reward/ Motivation Circuitry and Susceptibility to Addiction

There is evidence that reward and motivational circuitry function is influenced by individual differences such as sex and genotype. These factors may underlie motivational circuitry dysregulation that precedes adaptive changes due to repeated drug use and that enhance susceptibility to addiction and relapse.

Genes

A variety of polymorphisms in genes whose expression affects mesolimbic functioning have been associated with altered reward responsivity and vulnerability to addiction. The most studied polymorphisms include the dopamine transporter (DAT), the COMT enzyme that influences dopamine levels via dopamine catabolism, MAOA, another catabolic enzyme, and dopamine D_2 receptor gene polymorphisms [42]. For example, a common polymorphism in the COMT gene, the Val158Met polymorphism, is associated with substance abuse. The Val/Val genotype, which confers high enzyme activity and less synaptic dopamine, is more frequent in substance abusers and is associated with heroin addiction. The Met/Met allele is more frequent in different subsets of addicts, such as anxiety-associated alcoholics and both homozygous alleles are associated with novelty-seeking [42]. It is thought that homozygosity for either allele represents separate adaptive advantages: cognitive abilities vs. emotional resiliency [43].

The A118G polymorphism in the μ-opioid receptor gene is associated with altered μ-opioid system function, altered reward responsivity, and susceptibility to various addictions.

Motivational Circuitry and Pain Regulation

Pain modulatory circuitry overlaps extensively with reward and motivation circuitry, both anatomically and functionally. There is a good reason the systems are so

closely coupled—pain states can be conceptualized as motivational states and depending on the environmental context, pain modulation produces behavior most adaptive and appropriate for the organism and its context.

As mentioned earlier, opioids like morphine and heroin can induce powerful appetitive actions. They are obviously addictive, and studies have shown that they can enhance food and alcohol consumption. Injection of opioid agonists into the nucleus accumbens and ventral pallidum of rats increases objective facial expressions of "liking" in reaction to sucrose [29]. Because opioids are also potent analgesics and because they modulate motivational states, much research has sought to establish their role in the pain modulatory circuitry in the brain and how behavioral states influence, in a top-down manner, the modulation of pain via opioid receptor actions.

Before going into more depth about state dependent pain modulation, it will be helpful to first summarize how peripheral pain is processed through afferent pain pathways. Peripheral noxious stimuli activate primary afferent nociceptors, which project to the dorsal horn of the spinal cord and release glutamate and peptides onto second order neurons, activating them. The axons of the second order nociceptive dorsal horn neurons cross to the contralateral anterolateral quadrant and ascend through the spinal cord, sending out branches that terminate in the brainstem and thalamus. The thalamic neurons upon which the nociceptive terminals synapse then project to somatosensory cortex, anterior cingulate, and insula, which each process different components of the pain signals [44].

The beginnings of our understanding of the pain modulatory circuit were established when Wall [45] discovered that nociceptive dorsal horn neurons in various

laminae could be innervated by projections from supraspinal sites. Subsequent work demonstrated that PAG stimulation in rats prevented withdrawal reflexes to noxious stimulation [46-48]. This finding was extended to humans, where surgeons found that electrical stimulation of the PAG resulted in substantial pain relief [49]. Further investigations into the anatomy of this modulatory system established that hypothalamus, amygdala, and prefrontal cortex including anterior cingulate, send direct projections to the PAG. The PAG, in turn, modulates nociceptive inputs by sending connections through the rostral ventromedial medulla (RVM) and dorsolateral pontine tegmentum (DLPT). These two structures then project down through the spinal cord to the dorsal horn nociceptive neurons [44]. From these discoveries, it became clear that pain could be modulated in a top-down manner, originating from higher order processing areas like the prefrontal cortex.

Mu-opioid receptor-related neurotransmission is a primary and crucial component in the modulatory circuitry. The mu opioid receptor exists in all of the supraspinal structures of the pain modulatory system: insula, amygdala, hypothalamus, PAG, DLPT, RVM, as well as the spinal cord dorsal horn [44]. Mu opioid receptor agonist injection into any of these sites abolishes nociceptive behavioral reflexes [44]. Mu opioid receptor agonist analgesia is mediated by activation of supraspinal neurons that send projections through the RVM to the dorsal horn. This is supported by evidence from studies where inactivating the RVM interferes with morphine analgesia when morphine has been injected systemically or into supraspinal structures[44].

Clearly mu opioid receptors are involved in the pain modulatory circuit. Additional studies show that endogenous opioid release occurs to induce mu opioid

receptor signaling in the circuit. It appears that endogenous opioid release occurs at each component in order to activate (though not through direct activation; endogenous opioids probably act by disinhibition) the next downstream component. When mu opioid receptor agonists are injected into the posterior hypothalamus or basolateral amygdala, the resulting analgesia is reversed by injecting a mu opioid receptor antagonist into the PAG [50]. These endogenous opioids are thought to be enkephalins, as injecting an enkephalinase inhibitor into the RVM produces analgesia [51]. Indeed, a PET study in our lab confirmed that endogenous opioid activation regulates pain by showing for the first time in humans that a sustained pain challenge results in μ-opioid receptor related neurotransmission within the pain modulatory circuitry, and that this activation was associated with decreases in sensory and affective ratings of the pain experience [52].

The pain modulatory circuit can both inhibit and enhance nociceptive transmission. This dichotomy is the result of two populations of neurons, termed 'on cells' and 'off cells', which exist in the PAG, DLPT and RVM [44]. Both send projections that terminate on primary afferent nociceptor synapses in the dorsal horn. On cells facilitate nociceptive transmission while off cells inhibit it. The two populations of neurons are reciprocally active, and their firing patterns vary depending on the animal's environmental stimuli. When animals are exposed to noxious heat, RVM off cells pause their firing just before reflexive withdrawal, while at the same time on cells increase firing. In the absence of applied stimuli, RVM on and off cells take turns firing, each switching between active and inactive states in a reciprocal manner [44]. It is believed that the two populations share upstream connectivity, which regulates this reciprocal activity. Administration of mu opioid receptor agonists systemically, or directly into the

PAG or RVM induces an off cell state, in which off cell firing increases and is sustained over time. Withdrawal reflexes to noxious stimuli are inhibited. Blocking off cell activation prevents morphine analgesia. These results indicate that off cell activation is required for mu opioid receptor ligand-induced analgesia applied either systemically or supraspinally [44]. Prolonged application of noxious stimuli induces an on cell continuous firing state.

The on and off neuronal activity also varies according to an animal's arousal state. In awake rats, off cells fire intermittently and increase and sustain firing during slow-wave sleep, while on cell activity decreases [53].

The pain inhibitory and facilitatory aspects of the pain modulatory circuit probably evolved to allow adaptive decision-making in response to pain's motivational impetus, in accord with the environmental context. For example, in animals, the pain that results from injury elicits motivation to engage in instinctive behavioral responses such as licking, guarding, and reduced activity, which are adaptive in that they allow for healing. The pain also motivates the animal to learn to avoid the noxious stimulus in the future. However, there are situations in which these pain-reactive behaviors may not be adaptive. In the presence of a predator, an injured animal does not want to engage in recuperative behaviors or pain-induced vocalizations that might call attention to them or hinder escape. Likewise, eliciting pain behaviors in the presence of a competing male might threaten reproductive success. In these cases, inhibitory components of the pain modulatory system confer evolutionary advantage by suppressing pain and its accompanying behavioral responses so that the animal can behave adaptively [44].

The scientific evidence supports this scenario. Male rodents faced with a predator or an aggressive male exhibit naloxone-reversible analgesia [54]. When inescapable foot shock is paired with an initially neutral light or tone, eventually the light or tone alone can generate the motivational power to induce analgesia. The analgesia is blocked by injecting mu opioid receptor antagonists into the basolateral amygdala, PAG, and RVM [55]. The opioid-mediated pain modulatory system, along with the overlapping motivational network, is therefore activated when an organism must choose between responding to an anticipated threat or attending to an injurious noxious stimulus: the decision is based on weighing the costs and benefits of the possible behaviors.

The pain-modulatory opioid circuitry also is activated by and mediates appetitive motivational states. As mentioned previously, opioid agonist injection into NAcc or ventral pallidum can potentiate liking of sweet tastes [29] and can produce anti-nociception [56]. Naloxone-reversible analgesia is produced after feeding sucrose to animals or human infants [57, 58], as well as during the anticipation of a food reward [59]. Therefore, the pain-modulatory circuit plays a similar role in appetitive states as in aversive states: it induces analgesia so that in the face of injury, an animal can choose to approach survival-contingent rewards like food, despite competing pain-induced drives.

Placebo Analgesia as a Motivational State

Placebo analgesia is defined as pain relief induced by the belief and expectation that one will receive a potent analgesic when in fact only an inert agent is administered. The first study linking placebo analgesia and the pain-modulatory endogenous opioid

circuitry demonstrated that placebo analgesia was blocked by naloxone [60]. Expectancy, which is established verbally or by conditioning, is an important cognitive determinant of placebo analgesia. Because pain relief is rewarding via negative reinforcement, successful placebo administration can be considered a reward-predictive cue. Therefore, the expectancy established by placebo administration induces an appetitive motivational incentive for pain relief and accompanying opioid-mediated modulation of the pain. This scenario can be likened to that of the previously-mentioned opioid analgesia elicited by rodents' anticipation of food rewards; appetitive motivational states can engage the opioid-dependent pain modulatory circuitry to produce analgesia.

The notion that expectancy engages the endogenous opioid system to produce placebo analgesia is supported by a growing literature on brain imaging of placebo analgesia in humans. An early placebo analgesia imaging study found that in subjects in whom placebo analgesia was effective, the brain regions activated during placebo analgesia were largely the same ones activated by μ-opioid receptor agonist remifentanyl analgesia. These areas included the rostral anterior cingulate and areas of the pons near the periacqueductal grey [61]. A subsequent fMRI study found that activity in the pain modulatory circuit, including rostral anterior cingulate, insula and thalamus, was modified by placebo analgesia and was correlated with placebo-induced decreases in pain ratings. Additionally, anticipation of pain, prior to noxious stimulation, activated the DLPFC, rostral anterior cingulate and PAG, whose activity was correlated with placebo-induced reduction in pain, suggesting that expectation of pain relief from placebo engages the pain modulatory circuitry by recruiting DLPFC and PAG to dampen pain, presumably via endogenous opioid release in those regions [62]. The anterior cingulate

sends projections to the midbrain, including PAG, which places it in a favorable position to control opioid-mediated responses to spinal nociceptive signals [63]. The anterior cingulate is similarly activated by anticipation of rewards in humans and primates [64, 65], and it sends projections to the nucleus accumbens, which is also activated in response to both rewards and noxious stimulation and incorporates motives and actions. The assumption that endogenous opioid release within these regions was responsible for placebo analgesia was confirmed when Zubieta et al. [66] showed using PET imaging that placebo analgesia enhanced μ-opioid receptor- mediated neurotransmission in the rostral anterior cingulate, DLPFC, insula, and nucleus accumbens, and that these activations were associated with decreases in pain intensity, sensory and affective qualities of pain, and negative emotional state.

The preceding placebo analgesia imaging studies support the view that the activation of a network containing these and other structures in response to positive (rewards, placebo analgesia) and aversive (pain) stimuli suggests that, in the case of placebo analgesia, its serves to mediate the 'decision' to respond to or dampen incoming nociceptive signals through activation of endogenous opioid release [44]. Not all people make the 'decision' to dampen pain signals in response to placebo, however. In fact, there is a spectrum of placebo analgesic effectiveness among individuals, from high responders to nonresponders to nocebo (pain increases) responders. Clearly the pain modulatory and motivational circuitries do not operate identically among individuals in response to environmental inputs. Examining factors that contribute to this variation will broaden our understanding of normal functioning as well as dysregulation.

Individual Differences in μ-opioid receptor-mediated Pain and Placebo Analgesia

Early characterizations of placebo analgesia, while groundbreaking in their own right, did not capture the whole story of placebo analgesia, which can only be developed by accounting for individual factors that contribute to the variation in response to placebo analgesia. Because placebo analgesia is mediated by μ-opioid related neurotransmission in pain-modulatory and motivational regions, variations in the response to placebo analgesia likely stem from individual differences in μ-opioid related functioning. Factors known to affect μ-opioid related activity in response to stress and noxious stimuli include anticipatory and expectation-related cognitive functions, genotype, age, gender and reproductive hormones. Placebo analgesia imaging is a useful experimental model for engaging the motivational circuitry; therefore, examining individual differences in placebo analgesia responses is useful for understanding placebo analgesia in its own right, as well as in understanding normal variations within the more general motivational network. While there are many factors that influence functional variations in these systems, the following discussion will address only those factors relevant to the current body of work.

Cognition/Affect

The cognitive and affective strategies individuals employ in response to stressful situations like the sustained pain of the Zubieta lab's placebo studies provide a substantial portion of variance to the μ-opioid neurotransmission-mediated placebo analgesia response. In a follow-up analysis of the study mentioned above, 40-68% (depending on region) of the variance in regional endogenous opioid activity was accounted for by affective quality of pain, positive and negative internal affective state not specific to pain, and objective measures of individual pain sensitivity (volume of algesic substance infused to maintain pain at constant levels) [67]. Therefore, factors that influence how an individual will respond to placebo are affective responses to pain, general affective state, and individual pain sensitivity.

Sex

Sex differences in stress responsivity, pain tolerance and thresholds, and reward responding have been documented. In a sustained pain model similar to the one used in Zubieta's placebo analgesia studies, in which pain intensities were kept at a uniform and constant level for all subjects, men and women showed differences in the magnitude and direction of μ-opioid system responses to pain. Men showed greater magnitudes of μ-opioid activation in anterior thalamus, ventral basal ganglia and amygdala. Women, who were studied in the early follicular phase of their menstrual cycle when estrogen and progesterone levels are low, showed reductions in nucleus accumbens μ-opioid activity in response to pain. The results suggest that in women, the μ-opioid system is less active in

pain modulation in the follicular phase than it is in men, despite similar subjective ratings of intensity and pain-related affect between the sexes.

Chapter II

Positron Emission Tomography Measures of Sex Differences in Endogenous Opioid Neurotransmission during Placebo Analgesia and its Anticipation

The placebo effect, defined as therapeutic improvement upon administration of an inert substance given with the expectation of benefit, has been regarded historically as a confound to clinical trials and placebo-controlled studies. However, the placebo effect has received increasing attention for its intrinsic value as a tool for understanding how external information influences expectations and beliefs, and in turn, how these cognitive states translate into physiological responses in the brain and body.

Of particular relevance to placebo research is placebo analgesia, the relief from experimentally-induced pain when there is expectation of analgesia. In the context of reward and motivational mechanisms, placebo analgesia can be thought of as a reward state, in which relief from pain is a rewarding outcome. Consequently, just as natural rewards and punishments modulate motivational processes in order to elicit favorable and adaptive behaviors, so do pain and its relief via placebo recruit motivational brain mechanisms that allow the organism to respond appropriately and adaptively. Indeed, activity in brain areas that process and modulate pain (SI somatosensory cortex, thalamus, insula) co-occurs with activity in brain regions that respond to rewards and punishments (sublenticular extended amygdala, VTA, PAG, ventral striatum) after

noxious thermal stimuli are applied [35]. A substantial accumulation of literature has documented the involvement of midbrain dopamine in placebo and pain.

Recent reward research has managed to further dissect incentive states into anticipation periods and outcome periods. It was initially thought that rewards themselves induced dopamine release in the nucleus accumbens, but further examination has revealed that it is the actual anticipation of a reward or punishment, or the receipt of an unexpected reward or punishment, that results in accumbens dopamine release. The nucleus accumbens dopamine response is therefore triggered by perception of salient cues, both aversive and appetitive [38].

While dopamine is a central component of the reward response circuitry, endogenous opioids contribute to reward processing, and their modulatory role in the reward brain regions is intimately coupled with dopamine activity.

Recent placebo research has managed to tie together the role of opioids in reward and reward anticipation and the role of opioids in pain suppression via placebo. Placebo analgesia is attenuated after administration of naloxone, an opioid receptor antagonist [68], indicating that placebo analgesia is produced, at least in part, by endogenous opioid activation. Zubieta et al. [66] demonstrated for the first time with positron emission tomography (PET) that placebo analgesia is indeed mediated by activation of the endogenous opioid system through μ opioid receptors. The cognitive state of expectation of pain relief by placebo leads to endogenous opioid activation [69]. Expectations of analgesic effectiveness are correlated with endogenous opioid activation in the nucleus accumbens and periaqueductal gray, and the ratio between observed and expected efficacy is correlated with opioid activation in the amygdala and nucleus accumbens [70].

The element of expectation seems to play a crucial role in eliciting the placebo response, as its effect on endogenous opioid activation occurs in ventral striatum and amygdala, areas involved in processing incentive values and motivation. Expectation of pain and the possibility of relief thus induce a motivational state through opioid mechanisms, which in turn contribute to opioid mediation of placebo analgesia.

We have gained a greater understanding of the neurochemical and anatomical foundations of the placebo response, but this information can be interpreted with only partial accuracy and cannot be used for practical application until the substantial individual differences in placebo response and brain activation are understood. Zubieta et al. [67] observed that pain-related affect, pain sensitivity, and internal affective state contributed to the substantial individual differences in magnitude of opioid response. Scott et al. [71] demonstrated that individual variation in magnitude of dopamine release in the nucleus accumbens during placebo was correlated with variation in magnitude of fMRI BOLD response of the nucleus accumbens during reward anticipation.

Because the previous studies by Zubieta and Scott used only males, we are left with an incomplete picture of how individual variation contributes to placebo mechanisms. Therefore, it is important to determine what differences, if any, females contribute. Given the increasing literature on sex differences in behavioral and neural reward responsivity, pain processing, and opioid neurochemistry, an examination of sex differences and their neurochemical basis in placebo analgesia is a natural and crucial next step.

Many studies have documented sex differences in pain and analgesia. Much of the animal and human literature indicates that females are more sensitive than males to experimental pain, but this is only consistently observed for pain models of longer duration, and not brief pain [72]. Women of child-bearing age are diagnosed with pain-related disorders such as temporomandibular disorder (TMD) and fibromyalgia at a higher rate than men or women before gonadal maturation or after menopause [73]. Women also tend to experience greater analgesia and side-effects from μ-opioid receptor agonists than men [72]. Clinical pain also appears to fluctuate with plasma levels of gonadal steroids, with higher ratings in estradiol-low states [73].

These differences in pain and analgesia are thought to be at least in part mediated by sex differences in endogenous opioid activation and opioid receptor distribution and expression. Studies of postmortem brain tissue have demonstrated sex differences in μ-opioid receptor concentrations [74, 75]. PET imaging has shown that men and women exhibit distinctly different regional patterns of μ-opioid receptor activation during a sustained, deep-tissue pain challenge [76].

Estradiol is one likely mediator of sex-specific patterns of opioid system activation and organization. In a subsequent study using the same experimental pain paradigm as in Zubieta's 2002 study, Smith et al. [77] demonstrated that a high estrogen state in women was associated with greater activation of endogenous μ-opioid neurotransmission than in the same women in a low estrogen state. These findings are consistent with prior studies that suggest that estradiol has a regulatory influence on endogenous opioid activity and nociception. For example, Eckersell et al [78] have shown that estradiol treatment of ovariectomized rats leads to increased internalization of

μ-opioid receptors in the hypothalamus and amygdala, indicating enhanced endogenous opioid release. Other studies have demonstrated increases in mRNA and protein of both μ-opioid receptors and endogenous opioid peptides after treatment with estradiol in ovariectomized rats [79-83]. In humans, low estradiol states are associated with higher TMD-related pain ratings [84].

Given the knowledge that placebo response can studied in its separate components of anticipation of pain with placebo and receipt of pain with placebo and that there are demonstrated sex differences in both the anticipation of pain and in pain receipt itself in the medial prefrontal cortex and insula [85], we hypothesized that significant sex differences in patterns of opioid activation and psychophysical response would arise both in placebo and placebo anticipation. Additionally, we reasoned that one predictor of the sexually distinct patterns of activation would be estradiol levels in females.

Materials and Methods

Subjects

Volunteers were 39 healthy, right-handed men (n=20) and women (n=19), with a mean age of 28. Participants had no history of medical illness, psychiatric illness, substance abuse or dependence and no history of inheritable family illnesses. Volunteers were not taking psychotropic medications or hormonal treatments. Women had not taken hormonal birth control for at least 6 months prior to the study. Women were scanned during the follicular phase of their menstrual cycles (2 to 9 days after the onset of menses), as measured by plasma levels of estradiol (mean of 111 pg/mL) and

progesterone (< 3 ng/ml in all cases). All procedures were approved by the University of Michigan Investigational Review Boards.

