FUNCTIONAL CHARACTERIZATION OF THE *OPRM1* A118G SINGLE NUCLEOTIDE POLYMORPHISM IN MICE

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ABSTRACT

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Endogenous opioids acting at μ-opioid receptors (MOPR) mediate many biological functions. Pharmacological intervention at these receptors has greatly aided in the treatment of acute and chronic pain, in addition to other uses. However, the development of tolerance and dependence has made it difficult to adequately prescribe these therapeutics. A common single nucleotide polymorphism (SNP), A118G, in the MOPR gene can affect opioid function and, consequently, has been suggested to contribute to individual variability in pain management and drug addiction. Investigation into the role of A118G in human disease and treatment response has generated a large number of association studies across various disease states as well as physiological responses. However, characterizing the functional consequences of this SNP and establishing if it causes or contributes to disease phenotypes have been significant challenges. To clarify the functional mechanisms linking the *OPRM1* A118G SNP to addiction and analgesia phenotypes, we derived a mouse model possessing the equivalent nucleotide/amino acid substitution in the mouse *Oprm1* gene. I first evaluated MOPR expression and function using molecular and pharmacological techniques and, subsequently, investigated how these alterations affected basal and morphine-evoked responses using a variety of

behavioral tasks. In order to better understand the synaptic and circuit-level alterations conferred by this SNP, we employed voltage-sensitive dye imaging in hippocampal slice preparations to evaluate basal and opioid-stimulated neuronal responses. Mice harboring this SNP (A112G) demonstrated several phenotypic similarities to humans carrying the A118G SNP, including reduced mRNA expression and morphine-mediated antinociception. We found additional phenotypes associated with this SNP including significant reductions of receptor protein levels, morphine-mediated hyperactivity, and locomotor sensitization, as well as sex-specific reductions in the rewarding properties of morphine and the aversive components of naloxone-precipitated morphine withdrawal. Functionally, this SNP reduced opioid-stimulated excitatory responses in the hippocampus. Together, these findings extend our understanding of the functional consequences of this SNP and support evidence suggesting that this SNP results in a loss of receptor function. Further cross-species analysis will allow us to investigate mechanisms and adaptations present in humans carrying this SNP.

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CHAPTER 1: *OPRM1* SNP (A118G): INVOLVEMENT IN DISEASE DEVELOPMENT, TREATMENT RESPONSE, AND ANIMAL MODELS

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Abstract

Endogenous opioids acting at μ-opioid receptors mediate many biological functions. Pharmacological intervention at these receptors has greatly aided in the treatment of acute and chronic pain, in addition to other uses. However, the development of tolerance and dependence has made it difficult to adequately prescribe these therapeutics. A common single nucleotide polymorphism (SNP), A118G, in the μ-opioid receptor gene can affect opioid function and, consequently, has been suggested to contribute to individual variability in pain management and drug addiction. Investigation into the role of A118G in human disease and treatment response has generated a large number of association studies across various disease states as well as physiological responses. However, characterizing the functional consequences of this SNP and establishing if it causes or contributes to disease phenotypes have been significant challenges. In this manuscript, we will review a number of association studies as well as investigations of the functional impact of this gene variant. In addition, we will describe a novel mouse model that was generated to recapitulate this SNP in mice. Evaluation of models that incorporate known human genetic variants into a tractable system, like the mouse, will facilitate the understanding of discrete contributions of SNPs to human disease.

Keywords: alcohol; morphine; analgesia; dependence; pain; stress

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Terms:
Human SNP: A118G = Asn40Asp = N40D
Mouse SNP: A112G = Asn38Asp = N38D
Rhesus Macaque SNP: C77G = Pro26Arg = P26R

1. Introduction

The opioid system plays a role in diverse biological functions, including reward, analgesia, and stress responsivity (Kreek and Koob, 1998; Vaccarino and Kastin, 2000). Therapeutically, opioids are commonly prescribed for their effective analgesic properties. However, the response to treatments varies widely between individuals leaving many people taking the wrong dose, experiencing unbearable side effects, or receiving inadequate therapy. Additionally, chronic use is marred by habituation, tolerance, and the development of dependence, which occur in varying degrees depending on the individual. The ability to better predict clinical outcomes based on individual differences to opioid therapeutics would greatly reduce the trial and error of finding suitable drugs and doses, and could reduce the number of patients developing drug dependence.

Individual variability results from a complex interaction of genetic and environmental factors. However, linkage disequilibrium, genome-wide association, case-controlled, and family-controlled studies have demonstrated the heritability of complex behaviors and response to drug treatments, suggesting that specific genes or alterations in genes may be responsible for the differences in behavior. Common genetic variations among individuals include single nucleotide polymorphisms (SNPs), in which a single nucleotide of the genome is altered. SNPs occur every 100-300 base pairs and account for approximately 90% of human genetic variation. The nature of the change produced by the SNP greatly depends on which nucleotide is being altered and where this change occurs in the gene. For instance, synonymous SNPs will alter the nucleotide without changing the resulting amino acid (also called a “silent mutation”). Non-synonymous SNPs are

produced when the nucleotide substitution alters the resulting amino acid. Additionally, these alterations can occur in promotor, exonic, or intergenic regions and, consequently, may differentially affect transcription, processing, stability, translation, folding, transportation, and ultimately, function of the corresponding gene product.

In human populations, a commonly investigated SNP (rs1799971) occurs in exon 1 of the μ-opioid receptor gene (*OPRM1*), in which an adenine to guanine substitution (A118G) exchanges an asparagine for an aspartic acid at a putative N-glycosylation site (N40D). It is common in persons of European (15–30%) and Asian ancestry (40–50%), with lower prevalence in African American and Hispanic populations (1-3%) (Bergen et al., 1997; Gelernter et al., 1999; Tan et al., 2003). Despite the vast number of papers investigating the role of this SNP in human disease and drug responses, a consensus has yet to be reached on its functional consequences. The A118G SNP has been implicated in a wide variety of disorders, such as drug addiction and stress responsivity, and in treatment responses, including dependence and pain reduction; however, the mechanisms that mediate these alterations have not been determined. In this manuscript, we will review the relevant literature investigating the role of this SNP in human disease and treatment response, molecular and cellular function, and animal models that may help explain these effects. Indeed, several comprehensive reviews describe the role of pharmacogenetics, including *OPRM1* and other genes, in specific disease states and treatment responses, such as alcoholism/addiction (LaForge et al., 2000; Dick and Foroud, 2003; Enoch, 2003; Oslin et al., 2006; Anton et al., 2008; Haile et al., 2008) and pain (Lotsch et al., 2004; Skorpen et al., 2008; Kosarac et al., 2009); therefore, in this

review we focus on the elucidation of the functional significance of the A118G SNP in disease states both in humans and animal models.

2. The μ-opioid receptor (MOPR)

2.1. MOPR form and function

Early investigation of the endogenous targets of opioid drugs identified three main classes of opioid receptors: μ, δ, and κ. The cloning and characterization of the opioid receptors have impacted our understanding of their gene and protein structures. The MOPR is a member of the G-protein-coupled receptor (GPCR) family and interacts with (G_i/G_o) heterotrimeric G-proteins. Activation of the receptor and subsequent dissociation of the G-proteins results in the opening of G-protein-gated inwardly-rectifying K^+ (GIRK) channels, inhibition of voltage-gated Ca^{2+} channels, and reduction of adenylyl cyclase-mediated cAMP production, all of which serve to decrease membrane potential, neuronal excitability, and neurotransmitter release in addition to affecting second-messenger systems and gene expression.

Receptor activation is achieved through binding of endogenous or exogenous ligands. β-endorphin, the peptide encoded by proopiomelanocortin (*POMC*), has high affinity and selectivity for the MOPR and is considered the endogenous MOPR ligand. In addition, a separate class of peptides has been proposed as μ-selective: the endomorphins (Zadina et al., 1997). The preproenkephalin gene encodes enkephalin, the endogenous ligands for the δ receptor, which has modest affinity for the MOPR as well (Raynor et al., 1994). A large number of opioids and non-opioid ligands exist for this receptor; however,

those most commonly prescribed for their effective analgesic properties include morphine, codeine and oxycodone.

2.2. Genetic Variation in Human OPRM1

The human MOPR gene, *OPRM1*, [chromosome 6q24-q25] spans over 200kb with at least 9 exons and 19 different splice variants under the control of multiple promoters (Shabalina et al., 2009). The initial receptor subtype, MOPR-1, spanning approximately 80kb and containing 4 exons (http://genome.ucsc.edu), is abundantly expressed and has been most intensely studied. Its haplotype structure includes three large blocks with >100 polymorphisms reported (http://www.hapmap.org). In addition to the exon 1 A118G, there is *in vitro* functional evidence for only a few of these other polymorphisms. Two promoter polymorphisms, G–554A and A–1320G have been shown to affect transcription: G–554A decreases MOPR transcription but is extremely rare (MAF<0.001) and the A–1320G variant increases transcription, although the exact transcription factor binding to the site is unknown (MAF= 0.21) (Bayerer et al., 2007). In exon 3, G779A (R260H), G794A (R265H), and T802C (S268P) have been shown to decrease receptor coupling and signaling (Koch et al., 2000; Befort et al., 2001; Wang et al., 2001). Other *OPRM1* polymorphisms that have been identified and associated with pain or opioid dependence including a short tandem $(CA)_n$ repeat (Kranzler et al., 1998), C17T (A6V), which is found primarily in African Americans(Hoehe et al., 2000), and C440G (S147C), which is extremely rare in the general population (MAF< 0.006) (Glatt et al., 2007). To date, none of these polymorphisms have *in vitro* evidence supporting a functional consequence.

3. A118G and drug dependence

Mesolimbic dopamine (DA) neurons in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc) are part of a well-defined pathway involved in reward processing (Nestler, 2005). GABAergic interneurons in the VTA maintain a tonic inhibition over dopaminergic neurons. Binding of β-endorphin or morphine to MOPRs on these interneurons will decrease their activity, resulting in disinhibition of the DA neurons and elevations of DA in the NAc (Johnson and North, 1992). This dopamine influx has been associated with reward and reinforcement and is believed to contribute to the development of drug dependence (Wise and Bozarth, 1985; Di Chiara and Imperato, 1988).

A number of studies have examined *OPRM1* as a candidate for genetic contribution to the risk for substance dependence. The minor G118 allele has been associated with an altered susceptibility for developing drug dependence, with some studies suggesting that the SNP is a risk factor and others finding it to be protective, in addition to several studies that did not report any significant contribution of the G118 allele (Table 1). For instance, in a sample of 476 Caucasians grouped according to drug history – alcohol alone, alcohol and nicotine, or alcohol, nicotine, and illicit drug use and compared to two control groups – it was found that individuals homozygous for the A118 allele were present in greater frequency in the drug groups compared to controls. The absence of the G118 allele in the drug groups suggested it was protective against developing drug dependence (Schinka et al., 2002). Alternatively, in drug-dependent individuals in Eastern European and Russian populations, the G118 allele occurred more frequently (Zhang et al., 2006a). In addition, several studies using linkage disequilibrium

or haplotype analysis (Crowley et al., 2003; Luo et al., 2003), case- or family-controlled studies (Gelernter et al., 1999; Franke et al., 2001; Xuei et al., 2007), or meta-analyses of past studies (Arias et al., 2006) failed to detect a significant involvement of A118G in drug dependence.

3.1. Alcohol

Alcohol has been shown to affect a wide variety of transmitter systems; however, the rewarding and reinforcing aspects of alcohol intake seem to be mediated by the opioid system (Gianoulakis, 2004; Oswald and Wand, 2004). Indeed, acute alcohol administration has been shown to cause β-endorphin release measured in the plasma (Gianoulakis and Barcomb, 1987) or in reward-related brain regions (Rasmussen et al., 1998). MOPRs appear critical for mediating alcohol effects as MOPR knock-out mice show reduced ethanol intake and reward (Roberts et al., 2000; Hall et al., 2001). Likewise, antagonism of the receptor by naloxone in rats (Reid et al., 1986) and naltrexone in humans (O'Malley et al., 1992; Volpicelli et al., 1992) has been shown to reduce alcohol intake.

Studies investigating alcohol-dependence specifically have reported positive associations with the A118G SNP (Rommelspacher et al., 2001; Kim et al., 2004; Bart et al., 2005; Nishizawa et al., 2006; Miranda et al., 2010), no association (Bergen et al., 1997; Sander et al., 1998; Gscheidel et al., 2000; Kim et al., 2004; Loh el et al., 2004), or a protective effect (Town et al., 1999) in individuals possessing the G118 allele (Table 1). [For a detailed review of 12 of these clinical association studies, see (van der Zwaluw et al., 2007).].

A number of clinical studies have investigated altered effects of alcohol in individuals with the G118 allele. For instance, in a sample of 38 moderate and heavy drinkers without a history of alcohol problems or quit attempts, it was shown that G118 allele-carriers reported higher feelings of intoxication, stimulation, sedation, and happiness compared to A118 allele-carriers. Subjects carrying the G118 allele were also three times more likely to report a family history of alcohol use (Ray and Hutchison, 2004). In male heavy drinkers, G118 allele-carriers showed automatic approach tendencies for alcohol and other appetitive stimuli, but not for generally positive or negative stimuli (Wiers et al., 2009) and reported greater alcohol craving in a cue-reactivity task (van den Wildenberg et al., 2007). A recent study investigating the involvement of A118G in adolescent alcohol misuse found that a higher percentage of G118 allele-carriers tested positive for an alcohol use disorder, and that the G118 allele was associated with increased self-reports of drinking in order to enhance positive affect (Miranda et al., 2010). Functionally, G118 allele-carriers have demonstrated an increased BOLD response in fMRI in the orbitofrontal cortex, ventromedial prefrontal cortex, and striatum in response to alcohol and alcohol-related cues (Filbey et al., 2008). Additionally, a significant increase in dopamine receptor sensitivity, as measured by increases in apomorphine-induced growth hormone secretion, was found in G118 allele-carrying alcoholics following one week of alcohol abstinence (Smolka et al., 1999).

One of the more therapeutically relevant findings regarding the A118G SNP is in the treatment of alcoholism. It was discovered that individuals carrying the G118 allele were more likely to respond positively to naltrexone treatment (Crowley et al., 2003). In this study, there were 3.5 times more naltrexone-treated G118 allele-carriers, compared

with A118 allele-carriers, who did not relapse to heavy drinking. There were no differences in rates of abstinence, suggesting that individuals with the G118 allele may better handle alcohol exposure without fully relapsing to heavy drinking. There were no differences between genotypes for those receiving placebo treatments, demonstrating that the benefit of the G118 allele is specific for naltrexone treatment and does not confer an enhanced ability for the individual to refrain from relapse. Subsequent studies investigating the involvement of this SNP in naltrexone response have replicated these initial findings (Oslin et al., 2003; Anton et al., 2008; Kim et al., 2009; Oroszi et al., 2009). For instance, using a haplotype-based approach, the A118G locus, and not SNPs found in the same haplotype block, contributed to the improved response to naltrexone treatment in alcohol-dependent subjects with the G118 allele (Oroszi et al., 2009). However, other studies failed to find an association between the G118 allele and an improved response to naltrexone (Gelernter et al., 2007; Arias et al., 2008; Tidey et al., 2008) or nalmefene treatment (Arias et al., 2008).

The mechanisms underlying the interaction of the A118G SNP and the potential benefits of naltrexone in alcohol dependence are unknown. It is thought that the effect of naltrexone is mediated by its ability to block the elevations in β-endorphin induced by alcohol administration, which are thought to contribute to the subsequent euphoria. Indeed, it has been shown that naltrexone has a greater propensity to block alcohol-induced highs in individuals with the G118 allele (Ray and Hutchison, 2007), which may, in part, explain the reduction in rates of relapse. In contrast, it was shown that the urge to drink following naltrexone treatment was greater in G118 allele-carriers (McGeary et al., 2006). However, it should be noted that in this study, conducted in non-treatment seekers,

the urge to drink was evaluated following exposure to alcohol cues rather than alcohol administration.

Cortisol responses have also been implicated in drinking behavior. A study in non-treatment-seeking alcohol-dependent subjects found that G118 allele-carriers showed a trend towards decreased cortisol responses to stress, but elevated alcohol craving and intake following the stress (Pratt and Davidson, 2009). In animal models of addiction, discrete brain substrates have been identified for drug-, cue-, and stress-mediated reinstatement to drug-seeking behavior (Kalivas and McFarland, 2003). As such, it is possible that this SNP may confer benefits in certain situations, but could serve as a detriment (or not play a role) in others. [For additional information on alteration in stress-related responses, see Chapter 1, section 4.2 "OPRM1 A118G and physiological response: Stress".]