Scanning Protocols

PET scans were acquired with a Siemens ECAT EXACT scanner in three dimensional mode with septa retracted. Participants were positioned in the PET scanner gantry, and two intravenous lines were placed. A light forehead restraint was used to prevent head movement during the scan. [^{11}C] carfentanil was synthesized at high specific activity (>1000 Ci/mmol) by the reaction of ^{11}C-methyliodide and a nonmethyl precursor as previously described [86], with minor modifications to improve its synthetic yield; 10 to 15 mCi (370-555 MBq) were administered to each subject for each of the two PET scans. The two administrations were separated by 2 hours to allow for tracer decay. The maximum mass of carfentanil injected was less than 0.03 μg/kg per study, ensuring that the compound was administered in tracer quantities, i.e. subpharmacological doses. Fifty-five percent of the ^{11}C carfentanil dose was administered as a bolus and the remainder as a continuous infusion using a computer-controlled pump to achieve steady-state tracer levels. Nineteen sets of scans were acquired over 70 minutes with an increasing duration (30 s up to 10 min). Images were reconstructed using filtered back-projection with a Hanning 0.5 filter, and included both measured attenuation and scatter corrections. Dynamic images were coregistered to each other and the intercommisural line using automated computer routines [87]. Image data were then transformed on a pixel-by-pixel basis into two sets of parametric maps: (a) a tracer transport measure (K_1

ratio), and (b) a receptor-related measure, distribution volume ratio (DVR). To avoid the need for arterial blood sampling, the tracer transport and binding measures were calculated using a modified Logan graphical analysis [88], using the occipital cortex (an area devoid of μ-opioid receptors) as the reference region. With the protocol used, the Logan plot becomes linear by 10 minutes after the start of the radiotracer administration, with its slope being the DVR, a measure equal to the $(B_{max}/KD) + 1$ for this receptor site and radiotracer. B_{max}/KD (or DVR – 1) is the "receptor related" measure (μ-opioid receptor availability, or binding potential). K_1 and DVR images for each experimental period and MR images were coregistered to each other and to the International Consortium for Brain Mapping (ICBM) stereotactic atlas orientation [89].

MRI scans were acquired on a 1.5 Tesla scanner (Signa, General Electric, Milwaukee, WI). Acquisition sequences were axial SPGR IR-Prep MR (TE=5.5, TR=14, TI=300, flip angle=20 degrees, NEX=1, 124 contiguous images, 1.5 mm thickness), followed by axial T2 and proton density images (TR=4000, TE=20 and 100, respectively, NEX=1, 62 contiguous images, 3 mm thick). All MR scans were reviewed by a neuroradiologist to rule out gross structural brain abnormalities before PET scanning.

Data and Image Acquisition

Parametric maps of differences between conditions (pain-placebo) were generated by anatomically standardizing the MRI of each subject to the International Conference on Brain Mapping (ICBM) stereotactic atlas coordinates, with subsequent application of this transformation to the mu-opioid receptor binding maps [89]. Before nonlinear warping,

image data were prepared so that the side of the painful challenge (induced on the right or the left masseter muscle, in a counterbalanced design) was located on the same side of the image for all subjects. Image data are therefore presented as "ipsilateral" or "contralateral" to the painful stimulus, regardless of the actual location (right-left). Differences between conditions and subject groups were then mapped into stereotactic space using z-maps of statistical significance with SPM99 and Matlab software and using a general linear model and correction for multiple comparisons [90], but without global normalization (the data presented are based on absolute B_{max}/KD estimates). Only regions with specific μ-opioid receptor binding were included in the analyses (pixels with DVR values >1.2 times the mean global image value for μ-opioid receptor images as calculated with SPM 99). To compensate for small residual anatomic variations across subjects and to improve signal to noise ratios, a three-dimensional Gaussian filter (FWHM 6 mm) is applied to each scan. For each subtraction analysis of one sample, two-tailed t-statistic values were calculated for each pixel using pooled variances across pixels [91]. Areas of significant differences were detected using a statistical threshold that controls a type 1 error rate at P=0.05 for multiple comparisons, which was estimated using the Euler characteristic [91] based on the number of pixels in the gray matter and image smoothness [92]. This typically varies from z=4.4 to 4.6 in our studies for peak analyses, at a final resolution of approximately 10 mm. Z scores were also deemed significant if they reached statistical thresholds after correction for the size of the cluster under consideration [90].

Experimental Design

During pain scans and pain with placebo scans, subjects were administered a steady state of moderate muscle pain starting at 45 minutes after radiotracer injection until 65 minutes, which was adjusted to maintain pain levels around 40 on a scale of 0 (no pain) to 100 (most pain imaginable). Specifically, a computer-controlled delivery system infused medication-grade hypertonic saline (5%) into the left masseter muscle, a model of sustained, deep somatic pain. Specific details of the computer-controlled standardization of pain levels have been described previously [93, 94]. The first period of each scan consisted of periods of anticipation of either pain alone or pain with placebo starting at 5 minutes after radiotracer administration and lasting until 25 minutes into the scan. Isotonic (non-painful) saline was infused into the masseter muscle during this time. Pain intensity ratings were continually updated every 15 sec (scale of 0 to 100 as mentioned above) for both anticipation periods and pain administration periods. These values were recorded in the computer controller and averaged for statistical analyses. Prior to placebo phases, subjects were told that they may or may not receive a compound thought to reduce pain through the activation of internal pain control mechanisms. Placebo administration consisted of 1 mL i.v. infusions of isotonic saline every 4 minutes during the 20 minute pain period, with accompanying verbal and visual cues at each application.

Psychophysical questionnaires measuring the pain experience were administered at various times throughout the pain periods. The McGill pain questionnaire (MPQ),

which measures sensory and affective aspects of pain, was given after pain challenges [95]. The Positive and Negative Affectivity Scale (PANAS) was administered before and after each experimental phase (both anticipation and pain administration), and monitors the internal affective state of subjects [96]. The Profile of Mood States-Total Mood Disturbance (POMS-TMD) was given after pain challenges to measure degree of mood disturbance. Participants were also administered questionnaires before getting into the scanner in which they rated how effective they believed placebo would be and how much they wanted it to work (scale 0 to 100). After the entire experiment, participants were asked to rate how effective they believed the placebo had been in relieving their pain (scale 0 to 100).

Results

Significant differences in change in non-displaceable binding potential (BP_{ND}) from pain alone to pain plus placebo were found between males and females. In a SPM voxel-by-voxel analysis, an independent samples *t* test between males and females revealed differences in the change in μ-opioid BP_{ND} across conditions. At a threshold of > 20 voxels and $p \leq 0.01$, females showed evidence of increased μ-opioid receptor mediated activity (acute reductions in BP_{ND}) in the right amygdala (27, 0, -21; $z = 4.38$), hypothalamus (-2, 0, -12; $z = 5.88$), right nucleus accumbens (7, 15, -6; $z = 5.70$), left dorsal thalamus (-14, -11, 19; $z = 3.65$), and left anterior thalamus (-6, -15, 1; $z = 3.36$).

The males showed increased placebo-induced activation in the right dorsal caudate (10, 25, 10; $z = 3.92$), right ventral pallidum (9, -2, -3; $z = 3.55$), right dorsal

thalamus (10, -26, 20; z = 4.81), left amygdala (-33, 0, -27; z = 3.66), and left anterior cingulate (-5, 28, -4; z = 3.07).

	Region	x, y, z coordinates, mm	Cluster Size, mm^3	T score	P value
F > M					
	Right AMY	27, 0, -21	459	4.38	0.00
	HYPOTHAL	-2, 0, -12	377	5.89	0.00
	Right NAC	7, 15, -6	1033	5.70	0.00
	Left DORS THAL	-14, -11, 19	228	3.65	0.00
	Left ANT THAL	-6, -15, 1	67	3.36	0.00
M > F					
	Right CAUD	10, 25, 10	1761	3.92	0.00
	Right VENT PALL	9, -2, -3	346	3.55	0.00
	Right DORS THAL	10, -26, 20	692	4.81	0.00
	Left AMY	-33, 0, -27	183	3.66	0.00
	Left ACC	-5, 28, -4	162	3.07	0.00

Table 1. Placebo Regional Activation.

When an independent samples, one-tailed *t* test was performed, there were no significant differences between males and females for the change in psychophysical measures (MPQ sensory, MPQ affective, PANAS Negative, PANAS fear, PANAS Positive, POMS TMD, overall (0 to 20 minutes) average 15 second momentary VAS) from pain alone to pain with placebo. Males and females also did not differ in anticipated effectiveness of placebo, desired effectiveness of placebo, subjective effectiveness of placebo and the difference between anticipated and subjective effectiveness of placebo.

Measure	*Males (n=20) Pain Alone Minus Pain + Plbo*	*Females (n=19) Pain Alone Minus Pain + Plbo*	T	$p^{\dagger}$
MPQ Sensory Subscale	1.1 ± 6.4	-0.3 ± 5.9	-0.69	0.25
MPQ Affective Subscale	0.4 ± 1.7	0.7 ± 1.4	0.67	0.26
PANAS Negative Affect	0.1 ± 2.0	0.2 ± 3.5	0.12	0.46
PANAS Fear	0.3 ± 1.8	0.15 ± 1.4	0.22	0.41
PANAS Positive Affect	0.9 ± 4.4	1.4 ± 2.6	0.41	0.35
POMS-TMD	1.3 ± 7.6	2.3 ± 8.3	0.38	0.36
15 s momentary intensity VAS 0 to 20 minutes	7.4 ± 14.3	4.4 ± 12.0	-0.71	0.25

Table 2. Change in psychophysiological responses from pain alone to pain plus placebo, males vs. females. Data are expressed as mean ± SD. †Calculated using 2-sample, one-tailed *t*-tests for differences between males and females in changes from pain alone to pain with placebo.

When males and females were grouped together, paired samples, one-tailed *t* tests showed that there was a significant decrease in McGill Affect ($t = 2.06$) from pain alone to pain with placebo ($p = 0.02$), a significant decrease in PANAS Positive Affect ($t = 1.98$; $p = 0.03$), and a significant decrease in the average 15 second momentary intensity VAS ratings during the entire 20 minute pain period ($t = 2.58$; $p = 0.01$).

Measure	*All Subjects Pain*	*All Subjects Pain + Plbo*	T	$p^{\dagger}$
MPQ Sensory Subscale	13.8 ± 6.5	13.4 ± 7.7	0.39	0.35
MPQ Affective Subscale	1.5 ± 2.2	1.0 ± 1.8	2.06	**0.02**
PANAS Negative Affect	1.6 ± 2.8	1.5 ± 3.1	0.23	0.41
PANAS Fear	1.0 ± 2.2	0.8 ± 2.2	0.80	0.22
PANAS Positive Affect	8.9 ± 6.4	7.8 ± 7.1	1.98	**0.03**
POMS-TMD	14.1 ± 9.7	12.3 ± 7.6	1.41	0.08
15 s momentary intensity VAS 0 to 20 minutes	25.2 ± 13.9	19.7 ± 10.3	2.58	**0.01**

Table 3. . Psychophysiological responses during pain and pain plus placebo, All Subjects (n=39). Data are expressed as mean ± SD. †Calculated using paired, one-tailed *t*-tests for differences in ratings from pain alone to pain with placebo.

When females and males were examined separately, in paired samples, one-tailed *t* tests, females showed a significant decrease in MPQ affect (t = 2.11) and PANAS

positive affect (t = 2.33) from pain alone to pain with placebo ($p \leq 0.05$). There were no significant differences between ratings from pain alone to pain with placebo for overall intensity, overall unpleasantness, MPQ sensory, PANAS negative, POMS TMD, VAS early, VAS late or VAS overall in females. Males showed significant decreases in early (t = 2.86) and overall (t = 2.32) VAS scores ($p \leq 0.05$). There were no significant differences between ratings from pain to pain with placebo for overall intensity, overall unpleasantness, MPQ sensory, MPQ affective, PANAS Negative, PANAS Positive, POMS TMD, and VAS late in males.

Measure	***Females Pain***	***Females Pain + Plbo***	**T**	$p^{\dagger}$
MPQ Sensory Subscale	14.7 ± 7.1	15.1 ± 8.4	-0.23	0.41
MPQ Affective Subscale	2.1 ± 2.5	1.4 ± 2.3	2.11	**0.03**
PANAS Negative Affect	1.7 ± 3.1	1.5 ± 3.1	0.20	0.43
PANAS Fear	1.2 ± 2.9	0.9 ± 2.4	0.84	0.21
PANAS Positive Affect	7.9 ± 6.1	6.5 ± 6.8	2.33	**0.02**
POMS-TMD	13.1 ± 7.8	10.8 ± 5.9	1.19	0.13
15 s momentary intensity VAS 0 to 20 minutes	22.1 ± 15.3	18.6 ± 12.3	1.24	0.12

Table 4. Psychophysiological responses during pain and pain plus placebo, females (n=19). Data are expressed as mean ± SD.

Measure	***Males Pain***	***Males Pain + Plbo***	**T**	$p^{\dagger}$
MPQ Sensory Subscale	12.9 ± 5.8	11.9 ± 6.8	0.74	0.24
MPQ Affective Subscale	1.0 ± 1.8	0.7 ± 1.0	0.92	0.19
PANAS Negative Affect	1.5 ± 2.5	1.4 ± 3.1	0.11	0.46
PANAS Fear	0.9 ± 1.3	0.7 ± 2.1	0.36	0.36
PANAS Positive Affect	9.9 ± 6.8	9.0 ± 7.2	0.92	0.19
POMS-TMD	15.1 ± 11.3	13.8 ± 8.8	0.77	0.23
15 s momentary intensity VAS 0 to 20 minutes	28.1 ± 12.1	20.8 ± 8.2	2.32	**0.02**

Table 5. Psychophysiological responses during pain and pain plus placebo, males (n=20). Data are expressed as mean ± SD.

†Calculated using paired, one-tailed *t*-tests for differences in psychophysics measures during pain alone versus pain plus placebo.

BP_{ND} values from activated regions from the male>female and female>male voxel by voxel analyses were then extracted as ROIs for further analysis. Pearson correlations were conducted between the regional change in opioid binding from pain alone to pain with placebo in individual ROIs and the change in psychophysics measures from pain alone to pain with placebo. Correlations were conducted separately for males and females between the ROIs from the voxel by voxel analyses and measures of MPQ sensory, MPQ affective, PANAS negative, PANAS positive, POMS TMD, and average momentary 15 second VAS ratings for the entire 20 minute pain period. Each r value was then tested for significant difference between males and females by transforming each correlation coefficient with the Fisher Z-transform (($Z_f = 1/2 * \ln((1+R) / (1-R))$) and then estimating a z score using $z = (Z_{f1} - Z_{f2}) / SQRT(1/(N_1-3) + 1/(N_2-3))$.

In the right amygdala, placebo-induced μ-opioid system activation was correlated with the changes in PANAS Negative ratings in females (Males: r = -0.31, p = 0.19; Females: r = -0.65, p = 0.003; differences between groups, z = 1.31, p = 0.19), POMS TMD in both sexes (Males: r = -0.52, p = 0.02; Females: r = -0.63, p = 0.004; differences between groups, z = 0.47, p = 0.64), and McGill affect in males (Males: r = -0.70, p = 0.0007; Females: r = 0.00, p = 0.93; differences between groups, z = -2.49, p = 0.01).

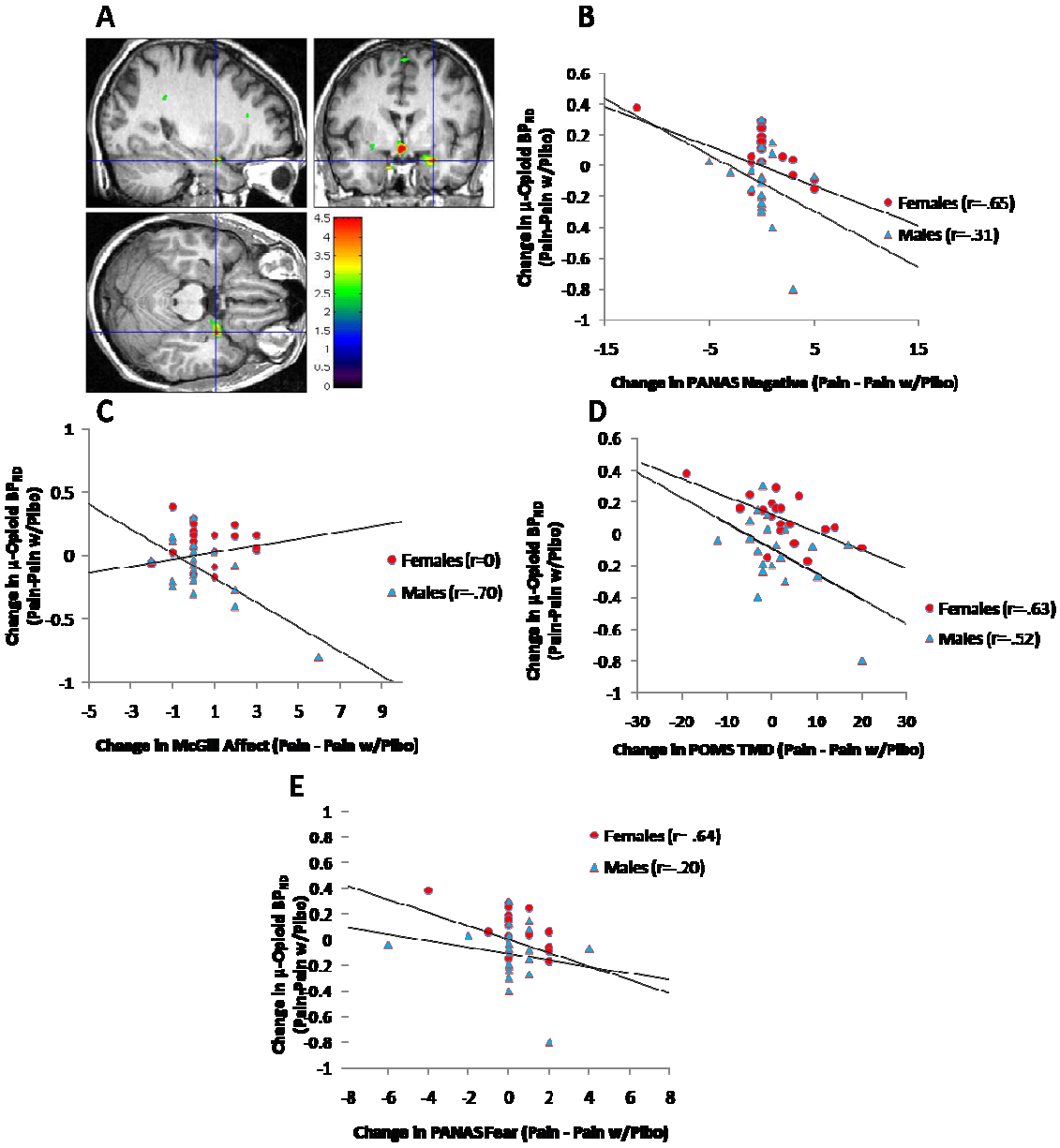

Figure 1. Sex differences in amygdala μ-opioid neurotransmission and the correlation with changes in psychophysical measures during placebo. A) Activation of endogenous opioid neurotransmission from pain alone to pain with placebo in right amygdala, females > males. B) Significant negative correlation between change in PANAS Negative and change in right amygdala μ-opioid BP_{ND} from pain alone to pain with placebo in females but not males. C) Significant negative correlation between change in McGill Affect from pain alone to pain with placebo and change in μ-opioid BP_{ND} in males but not females. D) Significant negative correlations between change in POMS-TMD and change in μ-opioid BP_{ND} in both sexes. E) Significant negative correlations between change in PANAS Fear and change in μ-opioid BP_{ND} in females but not males.

In the left dorsal thalamus, significant correlations were obtained with the change in PANAS positive in females (Males: r = -0.40, p = 0.08; Females: r = -0.54, p = 0.02;

differences between groups, z = 0.52, p = 0.60), McGill sensory in males (Males: r = 0.63, p = 0.003; Females: r = 0.38, p = 0.11; differences between groups, z = 0.98, p = 0.33), and VAS 0 to 20 in males (Males: r = 0.44, p = 0.05; Females: r = -0.11, differences between groups, p = 0.64; z = 1.67, p = 0.09). However, the correlations were not significantly different between the sexes.

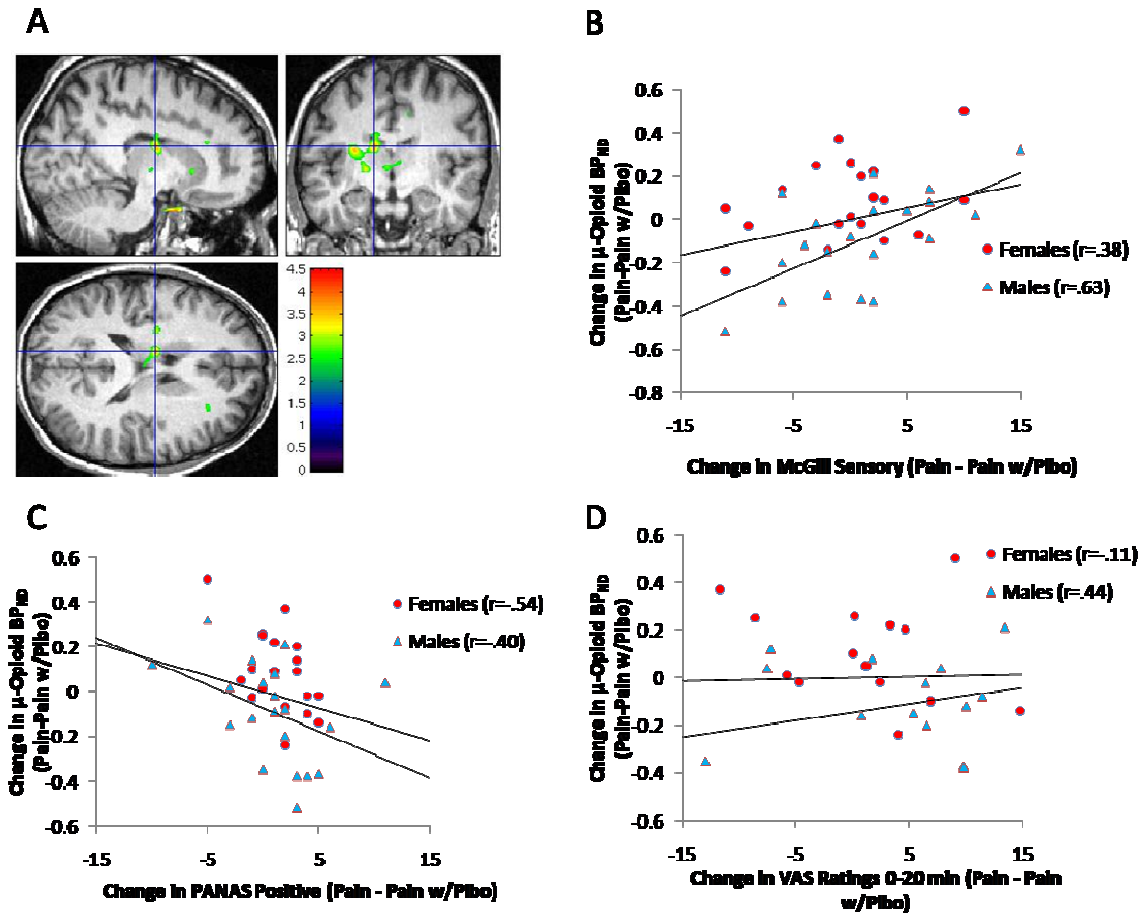

Figure 2. Sex differences in thalamus μ-opioid neurotransmission and the correlation with changes in psychophysical measures during placebo A) Activation of endogenous opioid neurotransmission from pain alone to pain with placebo in left dorsal thalamus, females > males. B) Significant positive correlation between change in McGill Sensory and change in left dorsal thalamus μ-opioid BP_{ND} from pain alone to pain with placebo in males but not females. C) Significant negative correlation between change in PANAS Positive from pain alone to pain with placebo and change in μ-opioid BP_{ND} in females but not males. D) Significant positive correlations between change in average VAS momentary ratings from 0 to 20 minutes and change in μ-opioid BP_{ND} in males but not females.

In the left anterior/inferior thalamus, significant correlations were obtained with the change in McGill sensory in females (Males: r = 0.24, p = 0.31; Females: r = 0.48, p = 0.04; z = -0.80, p = 0.42).

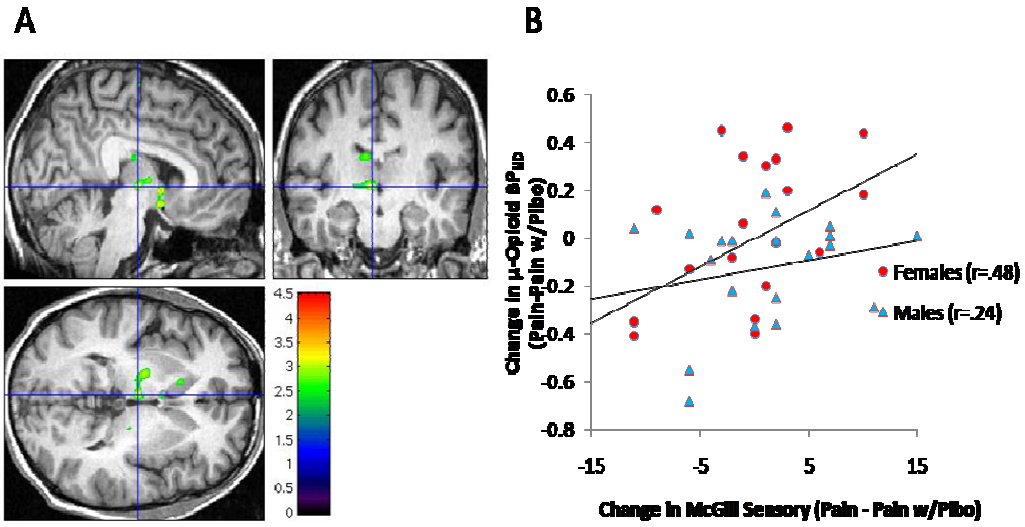

Figure 3. Sex differences in ventral thalamus μ-opioid neurotransmission and the correlation with changes in psychophysical measures during placebo A) Activation of endogenous opioid neurotransmission from pain alone to pain with placebo in left ventral thalamus, females > males. B) Significant positive correlation between change in McGill Sensory and change in left ventral thalamus μ-opioid BP_{ND} from pain alone to pain with placebo in females but not males.

In the left anterior cingulate, significant correlations were obtained with the change in PANAS positive in males (Males: r = -0.46, p = 0.04; Females: r = -0.12, p = 0.64; differences between groups, z = -1.08, p = 0.28), McGill sensory in males (Males: r = 0.45, p = 0.05; Females: r = -0.18, p = 0.45; differences between groups, z = 1.91, p = 0.06), and the VAS 0 to 20 in males (Males: r = 0.54, p = 0.01; Females: r = -0.06, p = 0.81; differences between groups, z = 1.91, p = 0.06).

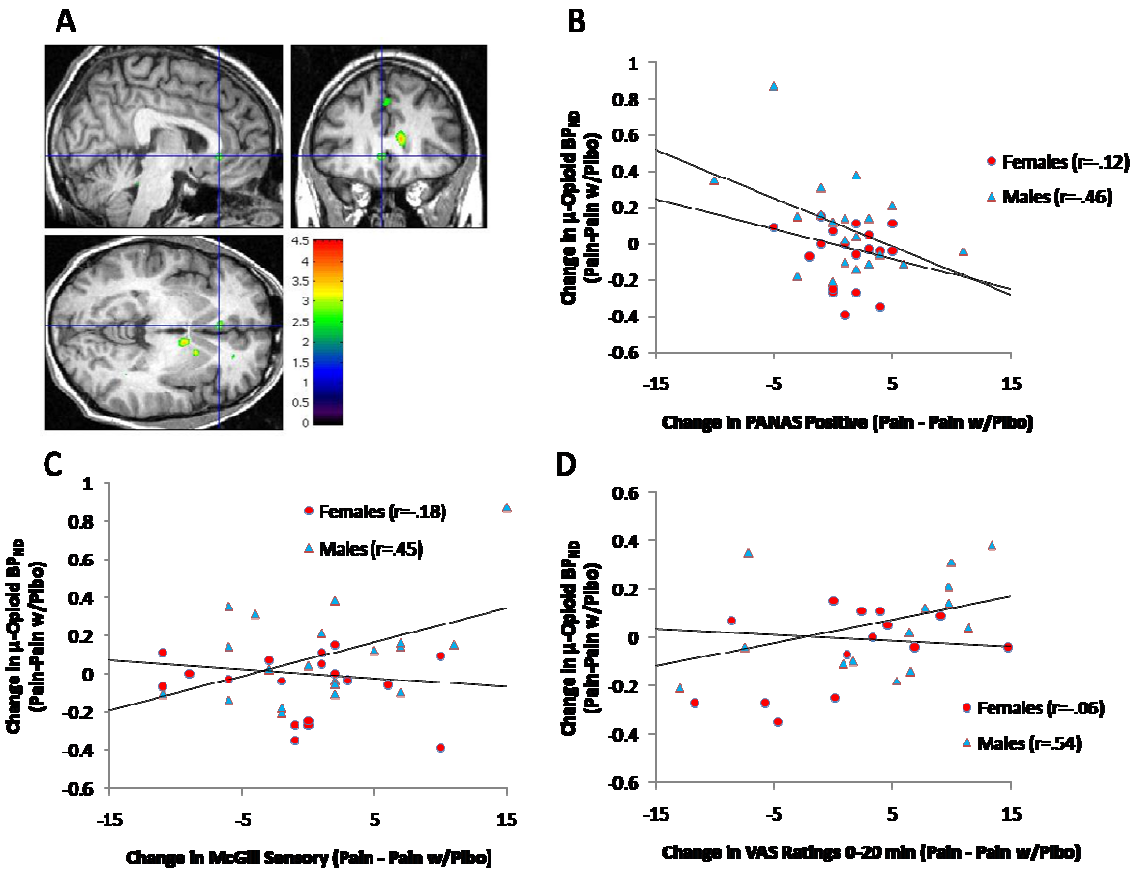

Figure 4. Sex differences in anterior cingulate μ-opioid neurotransmission and the correlation with changes in psychophysical measures during placebo. A) Activation of endogenous opioid neurotransmission from pain alone to pain with placebo in left anterior cingulate, males > females. B) Significant negative correlation between change in PANAS Positive and change in left anterior cingulate μ-opioid BP_{ND} from pain alone to pain with placebo in males but not females. C) Significant positive correlation between change in McGill Sensory from pain alone to pain with placebo and change in μ-opioid BP_{ND} in males but not females. D) Significant positive correlations between change in average VAS momentary ratings from 0 to 20 minutes and change in μ-opioid BP_{ND} in males but not females.

In the right ventral pallidum, significant correlations were obtained with the change in McGill sensory in males (Males: r = 0.60, p = 0.005; Females: r = -0.00, p = 0.99; z = 1.99, p = 0.05) and VAS 0 to 20 in males (Males: r = 0.55, p = 0.01; Females: r = -0.07, p = 0.77; z = 1.98, p = 0.05).

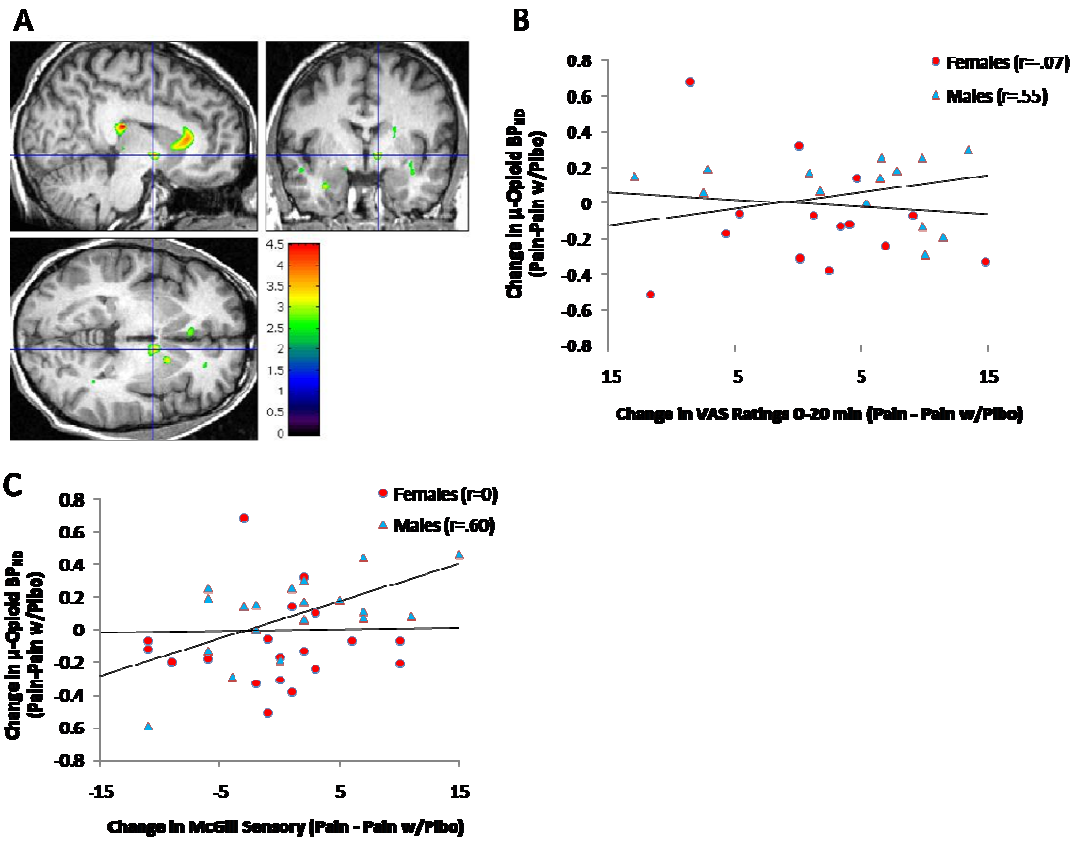

Figure 5. Sex differences in ventral pallidum μ-opioid neurotransmission and the correlation with changes in psychophysical measures during placebo A) Activation of endogenous opioid neurotransmission from pain alone to pain with placebo in right ventral pallidum, males > females. B) Significant positive correlation between change in average momentary VAS ratings over 20 minute pain period and change in right ventral pallidum μ-opioid BP_{ND} from pain alone to pain with placebo in males but not females. C) Significant positive correlation between change in McGill Sensory from pain alone to pain with placebo and change in μ-opioid BP_{ND} in males but not females.

In the left amygdala, significant correlations were obtained with VAS 0 to 20 in females (Males: r = -0.04, p = 0.87; Females: r = 0.56, p = 0.01; z = -1.93, p = 0.05).

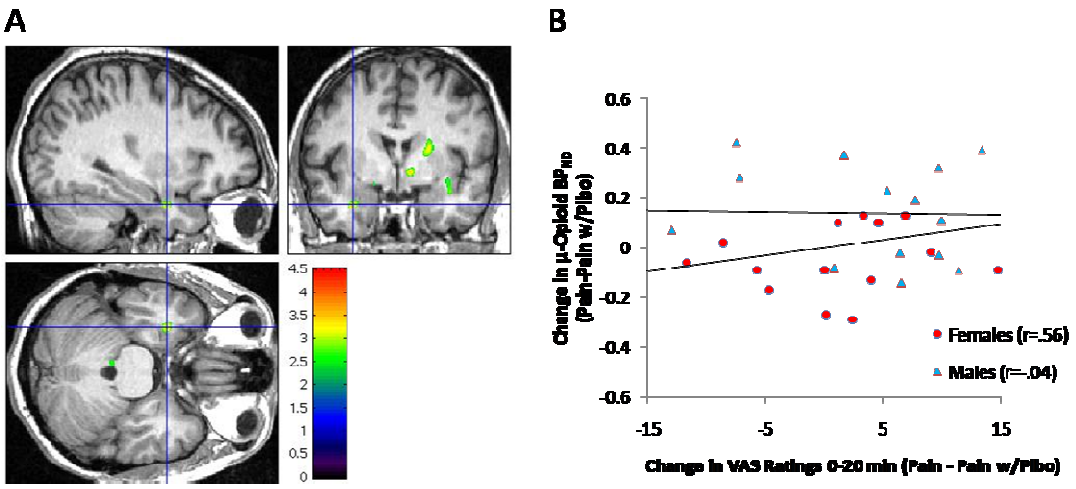

Figure 6. Sex differences in left amygdala μ-opioid neurotransmission and the correlation with changes in psychophysical measures during placebo A) Activation of endogenous opioid neurotransmission from pain alone to pain with placebo in left amygdala, males > females. B) Significant positive correlation between change in average momentary VAS ratings over 20 minute pain period and change in left amygdala μ-opioid BP_{ND} from pain alone to pain with placebo in females but not males.

A SPM simple regression analysis within the female group revealed a positive relationship between change in μ-opioid binding potential from pain alone to pain plus placebo and estradiol levels on the day of placebo administration in the following regions: right nucleus accumbens (13, 6, -11; z=6.15; p=0.00 corrected at voxel level), right ventral pallidum (22, 1, 1; z=4.96; p=0.012 corrected at voxel level), right posterior insula (z=3.46) and right anterior insula (z=3.88), and right anterior thalamus (2, -6, 2; z=6.54; p=0.00 corrected at cluster level).

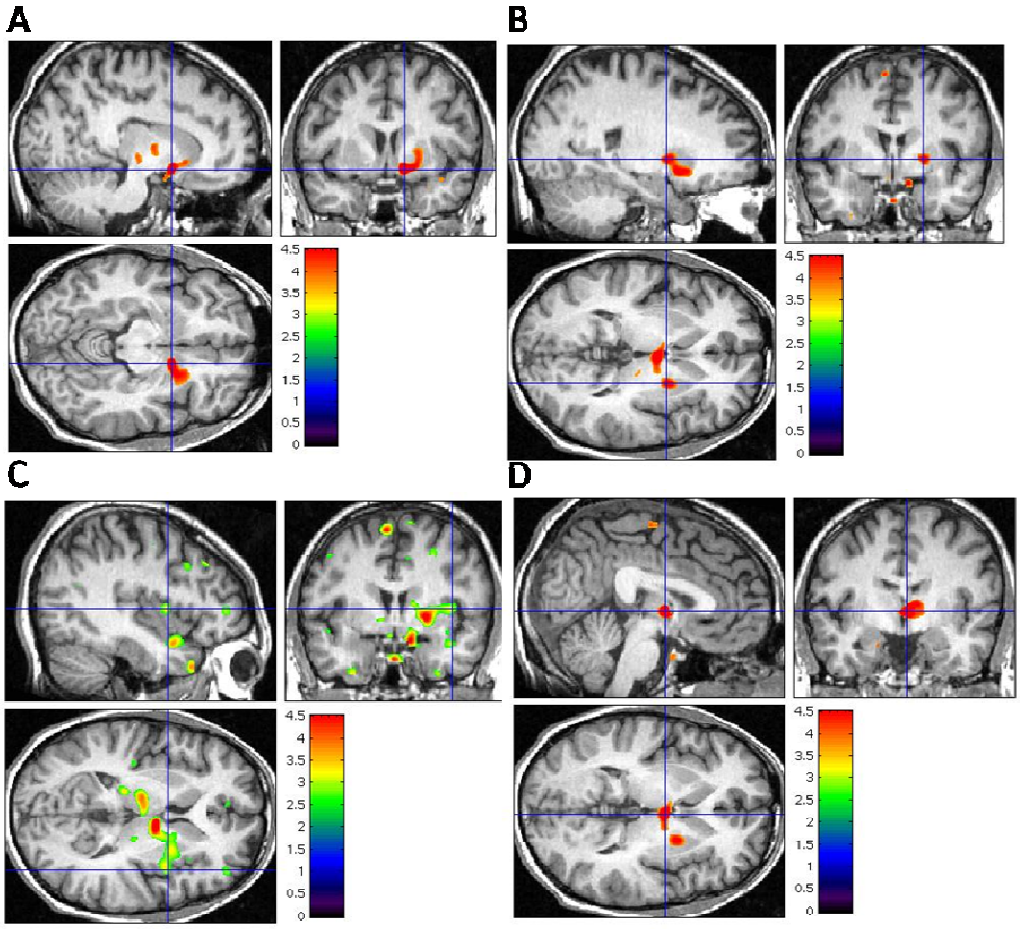

Figure 7. Correlations in Regional Change in Mu-Opioid Neurotransmission and Estradiol Levels in Females. A) Right Nucleus Accumbens, B)Right Ventral Pallidum, C) Right Anterior and Posterior Insula, D) Right Thalamus

We then examined sex differences in μ-opioid receptor related neurotransmission during the pain anticipation phase of the experiment. A SPM voxel-by-voxel independent samples *t* test between males and females revealed differences in the change in μ-opioid BP_{ND} from the pain anticipation phase to anticipation of pain with placebo. The females had decreased BP_{ND} (increase in activation) in the left nucleus accumbens/ventral pallidum (-19, 2, -6; t = 4.99) and the right anterior cingulate (12, 10, 37; t = 3.63). The males had decreased binding in the right caudate (12, 21, 5; t = 3.75) and nucleus coeruleus (-3, -26, -16; t = 3.25).