3.2. Heroin

Heroin directly stimulates MOPRs when converted to morphine in the brain and periphery. Similarly to alcohol, this results in activation of reward-related pathways, initially resulting in euphoria, but eventually leading to abuse and dependence. Studies evaluating the role of G118 allele in heroin dependence have reported positive associations (Szeto et al., 2001; Bart et al., 2004; Drakenberg et al., 2006; Kapur et al., 2007), negative associations (Bond et al., 1998; Tan et al., 2003), or no association (Shi et al., 2002; Glatt et al., 2007) (Table 1). For instance, one study found that approximately 90% of G118 allele-carriers were heroin users. Additionally, they found that preproenkephalin and preprodynorphin levels, which were reduced in heroin-dependent subjects, were even lower in G118 allele-carriers compared with A118 allele-

carriers (Drakenberg et al., 2006). Shi and colleagues found that the G118 allele was associated with elevated daily intake of heroin in dependent subjects, though they did not find a significant effect of genotype and heroin dependence (Shi et al., 2002). A positive response to initial drug exposure is typically associated with continued use and abuse. In a study investigating the relationship between initial drug response and *OPRM1* SNPs, the G118 allele was not associated with positive or negative subjective responses to first-time heroin use (Zhang et al., 2007), suggesting that differences in rates of dependence may not be explained by differences in initial euphoric experience.

3.3. Nicotine

The rewarding properties of nicotine are, in part, mediated by opioid transmission. For instance, it has been shown that nicotine stimulates the release of endogenous opioids in reward-related brain regions (Davenport et al., 1990; Pomerleau, 1998), blockade of MOPRs with systemic injections of naloxone blocks acute nicotine reward (Walters et al., 2005), MOPR null mutant mice do not show nicotine reward (Berrendero et al., 2002), and repeated treatment with nicotine elevates MOPR mRNA in the VTA (Walters et al., 2005). Human studies have reported an association of chromosome 6, which contains *OPRM1*, with nicotine dependence (Sullivan et al., 2004; Vink et al., 2004) (Table 1). Another study, however, found that the A118G SNP was not significantly associated with nicotine dependence, though it was in high linkage disequilibrium with haplotypes that did reach significance, suggesting there may be other SNPs nearby that confer an increased susceptibility for developing nicotine dependence (Zhang et al., 2006b). As in the case with alcohol, studies examining differences in response to nicotine administration have revealed some interesting associations. For instance, female G118

allele-carriers report attenuated rewarding effects of nicotine (Ray et al., 2006). In a separate study, nicotine was administered following the induction of a positive or negative mood via pictures and music. Smoking was reported as more rewarding to A118 allele-carriers when in a negative mood than it was for those with the G118 allele, suggesting that the A118 allele-carriers may respond more to the mood-enhancing properties of nicotine (Perkins et al., 2008). Accordingly, increases in cerebral blood flow to brain regions associated with cigarette craving were detected in smokers with the A118 allele compared to the G118 allele (Wang et al., 2008).

Consistent with alcohol studies, the A118G SNP may better predict treatment outcomes and relapse rates for those attempting to quit smoking rather than predict the susceptibility for developing nicotine dependence. Lerman and colleagues have shown that smokers with the G118 allele are significantly less likely to relapse and report fewer abstinence symptoms than smokers homozygous for the A118 allele (Lerman et al., 2004). A group in the UK reported that female G118 allele-carriers had significantly higher quit rates than female A118 allele-carriers; while the reverse was true for males (Munafo et al., 2007). It should be noted, however, that this study obtained DNA from only 50% of subjects, raising the possibility of ascertainment bias. This evidence supporting the role of the G118 allele in dependence phenotypes, such as liking and craving in chronic smokers, is more robust than evidence for association with the development of nicotine dependence.

3.4. Methamphetamine

The effects of methamphetamine (MA), which are predominantly mediated by dopaminergic and serotonergic systems, may also involve the endogenous opioid

systems. Indeed, work in animals has shown that naloxone administration can block behavioral sensitization to repeated MA exposure (Chiu et al., 2005). However, the few studies investigating the effect of the A118G SNP in MA dependence failed to find an association (Ide et al., 2004; Ide et al., 2006) (Table 1).

4. A118G and physiological response

4.1. Pain and analgesia

The relationship between altered pain thresholds and analgesic responses to opioid administration for the A118G SNP has been well characterized. In a variety of populations, the G118 allele has been associated with elevated pain responses and decreased pain thresholds (Sia et al., 2008; Tan et al., 2009) and a reduced response to morphine or other opioids for patients receiving treatment for post-operative or chronic pain (Klepstad et al., 2004; Fillingim et al., 2005; Chou et al., 2006a; Chou et al., 2006b; Coulbault et al., 2006; Janicki et al., 2006; Oertel et al., 2006; Reyes-Gibby et al., 2007; Campa et al., 2008; Hayashida et al., 2008; Landau et al., 2008; Sia et al., 2008; Tan et al., 2009) (Table 2). Additionally, in healthy volunteers carrying the G118 allele, higher concentrations of alfentanil, an opioid analgesic, were required for pain relief following electrical pain stimulation (Oertel et al., 2006); of interest, this dose did not increase respiratory depression, suggesting it may be safe to give a higher dose of opioid analgesics to patients that carry the G118 allele. Other studies, however, have reported divergent results, showing that G118 was associated with reduced pain responses. For instance, in healthy volunteers receiving different experimental pain procedures, the G118 allele was associated with reduced pain responses to pressure (Fillingim et al.,

2005). Additionally, males carrying the G118 allele rated thermal pain lower than those carrying the A118 allele. However, the G118 allele-carrying females reported higher pain scores following this thermal pain administration, consistent with previous literature demonstrating elevated pain responses (Fillingim et al., 2005).

Morphine-6β-glucuronide (M6G) is an active metabolite of morphine that has a greater analgesic potency but a reduced potency in affecting respiratory depression (Mantione et al., 2005). In G118 allele-carrying subjects, there was a reduced potency of M6G in eliciting an analgesic response, though there was no difference in M6G-induced respiratory depression (Romberg et al., 2005). A reduction in M6G-induced miosis was also found in G118 allele-carriers (Lotsch et al., 2002).

MOPRs also play a role in social pain, described as the feelings that result from social rejection, separation, or loss. Accordingly, the G118 allele was associated with increased self-reported sensitivity to rejection (Way et al., 2009). Subsequent fMRI measurement of neural responses in these individuals found greater activation in the dorsal anterior cingulate cortex and anterior insula while experiencing social rejection, suggesting a decrease in MOPR inhibitory modulation in G118 allele-carriers. These brain regions are associated with both physical and social pain (Eisenberger and Lieberman, 2004). Together, these data support a loss of function of the MOPR in individuals harboring the G118 allele in some, but not all, responses mediated by the same compounds.

4.2. Stress response

Activation of the hypothalamic-pituitary-adrenal (HPA) axis plays an integral role in responses to stress. Following a stressor, corticotropin-releasing factor (CRF) is

released from the paraventricular nucleus (PVN) of the hypothalamus, stimulating POMC synthesis in the pituitary and the release of two POMC metabolites: adrenocorticotropic hormone (ACTH) and β-endorphin. Acting on the adrenal glands, ACTH stimulates the release of glucocorticoids (cortisol in primates and corticosterone in rodents; CORT), which can then have many central and peripheral effects. MOPRs located on CRF neurons in the PVN serve to tonically inhibit HPA axis stimulation; thus, differences in endogenous opioid transmission or receptor activity can alter basal CORT levels or stress-mediated CORT responses. Just as genetic differences can affect response to drugs (pharmacogenetics), alterations in responses to an individual's own biologically active compounds (physiogenetics) are influenced by genetic differences (Kreek and LaForge, 2007). Indeed, individuals with the G118 allele have baseline elevations in CORT levels (Hernandez-Avila et al., 2003; Bart et al., 2006). Several studies have shown that the CORT-response to a behavioral stressor is lower in G118 allele-carriers (Chong et al., 2006; Pratt and Davidson, 2009).

In contrast, a greater CORT response has been observed in G118 allele-carriers following a naloxone challenge, which may suggest an elevated tonic inhibition of CRF by β-endorphin (Hernandez-Avila et al., 2003; Chong et al., 2006; Hernandez-Avila et al., 2007). Interestingly, one of these studies found population-specific effects in which G118 allele-carriers of European descent displayed elevations in CORT responses to naloxone, while G118 allele-carriers of East Asian descent did not (Hernandez-Avila et al., 2007). These population-specific differences could suggest that the A118G SNP is in high linkage disequilibrium (LD) with other SNPs that may mediate the observed alterations.

5. Functional relevance of A118G

Most studies to date have examined the functional consequences of this SNP *in vitro* using various cell culture systems. Initial studies identified an elevated binding affinity of β-endorphin, but not exogenous ligands, in the G118 variant to 3-fold higher than that of the A118 in AV-12 cells stably expressing the human MOPR (hMOPR) variants (Bond et al., 1998). Additionally, β-endorphin was found to be three times more potent in activating GIRK channels in *Xenopus* oocytes injected with *in vitro* transcribed mRNAs for the A118 or G118 variants (Bond et al., 1998). Together, these data suggested a gain-of-function of the MOPR as a consequence of the A118G point mutation. However, subsequent studies using other cell culture systems – COS cells (Simian fibroblasts) (Befort et al., 2001) or HEK 293 cells (Human embryonic kidney) (Beyer et al., 2004) – were less conclusive with regard to this altered function of the G118 allele. Another consequence of MOPR activation, Ca^{2+} inhibition, was investigated using rat sympathetic superior ganglion (SCG) neurons expressing either the A118 or G118 variant of the hMOPR. In these studies, the potencies of both DAMGO- and morphine-mediated Ca^{2+} current inhibition (but not morphine-6-glucuronide or endomorphin I) were increased in SCG neurons expressing the G118 variant (Margas et al., 2007), again suggesting enhanced response for some but not all opioid compounds.

A human post-mortem study examined allele-specific mRNA expression from heterozygous individuals with the A118G polymorphism and found significant reductions in mRNA transcribed from the G118 allele. Additionally, the authors transiently expressed both variants of the MOPR in CHO cells and showed a reduction in mRNA

and protein expression with the G118 allele (Zhang et al., 2005). The mechanisms underlying the decrease in expression is unclear. As the mutation occurs in a coding region, rather than a promotor, it might seem unlikely that transcription would be affected. However, using *in silico* tools (bioinformatics), it has been proposed that the A118G SNP may inactivate three transcription factor binding sites while creating two new ones, including a p53 site (Pang et al., 2009), suggesting that cis-acting factors could explain the alterations in expression. While transcriptional regulation using exonic sequence is rare, some examples do exist. Using Mfold technology, in which theoretical mRNA folding can be evaluated for different sequences, it was shown that the G118 variant demonstrated altered folding compared to other permutations which could affect mRNA stability (Zhang et al., 2005; Johnson et al., 2008).

Further evidence for a decrease in MOPR expression was demonstrated by studies showing a reduction in B_{max}, indicative of a lower receptor number, following [^{3}H]-DAMGO binding using both transient and stable expression of MOPR in AV-12 and HEK293 cells (Kroslak et al., 2007). Additionally, there was a decrease in agonist-mediated cAMP signaling for morphine, methadone, and DAMGO, but not β-endorphin, using stable expression in the two lines; this alteration in cAMP signaling was not seen in cell lines transiently expressing the receptor (Kroslak et al., 2007). Another study using HEK293 cells stably expressing the hMOPR also found a decrease in B_{max} using DAMGO binding in the G118 variant, though they did not find alterations in binding affinity or signal transduction (Beyer et al., 2004). Conversely, a recent study investigating MOPR expression, binding, and signaling in post mortem human tissue from G118 allele-carriers found decreased agonist-induced receptor signaling efficacy in

tissue from secondary somatosensory cortex, but not thalamus. However, there were no alterations in receptor expression or binding affinity (Oertel et al., 2009). Together, studies demonstrating that the G118 variant results in significantly reduced levels of MOPR expression and/or signaling suggest a loss-of-function of the mutation, while others reporting an increase in affinity and signaling suggest a gain-of-function.

6. Species-specific SNPs in *OPRM1*: Spontaneous and generated

6.1. Monkey orthologue (C77G)

Non-human primate research has been invaluable in the study of human disease and behavior. A conserved SNP, in which a cystine is replaced by a guanine at position 77 (C77G) resulting in a substitution of arginine with proline (R26P), occurs in the N-terminal arm of the monkey orthologue *OPRM1* and has been suggested to be comparable to the A118G SNP (Miller et al., 2004). In this initial characterization of 32 male and female macaques, 44% were homozygous for the C77-allele, 50% heterozygous, and 6% homozygous for the G77 allele. It was demonstrated that monkeys possessing the minor allele (G77), had lower CORT levels both at baseline and following dexamethasone suppression and subsequent ACTH challenge. In addition to lower CORT levels, the G77 allele was associated with an increase in aggression threat, which is the early communicative aspect of aggression occurring prior to actual physical actions. By expressing these receptor-coding regions in HEK-293, the authors were able to identify an elevated affinity of the G77 allele for β-endorphin (~3.5 fold), but not exogenous ligands, similarly to original *in vitro* work in the A118G SNP (Bond et al., 1998). Subsequent studies have shown an increase in attachment behavior in G77 allele-carrying

infants, who displayed increased distress vocalization during protracted periods of mother-infant separation and increased maternal contact during mother-infant reunion (Barr et al., 2008), possibly reflecting increased attachment reward through enhanced β-endorphin function. However, in light of the recent reports of increased social pain associated with the G118 allele (Way et al., 2009), an alternate explanation could be that the infants carrying the G118 allele had a greater sensitivity to maternal rejection, possibly through reduced MOPR function in the neocortex.

A haplotype containing the C77G SNP was shown to result in an increase in MOPR mRNA expression (Vallender et al., 2008). Though the human A118G SNP has been shown to reduce mRNA expression rather than increase it, this serves as further evidence that coding region SNPs may alter mRNA production, folding, or stability. Interestingly, in studying alcohol consumption in these macaques, a sex × genotype interaction was found, in which male carriers of the G77 allele showed elevated ethanol preference and consumption (Barr et al., 2007). In a subsequent study, macaques carrying the G77 allele showed both enhanced alcohol preferences following vehicle administration and greater reductions of alcohol preference following naltrexone administration (Barr et al., 2010).

6.2. Knock-in mouse model (A112G)

Despite information gained from human post mortem tissues, a full understanding of the *OPRM1* A118G polymorphism requires extensive biochemical characterization aligned with behavioral analysis, which is not feasible in human subjects. The mouse is a tractable model system to study behavioral effects of this SNP while at the same time allowing for detailed molecular and biochemical analysis in vivo. Homologous

recombination technology can now be used to generate point mutations in mice for those genes in which human SNPs have been identified. This approach, however, has not been fully utilized due in part to the labor-intensive procedures involved in building the complex targeting vectors required. Recent advances and the availability of bacterial artificial chromosome (BAC) vectors have streamlined this process, making the use of knock-in mice a natural progression to investigate human disease. To gain insight into the role of the A118G variant in humans, we have generated the equivalent point mutation in mice, A112G, which alters the same amino acid coding from an asparagine to aspartic acid at position 38 (Asn38Asp; N38D), eliminating an N-linked glycosylation site.

A number of molecular, biochemical, and behavioral alterations that resemble those previously identified in human and *in vitro* studies have been identified in G112 mice (Mague et al., 2009). For instance, the presence of the G112 allele results in decreased MOPR mRNA and protein expression. This decrease in receptor expression was also seen using [^{3}H]-DAMGO binding, though there were no alterations in affinity for β-endorphin or exogenous ligands (morphine, DAMGO, or naloxone). Mice with the G112 allele showed only a modest elevation in locomotor activity following acute morphine administration and failed to develop sensitization to repeated, intermittent injections. Similarly, G112 mice had reduced morphine-induced antinociceptive responses, though they showed similar signs of tolerance following repeated treatments. A sex × genotype interaction was found in measures of hedonia: female G112-carriers did not display a preference for morphine-associated environments nor did they demonstrate an aversion to environments associated with naloxone-precipitated withdrawal.