	Region	x, y, z coordinates, mm	Cluster Size, mm^3	T score	P value
F > M					
	Left VP	-19, 2, -6	1766	4.99	0.00
	Right ACC	12, 10, 37	1170	3.63	0.00
M > F					
	Right CAUD	12, 21, 5	936	3.75	0.00
	NUC COER	-3, -26, -16	351	3.25	0.00

Table 6. Placebo Anticipation Regional Activation

We then examined sex differences in the change in psychophysical measures from the pain anticipation phase to the pain with placebo anticipation phase. An independent samples, one-tailed *t* test revealed that there were no sex differences in the change in PANAS negative, PANAS positive, and PANAS fear ratings, or in ratings of anticipated effectiveness of placebo and desired effectiveness of placebo.

Measure	*Males (n=20) Ant. Of Pain Alone Minus Ant. of Pain + Plbo*	*Females (n=19) Ant. Of Pain Alone Minus Ant. Of Pain + Plbo*	T	$p^{\dagger}$
PANAS negative affect	1.7 ± 3.0	0.8 ± 4.0	-0.72	0.24
PANAS fear	1.5 ± 2.2	1.2 ± 3.3	-0.38	0.35
PANAS positive affect	0.8 ± 5.1	1.8 ± 2.7	0.83	0.21

Table 7. Change in psychophysiological responses from anticipation of pain alone to anticipation of pain plus placebo, males vs. females. Data are expressed as mean ± SD. †Calculated using 2-sample, one-tailed *t*-tests for differences between males and females in changes from anticipation of pain alone to anticipation of pain with placebo.

When males and females were grouped together, paired samples, one-tailed *t* tests showed that there was a significant decrease in PANAS Negative Affect ($t = 2.26$; $p = 0.02$), PANAS Positive Affect ($t = 1.95$; $p = 0.03$), and PANAS Fear ($t = 2.99$; $p = 0.00$).

Measure	*All Subjects Ant. Of Pain*	*All Subjects Ant. Of Pain + Plbo*	T	$p^{\dagger}$
PANAS Negative Affect	2.6 ± 3.7	1.3 ± 2.6	2.26	**0.02**
PANAS Fear	2.2 ± 3.3	0.8 ± 1.7	2.99	**0.00**
PANAS Positive Affect	8.6 ± 6.7	7.3 ± 6.7	1.95	**0.03**

Table 8. Psychophysiological responses during pain anticipation and pain plus placebo anticipation, All (n=39). Data are expressed as mean ± SD. †Calculated using paired, one-tailed *t*-tests for differences in ratings from anticipation of pain alone to anticipation of pain with placebo.

When females and males were examined separately, in paired samples, one-tailed *t* tests, females showed a significant decrease in PANAS positive affect ($t = 2.94$) from pain anticipation to pain with placebo anticipation ($p \leq 0.01$). There were no significant differences in PANAS negative affect or PANAS fear ratings between conditions. Males showed a significant decrease in PANAS negative affect ratings from pain anticipation to pain with placebo anticipation ($t = 2.49$; $p < 0.05$). There were no significant differences in PANAS positive affect or PANAS fear ratings.

BP_{ND} data from activated regions from the male>female and female>male voxel by voxel analyses for the change from pain anticipation to placebo anticipation were then extracted as ROIs for further analysis. Pearson correlations were conducted between the regional change in opioid binding from pain anticipation to pain with placebo anticipation in individual ROIs and the change in psychophysics measures from pain anticipation to pain with placebo anticipation. Correlations were conducted separately for males and females between the ROIs from the voxel by voxel analyses and measures of PANAS negative, PANAS fear, PANAS positive, anticipated effectiveness of placebo, and desired effectiveness of placebo. Each R value was then tested for significant difference between males and females using the test described above.

In the right anterior cingulate, a significant correlation was obtained with decrease in PANAS Fear ratings from pain anticipation to pain with placebo anticipation for males but not females (Males: r = 0.48, p = 0.03; Females: r = 0.20, p = 0.42; z = ?, p = 0.36).

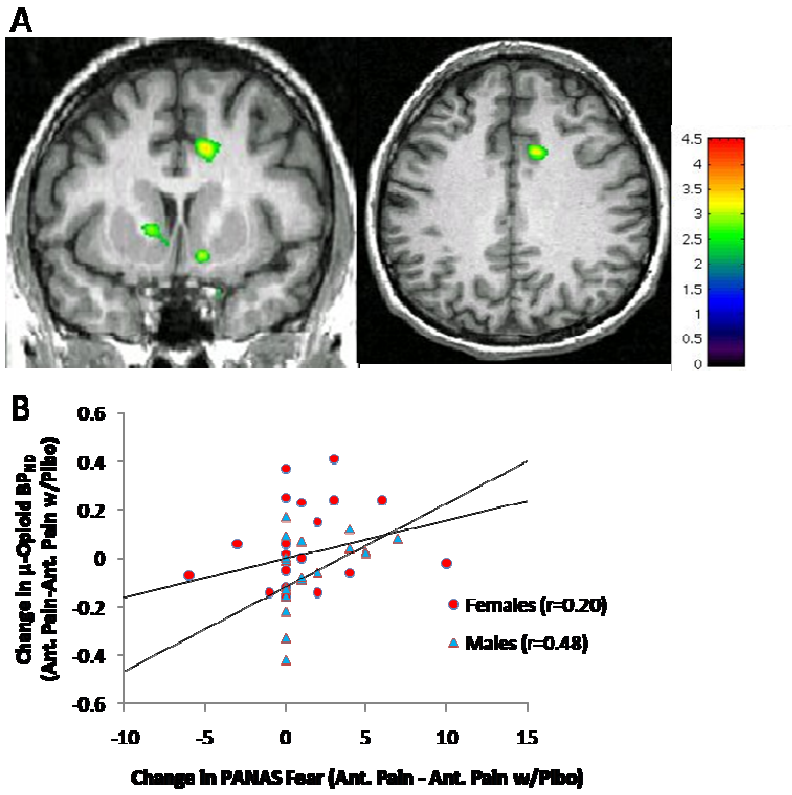

Figure 8. Sex differences in anterior cingulate μ-opioid neurotransmission and the correlation with changes in psychophysical measures during anticipation of placebo A) Activation of endogenous opioid neurotransmission from anticipation of pain alone to anticipation of pain with placebo in right anterior cingulate, females > males. B) Significant positive correlation between change in PANAS Fear and change right anterior cingulate μ-opioid BP_{ND} from anticipation of pain alone to anticipation of pain with placebo in males but not females.

In the right caudate, a significant correlation was obtained with the desired effectiveness of placebo for males (Males: r = 0.46, p = 0.04; Females: r = -0.04, p = 0.87; z = 1.54, p = 0.12).

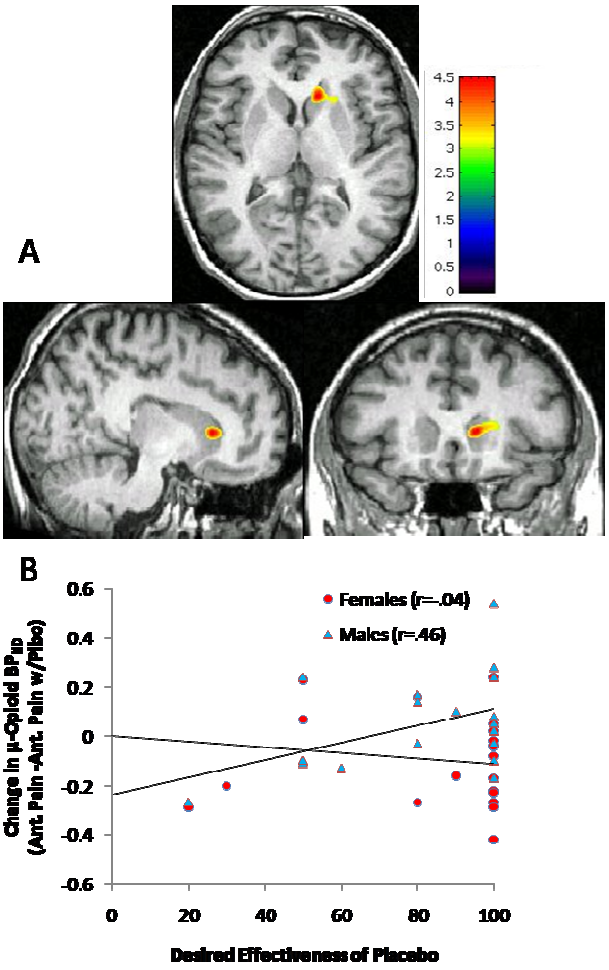

Figure 9. Sex differences in caudate μ-opioid neurotransmission and the correlation with changes in psychophysical measures during anticipation of placebo A) Activation of endogenous opioid neurotransmission from anticipation of pain alone to anticipation of pain with placebo in right caudate, males > females. B) Significant positive correlation between desired effectiveness of placebo and change in right caudate μ-opioid BP_{ND} from anticipation of pain alone to anticipation of pain with placebo in males but not females.

A SPM simple regression analysis within the female group revealed a positive relationship between change in μ-opioid BP_{ND} from pain anticipation without and with placebo and estradiol levels on the day of placebo anticipation and administration in the following regions: left amygdala (-25, 4, -21; t = 3.54; p = 0.01 uncorrected at voxel level), left dorsolateral prefrontal cortex (-28, 41, 22; t = 3.95; p = 0.009 uncorrected at cluster level), left insula (-34, 26, -7; t = 3.35; p = 0.002 uncorrected at voxel level), left

nucleus accumbens (-10, 6, -10; t = 4.49; p = 0.000 uncorrected at voxel level), right ventral posterior thalamus (1, -17, 2; t = 5.68; p = 0.001 uncorrected at cluster level), left temporal cortex (-55, 11, -25; t = 3.40; p = 0.002 uncorrected at voxel level), left centro-medial thalamus (-8, -19, 9; t = 5.18; p = 0.000 uncorrected at voxel level), right nucleus accumbens/putamen (22, 12, -6; t = 4.54; p = 0.002 uncorrected at cluster level), and right ventral posterior thalamus (12, -25, 9; t = 4.20; p = 0.000 uncorrected at voxel level).

Discussion

The current work demonstrates that males and females show significant differences in regional μ-opioid activation in response to anticipation and administration of placebo during an intensity-matched, sustained deep somatic pain challenge. The regions in which differences were found included some of the same regions in which sex differences were found in response to pain in an earlier study in our lab incorporating a similar experimental pain method [76]. Both the pain study and pain with placebo study had in common greater activation of left amygdala, right ventral pallidum, and right thalamus in males. In response to placebo administration, males additionally activated right caudate and right anterior cingulate to a greater extent than females. Conversely, the females showed a different pattern of response, activating to a greater extent than males right amygdala, hypothalamus, right nucleus accumbens, and dorsal and anterior parts of the left thalamus. These differing regional activations were also associated with sex-specific, placebo-induced changes in psychophysical measures monitoring sensory,

cognitive and affective aspects of the pain experience. The pain plus placebo challenges were preceded by non-painful anticipation periods, in which subjects expected pain with the possibility of placebo. Consistent with prior studies demonstrating engagement of pain-modulatory circuitry during anticipation of pain [62], the current study demonstrated activation of the μ-opioid pain-modulatory system, as well as sex differences in regional activation in response to placebo anticipation. Females activated the left ventral pallidum and right anterior cingulate to a greater extent, while males showed greater activation in the right caudate and nucleus coeruleus, along with associated changes in psychophysical ratings.

While we did not find any significant differences between males and females for the change in psychophysical responses from pain to placebo, or from anticipation of pain to anticipation of placebo, when we examined the groups separately, we found significant decreases in McGill affect and PANAS positive in females and significant decreases in early and overall VAS intensity scores in males in across the painful conditions. Although much of the clinical and pre-clinical pain literature suggests that females exhibit greater sensitivity to pain, other reports have also failed to find significant differences between the sexes for various measures of pain intensity [97, 98]. Other groups have argued that while there are probable sex differences in pain sensitivity, they are likely to be quite small and would require large groups of subjects to be detected [97, 99, 100]. Our observation that significant changes in psychophysics occur within each group separately supports the notion that sex differences do exist, but that the differences between groups are too small to be significant in our sample sizes.

Our findings of decreases in McGill affect and PANAS positive in females and VAS ratings in males suggest that the subjective experience of placebo analgesia is different between the sexes. The sex difference in placebo experience and interpretation appears to be mediated by more affect-related processes in females and by more evaluative or purely sensory processes in males. The finding of a decrease in positive affect from pain to placebo in females is surprising and may reflect an overall blunting of affect, because, although not significant, PANAS negative ratings also decreased in females.

In the pain and pain plus placebo anticipation phases, females' ratings of PANAS positive significantly decreased across conditions, while males' ratings of PANAS negative significantly decreased, suggesting the existence of differing cognitive and affective strategies between the sexes in mental preparations for pain and its relief.

The results of the male/female comparisons in μ-opioid related neurotransmission during placebo suggest that the sexes engage motivational and pain modulatory circuitry using unique, but overlapping pathways. Specifically, females uniquely engage the nucleus accumbens and hypothalamus to a greater degree during placebo than males. The differing psychophysical associations with placebo-related activation suggest that these unique pathways underlie sex differences in psychophysiological responses to pain and its relief via placebo. Our finding that estradiol levels are positively associated with regional increases in endogenous opioid activation during placebo and placebo anticipation suggests that sex-specific hormones influence acute neuropsychological responses to pain and placebo, and may also exert organizational effects on brain circuitry and functional connectivity between pain-modulatory regions. The

organizational effect of hormones like estradiol may underlie developmental establishment of sex-specific brain circuitry. The observed regional sex differences and the association with regional μ-opioid activity with estradiol levels may reflect evolutionarily established, sex-specific pain regulatory pathways that are functionally organized according to the divergent homeostatic needs of males (competing with other males for mates in the face of injury) and females (maintaining homeostasis during pregnancy-related pain). Similar analyses with male levels of testosterone would be useful to further develop such a theory.

Females, but not males, showed engagement of the hypothalamus during placebo. The hypothalamus is a critical component of the stress-modulatory hypothalamic-pituitary-adrenal (HPA) axis. Stress, including pain stress, induces corticotrophin releasing factor (CRF) release from the hypothalamus, which stimulates release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary into the bloodstream. ACTH then stimulates release of glucocorticoids from the adrenal glands, which can then feed back to the central nervous system, acting at glucocorticoid receptors located throughout the mesolimbic circuitry, including the hypothalamus [101]. Beta-endorphin, which acts at μ-opioid receptors, is synthesized in response to stress, and inhibits CRF release in the hypothalamus, blunting the subsequent stress response [102]. Estrogen is known to influence HPA stress responses. For example, in ovariectomized rats, low doses of estrogen inhibit HPA axis activity. It is possible that, as evidenced by HPA axis susceptibility to female reproductive hormones, females uniquely recruit the hypothalamus to induce placebo-related coping in response to sustained and stressful pain. The fact that placebo analgesia reduces affective pain ratings in females may mean

pain is a more stressful experience than for males, and perhaps reflects a greater need for endogenous opioid regulation of the HPA axis in order to elicit a placebo analgesic response.

Interestingly, males demonstrated a greater magnitude of μ-opioid activation in the locus coeruleus during pain plus placebo anticipation. The locus coeruleus, a major source of norepinephrine-containing neurons, has an excitatory effect on most of the brain, mediating arousal and stress responses [103]. μ-opioid activity in locus coeruleus inhibits the neurons that normally activate hypothalamic CRH release [104]. It is feasible that in males, placebo analgesia dampens stress reactivity elicited by pain via anticipatory mechanisms by the locus coeruleus. In women, the same dampening of the stress axis by placebo analgesia seems to occur during the actual receipt of placebo during pain, and via μ-opioid activity in the hypothalamus. The reason for this sexual dichotomy in stress regulation during placebo is unclear, but it points to engagement of unique stress-modulatory pathways in males and females via placebo-related endogenous opioid activation.

Greater nucleus accumbens activity in females was also observed during placebo. Estradiol levels were also associated with nucleus accumbens increases in endogenous opioid neurotransmission during placebo. Sex hormones may be in part responsible for this sex-specific activation.

Interestingly and unexpectedly, activation of the right amygdala was negatively associated with decreases in pain-related affect and internal affective states in both sexes. This may reflect a role of the amygdala as a mediator of affective coping during stressful sustained pain. In subjects for whom the placebo is not as effective in relieving pain,

right amygdala endogenous opioid neurotransmission may be recruited in a manner proportional to the amount of affective disturbance caused by placebo ineffectiveness, essentially as a compensatory or reactive mechanism. We included both placebo responders and nocebo responders in these analyses in order to capture the entire range of variance in placebo responses, and this amygdala recruitment may reflect reactions of nocebo responders and those who are only modest placebo responders.

The left dorsal thalamus was activated in response to placebo to a greater extent in females. This activation was significantly associated with increases in PANAS Positive affect in females but not males. This finding is consistent with the notion that placebo response in females is mediated in a more affective manner than males, and the left dorsal thalamus plays a dichotomous role in this unique sex-specific mechanism. Males, but not females, showed positive associations with decreases in McGill Sensory and VAS ratings in this same region, which suggests that while females show greater activation and it is associated with affective changes, activation of this region in males mediates more sensory aspects of the placebo experience. It seems this part of the thalamus is involved in mediating placebo responses in both sexes, but in a psychologically divergent manner, depending on sex.

While the dorsal region of the thalamus may mediate sensory responses to placebo in males, the ventral region may play a similar role in females. Females activated this region to a greater extent than males during placebo, and this activation was significantly associated with decreases in McGill sensory ratings in females but not males. This may reflect that some parts of the thalamus (like dorsal) mediate sex-specific placebo responses in both sexes, and that other parts (i.e. ventral) are recruited in an

exclusive and sex-dependent manner. It may be that the sexes process sensory aspects of pain relief using slightly different neural pathways within the thalamus; females in the ventral region and males in the dorsal region.

Consistent with the notion of diverging neural pathways for sex-specific processing of various psychological aspects of placebo, males showed significant associations with increase in PANAS Positive affect and endogenous activation within the left anterior cingulate, which they also activated to a greater extent than females. PANAS Positive affect changes were associated with left dorsal thalamus in females, and with anterior cingulate in males. The male-specific activations of the anterior cingulate and right ventral pallidum during placebo were also associated with decreases in VAS ratings of pain and McGill sensory ratings of pain. These regions may comprise part of a male-specific placebo subcircuit that is essential in mediating the male sensory bias.

Although the males showed greater activation in the left amygdala during placebo, females demonstrated a significant association with activation in this area and decrease in their VAS ratings for the 20 minute pain periods. The amygdala is a region that is responsive to emotional valence of stimuli, and this association may reflect its recruitment in female evaluations of pure intensity of pain; this may suggest that female evaluations of pain intensity contain an affective bias mediated by the amygdala.

Anticipation of pain and placebo are important determinants of the actual outcome of placebo analgesia. Sex differences in the anticipation of placebo provide more clues to how and why placebo analgesia is sexually divergent. Like placebo regional activation, the sexes showed unique areas of endogenous opioid activation in response to the anticipation of placebo with pain compared to the anticipation of pain

alone. Females activated the left ventral pallidum and right anterior cingulate to a greater extent than males, and males activated the right caudate and nucleus coeruleus to a greater extent. It is interesting to note that males uniquely activated the left anterior cingulate during placebo, while females activated this region (albeit contralaterally) during anticipation of placebo. Females may engage frontal regions and evaluative processes to a greater extent as they anticipate pain with placebo analgesia, but males engage these processes during actual receipt of pain with placebo. This differential recruitment of the same region at different times in the experimental process may underlie the male tendency to respond to placebo with more sensory-related evaluations.

Like the affective ratings during pain/placebo, affective changes in ratings from pain anticipation to anticipation of pain with placebo did not differ between the sexes. However, when males and females were examined separately, differences emerged. Females showed decreases in PANAS positive ratings, similar in nature to their pain/placebo ratings, while males showed decreases in PANAS negative ratings.

Females also had significant associations with estradiol and many regions within the pain modulatory and motivational circuit during placebo anticipation, including bilateral nucleus accumbens. Again, the sexually divergent patterns of activation during placebo anticipation and pain receipt with placebo seem to be at least in part due to hormonal influences.

Chapter III

Sex and Genotype Interaction in μ-opioid Neurotransmission for the μ-opioid Receptor A118G Polymorphism During PET Scanning of Placebo Analgesia and its Anticipation

A growing accumulation of research has attempted to characterize the neurochemical, neuroanatomical and psychophysical mechanisms underlying placebo analgesia. Placebo analgesia is defined as pain relief that occurs after receipt of an inert substance given under the guise of legitimate analgesic intervention. Belief in treatment efficacy and expectation of benefit are psychological states that determine how one responds to placebo administration. Brain regions that are activated during placebo analgesia reside in the motivational, reward and limbic networks: in particular, ventral striatum, including the nucleus accumbens, plays a crucial role in mediating placebo responses. Recent work in our lab demonstrated for the first time that both dopamine and endogenous opioids are released in the motivational circuitry, including the ventral striatum, during placebo analgesia. Additionally, this activation is associated with decreases in subjective ratings of sensory, affective, and cognitive aspects of pain.

However, there is substantial individual variation in the response to placebo: not all subjects exhibit large responses and some even show increases in pain in response to placebo administration. Clearly, there is work to be done to account for factors that confer individual variability in the response to placebo analgesia. Recent work in our lab established the existence of sex differences in neurochemical and psychophysical

measures of placebo analgesia, which seem to be at least in part driven by the reproductive hormone estradiol. These results were not unexpected given that substantial sex differences have been documented in many aspects of the pain experience: pain sensitivity, susceptibility to chronic pain, responsiveness to opiate analgesics, and even postmortem measures of μ-opioid receptor binding. Hormones like estradiol are known to interact with μ-opioid receptors, which could be a mechanism that accounts, in part, for the sex differences in pain modulation and placebo analgesia. However, genetic polymorphisms that alter μ-opioid receptor function are likely candidates for mediating individual variability in placebo response, and because of the close ties of μ-opioid receptors and estradiol activity, genetic variation in this receptor probably contributes to sex differences.

The A118G polymorphism in the μ-opioid receptor has been associated with both altered pain and reward responsivity and with sex differences in these areas. A118G is a single nucleotide polymorphism (SNP) that resides on exon 1 of the μ-opioid receptor. The functional significance of the SNP has not been completely established, but studies have suggested that the G variant is associated with decreased mRNA transcription and protein expression [105]. Another study found a decrease in agonist binding to receptors containing the G allele in cell cultures, indicating a decrease in receptor numbers [106]. Because of the involvement of μ-opioid receptor signaling in reward, stress, and analgesia, genetic variation in the receptor may predispose individuals to differential behavioral, pharmacological and neurochemical responses in opioid-mediated processes and regions. Indeed, evidence points to the polymorphism's association with drug dependence, stress responses, pain modulation and reward processing. Specifically, the

G allele is associated with lower pain thresholds, decreased response to morphine or other opioids, and increased pain in patients with chronic pain or post-operative pain [106-111].

In addition to its connection to pain mechanisms, the A118G polymorphism appears to affect reward responding in knock-in mice containing the variant. Mice containing the G variant have decreased receptor levels and demonstrate blunted reward and analgesic responses to repeated administration of morphine. Furthermore, a sex by genotype interaction for measures of hedonia revealed that females with the G variant failed to exhibit place-preference for environments associated with morphine [112].

In the context of reward and motivational mechanisms, placebo analgesia can be thought of as a reward state, in which relief from pain is a rewarding outcome. Consequently, just as natural rewards and punishments modulate motivational processes in order to elicit favorable and adaptive behaviors, so do pain and its relief via placebo recruit motivational brain mechanisms that allow the organism to respond appropriately and adaptively. Therefore the ability of humans to experience placebo analgesia can be thought of as an adaptive pyschophysiological mechanism, in which motivational brain processes are recruited to keep the organism in homeostatic balance and to initiate appropriate coping behaviors. The affective and sensory aspects of pain perception and the motivation to avoid aversive stimuli are psychological states intimately tied to opioid signaling in limbic and higher order associative brain regions.

Previous research in our lab has focused on examining individual differences in the placebo analgesia response. An initial study found that when the expectation of an analgesic agent, or placebo, was administered during a sustained pain challenge, PET

imaging of radiotracer displacement from μ-opioid receptors revealed significant activation of μ -opioid receptor mediated neurotransmission in several brain regions associated with cognitive and affective appraisal and motivational behavior [66]. Zubieta et al. [67] later observed that pain-related affect, pain sensitivity, and internal affective state contributed to the variation in magnitude of opioid release during this same challenge. Presumably, individual affective state before and during pain challenges influences brain neurochemistry and activity in the regions that govern placebo perception and response.

Psychophysiological, genetic, and neurochemical factors that influence individual differences in the functioning of these affective and motivational brain regions are likely to also mediate placebo responses. A separate PET study showed that men and women exhibit distinctly different regional patterns of μ-opioid receptor activation during a sustained, deep-tissue pain challenge similar to that described above [76]. A subsequent and similarly designed study found sex differences in regional μ-opioid mediated neurotransmission during the experience of placebo administration and the anticipation of placebo administration and that the female μ -opioid placebo responses were related to estradiol levels (Evans C. et al. 2010, in preparation).

Because hormonally influenced sex differences seem to play a large role in individual responding to placebo and placebo anticipation, we sought to examine whether this effect had a genetic component. Because of animal studies documenting A118G genotype differences in the analgesic and reward response to opiate treatment and because of the gene by sex interaction that indicated a blunted response in female carriers of the G variant, we hypothesized that there would be similar differences between

genotype and sex groups in μ -opioid mediated neurotransmission during the anticipation and experience of placebo analgesia.

Materials and Methods

Subjects

Volunteers were 34 healthy, right-handed men (n=20) and women (n=14), with a mean age of 28. Participants had no history of medical illness, psychiatric illness, substance abuse or dependence and no history of inheritable family illnesses. Volunteers were not taking psychotropic medications or hormonal treatments. Women had not taken hormonal birth control for at least 6 months prior to the study. Women were scanned during the follicular phase of their menstrual cycles (2 to 9 days after the onset of menses), as measured by plasma levels of estradiol (mean of 111 pg/mL) and progesterone (< 3 ng/ml in all cases). All procedures were approved by the University of Michigan Investigational Review Boards.

Genotyping

Subjects were divided into two groups according to whether they carried the rare (G) allele. Therefore, the group without the G allele were homozygous for the A allele (AA group; n = 24), and the group containing the G allele were either homozygous or heterozygous (AG/GG group; n = 10).

Scanning Protocols

PET scans were acquired with a Siemens ECAT EXACT scanner in three dimensional mode with septa retracted. Participants were positioned in the PET scanner gantry, and two intravenous lines were placed. A light forehead restraint was used to prevent head movement during the scan. [^{11}C] carfentanil was synthesized at high specific activity (>1000 Ci/mmol) by the reaction of ^{11}C-methyliodide and a nonmethyl precursor as previously described [86], with minor modifications to improve its synthetic yield; 10 to 15 mCi (370-555 MBq) were administered to each subject for each of the two PET scans. The two administrations were separated by 2 hours to allow for tracer decay. The maximum mass of carfentanil injected was less than 0.03 μg/kg per study, ensuring that the compound was administered in tracer quantities, i.e. subpharmacological doses. Fifty-five percent of the ^{11}C carfentanil dose was administered as a bolus and the remainder as a continuous infusion using a computer-controlled pump to achieve steady-state tracer levels. Nineteen sets of scans were acquired over 70 minutes with an increasing duration (30 s up to 10 min). Images were reconstructed using filtered back-projection with a Hanning 0.5 filter, and included both measured attenuation and scatter corrections. Dynamic images were coregistered to each other and the intercommisural line using automated computer routines [87]. Image data were then transformed on a pixel-by-pixel basis into two sets of parametric maps: (a) a tracer transport measure (K1 ratio), and (b) a receptor-related measure, distribution volume ratio (DVR). To avoid the need for arterial blood sampling, the tracer transport and binding measures were calculated using a modified Logan graphical analysis [88], using the occipital cortex (an

area devoid of mu-opioid receptors) as the reference region. With the protocol used, the Logan plot becomes linear by 10 minutes after the start of the radiotracer administration, with its slope being the DVR, a measure equal to the (B_{max}/KD) + 1 for this receptor site and radiotracer. B_{max}/KD (or DVR – 1) is the "receptor related" measure (μ-opioid receptor availability, or binding potential). K1 and DVR images for each experimental period and MR images were coregistered to each other and to the International Consortium for Brain Mapping (ICBM) stereotactic atlas orientation [52].

MRI scans were acquired on a 1.5 Tesla scanner (Signa, General Electric, Milwaukee, WI). Acquisition sequences were axial SPGR IR-Prep MR (TE=5.5, TR=14, TI=300, flip angle=20 degrees, NEX=1, 124 contiguous images, 1.5 mm thickness), followed by axial T2 and proton density images (TR=4000, TE=20 and 100, respectively, NEX=1, 62 contiguous images, 3 mm thick). All MR scans were reviewed by a neuroradiologist to rule out gross structural brain abnormalities before PET scanning.

Experimental Design

During pain scans and pain with placebo scans, subjects were administered a steady state of moderate muscle pain starting at 45 minutes after radiotracer injection until 65 minutes, which was adjusted to maintain pain levels around 40 on a scale of 0 (no pain) to 100 (most pain imaginable). Specifically, a computer-controlled delivery system infused medication-grade hypertonic saline (5%) into the left masseter muscle, a model of sustained, deep somatic pain. Specific details of the computer-controlled standardization of pain levels have been described previously [93, 94]. The first period

of each scan consisted of periods of anticipation of either pain alone or pain with placebo starting at 5 minutes after radiotracer administration and lasting until 25 minutes into the scan. Isotonic (non-painful) saline was infused into the masseter muscle during this time. Pain intensity ratings were continually updated every 15 sec (scale of 0 to 100 as mentioned above) for both anticipation periods and pain administration periods. These values were recorded in the computer controller and averaged for statistical analyses. Prior to placebo phases, subjects were told that they may or may not receive a compound thought to reduce pain through the activation of internal pain control mechanisms. Placebo administration consisted of 1 mL i.v. infusions of isotonic saline every 4 minutes during the 20 minute pain period, with accompanying verbal and visual cues at each application.

Psychophysical questionnaires measuring the pain experience were administered at various times throughout the pain periods. The McGill pain questionnaire (MPQ), which measures sensory and affective aspects of pain, was given after pain challenges [95]. The Positive and Negative Affectivity Scale (PANAS) was administered before and after each experimental phase (both anticipation and pain administration), and monitors the internal affective state of subjects [96]. The Profile of Mood States-Total Mood Disturbance (POMS-TMD) was given after pain challenges to measure degree of mood disturbance. Participants were also administered questionnaires before getting into the scanner in which they rated how effective they believed placebo would be and how much they wanted it to work (scale 0 to 100). After the entire experiment, participants were asked to rate how effective they believed the placebo had been in relieving their pain (scale 0 to 100).

Data Analysis

Parametric maps of differences between conditions (pain-placebo) were generated by anatomically standardizing the MRI of each subject to the International Conference on Brain Mapping (ICBM) stereotactic atlas coordinates, with subsequent application of this transformation to the mu-opioid receptor binding maps [52]. Before nonlinear warping, image data were prepared so that the side of the painful challenge (induced on the right or the left masseter muscle, in a counterbalanced design) was located on the same side of the image for all subjects. Image data are therefore presented as "ipsilateral" or "contralateral" to the painful stimulus, regardless of the actual location (right-left). Differences between conditions and subject groups were then mapped into stereotactic space using z-maps of statistical significance with SPM99 and Matlab software and using a general linear model and correction for multiple comparisons [113], but without global normalization (the data presented are based on absolute B_{max}/KD estimates). Only regions with specific mu-opioid receptor binding were included in the analyses (pixels with DVR values >1.2 times the mean global image value for mu-opioid receptor images as calculated with SPM 99). To compensate for small residual anatomic variations across subjects and to improve signal to noise ratios, a three-dimensional Gaussian filter (FWHM 6 mm) is applied to each scan. For each subtraction analysis of one sample, two-tailed t-statistic values were calculated for each pixel using pooled variances across pixels [91]. Areas of significant differences were detected using a statistical threshold that controls a type 1 error rate at P=0.05 for multiple comparisons, which was estimated

using the Euler characteristic [91] based on the number of pixels in the gray matter and image smoothness [92]. This typically varies from z=4.4 to 4.6 in our studies for peak analyses, at a final resolution of approximately 10 mm. Z scores were also deemed significant if they reached statistical thresholds after correction for the size of the cluster under consideration [113].

Results

In a SPM voxel-by-voxel analysis, an independent samples t test between subjects with and without the A118G polymorphism revealed differences in the change in μ - opioid binding potential from the pain alone to pain with placebo condition. At a threshold of > 20 voxels and $p \leq 0.01$, the AG/GG subjects had a significantly greater decrease compared to AA subjects in BP_{ND} (greater activation) in the right lateral amygdala, (27, -6, -22; t = 5.25), left anterior ventral thalamus (-14, -9, 9; t = 4.11), left posterior ventral thalamus (-3, -21, 0; t = 3.67), left ventral caudate (-16, 12, 0; t = 3.54), right ventral pallidum (8, 1, -11; t = 3.53 and 14, 1, -5; t = 3.99), left extended amygdala, (-19, -2, -12; t = 3.97), right dorsal caudate (12, 10, 6; t = 3.42), left amygdala (-25, -5, -18; t = 3.06), left dorsal cingulate (-10, 9, 45; t = 3.05), and right anterior insula (47, 2, -3; t = 3.08).

Compared to the AG/GG subjects, the AA subjects had a significantly greater decrease in BP_{ND} (greater activation) in the right and left amygdala (14, -1, -18; t = 4.78 and -32, -1, -17; t = 3.77) and the right nucleus accumbens (8, 13, -11; t = 4.37).

	Region	x, y, z coordinates, mm	Cluster Size, mm^3	T score	P value
AG/GG > AA					
	R. Lat. Amyg.	27, -6, -22	693	5.25	0.000
	L. Ant. Vent. Thal.	-14, -9, 9	1146	4.11	0.000
	L. Post. Vent. Thal.	-3, -21, 0	(part of prev. cluster)	3.67	0.000
	L. Vent. Caud.	-16, 12, 0	711	3.54	0.001
	R. Vent. Pallidum	14, 1, -5	548	3.99	0.000
	R. Vent. Pallidum	8, 1, -11	(part of prev. cluster)	3.53	0.001
	L. Vent. Pallidum	-6, -1, -12	356	3.72	0.000
	L. Ext. Amyg.	-19 -2, -12	210	3.97	0.000
	R. Dors. Caud.	12, 10, 6	275	3.42	0.001
	L. Amyg.	-25, -5, -18	112	3.06	0.002
	L. Dors. Cing.	-10, 9, 45	98	3.05	0.002
	R. Ant. Insula	47, 2, -3	226	3.08	0.002
AA > AG/GG					
	R. Amyg.	14, -1, -18	681	4.78	0.000
	R. Nac.	8, 13, -11	701	4.37	0.000
	L. Amyg.	-32, -1, -17	141	3.77	0.000

Table 9. Placebo Regional Activation.

We then examined whether μ -opioid release during placebo anticipation is affected by A118G genotype. In another SPM voxel-by-voxel analysis, an independent samples t test between AA and AG/GG subjects revealed differences in BP_{ND} from anticipation of pain alone to anticipation of pain with placebo in several regions. Compared to the AA subjects, the AG/GG subjects had a significantly greater decrease in BP_{ND} (greater activation) in the left nucleus accumbens (-18, 13, -3; t = 3.69), right medial anterior thalamus (3, -12, 5; t = 3.29), right amygdala/ventral pallidum (20, -4, -10; t = 3.33) and left anterior insula (-37, 15, -6; t = 2.96).

Compared to AG/GG subjects, AA subjects had a significantly greater decrease in BP_{ND} (greater activation) in the periaqueductal gray (0, -32, -19; t = 3.89) and in the right ventral pallidum/nucleus accumbens (8, 3, -14; t = 3.38).

	Region	x, y, z coordinates, mm	Cluster Size, mm^3	T score	P value
AG/GG > AA					
	L. Nac.	-18, 13, -3	228	3.69	0.000
	R. Med. Ant. Thal	3, -12, 5	282	3.29	0.001
	R. Amyg/ Vent. Pall.	20, -4, -10	201	3.33	0.001
	L. Ant. Insula	-37, 15, -6	82	2.96	0.003
AA > AG/GG					
	PAG	0, -32, -19	1049	3.89	0.000
	R. Vent. Pall/Nac.	8, 3, -14	458	3.38	0.001

Table 10. Placebo Anticipation Regional Activation.

When an independent samples, one-tailed *t* test was performed, there were no significant differences between AA subjects and AG/GG subjects for the change in psychophysical measures (MPQ Sensory, MPQ Affective, PANAS Negative, PANAS Fear, POMS TMD, 0 to 10 minute Early or 10 to 20 minute Late average 15 second momentary VAS ratings from pain alone to pain with placebo. There was a significant difference in change in PANAS Positive ratings between genotype groups (*t* = 1.77; p = 0.05): AA Subjects' positive affect ratings decreased from pain alone to pain with placebo, while AG/GG Subjects' ratings increased slightly.

Measure	*AA Alleles (n=24) Pain Alone Minus Pain + Plbo*	*AG/GG Alleles (n=10) Pain Alone Minus Pain + Plbo*	T	$p^{\dagger}$
MPQ Sensory Subscale	0.21 ± 5.96	0.20 ± 6.27	0.004	0.50
MPQ Affective Subscale	0.54 ± 1.64	-0.30 ± 0.82	1.53	0.07
PANAS Negative Affect	0.17 ± 1.79	-0.40 ± 4.74	0.51	0.31
PANAS Fear	0.42 ± 0.93	-0.50 ± 2.72	1.48	0.08
PANAS Positive Affect	1.79 ± 3.51	-0.60 ± 3.81	1.77	**0.05**
POMS-TMD	2.17 ± 6.94	-0.70 ± 10.45	0.94	0.18
15 s momentary intensity VAS 0 to 10 minutes	5.20 ± 13.06	3.42 ± 11.55	0.37	0.36
15 s momentary intensity VAS 10 to 20 minutes	4.77 ± 19.28	5.45 ± 14.78	0.10	0.50

Table 11. Change in psychophysiological responses from pain alone to pain plus placebo, AA alleles vs. AG/GG alleles. Data are expressed as mean ± SD. †Calculated using 2-sample, one-tailed *t*-tests for differences between genotype groups in changes from pain alone to pain with placebo.

When subjects from both genotype groups were combined, paired samples, one-tailed *t* tests showed that there was a significant decrease in PANAS Positive Affect (t = 1.71; p = 0.05), and a significant decrease in the average early (0 to 10 minutes) 15 second momentary intensity VAS ratings (t = 2.18; p = 0.02).

Measure	*All Subjects Pain*	*All Subjects Pain + Plbo*	T	$p^{\dagger}$
MPQ Sensory Subscale	13.76 ± 6.82	13.56 ± 7.86	0.20	0.42
MPQ Affective Subscale	1.41 ± 2.32	1.12 ± 1.89	1.15	0.13
PANAS Negative Affect	1.65 ± 2.92	1.65 ± 3.24	0.00	0.50
PANAS Fear	1.06 ± 2.32	0.91 ± 2.37	0.51	0.31
PANAS Positive Affect	9.68 ± 6.44	8.59 ± 7.07	1.71	**0.05**
POMS-TMD	14.29 ± 10.20	12.97 ± 7.87	0.96	0.18
15 s momentary intensity VAS 0 to 10 minutes	26.02 ± 13.30	21.35 ± 13.53	2.18	**0.02**
15 s momentary intensity VAS 10 to 20 minutes	25.05 ± 16.35	20.08 ± 12.91	1.62	0.06

Table 12. Psychophysiological responses during pain and pain plus placebo, All Subjects (n=34). Data are expressed as mean ± SD. †Calculated using paired, one-tailed *t*-tests for differences in ratings from pain alone to pain with placebo.