Since the A112G SNP alters MOPR expression, some of the behavioral outcomes could be explained by the reduction in protein levels. Indeed, studies investigating opioid responses in MOPR knockout heterozygous mice have found similar reductions in morphine-mediated antinociception using tail flick and hot plate tests (Sora et al., 1997) and sex-specific decreases in alcohol reward using voluntary ethanol consumption and place-conditioning paradigms (Hall et al., 2001). However, these sex-specific differences in ethanol reward were due in large part to elevated responses in wild type female mice compared to wild type male mice, with little differences reported between male and female heterozygous mice. Indeed in wild type male mice, no ethanol preference was observed; therefore, it is difficult to assess whether or not heterozygous MOPR knockout males displayed ethanol reward deficits. In addition to reducing expression levels, it should be emphasized that one consequence of the A112G knock-in mouse (and the A118G SNP) is the deletion of an N-linked glycosylation site. Glycosylation plays a role in receptor sorting, expression, trafficking, ligand binding, and signal transduction (Fan et al., 1997; Zhang et al., 2001; Rathz et al., 2002); alterations in these processes could affect MOPR function in ways distinct from protein level changes.

The A112G mouse model, similarly to the C77G non-human primate model, possesses analogous phenotypes as those reported in human studies and identifies new behaviors that have not been investigated. The A112G mouse model seems to replicate the loss-of-function phenotypes (e.g., decreased expression, reduced morphine-mediated antinociception, decreased hedonic reward), though it should be noted that decreases are not present in all morphine-mediated behaviors, suggesting that the alterations are dependent on other factors, including brain region, other neurotransmitter systems, and

sex. Future work will investigate the effects of the A112G SNP on alcohol consumption and stress responsivity to evaluate whether or not these animals display any gain-of-function phenotypes as suggested by human studies.

7. Conclusion

The A118G SNP in the human *OPRM1* gene has been studied intensely and implicated in a variety of disease states and treatment responses. In particular, alterations in receptor function resulting from this SNP are believed to contribute to the susceptibility for developing drug dependence. The strongest evidence for a beneficial effect of the A118G polymorphism stems from studies evaluating the response to naltrexone for alcohol and nicotine consumption, highlighting the importance of pharmacogenetics when devising treatment options to determine who may be more likely to benefit from a given therapy. In addition to choosing treatment options, understanding the mechanisms underlying these beneficial effects may allow us to develop therapies for individuals with the common A118 allele.

As acute overdose and long-term dependence are concerns when treating acute and chronic pain with opiates, it is important to minimize the dose prescribed in order to reduce the amount of drug the patient receives. The evidence showing a diminished response to opioid-mediated analgesia in G118 subjects illustrates the need for utilizing pharmacogenetics when developing treatment options. For instance, though G118 patients require higher doses of opioids for pain management, it has also been shown that they may be more resistant to the respiratory depressive effects of alfentanil than A118

allele-carrying individuals, suggesting that there may be less risk in providing these higher doses (Oertel et al., 2006).

Cross-species experiments allow investigators to validate both pharmacogenetic and physiogenetic phenotypes. Despite the potential of this approach to yield valuable information for treatment development, few mouse/human comparisons of SNPs have been reported. The derivation of the *Oprm1* A112G mouse will allow for such studies to investigate aspects of this SNP that have yet to be delineated in human populations. We know from human studies that the *OPRM1* A118G SNP can impact the response to treatment; however, we have learned from studies in mice that this SNP also influences sex-specific drug behaviors, differences that have been largely ignored in human association studies. Moreover, we anticipate that this mouse model may assist in drug design to generate effective analgesics and other opioid therapies without the risk of abuse liability.

Table 1. Association studies investigating *OPRM1* A118G involvement in drug dependence in humans

Direction of effect	Drug	Method	Finding	Population (number of subjects)	Reference
Protective	Substance dependence	Association	Greater frequency of A118 in drug groups	European American (476)	(Schinka et al., 2002)
Risk	Substance dependence	Haplotype analysis	Greater frequency of haplotype containing G118 in drug-dependent individuals	Eastern European and Russian (720)	(Zhang et al., 2006a)
No association	Substance dependence	Case-controlled association		European American (891)	(Gelernter et al., 1999)
No association	Substance dependence	Case- and family-controlled association		German Caucasian	(Franke et al., 2001)
No association	Substance dependence	Family-based association		European American (1923, from 219 multiplex alcohol dependent families)	(Xuei et al., 2007)
No association	Substance dependence	Haplotype analysis		European American and African American (213 opioid dependence and 196 "supercontrols")	(Crowley et al., 2003)
No association	Substance dependence	Linkage disequilibrium		European-American and African American (442 substance dependence and 234 control)	(Luo et al., 2003)
No association	Substance dependence	Meta-analysis		Caucasian (German, Finnish, Swedish, EA), African-American, Asian, Hispanic, Native-American (8000)	(Arias et al., 2006)
Risk	Alcohol	Case-controlled association	G118 associated with alcohol dependence	Swedish (467 alcohol-dependent and 170 healthy volunteers)	(Bart et al., 2005)
Risk	Alcohol	Case-controlled association	G118 associated with alcohol dependence	Japanese (64 alcohol dependent and 74 control)	(Nishizawa et al., 2006)
Risk	Alcohol	Association	G118 associated with more days drinking per month, but no significant increase in alcohol-dependence	Korean (112 alcohol dependent and 140 control)	(Kim et al., 2004)

Table 1 continued.

Direction of effect	Drug	Method	Finding	Population (number of subjects)	Reference
Risk	Alcohol	Association	Trend towards increase of G118 in alcohol-dependent subjects	German (327 alcohol-dependent, 340 control)	(Rommelspacher et al., 2001)
Risk	Alcohol	Association	G118 associated with increased alcohol use disorder (AUD) diagnoses in adolescents	Mostly Caucasian (27 AUD and 160 control)	(Miranda et al., 2010)
Protective	Alcohol	Association	A118 associated with risk for alcoholism	Mexican Americans (365 alcohol-dependent and 338 control)	(Du and Wan et al., 2009)
Protective	Alcohol	Association	A118 had two-fold greater risk for alcohol dependence	Caucasian (105 alcohol-dependent and 122 control)	(Town et al., 1999)
No association	Alcohol	Case-controlled association		German (327 alcohol dependent; and 340 control)	(Sander et al., 1998)
No association	Alcohol	Association		US-Caucasian, Finnish-Caucasian, Souithwestern American-Indian (791)	(Bergen et al., 1997)
No association	Alcohol	Association		German (327 alcohol-dependent and 340 control)	(Gscheidel et al., 2000)
No association	Alcohol	Association		Tawainese (158 alcohol-dependent, 149 control)	(Loh et al., 2004)
Risk	Heroin	Association	G118 associated with heroin dependence	Swedish (139 heroin-dependent and 170 control)	(Bart et al., 2004)
Risk	Heroin	Association	90% of G118 were heroin users	European Caucasian (118)	(Drakenberg et al., 2006)
Risk	Heroin	Association	2.5-fold higher frequency of G118 in opioid dependent subjects	Indian (126 opioid dependent and 156 control)	(Kapur et al., 2007)
Risk	Heroin	Association	G118 associated with heroin dependence	Chinese men (200 heroin dependent and 97 control)	(Szeto et al., 2001)
Protective	Heroin	Association	A118 associated with heroin in Indian, but not East Asian populations	Indian (20 dependent, 117 control) Malaysian (25 dependent, 131 control) Chinese (52 dependent, 156 control)	(Tan et al., 2003)

Table 1 continued.

Direction of effect	Drug	Method	Finding	Population (number of subjects)	Reference
Protective	Heroin	Association	A118 associated with heroin dependence in Hispanic subjects	African-American (46 dependent, 16 controls) Caucasian (60 dependent, 44 control) Hispanic (116 dependent, 78 control)	(Bond et al., 1998)
No association	Heroin	Association		Chinese (48 heroin-dependent, 48 control)	(Shi et al., 2002)
No association	Heroin	Family-based association		Chinese (1208 from 473 families with at least two siblings with opioid dependence)	(Glatt et al., 2007)
Risk	Nicotine	Linkage disequilibrium	QTLs on chromosome 6 associated with nicotine dependence	Dutch twins (536 DZ from 192 families)	(Vink et al., 2004)
Risk	Nicotine	Linkage analysis	Chromosome 6 associated with nicotine dependence	Caucasians from New Zealand (sibling pairs from 129 families with nicotine dependence)	(Sullivan et al., 2004)
No association	Nicotine	Haplotype analysis		Caucasians twins of European ancestry (688)	(Zhang et al., 2006)

Table 2. Association studies investigating *OPRM1* A118G in pain experience and opioid-mediated analgesia.

Drug	Method	Finding	Population (number of subjects)	Reference
Morphine	Pain Score and morphine consumption	Reported pain and morphine consumption lowest in AA and highest in GG	Singapore and Han Chinese women receiving morphine for post-cesarean pain (588)	(Sia et al., 2008)
Chronic morphine	Pain Score and morphine consumption	GG required more morphine; no difference in reported pain	Norwegian Caucasians receiving morphine for cancer pain treatment (207)	(Reyes-Gibby et al., 2007)
Chronic morphine	Morphine consumption	GG required more morphine to control pain	Norwegian Caucasians receiving morphine treatment for cancer pain (99)	(Klepstad et al., 2004)
Morphine	Morphine consumption	GG consumed more morphine that AA and AG; no difference in reported pain	Taiwanese patients receiving morphine following arthroplastic knee surgery (147)	(Chou et al,, 2006b)
Morphine	Morphine consumption	G118 required more morphine 24 hours following surgery, but not at 48; no differences in reported pain	Taiwanese female patients receiving morphine following total abdominal hysterectomy (80)	(Chou et al., 2006a)
Morphine	NRS and PPI	G118 were poor responders to morphine treatment	Italian Caucasian patients receiving morphine treatment for cancer pain (145)	(Campa et al., 2008)
Morphine	Morphine consumption	Trend towards an increase in morphine consumption in G118	Mostly Caucasian colorectal surgical patients (74)	(Coulbault et al., 2006)
Morphine or fentanyl	Opioid consumption	GG required more opioid at 24-hr post operation compared to AA and AG	Japanese patients receiving opioid treatment for open abdominal surgery pain (138)	(Hayashida et al., 2008)
Alfentanil	Electrical pain stimulation	GG required higher opioid concentration for pain relief; respiratory depression was not increased with the elevated dose	Healthy German volunteers (20)	(Oertel et al., 2006)
Opioid (Oxycodone, morphine, methadone, fentanyl patch, intrathecal pump)	Pain score (NRS) and evaluation of chronic pain presence x genotype interaction	G118 was less common in members of the chronic pain group; more A118 members were resistant to high-dose opioids.	Mostly Caucasian patients receiving opioid treatment for elecetive laproscopic abdominal surgery (101)	(Janicki et al., 2006)
No drug	Three experimental pain procedures: pressure, thermal, and ischemic	G118 had higher pressure-pain thresholds. Lower thermal pain in G118-allele men, higher in G118-allele women.	Healthy volunteers (mostly Caucasian) (167)	(Fillingim et al., 2005)
Morphine	Pain score and morphine consumption	G118 allele associated with increased pain, increased morphine consumption, but reduced nausea	Women of varying Asian ethnicities receiving voluntary cesarian section (994)	(Tan et al., 2009)

Dissertation Goals and Hypotheses

As detailed in this introductory chapter, the *OPRM1* A118G SNP has been implicated in a variety of disease states and treatment responses. However, the extent to which this SNP alters these traits and the mechanisms that may underlie these alterations have not been elucidated. In order to better understand the functional consequences of this SNP, we generated mice possessing an equivalent *Oprm1* polymorphism by introducing a point mutation that eliminated an N-linked glycosylation site in exon 1. We then utilized molecular, cellular, electrophysiological, and behavioral techniques to investigate the alterations in receptor expression and function that result from this SNP.

Clinical and *in vitro* studies have reported evidence supporting both gains and losses of function. By studying receptor expression and function in this mouse line, I hoped to clarify these discrepancies and determine the mechanisms by which receptor changes affect behavioral responses. Chapter 2 describes my initial evaluation of changes in MOPR expression and function in mice possessing the *Oprm1* SNP and the subsequent investigation of how these alterations affect basal and morphine-evoked responses. Based on the clinical and *in vitro* reports, we expected to find reductions in receptor expression and function.

I further explored receptor alterations conferred by this SNP in Chapter 3. Specifically, I employed voltage-sensitive dye imaging in hippocampus slice preparations to evaluate how this SNP might affect circuit function. Since we had previously shown decreases in receptor expression and reductions in morphine-mediated responses, we expected to find decreased MOPR-stimulated circuit alterations in mice harboring the *Oprm1* SNP.

These studies, together with clinical and *in vitro* findings from other labs, validate this mouse model of the A118G SNP and extend our understanding of the functional consequences of this SNP.

CHAPTER 2: MOUSE MODEL OF *OPRM1* (A118G) POLYMORPHISM HAS SEX-SPECIFIC EFFECTS ON DRUG-MEDIATED BEHAVIOR

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ABSTRACT

A single nucleotide polymorphism (SNP) in the human μ-opioid receptor gene (*OPRM1* A118G) has been widely studied for its association in a variety of drug addiction and pain sensitivity phenotypes; however, the extent of these adaptations and the mechanisms underlying these associations remain elusive. To clarify the functional mechanisms linking the *OPRM1* A118G SNP to addiction and analgesia phenotypes, we derived a mouse model possessing the equivalent nucleotide/amino acid substitution in the *Oprm1* gene. Mice harboring this SNP (A112G) demonstrated several phenotypic similarities to humans carrying the A118G SNP, including reduced mRNA expression and morphine-mediated antinociception. We found additional phenotypes associated with this SNP including significant reductions of receptor protein levels, morphine-mediated hyperactivity, and the development of locomotor sensitization in mice harboring the G112 allele. In addition, we found sex-specific reductions in the rewarding properties of morphine and the aversive components of naloxone-precipitated morphine withdrawal. Further cross-species analysis will allow us to investigate mechanisms and adaptations present in humans carrying this SNP.

INTRODUCTION

Mu-opioid receptors (MOPR) are integrally involved in the modulation of several pathways including pain, stress, and drug reward. Genetic mutations of the MOPR alter endogenous and exogenous opioidergic function, thus influencing behavior. A single nucleotide polymorphism (SNP) in exon 1 of the μ-opioid receptor gene (*OPRM1*), in which an adenine to guanine substitution (A118G) exchanges an asparagine for an aspartic acid at a putative N-glycosylation site (N40D), is common in persons of European (15–30%) and Asian ancestry (49–60%), with lower prevalence in African American and Hispanic populations (Bergen et al., 1997; Gelernter et al., 1999; Tan et al., 2003). The A118G SNP has been associated with an altered vulnerability to opioid addiction (Ray and Hutchison, 2004; Drakenberg et al., 2006; van den Wildenberg et al., 2007), a decreased response to opioid-induced analgesia (Chou et al., 2006b; Sia et al., 2008), and an enhanced response to therapies for alcohol (Ray and Hutchison, 2007; Anton et al., 2008) and nicotine addiction (Lerman et al., 2004). However, some association studies report divergent effects (Zhang et al., 2006b; Glatt et al., 2007), as well as sex-specific associations (Fillingim et al., 2005; Ray et al., 2006; Munafo et al., 2007), underscoring the need to understand the functional significance of this SNP.

Examination of the A118G variant in heterologous expression systems has yielded inconsistent results. Initial *in vitro* studies indicated that expression of the human G118 MOPR variant in AV-12 cells increases the binding affinity of β-endorphin to 3-fold higher than that of the human A118 MOPR and results in higher potency for activation of G protein-coupled potassium channels (Bond et al., 1998), suggesting a gain of function of the receptor. However, other studies report no differences in agonist

binding, functional coupling, or desensitization (Beyer et al., 2004). Using an allelic expression assay, Zhang and colleagues (Zhang et al., 2005) found a 1.5-fold reduction in allele-specific mRNA expression in post-mortem brain tissue and also a 10-fold reduction in protein levels in CHO cells expressing the G118 variant, supporting a loss of function of the receptor. More recent data support this claim, showing lower surface receptor expression, decreased forskolin-induced cyclic AMP activation, and lower agonist-induced MOPR activation in cell culture systems expressing the G118 allele (Kroslak et al., 2007). Discrepancies in the *in vitro* findings established the rationale for generating a mouse model to examine the molecular, pharmacological, and behavioral significance of this polymorphism in humans. Thus, we generated a mouse possessing the equivalent SNP (A112G), which corresponds to a similar amino acid (N38D) substitution. Due to high homology between mouse and human sequences at the nucleotide (86.9%) and amino acid level (92.3%), similar gene expression levels between human and mouse (Genomics Institute, Novartis Research Foundation; http://symatlas.gnf.org), as well as conserved chromosomal synteny (http://genome.ucsc.edu/cgi-bin/hgTracks), we generated the mouse equivalent of the human SNP rather than replacing the mouse *Oprm1* gene with the human *OPRM1* gene in exon 1.