When genotype groups were examined separately, in paired samples, one-tailed *t* tests, the AA allele group showed a significant decrease in PANAS Fear (t = 2.20), PANAS positive affect (t = 2.50), and VAS ratings for the early period (0 to 10 minutes) from pain alone to pain with placebo ($p \leq 0.05$). There were no significant differences between ratings from pain alone to pain with placebo for MPQ sensory, MPQ Affect, PANAS negative, POMS TMD, or VAS late in the AA allele group. There were no significant differences between ratings from pain to pain with placebo for any of the measures in the AG/GG group.

Measure	***AA Alleles Pain***	***AA Alleles Pain + Plbo***	**T**	$p^{\dagger}$
MPQ Sensory Subscale	14.17 ± 7.30	13.96 ± 8.20	0.17	0.44
MPQ Affective Subscale	1.58 ± 2.39	1.04 ± 1.81	1.62	0.06
PANAS Negative Affect	1.38 ± 2.73	1.21 ± 2.19	0.46	0.33
PANAS Fear	1.13 ± 2.56	0.71 ± 2.12	2.20	**0.02**
PANAS Positive Affect	9.00 ± 6.55	7.21 ± 6.71	2.50	**0.01**
POMS-TMD	14.21 ± 11.10	12.04 ± 6.42	1.53	0.07
15 s momentary intensity VAS 0 to 10 minutes	28.69 ± 11.97	23.49 ± 14.21	1.95	**0.03**
15 s momentary intensity VAS 10 to 20 minutes	26.60 ± 16.19	21.82 ± 13.52	1.21	0.12

Table 13. Psychophysiological responses during pain and pain plus placebo, AA alleles (n=24). Data are expressed as mean ± SD.

Measure	*AG/GG Alleles Pain*	*AG/GG Alleles Pain + Plbo*	T	$p^{\dagger}$
MPQ Sensory Subscale	12.80 ± 5.73	12.60 ± 7.32	0.10	0.46
MPQ Affective Subscale	1.00 ± 2.21	1.30 ± 2.16	-1.15	0.14
PANAS Negative Affect	2.30 ± 3.40	2.70 ± 4.95	-0.27	0.40
PANAS Fear	0.90 ± 1.73	1.40 ± 2.95	-0.58	0.29
PANAS Positive Affect	11.30 ± 6.18	11.90 ± 7.13	-0.50	0.32
POMS-TMD	14.50 ± 8.14	15.20 ± 10.66	-0.21	0.42
15 s momentary intensity VAS 0 to 10 minutes	19.62 ± 14.74	16.20 ± 10.66	0.94	0.19
15 s momentary intensity VAS 10 to 20 minutes	21.35 ± 16.99	15.90 ± 10.80	1.17	0.14

Table 14. Psychophysiological responses during pain and pain plus placebo, AG/GG alleles (n=10). Data are expressed as mean ± SD.

†Calculated using paired, one-tailed *t*-tests for differences in psychophysics measures during pain alone versus pain plus placebo.

We then examined group genotype differences in the change in psychophysical measures from the pain anticipation phase to the pain with placebo anticipation phase. An independent samples, one-tailed *t* test revealed that there were no genotype group differences in any of the measures.

Measure	*AA Alleles (n=24) Ant. Of Pain Alone Minus Ant. of Pain + Plbo*	*AG/GG Alleles (n=10) Ant. Of Pain Alone Minus Ant. Of Pain + Plbo*	T	$p^{\dagger}$
PANAS negative affect	1.04 ± 3.44	1.40 ± 3.31	-0.28	0.39
PANAS fear	1.33 ± 2.63	1.00 ± 2.36	0.35	0.37
PANAS positive affect	2.08 ± 3.84	-0.30 ± 5.23	1.48	0.08

Table 15. Change in psychophysiological responses from anticipation of pain alone to anticipation of pain plus placebo, AA alleles vs. AG/GG alleles. Data are expressed as mean ± SD. †Calculated using 2-sample, one-tailed *t*-tests for differences between genotype groups in changes from anticipation of pain alone to anticipation of pain with placebo.

When both groups were combined, paired samples, one-tailed *t* tests showed that there was a significant decrease in PANAS Negative Affect ($t = 1.99$; $p = 0.03$), PANAS Positive Affect ($t = 1.85$; $p = 0.04$), and PANAS Fear ($t = 2.06$; $p = 0.004$).

Measure	*All Subjects Ant. Of Pain*	*All Subjects Ant. Of Pain + Plbo*	T	$p^{\dagger}$
PANAS Negative Affect	2.53 ± 3.62	1.38 ± 2.80	1.99	**0.03**
PANAS Fear	2.06 ± 3.09	0.82 ± 1.82	2.86	**0.004**
PANAS Positive Affect	9.35 ± 6.75	7.97 ± 6.78	1.85	**0.04**

Table 16. Psychophysiological responses during pain anticipation and pain plus placebo anticipation, All subjects (n=34). Data are expressed as mean ± SD. †Calculated using paired, one-tailed *t*-tests for differences in ratings from anticipation of pain alone to anticipation of pain with placebo.

When genotype groups were examined separately, in paired samples, one-tailed *t* tests, the AA allele group showed a significant decrease in PANAS positive affect ($t = 2.66$) and PANAS Fear ($t = 2.48$) from pain anticipation to pain with placebo anticipation ($p \leq 0.01$). There were no significant differences in PANAS negative affect ratings between conditions. AG/GG alleles did not have significant changes in ratings for any of the measures.

Measure	*AA Alleles Ant. Of Pain*	*AA Alleles Ant. Of Pain + Plbo*	T	$p^{\dagger}$
PANAS Negative Affect	2.42 ± 3.54	1.38 ± 2.83	1.48	0.08
PANAS Fear	2.13 ± 3.18	0.79 ± 1.89	2.48	**0.01**
PANAS Positive Affect	9.13 ± 7.25	7.04 ± 6.33	2.66	**0.005**

Table 17. Psychophysiological responses during pain anticipation and pain plus placebo anticipation, AA alleles (n=24). Data are expressed as mean ± SD. †Calculated using paired, one-tailed *t*-tests for differences in ratings from anticipation of pain alone to anticipation of pain with placebo.

Measure	*AG/GG Alleles Ant. Of Pain*	*AG/GG Alleles Ant. Of Pain + Plbo*	T	*p*†
PANAS Negative Affect	2.80 ± 3.99	1.40 ± 2.88	1.34	0.11
PANAS Fear	1.90 ± 3.03	0.90 ± 1.73	1.34	0.11
PANAS Positive Affect	9.90 ± 5.70	10.20 ± 7.64	-0.18	0.43

Table 18. Psychophysiological responses during pain anticipation and pain plus placebo anticipation, AG/GG alleles (n=10). Data are expressed as mean ± SD. †Calculated using paired, one-tailed *t*-tests for differences in ratings from anticipation of pain alone to anticipation of pain with placebo.

We then performed a SPM voxel by voxel two-way ANOVA to determine if there were interactions between genotype and sex for the change in μ-opioid receptor BP from pain alone to pain with placebo. There was a significant gene by sex interaction for the right amygdala/hypothalamus (12, -4, -20; t = 4.54), right anterior thalamus (10, -4, 7; t = 4.53), left posterior thalamus (-17, -27, 10; t = 4.51), left nucleus accumbens (-12, 7, -8; t = 4.33), left ventral anterior thalamus (-9, -5, 3; t = 4.31), right lateral amygdala (23, 1, -24), right insula (32, 24, 7; t = 3.68), and right putamen (25, 8, -9; t = 3.30).

	Region	**x, y, z coordinates, mm**	**Cluster Size, mm^3**	**T score**	**P value**
Gene by Sex	R. Amyg./Hypothal.	12, -4, -20	492	4.54	0.000
	R. Ant. Thal	10, -4, 7	383	4.53	0.000
	L. Post. Thal.	-17, -27, 10	1360	4.51	0.000
	L. Nac.	-12, 7, -8	479	4.33	0.000
	L. Vent. Ant. Thal.	-9, -5, 3	380	4.31	0.000
	R. Lat. Amyg.	23, 1, -24	82	3.68	0.000
	R. Insula	32, 24, 7	266	3.68	0.000
	R. Putamen	25, 8, -9	200	3.30	0.001

Table 19. Regional μ-opioid activation during placebo; two-way ANOVA for gene x sex interaction

We then performed a SPM voxel by voxel two-way ANOVA to determine if there was a gene by sex interaction for μ -opioid receptor BP change from anticipation of pain

alone to anticipation of pain with placebo. There was a significant gene by sex interaction for the right posterior thalamus (18, -28, 15; t = 6.03), right lateral amygdala (27, -4, -15; t = 5.17), right ventral pallidum (10, -2, -6; t = 4.89), and left amygdala (-21, 2, -16; t = 4.15).

	Region	x, y, z coordinates, mm	Cluster Size, mm^3	T score	P value
Gene by Sex	R. Post. Thal.	18, -28, 15	1804	6.03	0.052
	R. Lat. Amyg.	27, -4, -15	1004	5.17	0.000
	R. Vent. Pallidum	10, -2, -6	(part of prev. cluster)	4.89	0.000
	L. Amyg.	-21, 2, -16	161	4.15	0.000

Table 20. Regional μ-opioid activation during placebo anticipation; two-way ANOVA for gene x sex interaction

In order to determine the direction of effects, we extracted each of the regions in which there was a significant gene by sex interaction in voxel by voxel SPM analyses and performed two-way ANOVAs and post-hoc comparisons using SPSS. For the change in μ -opioid binding potential from pain alone to pain with placebo, it was determined that both the overall model and sex by gene interaction were significant in the following regions: right anterior thalamus (model: F = 3.15; p = 0.039; interaction: F = 8.68; p = 0.006), right amygdala (model: F = 3.27; p = 0.035; interaction: F = 4.57; p = 0.041), right lateral amygdala (model: F = 3.08; p = 0.042; interaction: F = 5.67; p = 0.024), right putamen (model: F = 3.47; p = 0.028; interaction: F = 8.25; p = 0.007) and right insula (model: F = 4.90; p = 0.007; interaction: F = 13.25; p = 0.001). There was no significant effect of sex in any of the regions. There was a significant effect of genotype in the right amygdala (F = 5.98; p = 0.021). Bonferroni post-hoc comparisons between groups (AA Females, AA Males, AG/GG Females, AG/GG Males) for each region

revealed that in the right amygdala and right insula, AA Females had a significantly greater decrease in BP from pain alone to pain with placebo than AG/GG Females, whose BPs increased ($p = 0.024$ and $p = 0.012$, respectively). There were no other significant differences between groups in the post-hoc analyses for the remaining regions.

Region	*AA Females*	*AA Males*	*AG/GG Females*	*AG/GG Males*
R. Ant. Thal	0.10 ± 0.16	-0.10 ± 0.30	-0.12 ± 0.22	0.21 ± 0.13
R. Amyg.	0.19 ± 0.16	0.03 ± 0.27	-0.24 ± 0.29	0.01 ± 0.23
L. Vent. Ant. Thal	0.05 ± 0.19	-0.06 ± 0.28	-0.10 ± 0.20	0.22 ± 0.28
L. Post. Thal	0.03 ± 0.13	-0.05 ± 0.14	-0.19 ± 0.25	0.03 ± 0.21
R. Lat. Amyg.	0.08 ± 0.17	-0.12 ± 0.25	-0.01 ± 0.26	0.20 ± 0.12
R. Putamen	0.13 ± 0.13	-0.07 ± 0.20	-0.05 ± 0.07	0.12 ± 0.18
R. Insula	0.15 ± 0.14	0.00 ± 0.10	-0.11 ± 0.19	0.12 ± 0.18

Table 21. Sex/Genotype Differences in Placebo-Induced Changes in Regional μ-Opioid Receptor BP. Data are expressed as the mean ± SD. Means represent mean change in BP pain alone to pain with placebo in regions in which there was a significant genotype x sex interaction as performed by a two-way ANOVA in SPM.

Region	*AA Females*	*AA Males*	*AG/GG Females*	*AG/GG Males*
R. Post. Thal	0.15 ± 0.09	-0.11 ± 0.17	-0.08 ± 0.09	0.08 ± 0.13
R. Lat. Amyg.	0.07 ± 0.16	-0.07 ± 0.18	-0.16 ± 0.21	0.13 ± 0.15
R. Vent. Pallidum	0.07 ± 0.16	-0.07 ± 0.18	-0.1 6± 0.21	0.13 ± 0.15

Table 22. Sex/Genotype Differences in Placebo Anticipation-Induced Changes in Regional μ-Opioid Receptor BP. Data are expressed as the mean ± SD. Means represent mean change in BP from anticipation of pain alone to anticipation of pain with placebo in regions in which there was a significant genotype x sex interaction as performed by a two-way ANOVA in SPM.

For the change in μ -opioid binding from pain anticipation to pain with placebo anticipation, it was determined that both the overall model and sex by gene interaction were significant in the following regions: right posterior thalamus (model: $F = 7.81$; $p = 0.001$; interaction: $F = 15.89$; $p = 0.000$), right lateral amygdala (model: $F = 3.54$; $p = 0.026$; interaction: $F = 10.60$; $p = 0.003$), and right ventral pallidum (model: $F = 3.54$; $p = 0.026$; interaction: $F = 10.60$; $p = 0.003$). There were no significant effects of sex or genotype for any of the regions. Bonferroni post-hoc comparisons between groups (AA Females, AA Males, AG/GG Females, AG/GG Males) for each region revealed that in the right posterior thalamus, AA Females had a significantly greater decrease in BP from pain anticipation to placebo anticipation than AG/GG Females and AA Males, whose BPs decreased ($p = 0.039$ and $p = 0.001$, respectively).

We then sought to determine if there were similar effects of genotype and sex in subjective ratings of psychophysical measures of the pain and placebo experience. For the reduction in ratings from pain alone to pain with placebo, a two-way ANOVA in SPSS revealed that there was no significant effect of sex, genotype or sex by genotype interaction in any of the psychophysical measures we used. (McGill sensory and affect, POMS TMD, PANAS fear, negative and positive, VAS intensity, unpleasantness, 0 to 20, 0 to 10, 10 to 20, anticipation of effectiveness, effectiveness, desire to work and anticipation/effectiveness differential).

For the reduction in ratings from pain anticipation to placebo anticipation, a two-way ANOVA in SPSS revealed that there was no significant effect of sex, genotype or

sex by genotype interaction in any of the psychophysical measures we used (PANAS fear, positive and negative).

For each of the regions mentioned above with significant ANOVA models and interactions, we performed Pearson correlations between change in BP and the females' average estradiol levels for both genotype groups. In the right anterior thalamus, there was a significant correlation between estradiol and BP change from pain to placebo for AA females ($r = 0.86$; $p = 0.003$) and a significant negative correlation between estradiol and BP change for AG/GG females ($r = -0.88$; $p = 0.048$). In the right lateral amygdala, there was a significant correlation between estradiol and BP change from pain to placebo for AG/GG females ($r = 0.94$; $p = 0.017$) and a negative but non-significant correlation for AA females ($r = -0.35$; $p = 0.36$). There were no significant correlations between estradiol and BP change in the other 10 regions. An independent samples T-test revealed that there were no significant differences in estradiol levels between AA and AG/GG females.

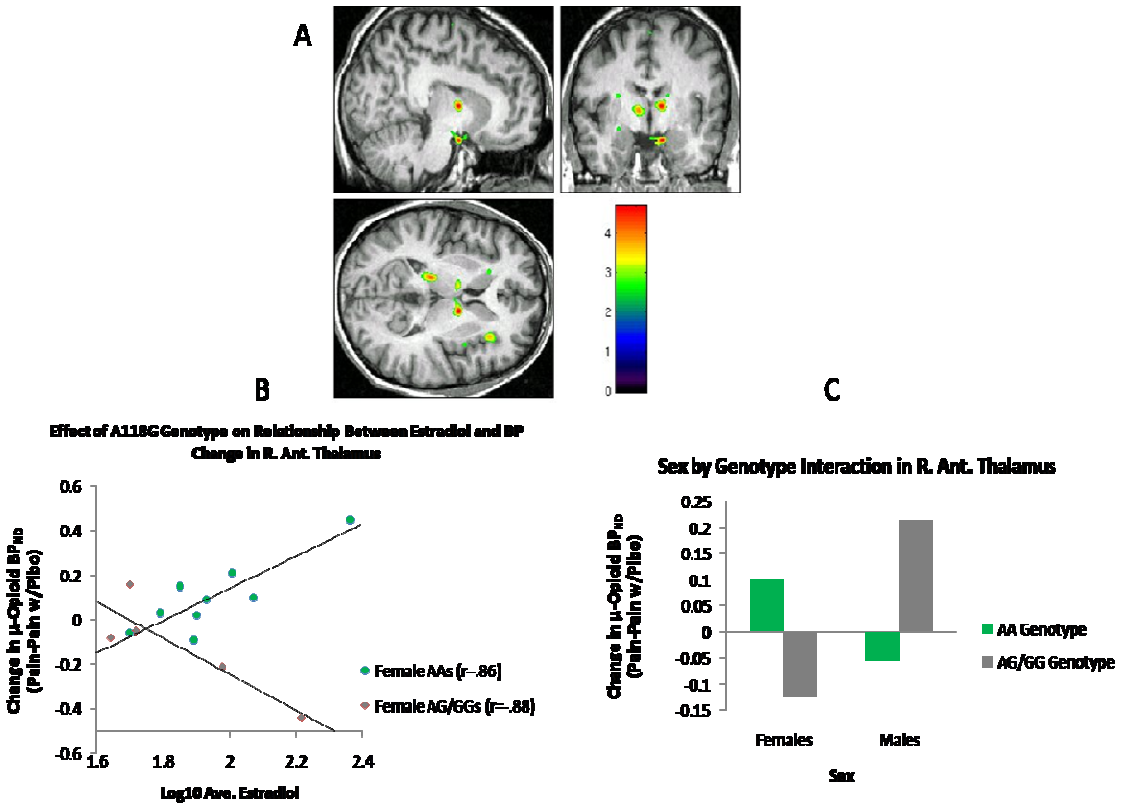

Figure 10. Sex by genotype interaction in the right anterior thalamus and correlations with estradiol levels. A) Right Anterior Thalamus. B) Opposite directions of the correlation of estradiol levels and right anterior thalamus change in μ-opioid receptor BP from pain alone to pain with placebo for females with and without G allele. C) Sex by genotype interaction in change in μ-opioid receptor BP from pain alone to pain with placebo in right anterior thalamus.

Discussion

We found a significant effect of A118G genotype and a sex by genotype interaction in regional μ-opioid receptor mediated neurotransmission during both the anticipation of pain with placebo administration and during the experience of pain with placebo administration. Further, we found opposing effects of female genotype on the relationship between estradiol levels and μ-opioid related neurotransmission in the amygdala and thalamus.

During the anticipation of pain with placebo, subjects with the G allele showed significantly greater μ-opioid mediated activation than those without it in the left nucleus accumbens, right medial/anterior thalamus, right amygdala/ventral pallidum, and left anterior insula. Conversely, subjects lacking the G allele had significantly greater activation in the periacqueductal gray and the right ventral pallidum/nucleus accumbens.

During placebo with pain administration, subjects with the G allele demonstrated significantly greater μ-opioid activation in bilateral amygdala, left anterior and posterior thalamus, bilateral ventral pallidum, left ventral and right dorsal caudate, left dorsal cingulate, and right anterior insula. Subjects without the G allele had greater activation in bilateral amygdala and the right nucleus accumbens.

Consistent the finding that knock-in mice with the A118G polymorphism demonstrated a sex by genotype interaction in morphine reward [112], we also found a sex by genotype interaction in mu-opioid mediated regional activation during the anticipation of placebo and during the administration of placebo. During placebo anticipation, we found sex by genotype interactions in the right posterior thalamus, right lateral amygdala, and the right ventral pallidum. The pattern of activation in each sex and genotype group was similar in all three regions: females lacking the G allele showed activation, while females containing the G allele deactivated. The opposite pattern occurred in males: male G allele carriers activated while males without the G allele deactivated.

We observed an identical pattern in several brain regions during placebo administration. These regions included the right anterior thalamus, right amygdala, right lateral amygdala, right putamen and right insula.

In order to account for the sex differences, we examined the effect of female A118G genotype on the relationship of estradiol and mu-opioid receptor mediated neurotransmission in the regions in which we found sex by genotype interactions. We found that in the right anterior thalamus, females without the G allele had significant positive associations of estradiol and mu opioid release during placebo, while female G allele carriers had a negative relationship between estradiol and mu opioid neurotransmission. In the right lateral amygdala, we found the opposite pattern: females lacking the G allele had a negative (albeit insignificant) association of estradiol with mu-opioid BP, while female G allele carriers had a highly significant positive relationship. Mague et al. [112] interpreted the polymorphism as conferring a loss of function, and they observed that carriers of the G allele seemed to have a blunted response to morphine reward and analgesia. In this context, our results similarly suggest that, at least in females, having the G allele seems to result in deactivation during placebo and placebo anticipation, perhaps rendering these individuals less effective placebo responders. However, we did not find any significant interactions between sex and genotype in psychophysical measures of the placebo experience. This is probably because the polymorphism has a very small biological effect that can be detected at the neurochemical level, but that may not translate into detectable differences in conscious processing.

Taken together, these findings indicate that the A118G polymorphism in the μ-opioid receptor is associated with altered functioning of the μ-opioid system during placebo analgesia, and that sex and genotype interact to introduce further variation in the

system. The sex and genotype interaction may be at least partially mediated by estradiol levels in females.

Chapter IV

PET Measures of Dopamine and Endogenous Opioid Neurotransmission in Smokers During Cigarette Smoking and Relation to A118G Genotype

Addiction research has established the importance of the concerted actions of dopamine and endogenous opioids in the initiation and maintenance of dependence on addictive substances, including nicotine. Both animal and human studies have shown that dopamine is released in reward and addiction-mediating brain regions in response to nicotine administration. Rat microdialysis studies demonstrate that endogenous dopamine transmission increases in the nucleus accumbens and dorsal caudate after acute nicotine administration [114]. [^{11}C]-raclopride positron emission tomography (PET) scanning in humans has shown ventral striatal dopamine (DA) release after smoking [115, 116].

Evidence for endogenous opioid activation in response to nicotine administration is more indirect. In an initial study, the nonselective opioid receptor antagonist, naloxone, diminished tobacco smoking and craving in humans [117]. Subsequent attempts to replicate this finding were met with mixed results: one group found that naloxone administration did not affect number of cigarettes smoked, number of puffs, or levels of expired carbon monoxide [118], and another study found that naltrexone had no effect on nicotine intake or enjoyment, although the perceived difficulty of abstaining from smoking was decreased [119]. However, Gorelick et al. [120] found that naloxone

administration reduced the number of cigarettes smoked, and Wewers et al.[121] demonstrated that chronic naltrexone administration reduced plasma nicotine levels, number of cigarettes smoked and satisfaction from smoking.

Animal studies have shown increases in opioid peptide metabolites [122], mRNA content [123], and endogenous opioid release in the central nervous system in response to nicotine administration [124]. A recent microdialysis study in rats measured increases in met- and leu-enkephalin levels in the striatum in response to nicotine administration (Li et al 2008, unpublished data). Long-acting opioid antagonists or mixed agonists-antagonists blocked nicotine-induced dopamine release in the rat nucleus accumbens and nucleus of the stria terminalis [125, 126], evidence suggestive of joint actions of dopamine and opioids in mediating nicotine's neurobiological effects.

In addition to the known dopamine release that occurs in response to cigarette smoking in smokers, along with the purported release of endogenous opioids, genetic polymorphisms probably introduce individual variation into the degree of neurotransmission that occurs. In particular, the A118G polymorphism in the μ-opioid receptor is known to affect several aspects of smoking, addiction, and reward related behavior in both humans and animals [127].

Here, with a new, larger sample of 20 male tobacco smokers, we extended the findings from our previous PET imaging pilot study that showed tobacco smoking activates both dopamine and endogenous opioid neurotransmitter systems [116]. We examined changes in DA D2 and μ-opioid receptor-mediated neurotransmission from a denicotinized cigarette smoking condition to an average nicotine cigarette smoking condition during positron emission tomography (PET) scanning. Activation of these

neurotransmitter systems is indicated by reductions in *in vivo* availability, or binding potential (BP), of DA D2 and μ-opioid receptors, which are measured by PET scanning of [^{11}C] raclopride for DA receptors and [^{11}C] carfentanil for μ-opioid receptors.

Materials and Methods

Subjects

Twenty healthy right-handed male volunteers, between the ages of 21 and 35 (mean ± SD: 25.8 ± 4.2), who smoked 5-30 cigarettes per day (mean ± SD: 18.4 ± 6.2) were recruited by advertisement. Subjects did not have histories of psychiatric or physical illness, were not substance-dependent except for nicotine, had not abused substances in the past year and had not taken psychoactive substances within the past month. All subjects were medication free. Subjects completed the Fagerstrom test to determine their level of tobacco dependence, and only volunteers whose scores were at or above 5 (scale of 1 to 10, where 10 is the highest) were included in the study. Written informed consent was obtained after the study procedures were explained to the subjects. The study was reviewed and approved by the Institutional Review Board for Human Subject Research and the Radioactive Drug Research Committee at the University of Michigan.

Subjects were instructed to abstain from smoking overnight the night before each scan, starting approximately 12 hours before the start of the 8:30 AM scan. They arrived at the PET suite at the University of Michigan Hospital Nuclear Medicine Division at 7:30 AM on both scan days and adherence to smoking abstinence was confirmed by breath carbon monoxide (CO) testing. Subjects exhaled air into a CO detector

(Vitalograph Breath CO Model BC1349, Vitalograph Inc., Lenexa, KS), which measures CO in parts per million (p.p.m.). Subjects who exhaled air CO levels of greater than 10 p.p.m. were given a re-interview to determine whether they had complied with the 12-hour abstinence. All subjects appeared compliant with the requirement.

Genotyping

10 cc of arterial blood was drawn from all subjects prior to scanning for genotyping analyses. Blood was sent to the Michigan Center for Translational Pathology (MCTP) laboratory Biorepository for genomic DNA extraction and purification. Extracted DNA was amplified via PCR using Roche's High Fidelity PCR kit according to manufacturer's instructions. Primer sequences were as follows: Forward: AGAGGAGAATGTCAGATGCTCAGC (5'- 3') and Reverse: ATGGAGTAGAGGGCCATGATCGTG (5'- 3'). Amplified product of 430 bp was confirmed using 1% agarose gel electrophoresis. Samples that were amplified successfully were sent to the University of Michigan Sequencing Core for sequencing using the same primers used for PCR amplification. Sequence chromatograms were evaluated to manually determine the A118G allele at position ~283 within the amplified sequence for each subject, using FinchTV 1.4.0 software (Geospiza, Inc.). Subjects were divided into two groups according to whether they carried the rare (G) allele. Therefore, the group without the G allele were homozygous for the A allele (AA group; n = 14), and the group containing the G allele were either homozygous or heterozygous (AG/GG

group; n = 5). One subject's sequencing results were indeterminable and he was left out of genotyping analyses.

Experimental Design

Subjects were scanned under 3 different conditions: baseline, denicotinized cigarette smoking and average nicotine smoking, with 2 radiotracers, [^{11}C]-raclopride and [^{11}C]-carfentanil, targeting DA D2/3 and μ-opioid receptors, respectively. Subjects rested quietly during the first 45 minutes for baseline measures, then smoked two denicotinized cigarettes at 45 and 55 minutes after the start of the 90 minute scan. During a subsequent, identical 90 min scan, subjects again rested quietly for the first 45 minutes for baseline measures, and then smoked two average nicotine cigarettes at 45 and 55 minutes. All subjects received both radiotracers, in a randomized and counterbalanced order on two separate days, for a total of four scans.

Subjects were instructed to smoke as they normally would. Volume of each puff was not controlled for. The order of smoking conditions was not randomized in order to avoid carryover effects of the average nicotine content cigarettes.

Visual analog scales (VAS) were administered before and after each smoking session to obtain subjective ratings of 'craving for a cigarette', 'relaxation', 'nervousness', 'wakefulness', and 'sickness' on a scale of 0 (none) to 5 (most ever). The Positive and Negative Affective Scale (PANAS) and the Profile of Mood States (POMS) were also administered before and after each smoking session to obtain ratings of subjective affective states affected by smoking.

Scanning Protocol

PET scans were acquired with a Siemens HR+ scanner in three-dimensional (3-D) mode (reconstructed FWHM resolution ~5.5mm in-plane and 5.0mm axially), with septa retracted and scatter correction. Participants were positioned in the PET scanner gantry, and two intravenous (antecubital) lines were placed. A light forehead restraint was used to eliminate intrascan head movement. [^{11}C] carfentanil was synthesized at high specific activity (>2000 Ci/mmol) by the reaction of [^{11}C] methyliodide and a nonmethyl precursor as described previously [128]. [^{11}C] raclopride was synthesized at high specific activity (>2000 Ci/mmol) by the reaction of O-desmethyl raclopride with ^{11}C-methyl triflate. In each of the two scans, 10–15 mCi was administered, with a mass of carfentanil injected of 0.048 ± 0.037 μg/kg per scan and a total mass of raclopride of 0.089 ± 0.047 μg/kg per scan. This ensured that the compounds were administered in tracer quantities, that is, subpharmacological doses occupying less than 1% of the available receptors. Fifty percent of the radiotracer doses were administered as a bolus, and the remaining 50% by continuous infusion for the remainder of the study.

Image and Data Acquisition

Images were reconstructed using iterative algorithms (brain mode; FORE/OSEM four iterations, 16 subsets; no smoothing) into a 128x128 pixel matrix in a 28.8 cm diameter field of view. Attenuation correction was performed through a 6-min

transmission scan (^{68}Ge source) obtained before the PET study, also with iterative reconstruction of the blank/transmission data followed by segmentation of the attenuation image. Small head motions during emission scans were corrected by an automated computer algorithm for each subject before analysis, and the images coregistered to each other with the same software [87]. Time points were then decay-corrected during reconstruction of the PET data. Image data were then transformed on a voxel-by-voxel basis into two sets of parametric maps: (a) a tracer transport measure (K_1 ratio), and (b) a receptor-related measure (distribution volume ratio, DVR). To avoid the need for arterial blood sampling, these measures were calculated using a modified Logan graphical analysis [88] using the occipital cortex (an area devoid of μ-opioid receptors) or the cerebellum (devoid of DA D2 receptors) as the reference regions. With the partial bolus, continuous infusion radiotracer administration protocol used, the Logan plot becomes linear by 5–7 min after the start of radiotracer administration, allowing the calculation of receptor measures early after each tracer administration. The slope of the Logan plot is equal to the $(B_{max}/K_d) + 1$ for this receptor site (receptor concentration divided by its affinity for the radiotracer) and it has been referred to as the DVR. B_{max}/K_d (or DVR - 1) is the 'receptor related' measure (BP, or receptor availability in vivo; B_{max} = concentration of receptors, K_d = receptor affinity for the radiotracer). As changes in B_{max}/K_d will cause a change in the slope of the Logan plot, we measured DVR during both the early and late phases of each scan. The slope during the early phase was estimated from 5 to 40 min post-injection, whereas the slope for the second phase was estimated from 45 to 90 min post-injection. Anatomical MRI scans were acquired before PET scanning on a 1.5 Tesla scanner (Sigma, General Electric, Milwaukee, WI).

Acquisition sequences were axial SPGR IR-Prep MR (TE = 5.5, TR = 14, TI = 300, flip angle = 20°, NEX = 1, 124 contiguous images, 1.5mm thickness), followed by axial T2 and proton density images (TE = 20 and 100, respectively; TR = 4000, NEX = 1, 62 contiguous images, 3mm thickness). K_1 and DVR images for each experimental period and MR images were coregistered to each other and to the International Consortium for Brain Mapping (ICBM) stereotactic atlas orientation. Statistical parametric maps of differences between conditions (denicotinized vs. average nicotine) were generated by anatomically standardizing the T1-SPGR MRI of each subject to the ICBM stereotactic atlas coordinates, with subsequent application of this transformation to the DA D2 and μ -opioid receptor binding maps. The accuracy of coregistration and nonlinear warping algorithms was confirmed for each subject individually by comparing the transformed MRI and PET images to each other and the ICBM atlas template.

Data Analysis

Differences within subjects and between conditions (effects of nicotine) were mapped into stereotactic space using z maps of statistical significance with SPM'99 and Matlab software, with a general linear model and correction for multiple comparisons. No global normalization was applied to the data, and therefore the calculations presented are based on absolute B_{max}/K_d estimates. To compensate for small residual anatomic variations across subjects and to improve signal to noise ratios, a 3-D Gaussian filter (FWHM 6mm) was applied to each scan. For each subtraction analysis, one-sample, two-tailed t-statistic values were calculated for each pixel using a smoothed pooled variance

across pixels. Significant differences and correlations were detected using a statistical threshold that controls a Type-I error rate at $p = 0.05$ for multiple comparisons, estimated using the Euler characteristic and the number of pixels in the gray matter and image smoothness [91]. Z scores were also deemed significant if they reached statistical thresholds after correction for the size of the cluster under consideration [113]. Correlation coefficients described in the text were calculated by extracting from the image data the values of voxels contained in an area where significant differences were obtained in the voxel-by-voxel analysis, down to a threshold of $p = 0.01$. Correlations between activation of neurotransmission and the changes in psychophysical measures levels were further calculated with two-tailed Pearson's correlations at $p < 0.05$.

Results

Decreased DA D2/3 BP_{ND} from denicotinized cigarette smoking to average nicotine cigarette smoking was observed in the left dorsal caudate (-14, 6, 11; T = 3.77), left and right ventral putamen (-26, 3, -8; T = 4.27; 28, 2, 1; T = 4.25, respectively), and right caudate (17, 18, 1; T = 3.92).

	Region	x, y, z coordinates, mm	Cluster Size, mm^3	T score	P value
After Denic > After Ave. Nic					
	Left Vent. Pallidum	-26, 3, -8	2957	4.27	0.000
	Left Dorsal Caud.	-14, 6, 11	Part of prev. cluster	3.77	0.001
	Right Vent. Pallidum*	28, 2, 1	988	4.25	0.000
	Right Caud.*	17, 18, 1	1033	3.92	0.000

Table 23. Dopamine D2/D3 Regional Activation After Smoking. (n=20), *Areas in which G allele carriers demonstrated significantly greater magnitudes of release after smoking than AA subjects.

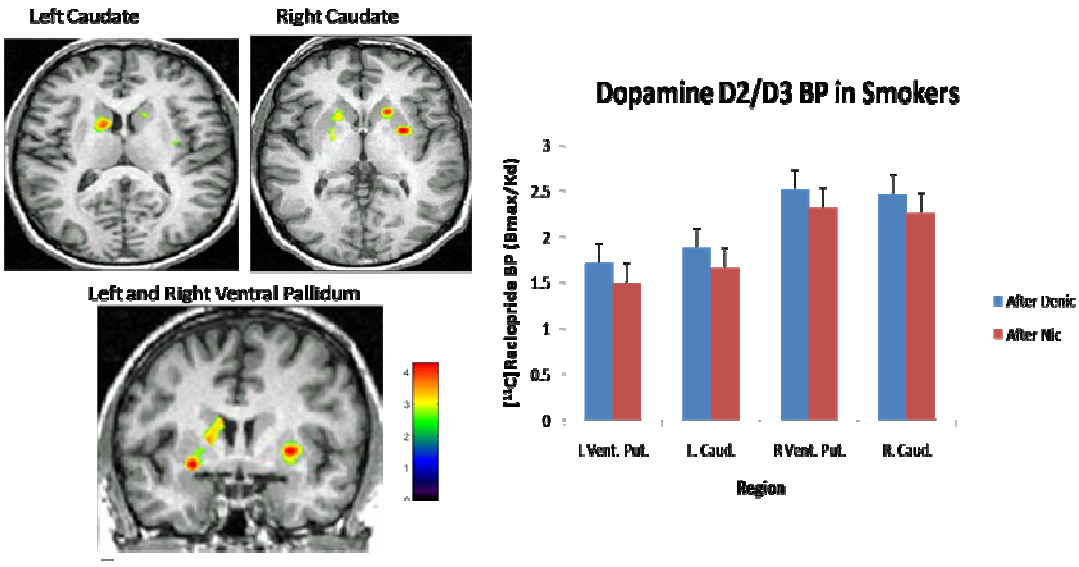

Figure 11. Dopamine D2/D3 Regional Activation After Smoking

The binding potentials for the four regions were extracted for analysis of the effect of A118G genotype on decreased BP after smoking. An independent samples, two-tailed *t* test revealed that carriers of the G allele demonstrated larger magnitudes of dopamine release in response to smoking than those homozygous for the A allele in the right caudate and right ventral pallidum ($t = 3.03$; $p = 0.008$ and $t = 3.91$; $p = 0.001$). A voxel by voxel whole brain SPM analysis using an independent samples *t* test did not reveal any other differences between genotype groups.

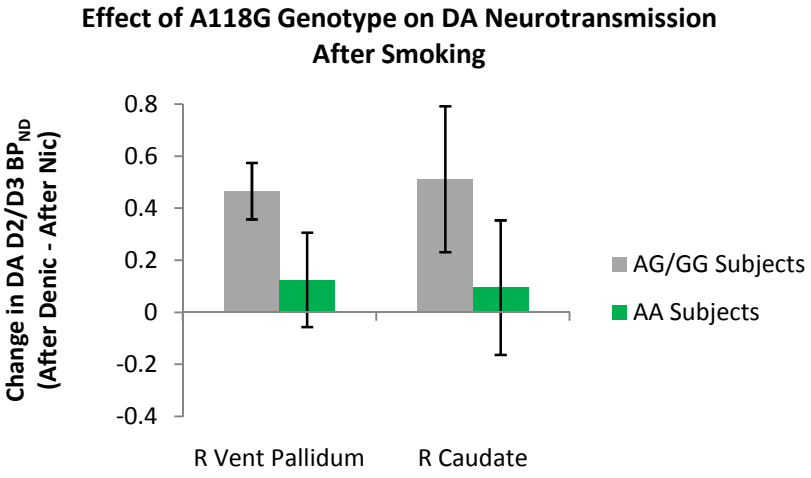

Figure 12. Effect of A118G Genotype on DA Neurotransmission After Smoking

Decreased μ-opioid BP_{ND} was observed in the left hypothalamus (-4, -2, -12; T = 6.42), left ventral-lateral prefrontal cortex (VLPFC) (-37, 46, -14; T = 4.47), right dorsal thalamus (5, -16, 13; T = 5.81,), right nucleus accumbens (11, 4, -3; T = 6.45), right dorsal cingulate (5, 14, 61; T = 3.77), and left insula (-43, 13, 4; T = 2.81). Increased μ-opioid BP_{ND} was observed in the left amygdala (-21, 2, -26; T = 4.75) and left ventral putamen (-23, 10, -16; T = 3.48).

	Region	x, y, z coordinates, mm	Cluster Size, mm^3	T score	P value
After Denic > After Ave. Nic					
	Right Nac	11, 4, -3	8363	6.45	0.000
	Left Hypothal.	-4, -2, -12	Part of prev cluster	6.42	0.000
	Right Dors. Thal.	5, -16, 13	Part of prev cluster	5.81	0.000
	Left VLPFC	-37, 46, -14	1887	4.47	0.000
	Right Dors. Cing.	5, 14, 61	1210	3.77	0.001
	Left Insula	-43, 13, 4	1232	2.81	0.006
After Ave. Nic > After Denic					
	Left Amyg.	-21, 2, -26	388	4.75	0.000
	Left Vent. Putamen	-23, 10, -16	357	3.48	0.001

Table 24. Mu-opioid regional activation after smoking

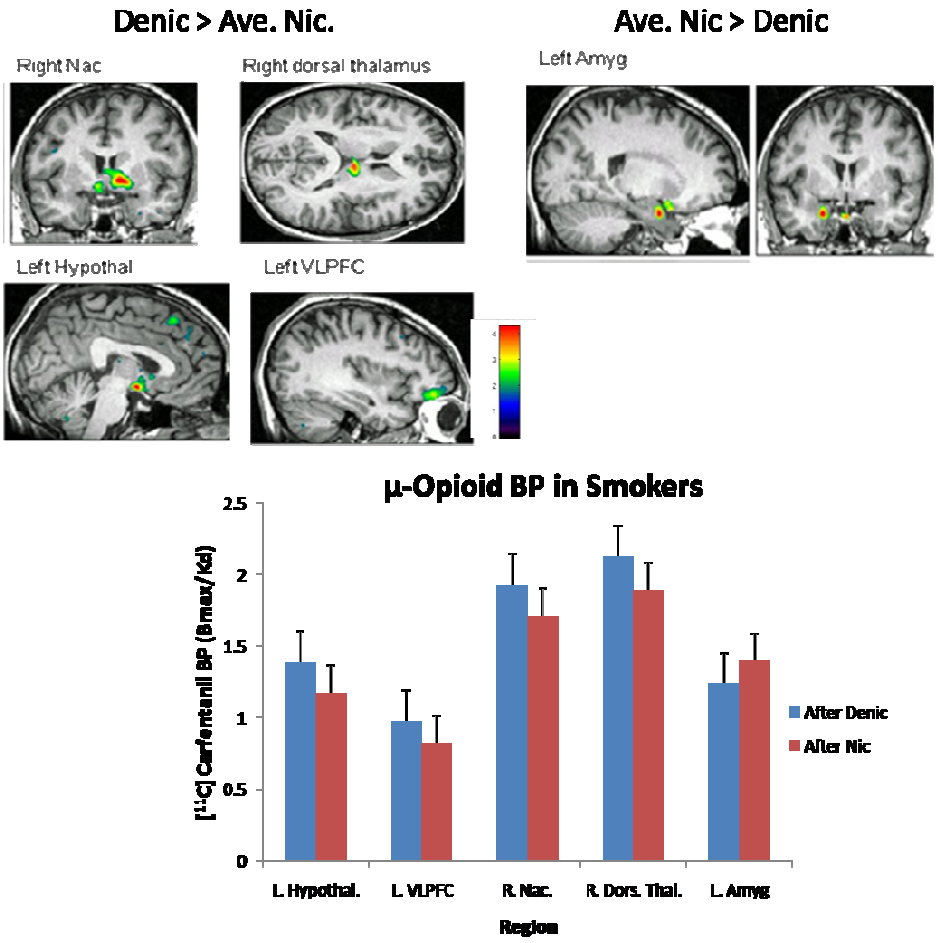

Figure 13. µ-opioid regional activation during smoking

A whole brain, voxel by voxel SPM analysis using an independent samples *t* test was then performed to determine if there were differences in endogenous opioid neurotransmission after smoking between genotype groups.