RESULTS

Generation of the A112G mouse The derivation of the *Oprm1* A112G mouse was accomplished using a bacterial artificial chromosome (BAC) containing the entire *Oprm1* locus derived from C57BL/6 mouse DNA (PAC/BAC Resource, Oakland, CA) (**Fig. S1**). Mating A112G heterozygous mice produced offspring of each genotype (A/A: 32.6%, A/G: 47.7%, and G/G: 19.7%, n = 700). Though G/G births were below expected Mendelian rates ($\chi^2 = 24.6$), there were no noticeable deficits in overall size or health, nor were there differences in rates of perinatal mortality between genotypes or sexes (unpublished observations). A/A and G/G homozygous mice of both sexes were used for all molecular, biochemical, and behavioral assays.

MOPR expression and function We evaluated the expression and function of the MOPR using a variety of molecular and pharmacological techniques. MOPR mRNA was reduced in G/G mice in several brain regions related to pain, stress, and reward (main effect of genotype, $F_{1,77} = 71.018$, $p < 0.0001$; **Fig. 1a**). Using primers designed to anneal to different regions of the *Oprm1* gene both 5' and 3' of the modified SNP, we found similar reductions in mRNA (primer × genotype interaction, $F_{2,42} = 3.416$, $p = 0.04$; **Fig. S2**). The A to G substitution in these mice eliminates one of the four putative N-glycosylation sites; thus, the observed decrease in MOPR protein size in G/G mice may reflect the reduction in the extent of N-linked glycosylation (**Fig. 1b**). In addition to a lower molecular weight, total MOPR protein levels were reduced in G/G animals in the thalamus, a region highly enriched in these receptors ($t_{20} = 3.881$, $p = 0.0009$; **Fig. 1c**). Whole brain saturation binding using [^{3}H]DAMGO showed decreases in receptor number (B_{max}) in G/G animals (effect of genotype $F_{1,8} = 8.161$, $p = 0.02$; **Table 1**). These data are

in accordance with previous studies showing decreased cell-surface [^{3}H]DAMGO binding in AV-12 and HEK293 cells stably expressing the G118 variant (Beyer et al., 2004; Kroslak et al., 2007). K_d values of [^{3}H]DAMGO for the MOPR were similar among the four groups of mice (**Table 1**). Analysis of brain region-specific binding using a single concentration of [^{3}H]DAMGO (3 nM) revealed decreased specific receptor binding in the thalamus of G/G animals compared with their A/A counterparts (t_{20} = 3.170, p = 0.005; **Fig. 1d**). Using whole brain membranes, we determined the binding affinities of β-endorphin, morphine, and naloxone by competitive inhibition of [^{3}H]DAMGO binding and found no alterations between genotypes or sexes (**Table 1**).

Behavioral responses to acute morphine administration In C57BL/6 mice, acute morphine elevates locomotor activity(Crawley et al., 1997); accordingly, we observed a robust increase in locomotor activity in A/A mice over the course of a 120-min session following saline or morphine administration. In contrast, G/G mice failed to exhibit morphine-mediated hyperactivity (time × treatment × genotype interaction, $F_{11,649}$ = 11.108, p < 0.0001; **Fig. S3a**). There was no difference in locomotor activity between genotypes during the 30-min baseline test or following saline administration, suggesting that the alterations in activity are specific to morphine effects and not reflective of a general locomotor deficit. Additionally, there was no difference in activity between males and females in either genotype or treatment group (treatment × genotype interaction, $F_{1,59}$ = 16.076, p = 0.0002; **Fig. S3b**). Since morphine can have hypolocomotor actions at high doses, it is possible that the decrease in activity in the G/G animals could result from a heightened sensitivity to morphine. Thus, we evaluated the locomotor response to a low dose of morphine (1 mg/kg). Neither of the genotypes or sexes displayed elevated activity

in response to a low-dose morphine administration, indicating that the A112G SNP does not confer an enhanced sensitivity (**Fig. S3c,d**). Morphine has been shown to elicit enhanced locomotor-activating effects (behavioral sensitization) with repeated, intermittent administrations (Babbini and Davis, 1972; Mickiewicz et al., 2009). Indeed, A/A animals showed behavioral sensitization following repeated morphine injections, while G/G animals did not (day × genotype × treatment interaction, $F_{6,138} = 9.688$, $p < 0.0001$; **Fig. 2**). Under these conditions, in which animals habituated to the testing chambers, morphine elevated locomotor activity in the G/G animals, though this response was greatly reduced compared to A/A animals (**Fig. 2**).

Opiate analgesics are widely used for pain management, but individual differences in opiate-sensitivity can alter effective treatment. Clinical findings demonstrate that individuals carrying the G118 allele report greater pain sensation (Sia et al., 2008) and require higher doses of morphine to alleviate pain following surgery (Chou et al., 2006a; Chou et al., 2006b; Reyes-Gibby et al., 2007; Sia et al., 2008). Therefore, we used the hot-plate assay to evaluate basal nociceptive responses and morphine-mediated antinociception in mice with the A112G SNP. Using a cumulative dosing paradigm, in which animals were injected with increasing doses of morphine and evaluated for morphine-mediated antinociception at 30-min intervals (Sora et al., 1997), G/G mice showed a significantly lower maximal possible effect of morphine (%MPE) at higher doses (genotype × dose interaction, $F_{1,63} = 5.348$, $p = 0.02$; **Fig. 3a**). There were no baseline differences in hind-paw lick latency, suggesting that the G/G mice do not have a decreased pain threshold. However, when testing at a higher temperature (58°C), a difference was detected in baseline jumping behavior (main effect of genotype, $F_{1,63} =$

5.348, p = 0.02, **Fig. S4a)** along with a decrease in morphine-mediated antinociception (main effects of genotype, $F_{1,30} = 24.310$, $p < 0.0001$ and sex, $F_{1,30} = 4.356$, $p = 0.05$; **Fig. S4b**). Following 7 days of twice daily morphine injections (10 mg/kg), all animals showed a reduced effect of morphine (day × genotype × treatment interaction, $F_{1,48} = 4.801$, $p = 0.03$; **Fig. 3b**), suggesting that although the acute antinociceptive properties of morphine are diminished, tolerance to repeated administration remains intact.

The use of morphine as an analgesic is limited by the abuse liability of the drug engendered by its ability to activate the reward pathway. In mice, the rewarding properties of morphine can be demonstrated through the development of a conditioned place-preference to environments paired with morphine. As expected, A/A animals showed a robust preference for morphine-paired environments. G/G males showed a preference for morphine-paired environments equivalent to that of the A/A mice. In contrast, G/G females did not show a preference for the morphine-paired environment (treatment × genotype × sex interaction, $F_{1,44} = 3.958$, $p = 0.05$; **Fig. 4**). The variable effect of the A118G SNP in males and females has been reported in clinical studies of nicotine addiction and pain response (Fillingim et al., 2005; Ray et al., 2006; Munafo et al., 2007), but not for opioid reward (Compton et al., 2003).

Behavioral responses to withdrawal from chronic morphine exposure Chronic morphine exposure can cause both physical and psychological dependence. Following chronic morphine administration, male and female mice of both genotypes demonstrated physical dependence, as measured by the presence of somatic signs following naloxone-precipitated morphine withdrawal (main effect of sex, $F_{1,18} = 7.537$, $p = 0.01$, with no contribution of genotype; **Fig 5a**). Psychological dependence was measured using a

similar conditioning paradigm as was used to evaluate reward. Animals were implanted with subcutaneous morphine or placebo pellets three days prior to receiving a single naloxone (0.1 mg/kg, s.c.) administration in one chamber of a two-chamber conditioning apparatus. All mice avoided environments associated with naloxone-precipitated morphine withdrawal (main effect of treatment, $F_{1,76} = 20.206$, $p < 0.0001$; **Fig. 5b**). Further analysis, however, shows that A/A females spent significantly less time on the side of the chamber paired with naloxone-precipiated morphine withdrawal than did G/G females (chronic treatment × genotype × sex interaction, $F_{1,76} = 4.810$, $p = 0.03$; **Fig. 5b**). In contrast, there were no place aversion differences between male A/A and G/G mice. Interestingly, the placebo-treated G/G females also avoided naloxone-paired environments compared to placebo-treated A/A females, while there were no differences in males between genotypes. Together, these studies demonstrate that, in contrast to the physical withdrawal signs, the psychological aversion associated with acute withdrawal in morphine-dependent mice is altered by the G112 allele in females only.

DISCUSSION

The A118G SNP has been implicated in a variety of pain sensitivity and drug addiction phenotypes in humans. Specifically, carriers of the G118 allele show an elevated sensitivity to pain and a reduced analgesic response to opioid administration. Additionally, the G118 allele has been associated with increased efficacy of treatments for alcohol and nicotine dependence. An understanding of the mechanisms underlying these alterations is essential for developing alternative pain therapies for carriers of the G allele or treatments for addiction that take advantage of the apparent benefit conferred by this SNP. To clarify the functional mechanisms linking the *OPRM1* A118G to some of these phenotypes, we developed a knock-in mouse that possesses the mouse-equivalent SNP in the MOPR gene (*Oprm1* A112G).

Functional knock-in technology using Cre-loxP homologous recombination allows for the generation of mouse models of human mutations or polymorphisms (Roebroek et al., 2006). To prevent interference with normal transcriptional control, most models have removed the selection marker resulting in a residual loxP site in the targeted gene, which has not been shown to alter expression (Chen et al., 2006). In the present mouse model, the G112-targeted allele did reduce both mRNA and protein expression in some brain regions; however, SNPs in transcribed regions, specifically the A118G SNP, have been shown to affect mRNA processing and turnover (Zhang et al., 2005; Johnson et al., 2008). Thus, while we cannot rule out the potential effect of the loxP site as contributing to reductions in mRNA and protein, the fact that these mice displayed similar molecular and behavioral phenotypes to human carriers of the G118 allele

provides evidence that this SNP indeed has functional consequences and that this mouse could serve as a valuable tool in identifying the effects of these changes.

It has been contested whether the A118G SNP confers a gain or loss of function. Studies reporting elevations in biochemical or behavioral traits (e.g., increases in maternal attachment in primates (Barr et al., 2008) or cortisol responses in humans (Chong et al., 2006)) typically cite elevations in β-endorphin binding (Bond et al., 1998) as a potential mechanism. Alternatively, studies reporting deficits in behavior (e.g., decreased nicotine reward (Ray et al., 2006)) typically cite decreases in MOPR expression (Zhang et al., 2005) as explanation for the effects. In the present study, we found evidence suggesting that the consequences of this SNP cannot be evaluated as a simple gain or loss of function. We did not find evidence suggesting altered affinity to MOPR agonists, though we did corroborate studies showing decreased MOPR expression by demonstrating decreases in mRNA, protein, and receptor number. In line with decreased MOPR levels, G/G mice showed deficits in the hyperlocomotor and antinociceptive actions of acute morphine administration; however, not all behaviors showed deficits despite these reductions. This is most evident in the conditioned reward and aversion studies in which only females demonstrated an altered behavioral response. On the other hand, physical morphine withdrawal signs were similar between genotypes for both sexes. Previous studies have demonstrated a disassociation between the physical and aversive components of precipitated morphine withdrawal (Harris and Aston-Jones, 1993; Schulteis et al., 1994), suggesting that the alterations caused by this SNP is dependent on the circuitry involved.

Distinctions between genotypes do not appear to be dependent on the timing or duration of morphine administration. G/G mice showed a significant, albeit diminished, antinociceptive effect of acute morphine treatment. This effect decreased with repeated morphine exposure, demonstrating that these mice develop tolerance similar to A/A mice. Furthermore, G/G mice showed a significant, yet diminished, hyperlocomotor effect of acute morphine treatment. However, repeated morphine exposure did not increase this response, demonstrating that G/G mice do not develop locomotor sensitization. Therefore, the mechanisms underlying the development of tolerance and sensitization may be differentially influenced by the *Oprm1* SNP. Loss of the delta-opioid receptor, for instance, results in elevated sensitization and diminished tolerance to morphine (Chefer and Shippenberg, 2009). Though we did not investigate changes in the expression and function of other opioid receptors, it is possible that compensatory upregulation or altered dimerization of these receptors could contribute to some of the altered behaviors of the G/G mice.

Morphine has varying potencies in males compared with females, depending on the assay (for review, see (Craft, 2008)). Recently, differences in MOPR receptor levels in rat brain have been identified as essential for sex differences in morphine analgesia (Loyd et al., 2008). Estrogen modulation of MOPRs is supported by *in vivo* positron emission tomography (PET) imaging studies with [^{11}C] carfentanil. Pre-menopausal women have approximately 25% greater availability of MOPRs than men in cortical and subcortical areas (Zubieta et al., 1999; Zubieta et al., 2002), a difference that disappears after menopause (Zubieta et al., 1999). The sex-differences we observed in morphine reward and withdrawal, however, cannot be explained by altered levels of MOPRs, as

these reductions were equivalent across sexes. To further explore hormonal modulation in this phenotype, studies requiring estrogen depletion of females or feminization of males harboring this SNP will be required. To date, human genetic studies have not been designed *a priori* with adequate power to examine the sex-dependent effects of the *OPRM1* A118G SNP on behavior. The current data suggest such analysis is warranted.

Genetic association studies in psychiatry and addiction are plagued by nonreplications. However, there is a critical mass of positive studies linking the *OPRM1* A118G SNP with opioid, alcohol, and nicotine dependence, and subsequent treatment responses. Data obtained from the A112G mice provide compelling evidence that this type of a translational cross-species model is important for complete functional characterization of genetic variants. Future studies utilizing this mouse model could serve as a valuable tool in determining the mechanisms underlying responses to a variety of drugs of abuse and in developing personalized therapies based on genotype.

MATERIALS AND METHODS

Animals All mice (8–15 weeks, 18–30 g) were group housed and maintained on a 12-h/12-h light/dark cycle with food and water available *ad libitum* in accordance with the University of Pennsylvania Animal Care and Use Committee. For a complete description of the derivation of *Oprm1*^{tm1Jabl} mice, see supporting information. All experimental testing sessions were conducted between 0800 hours and 1700 hours, with animals randomly assigned to treatment conditions and tested in counterbalanced order. Both male and female mice were used in all studies except for morphine locomotor sensitization, in which only males were utilized. Male and female data were combined when there were no statistical contributions of sex. Separate, näive cohorts were used for behavioral experiments, except for acute locomotor (**Fig. S3**), hot-plate (**Fig. S4**), and physical withdrawal (**Fig. 5a**) studies, in which one cohort of animals was used for all three experiments conducted in the order listed and separated by at least one week. Separate cohorts of animals were used and the data combined for the following experiments: CPP (**Fig. 4**), CPA (**Fig 5**), acute locomotor activity (**Fig. S3**), and 58° C hot-plate (**Fig. S4**), as there were no statistical differences within groups measured between cohorts.

Drugs For acute drug administration, morphine sulfate was obtained from NIDA Drug Supply (Research Triangle Park, NC) and naloxone hydrochloride was obtained from Sigma Aldrich. Both drugs were dissolved in 0.9% saline and administered subcutaneously at a volume of 0.1 mL/10 g body weight. Morphine dependence was achieved by subcutaneously implanting a single placebo (cellulose) or morphine (25 mg

morphine base) pellet (NIDA Drug Supply, Research Triangle Park, NC) in the dorsal surface of mice under general isoflurane anesthesia for three days prior to testing.

Quantitative real-time PCR For RNA isolation and cDNA synthesis, mice were killed by cervical dislocation and brains were rapidly removed and dissected on ice. Brains were first sliced using a mouse brain matrix into 1mm slices. Specific regions were identified and macrodissected using their approximate mouse stereotaxic coordinates (AMYG and HIPP, bregma −1.2mm; BNST and CTX, bregma +0.26mm; NAc, bregma +1.10mm; VTA, bregma −3.64mm). The hypothalamus was removed from the ventral side of the brain prior to placement in the mouse brain matrix. RNA was isolated from brain tissue using TRIzol/chloroform in conjunction with an RNeasy Mini kit (Qiagen). cDNA was synthesized using Oligo dT primer (Operon) and Superscript II reverse transcriptase (Invitrogen). Taqman QPCR multiplex reactions were assembled using the Taqman Universal PCR Master Mix (Applied Biosystems) along with 300nM primers (final concentration). All quantitative real-time polymerase chain reactions (QPCR) were run using the Stratagene MX3000 and MXPro QPCR software with cycling parameters set at 95°C for 10min followed by 40 cycles of 95°C (30s) and 60°C (1min). All reactions were performed in triplicate and the median cycle threshold was used for analysis. The mRNA levels of target genes were normalized to the housekeeping gene, TATA binding protein (TBP). Primers used in **Fig 1** were found 3' of the knock-in and spanned exon 1 and exon 2 (5': caccatcatggccctctatt; 3': caaaatgaagactgccacca).

Brain membrane preparation Frozen mouse whole brains or thalami were homogenized in ~8-volume 25 mM Tris-HCl buffer/pH7.4 containing 1 mM EDTA and 0.1 mM PMSF on ice and then centrifuged at ~100,000g for 30 min. Pellets were twice

rinsed with 25 mM Tris-HCl buffer and re-suspended in 0.32 M sucrose in 50 mM Tris-HCl/pH7. Suspended membranes were passed through a 26.5G needle for 5 times and then frozen at -80^{o}C.

Western Blot Membranes were prepared from thalami of A/A mice, G/G mice, and MOPR knock-out mice. The MOPR knock-out mice used were originally developed in the lab of Dr. John Pintar by disruption of exon 1 of the *Oprm1* gene through homologous recombination (Schuller et al., 1999). Membrane proteins were loaded (15 μg per lane) for SDS-PAGE and western blot was performed with the MOPR antibody, anti-μC [against the MOPR (383-398) peptide] (1:5,000, final 0.26 μg/ml), followed by goat anti-rabbit IgG conjugated with HRP (1:5,000), and then reacted with enhanced chemiluminescence (ECL) western blotting detection reagents (Huang et al., 2008). Images were captured with a FujiFilm LAS-1000 Imaging System. After a brief wash, the same blot was then incubated with mouse anti-GAPDH-HRP-conjugated (1:10,000) (Abcam) followed by ECL reagents. Quantification of MOPR-immunoreactivities was carried out by densitometry analysis with the ImageGauge software for Fuji Imaging System. MOPR immunoreactivity in each lane was normalized against that of GAPDH.

MOPR Ligand Binding Binding assays were performed as previously described (Liu-Chen et al., 1995) with some modifications. Mouse brain membranes were incubated at room temperature for 30 min with 100 mM NaCl and 100 μM GDP in 50 mM Tris-HCl buffer (pH7.4) containing 1 mM EDTA (TE buffer) and washed three times with the TE buffer. Binding was performed in TE buffer containing 5 mM $MgCl_2$ for 2.5~3 hrs at room temperature in order to convert receptors to high affinity states. Saturation binding of [^{3}H]DAMGO to MOPRs in whole brain membranes was performed with seven

concentrations of [^{3}H]DAMGO (ranging from 0.1 to 8.0 nM), and K_d and B_{max} values were determined by non-linear regression curve fit with one site binding (GraphPad Prism). For binding to MOPR in thalamus membranes, a single concentration of [^{3}H]DAMGO was used (3 nM). Competitive inhibition of [^{3}H]DAMGO (1.0 nM) binding by β-endorphin, morphine or naloxone was performed in the absence or presence of various concentrations of each ligand. K_i values were determined by non-linear regression curve fit of one site competition (GraphPad Prism). Nonspecific binding was measured in the presence of naloxone (1.0 μM). Each binding assay was carried out in duplicate in a final volume of 0.5 ml with 0.2–0.4 mg protein/tube for whole brain binding or 0.1 mg protein/tube for thalamus binding. Incubations were terminated by filtration through Whatman GF/B filters under vacuum.

Locomotor Activity Locomotor activity was analyzed in a "home cage" activity monitoring system (MedAssociates). The testing cage, which was identical in dimension to the home cage, was placed in a photobeam frame (30 × 24 × 8 cm) with two levels of sensors arranged in an 8-beam array strip. A small amount of fresh bedding was scattered on the cage floor. *Locomotor sensitization*: animals were tested every 2–3 days for 120 minutes following an injection of saline or morphine (10 mg/kg, s.c.). On treatment days 1–3, all animals were administered saline. On days 4–9, animals received either morphine or saline, according to their treatment group. Beam break data were read into MedAssociates personal computer-designed software and monitored at 10-minute intervals.

Hot-Plate The nociceptive threshold for analgesia was examined with a hot-plate analgesia meter (Columbus Instruments). The hot-plate provided a constant 55° C (**Fig.**

3) or 58° C (**Fig. S4**) surface, temperatures low enough to avoid harming the mice, but high enough to be uncomfortable for a saline-treated animal. A small plastic cage surrounding the hot-plate prevented the animal from leaving the plate surface. *Cumulative dosing* (**Fig. 3a**): animals were first placed on the hot-plate and the latency to lick the hind-paw or jump was recorded. Upon displaying one of these behaviors or upon reaching the predetermined cut-off time (60 s), the animals were immediately removed from the hot-plate, injected with the first dose of morphine, and returned to their home cage. Animals were retested on the hot-plate and immediately injected with the next morphine dose at 30-minute intervals. Animals received doses of 0, 1, 2, 7, 20, and 20 mg/kg; any animals that did not complete the 60-second trial without licking or jumping at the highest dose received an additional 20 mg/kg injection at the usual dosing schedule. *Tolerance* (**Fig. 3b**): animals were injected twice daily for 8 days with morphine (10 mg/kg) or saline, according to treatment group, and tested for antinociception 30 minutes following the a.m. injection. The latency to lick the hind-paw or jump was recorded and a maximum test-duration set at 60 seconds.

Conditioned Place Preference Place-conditioning boxes consisted of two chambers (20 × 20 × 20 cm), one with stripes on the wall and a metal grid floor and the other with gray walls and a metal patterned floor. A partition with an opening separated the two chambers in each box, but allowed access to either side of the chamber. This partition was closed off during the pairing days. *Preconditioning Phase (day 1):* Animals were placed in the boxes and allowed to roam freely throughout both chambers for 15 min; time spent in each chamber was recorded. These data were used to separate the animals into groups of approximately equal bias. *Conditioning Phase (days 2–9):* Animals received 8 days of

once-a-day pairings in which an animal was injected with morphine (10 mg/kg) or saline and then immediately placed into one chamber for 30 minutes. On the following day, animals were injected with either saline or morphine, depending on what they had received on the previous day, and placed in the opposite chamber. Drug pairings were divided such that half of the animals received morphine injections on odd days while the other half received morphine on even days; non-drug paired animals received saline injections throughout the conditioning phase. Drug-paired chambers were randomized among all groups. *Testing Phase (day 10):* Animals were all given a saline injection and allowed to roam freely between the two chambers; the amount of time spent in each chamber was recorded.

Conditioned Place Aversion The CPA test was performed similarly to the CPP test with the following differences. *Preconditioning phase (day 1)*: Three days following implantation of morphine or placebo pellets, animals were placed in the boxes and allowed to explore both chambers in order to test for preexisting biases. *Conditioning phase (days 2–3):* On day 2, all animals were injected with saline and confined to one chamber for 30 minutes. On day 3, all animals were injected with naloxone (0.1 mg/kg, s.c.) and confined to the opposite chamber for 30 minutes. *Test phase (day 4)*: Animals were injected with saline and allowed to explore both chambers; the amount of time spent in either chamber was recorded.

Somatic Withdrawal Signs Three days following implantation of morphine or placebo pellets, animals were placed on cotton pads inside of a clear plastic cylinder with an open top. Animals were allowed to habituate for 30 minutes prior to receiving a subcutaneous injection of naloxone (0.1 mg/kg). The number of occurrences of jumping, paw tremor,

genital licking, backing up, and gnawing was recorded. Additionally, the presence or absence of the following symptoms was recorded in 5-minute bins for the 30-minute test: ptosis, resting tremor, diarrhea, and teeth chatter. Withdrawal scores were calculated as the sum of all occurrences of somatic signs displayed during the 30-minute test.

Analysis When comparing multiple effects, analyses were performed using two- or three-way analysis of variance (ANOVA) tests (repeated measures tests were utilized when comparing multiple time points) with significant *F* values reported in the text. Bonferroni/Dunn post-hoc tests were used to compare significant interactions between main effects. When comparing differences between genotypes for only one effect, unpaired *t*-tests were utilized with significant *t* values reported in the text. Statistical significance was set at $p \leq 0.05$.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. MOPR expression is decreased in A112G knock-in mice. (a) MOPR mRNA, as measured by Real Time RT-PCR and normalized against TATA binding protein (TBP), in the periaqueductal grey (PAG), hypothalamus (Hypo), ventral tegmental area (VTA), nucleus accumbens (NAc), and cortex (Ctx) (mean ± SEM, n = 7-8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, † $p < 0.0001$ compared to A/A, Bonferroni/Dunn). **(b)** A representative immunoblot of MOPR in membranes prepared from thalami of A/A mice, G/G mice, and MOPR -/- mice and probed with the MOPR antibody shows decreased molecular weight of MOPR protein in G/G mice. **(c)** Quantification of MOPR-immunoreactivities, normalized against GAPDH (mean ± SEM, $n = 11$; *** $p < 0.001$ compared to A/A). **(d)** Binding of [^{3}H]DAMGO (3 nM) in thalamus membranes (0.1 mg/tube). Data are presented as specific binding/tube (dpm) for each sample run in duplicate (mean ± SEM, $n = 11$; ** $p < 0.01$ compared to A/A).

Figure 2.1

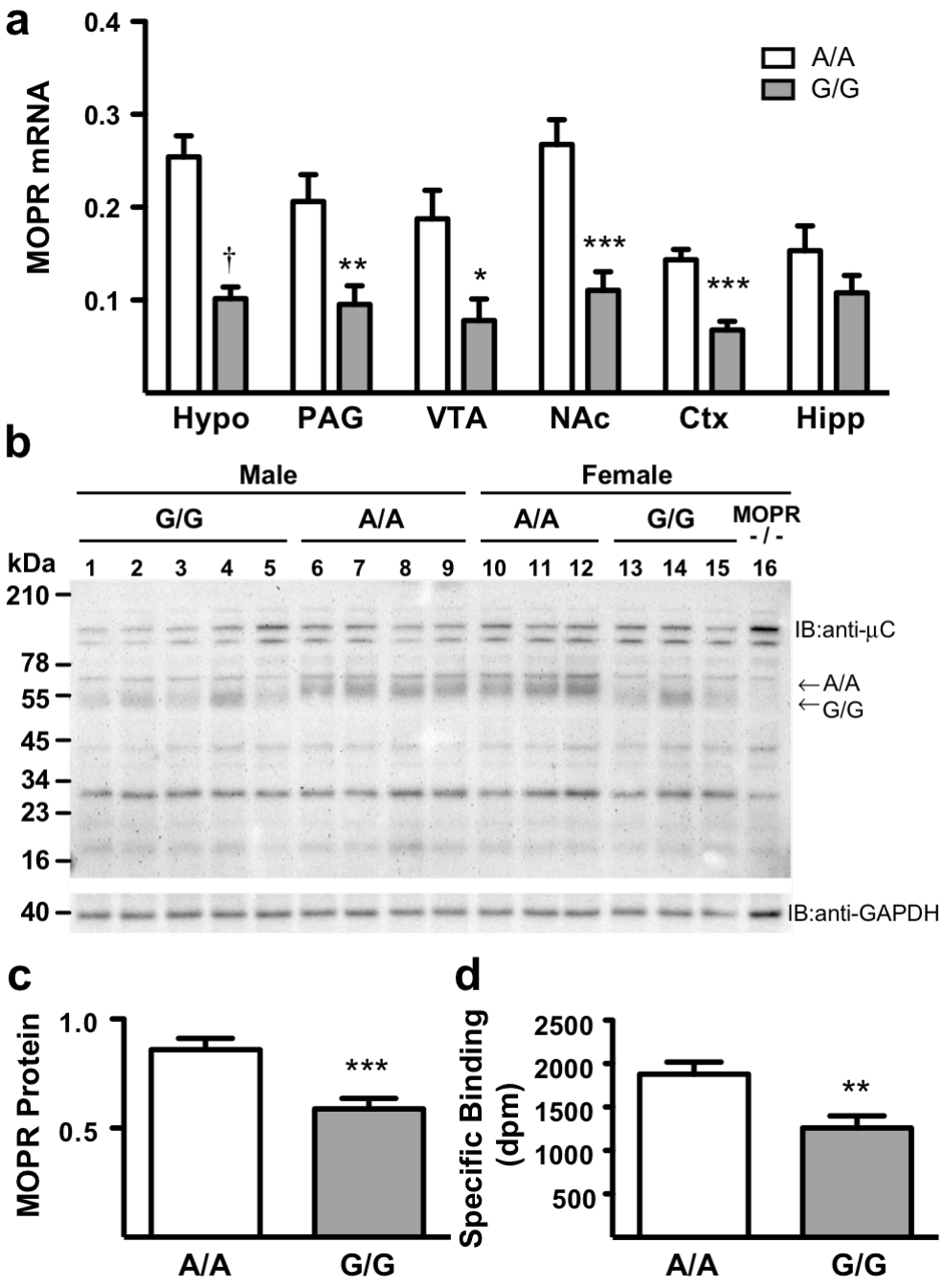

a
MOPR mRNA
0.4
0.3
0.2
0.1
A/A
G/G
†
**
*
***
***
Hypo
PAG
VTA
NAc
Ctx
Hipp
b
Male
Female
G/G
A/A
A/A
G/G
MOPR -/-
kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
210
78
55
45
34
23
16
40
IB:anti-μC
←A/A
←G/G
IB:anti-GAPDH
c
MOPR Protein
1.0
0.5
***
A/A
G/G
d
Specific Binding (dpm)
2500
2000
1500
1000
500
**
A/A
G/G

Figure 2. Morphine-mediated hyperlocomotion is blunted in G/G mice. Saline was administered to all groups on days 1–3 and morphine was administered on days 4–9 (saline control groups received saline injections on all 9 days). Results are presented as total activity counts for the 120-min post-injection test (mean ± SEM, n = 6-7; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, † $p < 0.0001$ compared to saline-injected controls; $^{+}$ $p < 0.01$, $^{++}$ $p < 0.0001$ compared to the average of days 1–3, Bonferroni/Dunn).

Figure 2.2

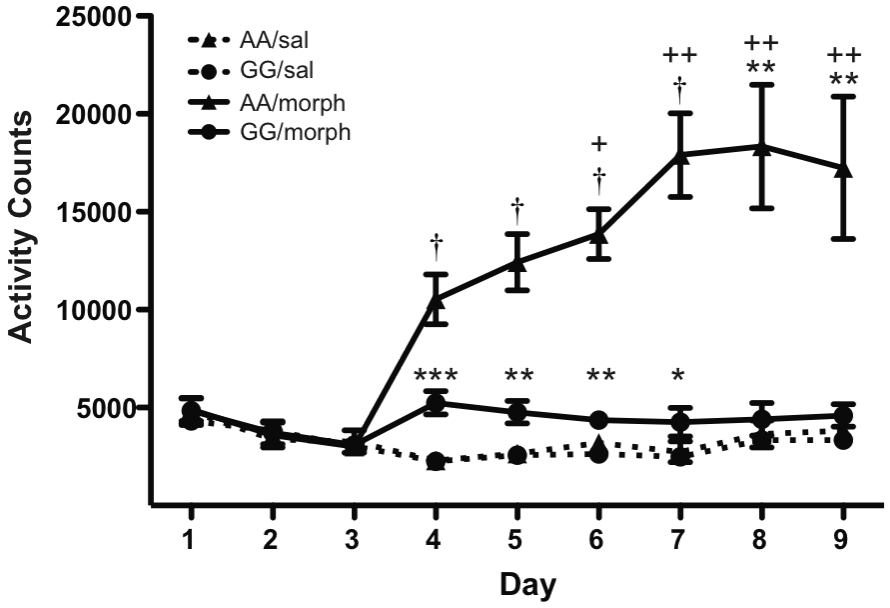

25000
20000
15000
10000
5000
Activity Counts
AA/sal
GG/sal
AA/morph
GG/morph
1
2
3
4
5
6
7
8
9
Day

Figure 3. Morphine-mediated antinociception is decreased in G/G mice while tolerance to repeated exposure remains intact. (a) Morphine-mediated antinociception, as measured by hind-paw lick latency on a 55º C hot-plate assay using a cumulative-dosing paradigm, was significantly reduced in G/G mice. Results are presented as percent maximal possible effect (MPE) [(morph jump latency – saline jump latency)/(total time – saline jump latency) × 100] (mean ± SEM, n = 18; *** $p < 0.001$, † $p < 0.0001$ compared to G/G mice). **(b)** Tolerance to morphine-mediated (10 mg/kg) hot-plate antinociception was present in both A/A and G/G mice. Results are presented as percent maximal possible effect (MPE) [(morph jump latency – baseline jump latency)/(total time – baseline jump latency) × 100] (mean ± SEM, n = 12-14; * $p < 0.05$, ** $p < 0.01$ compared to saline-treated controls; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ compared to Day 8, † $p < 0.0001$ compared to G/G mice treated with morphine, Bonferroni/Dunn).