	Region	x, y, z coordinates, mm	Cluster Size, mm^3	T score	P value
AG/GG > AA					
	Right Med. Temp Gyrus	50, 9, -24	239	5.18	0.00
	Right Sup. Frontal Gyrus	9, -13, 64	349	4.91	0.00
	Right Temp. Pole	36, 20, -44	81	4.72	0.00
	Left Vent. Post. Thalamus	-10, -25, 2	380	4.48	0.00
	Right Vent. Pallidum	10, -1, -2	303	4.46	0.00
	Left Putamen/Nuc. Accumbens	-19, 7, -12	211	3.75	0.01
	Left Vent. Pallidum	-9, -2, -5	42	4.36	0.00
	Left Putamen/Nuc. Accumbens	-18, 9, -3	89	4.05	0.00
	Left Ant. Cing.	-14, 34, 19	648	4.02	0.00
AA > AG/GG					
	Left Nuc. Accumbens	-12, 9, -5	82	4.88	0.00
	Right Nuc. Accumbens	8, 9, -5	2481	4.73	0.00
	Left Dorsal Ant. Thalamus	-6, -4, 12	Part of prev. cluster	3.55	0.001
	Left OFC	-11, 38, -23	413	4.12	0.00

Table 25. A118G Genotype Effect on Regional Mu-Opioid Neurotransmission After Cigarette Smoking

For the raclopride scans, PANAS Fear significantly increased from after denicotinized smoking to after average nicotine smoking ($p = 0.05$). There were no significant changes between these two conditions for any other of the PANAS subscales. VAS ratings of sickness also increased significantly between conditions ($p = 0.03$), while all other VAS measures were not significantly different. POMS Vigor significantly

decreased from denicotinized smoking to nicotinized smoking ($p = 0.05$). No other POMS subscales were significantly different between conditions.

Decreased DA D2/3 BP_{ND} from denicotinized cigarette smoking to average nicotine cigarette smoking (dopamine activation) in the left caudate was positively correlated with a decrease in the Serenity subscale of the PANAS. ($r = 0.561$; $p = 0.01$) and was negatively correlated with a decrease in VAS sickness ($r = -0.601$; $p = 0.01$).

For the carfentanil scans, VAS ratings of craving significantly decreased from denicotinized cigarette smoking to average nicotine cigarette smoking ($p = 0.03$). There were no other significant changes between conditions for other VAS, PANAS, or POMS subscales.

Decreased μ-opioid BP_{ND} from denicotinized cigarette smoking to average nicotine cigarette smoking (endogenous opioid activation) in the left hypothalamus and in the left VLPFC was positively correlated with decreases in PANAS Shyness between conditions ($r = 0.455$; $p = 0.04$ and $r = 0.527$; $p = 0.02$, respectively). Decreased μ-opioid BP_{ND} was negatively correlated with increases in POMS Vigor ($r = -0.459$; $p = .042$). Increased μ-opioid BP_{ND} from denicotinized cigarette smoking to average nicotine cigarette smoking (endogenous opioid deactivation) in the left ventral putamen was negatively correlated with increases in PANAS Fatigue ($r = -0.449$; $p = 0.047$).

Discussion

These results replicate and extend our previous findings of modulation of both DA D2/3 and μ-opioid receptor-mediated neurotransmission during smoking. We have

demonstrated the feasibility and importance of studying both systems together using molecular imaging, as they are both functionally and behaviorally related in the regulation of reward and the addictions. Dopamine and endogenous opioids are also implicated in mediating responses to natural rewards, emotion, attachment, and stress. This methodology can be extended to the study of individual differences and their pathological disruption in these processes.

In the pilot study, Scott et al. observed reductions in raclopride BP from denicotinized cigarette smoking to average nicotine smoking in the left ventral basal ganglia [116]. We extended these findings in the current study with a larger sample, and consistent with the pilot study as well as other human and animal studies, we found that smoking average nicotine cigarettes results in decreased raclopride BP in the basal ganglia, specifically the left dorsal caudate, left and right ventral pallidum, and the right caudate. Dopamine release in these regions may mediate the rewarding aspects of nicotine and the saliency and reinforcing effects of nicotine-related stimuli, as prior animal studies have shown that ventral striatal and nucleus accumbens dopamine release is involved in the reinforcing aspects of nicotine [129]. A PET study in smokers found that raclopride displacement after smoking in the caudate and posterior putamen is associated with the pleasurable effects of smoking [130].

Also consistent with the Scott et al. pilot study, we observed decreased carfentanil BP from the denicotinized smoking to average nicotine conditions in several regions related to reward and stress. Scott et al. observed decreased carfentanil BP in the dorsal and rostral anterior cingulate, which are involved with reward anticipation [131], anticipation and evaluation of rewarding versus nonrewarding events [132], as well as

affect and antinociception [52, 133]. The present study also revealed activation in the dorsal anterior cingulate (although not in the rostral cingulate), as well as in several additional areas: left insula, left hypothalamus, left VLPFC, right dorsal thalamus, and right nucleus accumbens. Although the present study found carfentanil BP reduction in only one region in common with that of the Scott study, our additional results are likely due to the larger sample size of the current study, and presumably present a more complete picture of μ-opioid activity in response to nicotine intake.

A somewhat surprising finding, consistent across both the pilot and present studies, was an increase in carfentanil BP_{ND} from denicotinized to average cigarette smoking in the amygdala. This finding may be indicative of a non-specific, nicotine-independent effect of smoking. Because subjects were blind to the nicotine content of the cigarettes, it is feasible that smoking a denicotinized cigarette confers many of the same neurochemical and psychophysiological effects as that of a regular cigarette. Indeed, measures of craving significantly decreased after denicotinized smoking. This effect is placebo-like in nature, and is probably a result of the saliency and positive associations connected to cigarettes, regardless of nicotine content. Future studies should seek to tease apart the specific and non-specific effects of cigarette smoking in order to draw a more comprehensive conclusion about the neurochemical and psychophysiological bases of the reinforcing effects of smoking. One way to achieve this would be to measure the change in BP_{ND} from the baseline, non-intervention condition to the denicotinized smoking condition. It is likely that opioid and dopamine activation would be observed throughout the motivational circuitry, especially in the nucleus accumbens, and that this activation would be overlapping but regionally distinct from the activations attributed to

nicotine-related aspects of smoking.

A118G genotype was also found to affect μ-opioid and dopamine activity in response to smoking. Specifically, smokers carrying the G allele showed significantly larger magnitudes of dopamine activity after smoking in the right ventral pallidum and right caudate. Studies have shown that the polymorphism results in reduced receptor mRNA expression and protein levels [106, 112] and increased binding affinity of β-endorphin and higher potency of receptor activation [134], perhaps as a compensatory mechanism for reduced receptor expression. Other studies have shown that carriers of the G allele are more sensitive to the rewarding aspects of alcohol, including intoxication, stimulation, sedation, and happiness [127]. It may be that the enhanced dopamine activation shown by G allele carriers in response to smoking indicates a similar enhanced sensitivity to the effects of nicotine, as well as a compensatory mechanism for lower numbers of μ-opioid receptors. Since smoking results in endogenous opioid release, which acts to disinhibit dopamine neurons in the VTA, dopamine neurotransmission could also be altered in relation to the decreased expression of μ-opioid receptors in G allele carriers.

For μ-opioid related neurotransmission after smoking, G allele carriers had a more diffuse pattern of regional activation than subjects homozygous for the A allele, whose activation was concentrated only in the nucleus accumbens, thalamus and orbitofrontal cortex. This pattern may also reflect a compensatory mechanism in that G allele carriers must release a greater amount of endogenous opioids in more regions to account for the loss of receptor expression.

The regions that were activated in response to nicotine form part of the circuitry

involved in mediating the cognitive, emotional, motivational, and subjective effects of drugs of abuse. The left ventral lateral prefrontal cortex and dorsal anterior cingulate have been found to have lower gray matter volumes in smokers compared to nonsmokers [135]. The insula is thought to play a role in awareness of bodily states like urges and cravings, and a recent retrospective study of smokers with insular lesions found that their cigarette addictions were more easily disrupted than in smokers with lesions in other brain regions [136]. The left VLPFC is involved in executive functions like response inhibition [137] and working memory [138], which are impaired in smokers [139, 140]. The anterior cingulate is implicated in anticipation of reward, response to cigarette cues [141], resisting cigarette craving [142] and evaluating decision-making outcomes. The thalamus contains one of the highest densities of nicotinic acetylcholine receptors in the brain [143]; activation of these receptors leads to subsequent dopamine release in the nucleus accumbens.

Taken together, this study showed *in vivo* activation of dopamine and endogenous opioid neurotransmitter systems in response to cigarette smoking in human smokers, and that a polymorphism shown to affect receptor expression and binding, as well as reinforcing effects of nicotine and alcohol, affects regional activation in both neurotransmitter systems after smoking.

Chapter V

Conclusion

This work shows that individual differences related to genetics and sex contribute substantial variation to the μ-opioid response to behaviors that engage the motivational system, in both healthy and addicted humans. This research provides substantial evidence to convincingly make the case that the methodology used in research and clinical interventions should be expanded to include assessments of individual factors that could affect results or treatment outcome. Generic study methods and treatments may not address the full range of variation in behavioral responses or pathology. Additionally, genotyping particular risk-conferring polymorphisms or taking sex into account may contribute to the ability to predict pain or addiction treatment outcomes or susceptibility to these conditions.

First, we found substantial sex differences in regional μ-opioid related neurotransmission in response to both placebo analgesia and the anticipation of placebo analgesia. These sex differences occurred throughout the motivational circuitry and notably in the nucleus accumbens and hypothalamus. Furthermore, several of the regions in which there were sex-specific responses showed sexually divergent associations with pain-related measures of psychophysical reactions to pain and placebo. This finding provides further evidence for both a sexual dimorphism in brain regions and circuitry as

well as various emotional and cognitive aspects of the pain and placebo experience. It appears that the structural differences between males and females confer sex-specific affective and cognitive coping strategies in reaction to pain and in initiation of placebo responding. Conversely, innate sex-specific psychological responses to pain and expectation of relief may engage differing modulatory pathways. Reproductive hormones appear to at least partially account for these differences, as estradiol levels were associated with regional μ-opioid activity during anticipation of placebo and during experience of placebo.

The second set of experiments expanded on the previous findings of sex differences in placebo response and attempted to explore whether these differences could be accounted for in part by a genetic polymorphism within the μ-opioid receptor that presumably affects μ-opioid signaling within the motivational system. We found differences in μ-opioid regional activation and psychophysical responses during both the anticipation of placebo and during the experience of placebo analgesia between subjects with and without the polymorphism. Additionally, this effect was further influenced by sex, such that sex and genotype interacted to produce sex/genotype specific neurochemical responses to placebo anticipation and placebo receipt.

The last data chapter addressed the same neurochemical system, but in a different subject population using a different experimental method to engage activation within the motivational circuitry. We found that cigarette smokers exhibit release of both dopamine and endogenous opioids in response to cigarette smoking. It is important to characterize individual variation in μ-opioid functioning in both healthy and addicted populations in

order to develop a comprehensive picture of normal function and individual vulnerability factors.

The characterization of altered μ-opioid receptor related neurotransmission according to A118G genotype and sex, in both placebo analgesia and smoking, may provide one possible mechanism by which certain individuals become susceptible to dysregulation of the system. Additionally, these results may provide a template for creating a "vulnerable" individual profile. In the Introduction section, it was mentioned that chronic pain may put a subset of individuals at greater than normal risk for addiction. The frequent occurrence of comorbid psychiatric disorders with chronic pain and addiction suggests common etiological mechanisms in some people. As this body of work has demonstrated, one likely candidate for a system whose dysregulation leads to pain and addiction comorbidities is the motivational brain network.

Work here and elsewhere suggests that having the G allele of the A118G polymorphism confers different functional consequences for males versus females. In females, for example, the G allele is associated with increased likelihood of abstinence from smoking after quitting, reduced reinforcing value of nicotine, reduced reward responsivity to morphine in female AG/GG rats [112, 127, 144]. In a pain study of humans, female G allele carriers had higher heat pain ratings than homozygous A females or male G allele carriers [145]. The current work indicated a regionally specific deactivation of μ-opioid mediated neurotransmission during placebo analgesia and its anticipation for AG/GG females.

Studies to date have failed to come to a consensus on the specific mechanisms by which the A118G polymorphism alters receptor expression, signaling, analgesic

response, and addictive behaviors. Similarly, it is not entirely clear what mechanisms can account for the genotype-related differences we observed in μ-opioid neurotransmission, in the general placebo analgesic response and during smoking, as well as in the sex-specific effects. However, some reasonable conjectures can be made using the evidence that exists thus far.

Several studies have found that in cell cultures, G118 receptor variants have increased binding affinity for β-endorphin, but not other ligands [134, 146]. Additionally, one study found substantially stronger signaling by β-endorphin in G118 receptor variants [134]. Other studies are consistent in showing decreased mRNA and protein expression of G118 receptor variants in both human post mortem tissue and in transfected cells, as well as decreased B_{max} in cell cultures with the G118 receptor [105, 106, 147]. These results at first glance appear contradictory, but they suggest that receptors containing the G allele have enhanced β-endorphin signaling capacity, and their diminished receptor expression may reflect a compensatory mechanism by which endogenous opioid activity is G allele carriers maintains a homeostatic balance.

Interestingly, another study found that in brain tissue from post mortem G-allele carriers, agonist-induced signaling, but not receptor expression or binding affinity, was diminished in tissue from secondary somatosensory cortex but not thalamus [148]. The fact that A118G genotype can have regionally specific effects on receptor function may account for inconsistencies in previous studies, as well as the regional differences in μ-opioid neurotransmission we observed in subjects undergoing placebo analgesia and cigarette smoking. Regional variation in receptor function may also account for the differences we observed in measures of internal affect, mood disturbance, and pain

intensity between genotype groups. For example, greater magnitude of activation was observed in the thalamus in G allele carriers in placebo during pain anticipation and during pain; this observation is consistent with the study that found G carriers did not have altered signaling within the thalamus. The functional effects of the A118G polymorphism may not be as simple as a gain or loss of function, and are not consistent across regions, as the literature has shown, and thus it is not surprising that there is no straightforward pattern in activation differences between the genotype groups.

The A allele carriers, but not G carriers, had placebo-induced decreases in pain intensity. This finding may point to a superior placebo response in A carriers, and it is consistent with many studies that have found the G allele to be associated with substance dependence and reduced reward responsivity. The greater number and wider dispersal of regions activated in response to placebo in G carriers may not necessarily be contradictory to the assumption that they are inferior responders. It may instead indicate that they have a greater need for pain relief (i.e. pain unpleasantness is more salient), and that there is a compensatory widespread release of endogenous opioids. If it is true that the G allele receptors are less prevalent throughout the brain, more endogenous release may be necessary to compensate for the reduced expression.

There is also the issue of sex differences and hormone interaction with genotype. The findings of differences between genotype groups may be less than clear cut because of the inclusion of both sexes in these analyses. Indeed, when sex and genotype interactions were explored, a consistent pattern emerged across several regions: female carriers of the G allele deactivated in response to placebo during pain anticipation and during pain, while male G carriers showed the opposite response. Estrogen levels of

female A carriers and female G carriers were correlated with BP_{ND} in opposite directions, suggesting estrogen may have divergent effects at the receptor, depending on the variant. Estrogen is generally believed to have a dampening effect on pain, and is correlated with greater μ-opioid activation during placebo. Perhaps reduced availability of receptors in G allele carriers results in fewer locations for estrogen to contribute to opioid signaling. It would be beneficial to examine baseline μ receptor availability in both genotype groups, in order to confirm the feasibility of such a scenario.

Taken together, these studies suggest that having the G allele as a female confers a general dampening of the reward/motivational/pain-modulatory circuitry. It is unclear whether this might result in a phenotype that is vulnerable to combined chronic pain and addiction, or one or the other, especially considering that many other gene variants, environmental context, and psychosocial factors contribute to and interact with each other to form the etiological basis of these diseases. However, modeling sex and A118G genotype interactions can be applied as a tool in future research studies and may be used to clarify the vulnerability or resiliency patterns of this particular phenotype.

The results from these studies advance the field in the following ways:

1) They provide a better understanding of how sex and genotype influence physiological mechanisms of placebo analgesia; this leads to a better grasp of how to design clinical trials and studies in a way that separates experimental or treatment effects from placebo effects

2) They provide a better understanding of how sex and genotype influence placebo analgesia so that it can be harnessed in the treatment of pain, either by itself or as an adjunct to pharmacological analgesia, and so these treatments can be personalized
3) They provide a more complete picture of how sex and genotype influence the more generalized μ-opioid mediated pain regulatory system, which will lead to more individually tailored treatment of chronic pain diseases, post-surgical pain, and injury-related pain.
4) They expand our knowledge about how A118G genotype affects general functioning of motivational circuitry during a challenge that engages it, in both healthy and addicted subjects and it provides template for future studies of the system in controlling for various individual factors

Future Directions

The present body of work, while adding to our knowledge of neurochemical and psychophysical functions of the motivational circuitry, leaves many questions unanswered while creating several new questions.

Estrogen appears to partly mediate female neurochemical responses to placebo analgesia. If sex-specific responses to placebo analgesia are in part hormonally mediated, it is critical to examine how male reproductive hormones, namely testosterone, affect neurochemical placebo responses. While measuring hormonal levels and correlating them with endogenous opioid release during placebo suggests a relationship, a further step in would involve manipulating hormone levels in reproductive-age women and measuring

placebo responses in both low and high estrogen and/or progesterone states. Alternatively, post-menopausal women with and without hormone replacement therapy could be used to examine these effects as well.

The A118G genotype is associated with opposite directions of correlations between estradiol and regional μ-opioid activity in placebo analgesia. While we did not find any significant interactions between genotype and estradiol levels, a significant interaction may emerge in studies in which reproductive hormone levels are manipulated, or in women who are studied twice during their cycles. Such a finding might provide a more definitive mechanism by which A118G genotype affects female μ-opioid activity in placebo analgesia. Examining interactions between A118G genotype and testosterone levels in males would also help explain the sex by genotype interaction we found.

Given the effects of sex, genotype, and genotype by sex interactions we found within the motivational μ-opioid mediated network in healthy volunteers, it would be helpful to extend these analyses to smokers. A118G genotype affects endogenous opioid and dopamine release during smoking in males, but it likely also interacts with sex in this experimental model. There are also probably sex differences in the neurochemical response to smoking, as there are documented sex differences in the reinforcing effects of nicotine and in smoking cessation [144], as well as significant sex by A118G genotype interactions for the reinforcing effects of nicotine [127]. Future studies should seek to expand on the current work by addressing these possibilities.

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