Figure 2.3

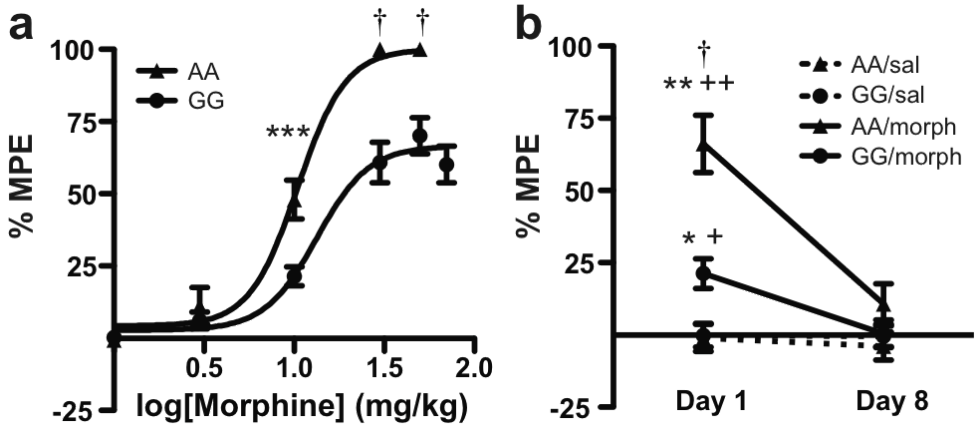

a
100
75
50
25
-25
% MPE
AA
GG
***
† †
0.5
1.0
1.5
2.0
log[Morphine] (mg/kg)
b
100
75
50
25
-25
% MPE
** ++
†
* +
AA/sal
GG/sal
AA/morph
GG/morph
Day 1
Day 8

Figure 4. Female G/G mice failed to show a conditioned place-preference to morphine-paired environments (10 mg/kg). Results are presented as the difference in time spent in drug-paired environments compared to non drug-paired environments on the test day minus the difference in time from the preconditioning day (mean ± SEM, n = 6–8; * $p < 0.05$, ** $p < 0.01$ compared to saline-treated controls; $^{+}$ $p < 0.05$ compared to morphine-treated A/A females and G/G males, Bonferroni/Dunn).

Figure 2.4

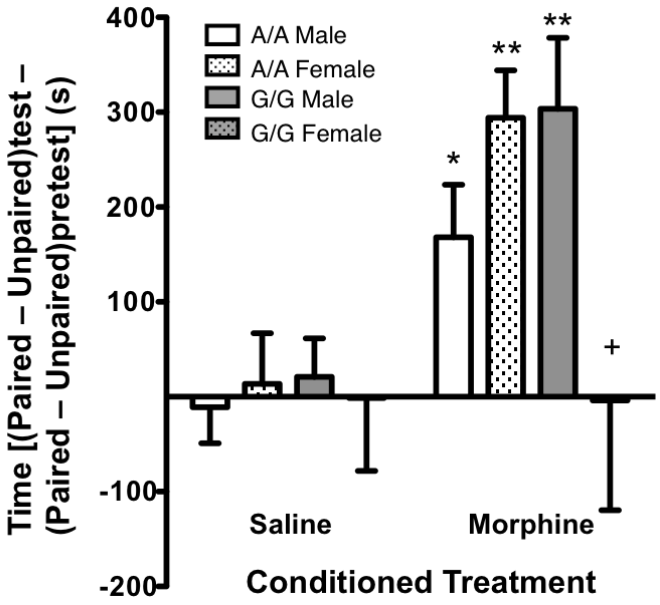

400
300
200
100
-100
-200
A/A Male
A/A Female
G/G Male
G/G Female
*
**
**
+
Saline
Morphine
Conditioned Treatment
Time [(Paired – Unpaired)test – (Paired – Unpaired)pretest] (s)

Figure 5. Dissociation of the physical and affective components of naloxone-precipitated morphine withdrawal (a) A/A and G/G mice displayed similar somatic signs of naloxone-precipitated (0.1 mg/kg) withdrawal. Results are presented as the withdrawal score calculated by summing the total number occurrences of jumping, paw tremor, genital licking, backing up, gnawing, ptosis, resting tremor, diarrhea, and teeth chatter (mean ± SEM, n = 5–6; * $p < 0.05$ compared to male A/A and G/G). (**b**) Naloxone-precipitated morphine withdrawal-induced place aversions were reduced in G/G females. Additionally, placebo-treated G/G females displayed aversion to naloxone-paired environments compared to A/A females. Results are presented as the difference in time spent in drug-paired environments compared to non drug-paired environments on the test day minus the difference in time from the preconditioning day (mean ± SEM, n = 8–12; * $p < 0.05$ compared to morphine-treated A/A females; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.0001$ compared to placebo-treated A/A females, Bonferroni/Dunn).

Figure 2.5

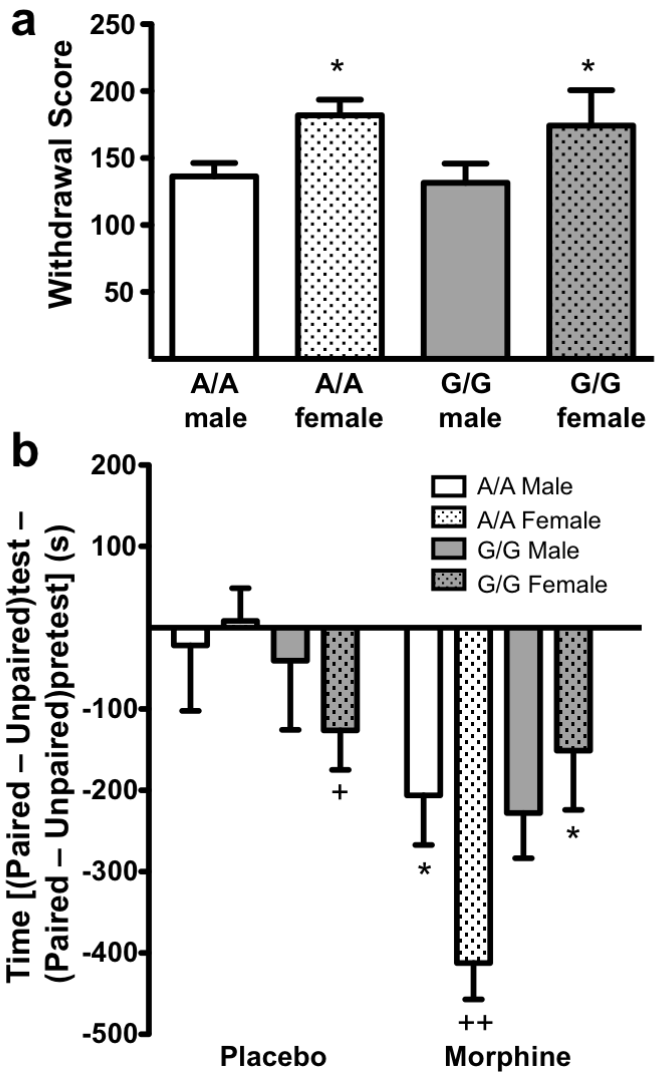

a
Withdrawal Score
250
200
150
100
50
*
*
A/A male
A/A female
G/G male
G/G female
b
Time [(Paired – Unpaired)test – (Paired – Unpaired)pretest] (s)
200
100
-100
-200
-300
-400
-500
A/A Male
A/A Female
G/G Male
G/G Female
+
*
*
++
Placebo
Morphine

TABLE LEGENDS

Table 1. K_d and B_{max} values were calculated from saturation binding of [^{3}H]DAMGO using whole brain membranes. Competitive inhibition by β-endorphin, morphine and naloxone of [^{3}H]DAMGO (1.0 nM) binding was conducted to determine K_i values. For each independent experiment, two mouse brains were pooled (0.2–0.4 mg membrane proteins/tube) for one saturation curve and three competition curves. Each value represents the binding for three independent experiments performed in duplicate (mean ± SEM; * $p < 0.05$, compared to A/A).

Table 2.1 Expression Levels and Ligand Binding Affinities of MOPR in A/A and G/G mice.

	[^{3}H] DAMGO		β-Endorphin	Morphine	Naloxone
	B_{max} (fmol/mg protein)	K_d (nM)		K_i (nM)	
A/A male	158 ± 11.7	0.29 ± 0.03	1.9 ± 0.06	2.4 ± 0.16	2.9 ± 0.04
A/A female	182 ± 5.0	0.26 ± 0.03	2.1 ± 0.28	2.7 ± 0.25	3.2 ± 0.12
G/G male	142 ± 13.7*	0.33 ± 0.02	1.6 ± 0.11	2.9 ± 0.23	2.7 ± 0.32
G/G female	114 ± 22.5*	0.33 ± 0.03	1.8 ± 0.18	2.8 ± 0.31	3.0 ± 0.27

SUPPORTING INFORMATION

Materials and Methods

Derivation of *Oprm1*^{tm1Jabl} mice The construction of the A112G allele was accomplished by using a bacterial artificial chromosome (BAC) containing the entire *Oprm1* locus derived from C57BL/6 mouse DNA (obtained from the PAC/BAC resource in Oakland, CA; (clone RP23)). The region containing exon 1 and flanking introns was used as the template for site-directed mutagenesis. The polymorphism in exon 1 was constructed by changing the adenosine (A) nucleotide at position 112 of the mouse, which corresponds to position 118 in the human sequence, to a guanosine (G). A second mutation (T108C) was introduced which abolished a BstXI restriction site in order to identify the mutated clones without affecting the aspartic acid encoded by the codon. Mutations were verified by sequencing and restriction analysis. For the generation of the targeting vector, two PCR fragments were cloned into plasmid pL452 (containing loxP sequences and a Neomyocin-resistant cassette for later selection) flanking the loxP sites. The first fragment (1.3kb) contained exon 1 with the point mutation and was cloned by KpnI-EcoRI. The second fragment (650bp) corresponded to an intronic DNA sequence located downstream of the fragment containing exon 1 and was cloned by NotI-SacII. A plasmid was generated to retrieve a 10kb fragment containing the MOPR gene. A fragment containing the modified exon 1, the neo cassette flanked by loxP sequences, and the downstream fragment was recovered from vector pL452-MUT and electroporated into EL250 cells containing the MOPR plasmid. This homologous recombination led to the introduction of the point mutation, loxP sites, and neo cassette into the final targeting construct. 100 mg of this vector was linearized with XmaI and electroporated into

C57BL/6 ES cells (Chemicon Inc.). Putative recombinants were selected by neomycin resistance. Initial screening of resistant clones was performed by PCR. Genomic DNA from clones growing in 96-well plates served as the template for a first PCR reaction using the primers NEO-F-KK-1 and PROBE-R. One μl from the first PCR product was the template for a nested PCR using the primers NEO-F-KK-2 and PROBE-R-KK-nested. Six clones out of 192 were identified as positive by PCR and, following re-screening by Southern blot, one clone was confirmed as correctly targeted. ES cells were injected into C57BL/6 blastocysts, which were then implanted into pseudopregnant females. Thirteen male chimaeras were obtained. Chimeric animals were mated with C57BL/6 mice to produce heterozygous animals, which were then crossed with mice expressing Cre recombinase to remove the neo cassette. Heterozygous offspring were then mated to produce mice of all genotypes.

Quantitative real-time PCR Primers used in **Fig S2** were in exon 1 5' from the knock-in (5': ctggaacccgaacactcttg; 3': gcaacttgcaggagctaagg), spanned exon 2 and exon 3 (5': cctctcttctgccattggtc; 3': tgaaggcgaagatgaagaca), or spanned exon 3 and exon 4 (5': tcccaacttcctccacaatc; 3': tagggcaatggagcagtttc).

Locomotor Activity Mice were placed in the chamber and baseline activity was recorded for 30 minutes. Animals were then injected with saline or morphine (1 or 10 mg/kg, s.c.) and placed back into the chambers and activity was recorded for 120 minutes.

Antinociception For baseline and acute morphine antinociception (**Fig. S4**), animals were exposed to the hot-plate (58° C) and the latency to jump was recorded. Twenty-four hours later, the test was repeated 15 minutes following an acute morphine (10 mg/kg, s.c.) or saline injection. The cut-off time was set at 120 seconds.

Figure Legends

Figure S1. Generation and validation of *Oprm1*^{tm1Jabl} mice (a) Schematic diagram of the strategy used to replace exon 1 of the *Oprm1* gene with the *Oprm1* Asp38Asn variant. For the variant, a point mutation has been made (A112G) which exchanges an asparagine at position 38 for an aspartic acid. **(b)** Southern blot analysis of genomic DNA from one embryonic stem-cell clone following digestion with BclI and hybridization with a flanking genomic fragment as a probe, indicated in A, was used to detect homologous recombination in the *Oprm1* locus. The 10.9 kb wild type (+/+) and 3 kb (+/Asp) variant DNA bands are indicated. **(c)** Sequencing the targeting vector confirms the A112G point mutation that leads to the Asn38Asp amino acid change. **(d)** Gel electrophoresis of DNA PCR products depicts wild-type (A/A), heterozygous (A/G), and homozygous (G/G) mice. The difference in molecular weight for the wildtype (431 kb) and the mutant (327 kb) bands results from intronic DNA lost during generation of the targeting vector.

Supplementary Figure 2.1

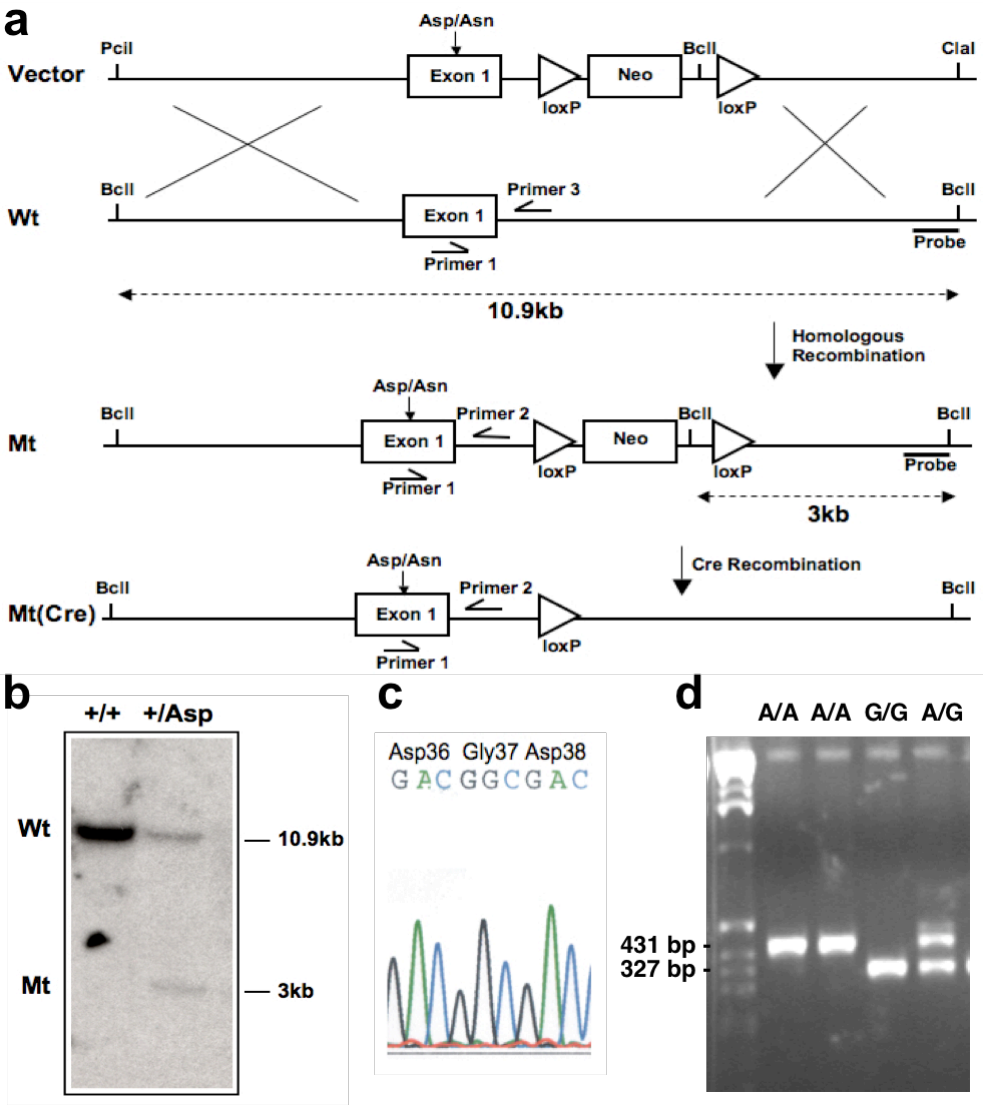


Figure S2. MOPR mRNA is reduced in NAc when using primers targeting different exons as measured by Real Time RT-PCR and normalized against TATA binding protein (TBP) (mean ± SEM, $n = 8$; ** $p < 0.01$, *** $p < 0.001$ compared to A/A, Bonferroni/Dunn).

Supplementary Figure 2.2

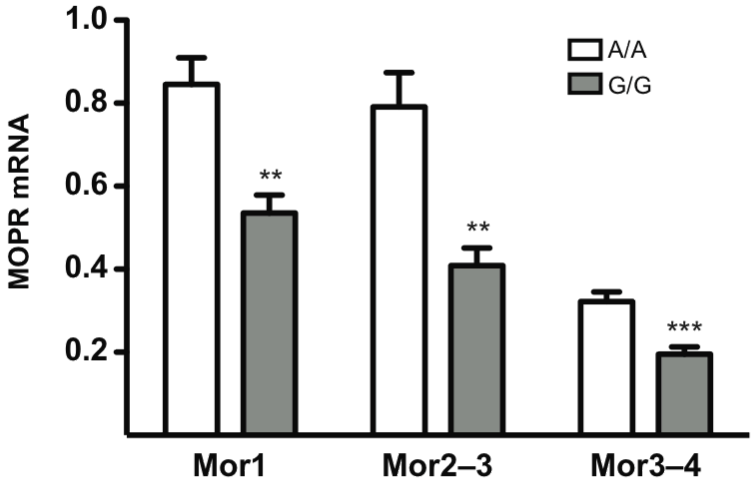


Figure S3. Reduction in locomotor-activating effects of morphine (a) Following 30 minutes of drug-free exploration, morphine administration (10 mg/kg) elevated locomotor activity only in A/A mice. Results are presented as activity counts in 10-min bins (mean ± SEM, n = 16–18; ** $p < 0.01$, *** $p < 0.001$, † $p < 0.0001$ compared to G/G mice receiving morphine and A/A and G/G mice receiving saline, Bonferroni/Dunn). **(b)** Total activity count alterations following an acute saline or morphine injection, as shown in A, are not sex-dependent. (mean ± SEM, n = 8–9; † $p < 0.0001$ compared to G/G mice receiving morphine and A/A and G/G mice receiving saline, Bonferroni/Dunn). **(c)** A low-dose administration of morphine (1 mg/kg) did not alter activity during the 2-h test. Results are presented as activity counts in 10-min bins and **(d)** over the entire session (mean ± SEM, n = 6–8).

Supplementary Figure 2.3

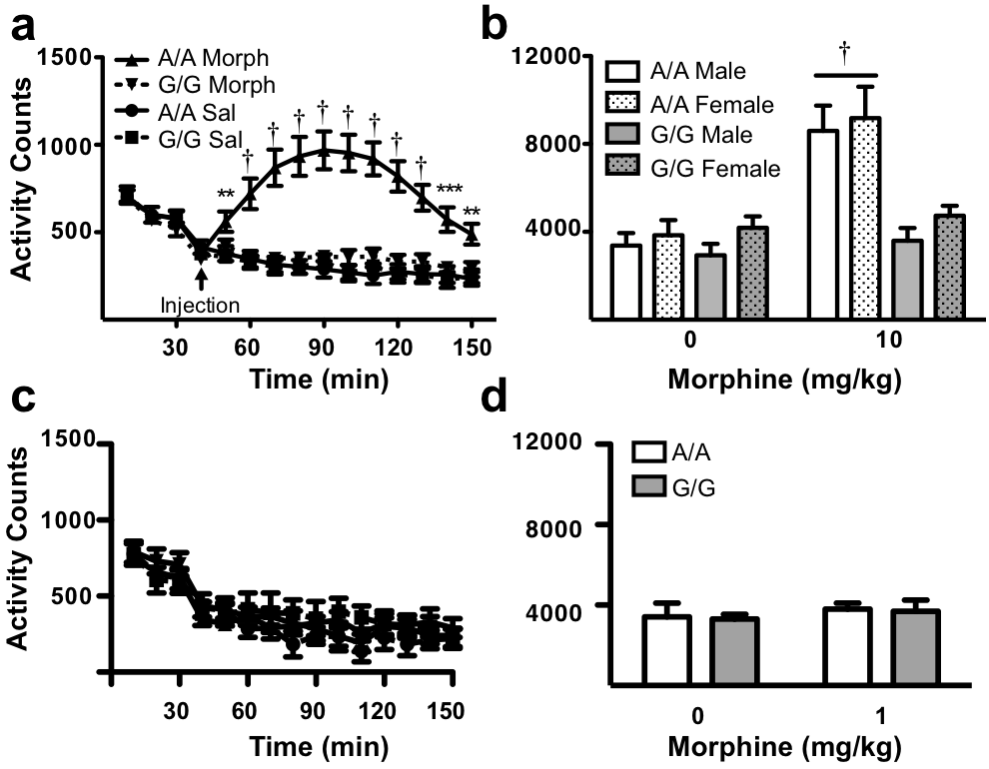
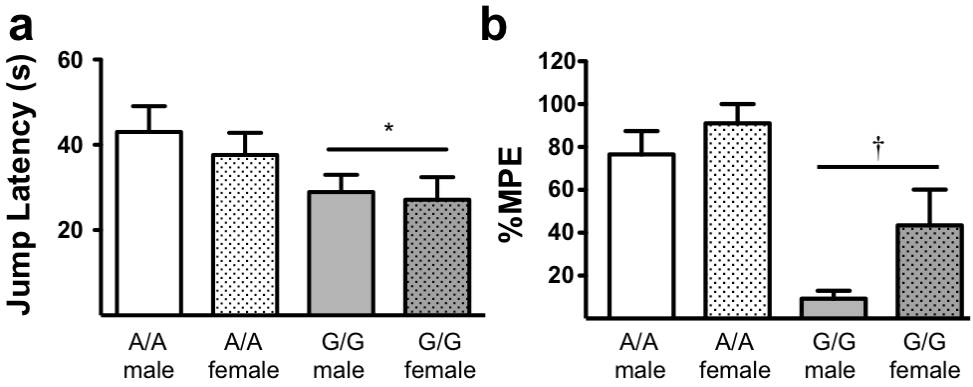


Figure S4. Altered hot-plate responses (a) Baseline nociception, as measured by jump latency on a 58º C hot-plate assay, was significantly reduced in G/G mice. Results are presented as the latency to jump (in seconds) following placement on the hot plate (mean ± SEM, n = 16–18; * $p < 0.05$). **(b)** Morphine-mediated (10 mg/kg) hot-plate antinociception was decreased in G/G mice. Results are presented as percent maximal possible effect (MPE) [(morph jump latency – average saline jump latency)/(total time – average saline jump latency) × 100] (mean ± SEM, n = 7–9; † $p < 0.0001$).

Supplementary Figure 2.4



CHAPTER 3: ALTERED HIPPOCAMPUS NETWORK FUNCTION IN MICE WITH *OPRM1* A118G SNP

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ABSTRACT

A single nucleotide polymorphism (SNP) in the human μ-opioid receptor gene (*OPRM1* A118G) has been widely studied for its association in a variety of drug addiction and pain sensitivity phenotypes; however, the extent of these adaptations and the mechanisms underlying these associations remain elusive. To clarify the functional mechanisms linking the *OPRM1* A118G SNP to altered phenotypes, we derived a mouse model possessing the equivalent nucleotide/amino acid substitution in the *Oprm1* gene. These mice have reduced levels of MOPR expression in some, but not all, brain regions: specifically, the levels of MOPRs in the hippocampus are not different between genotypes. The hippocampus, which contains excitatory pyramidal cells whose activity is highly regulated by a dense network of inhibitory neurons, serves as an ideal structure to evaluate how putative receptor function abnormalities may influence alterations in circuit function. Therefore, to investigate whether this SNP impacts a functional response in the absence of reduced receptor levels, we utilized voltage-sensitive dye imaging in hippocampal slices before and after MOPR stimulation with DAMGO. Utilizing several analytical methodologies, we found that MOPR activation increased excitatory responses in wild-type animals, an effect that was significantly reduced in animals possessing the *Oprm1* SNP. These data further support claims that this SNP results in a loss of receptor function.

INTRODUCTION

Mu-opioid receptors (MOPR) are integrally involved in the modulation of several pathways including pain and drug reward. Genetic mutations of the MOPR alter endogenous and exogenous opioidergic function, thus influencing behavior. A single nucleotide polymorphism (SNP) in exon 1 of the μ-opioid receptor gene (*OPRM1*), in which an adenine to guanine substitution (A118G) exchanges an asparagine for an aspartic acid at a putative N-glycosylation site (N40D), has been associated with an altered vulnerability to opioid addiction (Ray and Hutchison, 2004; Drakenberg et al., 2006; van den Wildenberg et al., 2007), a decreased response to opioid-induced analgesia (Chou et al., 2006a; Sia et al., 2008), and an enhanced response to therapies for alcohol (Ray and Hutchison, 2007; Anton et al., 2008) and nicotine addiction (Lerman et al., 2004). *In vitro* studies using a variety of cell lines expressing the G118 allele have reported increased affinity of the receptor to the endogenous opioid β-endorphin (Bond et al., 1998) and elevated ability of exogenous opioids to inhibit calcium currents (Margas et al., 2007); however, these results were dependent on the cell type and transfection method utilized and other studies have reported no differences in agonist binding, functional coupling, or desensitization (Befort et al., 2001; Beyer et al., 2004). Conversely, studies have reported decreases in MOPR mRNA (Zhang et al., 2005) and cell surface expression of the receptor (Beyer et al., 2004; Zhang et al., 2005; Kroslak et al., 2007), suggesting a loss of receptor function.

Discrepancies in the *in vitro* findings established the rationale for developing a mouse model to examine the molecular, pharmacological, and behavioral significance of this polymorphism in humans. Mice possessing the equivalent SNP (A112G) have

decreases in MOPR expression and morphine-evoked behaviors, in addition to sex-specific deficits in the rewarding properties of morphine (see Chapter 2; Mague et al., 2009). It was not determined, however, whether these behavioral effects resulted solely from a decrease in receptor availability or if the SNP altered receptor functionality independently from expression differences. For instance, both male and female mice homozygous for the G112 allele (G/G) showed equivalent decreases in levels of MOPR mRNA and protein expression in reward-related brain regions; however, only the female mice showed decreases in morphine-conditioned place preference and naloxone-precipitated morphine withdrawal-induced aversions.

Either a change in receptor number or receptor function could alter circuit-level activity due to disregulated opioidergic modulation of target cells. In order to investigate if alterations in receptor function were responsible for these changes, we evaluated circuit function in the hippocampus, a region displaying similar MOPR expression between genotypes and sexes. Structurally, the CA1 region of the hippocampus consists of a single lamina of glutamatergic pyramidal cell bodies [stratum pyramidae (SP)], which have distal apical dendrites that form synapses with entorhinal cortex axons of the perforant path [stratum lacunosum moleculare (SLM)], proximal apical dendrites that form synapses with Shaffer collateral axons from CA3 [stratum radiatum (SR)], and basal dendrites which receive input from other pyramidal neurons [stratum oriens (SO)]. These excitatory synapses are densely innervated by a variety of inhibitory interneurons, which can be identified by the layer in which the soma resides, layers to which the axons project, and the molecular constituents they express (for review, see Klausberger and Somogyi, 2008). MOPRs are predominantly found on somatodendritic and axonal aspects

of fast-spiking, parvalbumin (PV)-containing GABAergic basket cells (Drake and Milner, 1999, 2002). PV cell bodies comprise approximately half of the GABAergic neurons in SP and a smaller portion of the interneurons in the adjoining areas of SO and SR (Kosaka et al., 1987; Freund and Buzsaki, 1996) and their axons project to SLM, SR, and SO (Drake and Milner, 2002).

GABAergic modulation by these interneurons causes local inhibition of excitatory responses in CA1. Specifically, activation of PV cells (e.g., SR stimulation by input from Schaffer collaterals of CA3) leads to strong repolarization of the target pyramidal neurons. This strong perisomatic GABAergic inhibition can induce fast changes in neuronal polarity and gate cell firing at high frequencies (Csicsvari et al., 2003; Uhlhaas and Singer, 2010). Since each PV basket cell targets many pyramidal cell afferents, they are well-situated to modulate CA1 output. Indeed, oscillatory activity in CA1 reflects pyramidal neuron synchronization resulting from the interactions between these cells and the highly interconnected network of GABAergic interneurons (Bartos et al., 2007), particularly the fast-spiking PV cells (Cardin et al., 2009; Lodge et al., 2009). Thus, regulation of excitatory output by PV neurons may, in part, underlie network synchrony and gamma-band oscillatory activity (Whittington and Traub, 2003), which has been shown to influence memory storage and retrieval (Montgomery and Buzsaki, 2007; Montgomery et al., 2009). Activation of MOPRs, by endogenous or exogenously-applied opioids, located on somata and axonal aspects of the PV interneurons hyperpolarizes these cells and decreases GABAergic neurotransmission, thereby disinhibiting glutamatergic neurons and providing net excitatory activity (Neumaier et al., 1988; Glickfeld et al., 2008). This loss of GABAergic modulation induced by MOPR activation

has been shown to reduce high-frequency gamma-band oscillations in the hippocampus (Whittington et al., 1998) and cortex (Sun et al., 2006; Zuo et al., 2007).

CA1 GABAergic modulation by MOPR stimulation has also been shown to be necessary for the rewarding effects of morphine. GABA agonists administered into the CA1 region of the hippocampus – functionally bypassing morphine-mediated $GABA_A$ inhibition – reduced preferences to morphine-paired environments. Conversely, $GABA_A$ antagonists administered directly into the CA1 – functionally mimicking MOPR stimulation – elicited a preference to environments paired with sub-threshold doses of morphine (Rezayof et al., 2007). Together, these experiments demonstrate the necessity of GABAergic modulation by morphine in the CA1 for the expression of morphine reward. In previous studies of the A112G mice, we found a sex-specific deficit in the rewarding properties of morphine, in which the G/G male mice preferred morphine-paired environments equivalently to A/A animals, whereas the G/G females did not. The lack of concomitant MOPR expression-level differences in reward-related brain regions suggested that receptor-signaling differences could be mediating these alterations. Specifically, sex-specific alterations in GABAergic modulation of excitatory responses due to aberrant MOPR regulation could alter hippocampal network activity and, subsequently, disrupt morphine-conditioned preferences.

In order to better understand the synaptic and circuit-level alterations conferred by the A112G SNP, we employed voltage-sensitive dye imaging techniques in hippocampus slice preparations to evaluate basal and opioid-stimulated neuronal responses. The activity of pyramidal cells provides a read-out of net circuit effects on hippocampal output neurons. CA1 pyramidal cells supply an especially clear view of inhibition

because they do not generate recurrent excitation; accordingly, excitatory postsynaptic potentials (EPSPs) induced by afferents are followed almost exclusively by locally induced inhibitory postsynaptic potentials (IPSPs). As a result, CA1 IPSPs in the pyramidal cells form a temporally distinct and therefore measurable VSDi component (Ang et al., 2005; Carlson and Coulter, 2008). In these studies, we found that baseline net circuit activity elicited by a single excitatory stimulus was similar between wild-type A/A mice and the G/G mice. However, while DAMGO administration increased net activity in slices from A/A mice, these effects were significantly attenuated in G/G mice, suggesting a loss of function of the MOPR. These data, which support clinical findings of decreased responses to opioidergic modulation, are the first to show functional receptor deficits resulting from this SNP irrespective of expression-level changes.

MATERIALS AND METHODS

Animals. All experiments utilized adult male and female mice (10-20 weeks of age; 20-35 g) homozygous for the A112 (wild-type) or G112 (knock-in) allele [for detailed description of generation of *Oprm1*^{tm1Jabl} mice, see Chapter 2 (Mague et al., 2009). Briefly, we used site-directed mutagenesis in a bacterial artificial chromosome containing the C57BL/6 mouse *oprm1* to eliminate an equivalent N-linked glycosylation site to the A118G SNP found in humans by replacing the adenine at nucleotide position 112 with a guanine, resulting in an aspartic acid substitution of asparagine at amino acid position 38]. These mice were maintained on a C57BL/6 background and were bred, group housed, and maintained on a 12 h light/dark cycle with food and water available *ad libitum* in accordance with the University of Pennsylvania Animal Care and Use Committee.

Voltage-sensitive dye imaging (VSDi). VSDi experiments were performed according to previous studies [(Ang et al., 2005, 2006); for detailed methodology, see (Carlson and Coulter, 2008)]. Briefly, mice were decapitated following isoflurane anesthesia. The brain was removed and horizontal hippocampal slices (350 μm) were cut using an Integraslice 7550 PSDS vibrating microtome (Campden Instruments, Lafayette, IN) in ice-cold sucrose artificial cerebrospinal fluid (ACSF), in which NaCl was replaced with an equiosmolar concentration of sucrose. ACSF consisted of 130 NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 10 mM glucose, 1 mM MgCl2, 2 mM CaCl2 (pH 7.2-7.4 when saturated with 95% O2/5% CO2). Slices were then transferred to a static

interface chamber (34°C) for 30 min and kept at 22-25°C thereafter. The osmolarity of all solutions was 305-315 mOsm.

Slices were stained for 20 min with 0.125 mg/ml (in ACSF) of the voltage sensitive dye di-3-ANEPPDHQ (D36801, Invitogen), and imaged in an oxygenated interface chamber using an 80 x 80 CCD camera recording at a 1 kHz frame rate (NeuroCCD: RedShirtImaging, Decatur, GA). Epi-illumination was provided by a custom LED illuminator. Compared to the more commonly used photodiode array, the CCD chip well size (215,000 electrons) requires use of relatively low light-intensities, thereby minimizing photodynamic damage. Schaffer collateral stimulation using a single 20-μA, 200-μs pulse was administered with the electrode placed in SR near the CA3/CA1 border (Figure 1a,b). This stimulation paradigm was utilized to highlight influences of PV interneurons, as these cells have been shown to respond with high reliability to initial, but not repeated, afferent input (Pouille and Scanziani, 2004; Spruston, 2008). A field-recording electrode was also placed in SR to monitor population responses following stimulation; these data, however, were not analyzed or included in this manuscript. After initial electrode-placement and evaluation of population responses, the slice was allowed to recover for at least 5 min prior to testing. Baseline responses elicited by 12 single-stimulus trials, each separated by 20 s, were recorded during bath application of ACSF. Following these recordings, the control ACSF was replaced by ACSF containing the selective MOPR agonist [d-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin [DAMGO; 1 μM (Sigma-Aldrich)], which bathed the slice for at least 10 min prior to the presentation of 12 single-stimulus trials of 20-μA, 200-μs pulses.

Data analyses. VSD data was analyzed in IGOR (Wavemetrics, Lake Oswego, OR) on 12-trial-averages as previously described (Ang et al., 2005, 2006). Briefly, fluorescence-changes were calculated as the percent change in fluorescence divided by the resting fluorescence ($\%\Delta F/F_0$). Fitted double exponentials were subtracted from the normalized fluorescence to compensate for photobleaching. ROI Quantification: Local VSD signals were quantified from visually-identified regions of interest (ROIs), including SR and SO (Figure 1b,c). To evaluate the extent of spatial activation, we measured the *active area* in each ROI by determining the percentage of pixels that exhibited depolarization corresponding to responses greater than 3 standard deviations above noise levels following stimulation. Next, using two-dimensional (2D) traces showing changes in fluorescence over time averaged between all pixels within each ROI (Figure 1d), we determined the *peak amplitude* of the excitatory response corresponding to the greatest positive change in fluorescence. Lastly, we identified the *tau*, which describes the duration of the excitatory event, by calculating the time to return to baseline from peak excitation using nonlinear regression analyses in IGOR (Figure 1d). Raster Plot Quantification: Raster plots were generated by drawing a line through the peak of the response from SO to the SLM over the slice image and plotting the fluorescence signal from those pixels that fall under the line for all sampling points in time (Figure 1e). The *peak amplitude* and *tau* were calculated from 2D traces drawn for average pixel-changes corresponding to SO, SR, and SLM (Figure $2b_i$).

To determine net excitatory changes resulting from DAMGO administration, we employed two methods of analysis: a baseline-normalized method that compared the values obtained above before and after drug application and a raster plot subtraction

method that compared pixel-changes prior to quantification. For the first method, we normalized the DAMGO-mediated response with respect to each animal's baseline response for each of the parameters (e.g., area, amplitude, and tau) in the SR and SO for both ROI and raster plot quantifications [(DAMGO – Baseline)/Baseline × 100]. The second method involved subtracting the DAMGO raster plot (Figure $2a_{ii}$) from the basal raster plot (Figure $2a_{i}$), resulting in a representation of the alteration in inhibitory regulation as a result of MOPR stimulation (Figure $2a_{iii}$). From 2D traces corresponding to average subtracted pixel-changes for SO, SR, and SLM, we determined 1) the *peak amplitude* of disinhibition, determined by the greatest change in fluorescence ($\%\Delta F/F_0$), 2) the *duration* of disinhibition, measured as time (ms) that the loss of inhibition remained elevated, and 3) the *area under the curve (AUC)*, which summed the subtracted changes in fluorescence for a 50-ms window following the stimulation (Figure $2b_{iii}$). Statistical analyses were performed with GraphPad Prism 5.0 software package (GraphPad Software, San Diego, CA). Differences between groups (genotype and sex) were assessed using two-way ANOVAs.

RESULTS

Quantification of baseline responses

To evaluate differences in circuit responses to afferent activity, compound population responses in CA1 were induced with a single 20-μA, 200-μs pulse delivered to Schaffer collateral axons passing through SR of CA1. VSDi of area CA1 recorded an evoked fast depolarization followed by a rapid repolarization (Figure 1c–e), reflecting responses at the single-cell level (Ang et al., 2005, 2006; Carlson and Coulter, 2008). As previously validated, these alterations in fluorescence depict net functional changes in neuronal activity, which have been shown to be comparable to AMPA/NMDA-mediated EPSPs and GABA-mediated IPSPs measured by intracellular electrophysiologal techniques (Ang et al., 2005, 2006; Carlson and Coulter, 2008). The initial depolarization, which directly activated CA1 dendrites and local interneurons, propagated to distal regions of SR and outwards towards SLM and SO and was followed by a longer hyperpolarization. This can be visualized spatially in snapshots of the averaged peak excitatory (Figure 1c_i) or inhibitory (Figure 1c_{ii}) responses and temporally as 2D traces of fluorescence changes over time (Figure 1d) or raster plots of activity, which show changes in fluorescence across space and time (Figure 1e).

We employed several methods of analysis in an effort to evaluate different aspects of circuitry responses that may be altered as a result of the MOPR SNP. To examine average pixel-changes within ROIs, we measured the area, amplitude, and tau (duration) of the response. Raster plots depict the fluorescence-change over time of pixels imaged along a line drawn through the peak area of activation/inhibition of the SO, SR, and SLM. We analyzed the amplitude and the duration of the responses for these raster plots

to more directly assess the peak responses, as these data exclude areas within ROIs that did not change as a result of the stimulation. Though this method precludes examination of the area of activation, it does provide a uniform method for determining response amplitudes and kinetics.

A112 and G112 animals, both male and female, displayed similar VSDi responses to Schaffer collateral stimulation under basal conditions. In the SR (Figure 3), there was a trend toward a decrease in basal responses in the G112 animals when measuring the peak amplitude of the ROI (Figure 3b) and a significant decrease in G112 animals when evaluating the peak amplitude of the raster plot (main effect of genotype, $F_{1,19} = 5.26$, $p < 0.05$; Figure 3e). Similar to the SR, there were trends in the SO that were not significantly different (Figure 4). For instance, the females of both genotypes had slightly lower baseline areas of activation (Figure 3a) and peak amplitudes for both ROI (Figure 3b) and raster plot (Figure 3e) quantifications. All other attributes were virtually identical between groups. Taken together, despite the presence of some minor trends, the baseline responses were similar for all groups, suggesting that the A112G SNP did not substantially alter basal circuit function of the hippocampus.

Analysis of DAMGO-mediated response-changes

In order to determine if the A112G SNP alters hippocampal circuit activity during MOPR stimulation, we first examined responses following application of the highly specific MOPR agonist DAMGO (1 μM) and normalized these to each animal's basal response. In accordance with previous studies (McQuiston and Saggau, 2003; McQuiston, 2007) and expectations for agents that inhibit GABAergic release, we found

increases in neuronal activation following DAMGO administration in wild-type mice (Figure 2). In contrast, this effect was greatly reduced in G/G mice, suggesting a loss of receptor function resulting from this SNP.

Specifically, in the SR there was a significant reduction in the ability of DAMGO to prolong the excitatory event in G/G animals (Figure 4). The tau increased by ~40% following DAMGO application in wild-type mice, but was unaffected in the G/G animals, both for the ROI (main effect of genotype, $F_{1,19} = 9.167$, $p < 0.01$; Figure 5c) and raster plot quantifications (main effect of genotype, $F_{1,19} = 18.92$, $p < 0.001$; Figure 5f). There were no significant alterations in the amplitude or the area of activation, suggesting a dissociation between the initial peak response and the kinetics. In the SO, there was a trend towards a selective decrease in G112 females for most of the parameters measured, though these differences were not significant (Figure 6).

A problem with normalizing the DAMGO responses to the baseline responses is that in the event that an animal displayed low or an absence of a baseline response, the normalized result was either exponentially high or undefined, respectively, as a result of dividing by this low or zero value corresponding to the baseline response. For a few animals, in which the baseline response in the SO was small, we were unable to include the normalized data as they were orders of magnitude higher than the average. There were also a few animals, in which the baseline response in the SO was negligible, that a value was unable to be ascertained; this could, in part, explain why trends in SO were not significant. As such, we sought to find a method of analysis that could compare baseline and DAMGO-mediated responses before determining values. To that end, we subtracted the raster plot pixel-changes following DAMGO application from the raster plot

responses observed under basal conditions in order to highlight the loss of inhibition due to MOPR-stimulated GABA inactivation. Since MOPR stimulation with DAMGO effectively decreases GABA transmission, any increase in excitatory events must have occurred due to this reduction in inhibitory modulation: the subtracted raster plots illustrate this MOPR-mediated loss of inhibition. An advantage of this novel approach is that it allowed us to more reliably compare drug treatment effects across genotypes and sexes. By subtracting the actual pixel responses between sessions, we eliminated the requirement for excessive numerical transformations and were able to analyze 2D traces quantified directly from the subtracted plot.

Comparing the subtracted raster plots between genotypes and sexes showed that while all groups showed an initial decrease in inhibitory modulation following DAMGO application, the wild-type animals had an elevated and prolonged response compared to the G/G mice (Figure 7a). Indeed, these observations were supported by quantification of raster plots for each of the regions within CA1. In order to identify differences between groups, we used a 2D trace of the subtracted pixel-changes over time for each region of CA1 and measured the peak amplitude and duration of the response in addition to the area under the curve (AUC) (Figure 7b). In the SO, there were significant reductions in the ability of DAMGO to disinhibit excitatory responses both in G/G animals and in females, without an interaction between these effects. The G/G genotype and the females showed reduced disinhibition compared to their respective counterparts for the peak amplitude (main effects of genotype, $F_{1,19} = 7.45$, $p < 0.05$ and sex, $F_{1,19} = 6.30$, $p < 0.05$; Figure 7b$_i$), duration (main effects of genotype, $F_{1,19} = 22.58$, $p < 0.001$ and sex, $F_{1,19} =$

6.05, $p < 0.05$; Figure $7b_{ii}$), and the AUC (main effects of genotype, $F_{1,19} = 20.68$, $p < 0.001$ and sex, $F_{1,19} = 9.94$, $p < 0.01$; Figure $7b_{iii}$).

We found a similar pattern in the SR, in which there was a significant reduction in the ability of DAMGO to disinhibit excitatory responses in G/G animals, as demonstrated by decreases in the peak amplitude (main effect of genotype, $F_{1,19} = 13.76$, $p < 0.01$; Figure $7b_{iv}$), duration (main effect of genotype, $F_{1,19} = 47.12$, $p < 0.0001$; Figure $7b_{v}$), and the AUC (main effect of genotype, $F_{1,19} = 22.37$, $p < 0.001$; Figure $7b_{vi}$). There was also a main effect of sex for the duration of response, in which the females of both genotypes showed reduced disinhibition compared to their male counterparts (main effect of sex, $F_{1,19} = 10.67$, $p < 0.01$; Figure $7b_{v}$); there was not, however, an interaction between genotype and sex main effects. Another advantage of this analysis was that it allowed us to evaluate differences in SLM, a region that, due to its lower basal responses, we could not otherwise have analyzed. Though the responses for all groups were lower in this region compared to the SR and SO, there was still a significantly reduced disinhibition in the SLM for the G/G animals for the peak (main effect of genotype, $F_{1,19} = 6.30$, $p < 0.05$; Figure $7b_{vii}$), duration (main effect of genotype, $F_{1,19} = 8.42$, $p < 0.05$; Figure $7b_{viii}$), and AUC (main effect of genotype, $F_{1,19} = 18.76$, $p < 0.001$; Figure $7b_{ix}$).

DISCUSSION

MOPR stimulation in the hippocampus increases net excitatory activity by decreasing GABAergic inhibition from local interneurons, which likely results in disruption of pyramidal cell firing synchrony and an alteration in hippocampal function (Faulkner et al., 1998). A common SNP in the gene encoding the MOPR has been shown to alter a variety of behaviors and drug responses in clinical populations (for review, see Chapter 1; Mague and Blendy, 2010) and in animal models (Barr and Goldman, 2006; Mague et al., 2009; Ramchandani et al., 2010). Neither the extent of these changes nor the mechanisms mediating the effects are completely understood. We used VSDi techniques to investigate circuit changes in the hippocampus, a region that showed similar MOPR expression levels between genotypes and sexes in A112G mice, in order to determine if functional alterations resulting from this SNP could better inform results from previous clinical and preclinical studies. Additionally, we developed a novel method of VSDi analysis to highlight the disinhibitory actions of MOPR activation. Overall, we found that the augmentation of excitatory responses elicited by DAMGO administration in wild-type animals was reduced in animals homozygous for the G112 allele. This reduction was particularly striking in raster plot subtraction analyses in which DAMGO-mediated responses of individual pixels were subtracted from basal responses, revealing the loss of inhibition caused by the MOPR activation.

Previous work using similar methodology has evaluated GABAergic mediation of MOPR-stimulated response-augmentations in hippocampal slices (McQuiston and Saggau, 2003; McQuiston, 2007). In these studies, DAMGO application increased neuronal activation throughout the CA1 regardless of the specific area stimulated (e.g.,

SR, SO, SLM). These responses were mediated by either $GABA_A$ (McQuiston and Saggau, 2003) or $GABA_B$ (McQuiston, 2007) receptors, depending on the duration of the simulation paradigm utilized. In the present studies, the normalized data showed moderate increases in the SR and more substantial elevations occurring in the SO. This could be due to ceiling effects in the SR: since stimulation of the SR caused a greater basal activity in this region compared to SO, it is not surprising that there were greater increases in the SO when comparing as percentages of baseline responses. Indeed, in previous studies, the augmentation of responses was lower in the SR compared to SO when stimulation was directed to the SR (McQuiston and Saggau, 2003). Raster plot subtraction analyses, however, showed that dendritic disinhibition was equivalent between the SO and SR, but less robust in the SLM. The disparity between the DAMGO normalization and raster plot subtraction methods highlights the limitations of the former method of analysis. Larger basal responses may lower the potential for augmentation by DAMGO administration. Likewise, smaller basal responses might allow for greater augmentation; however, this could also increase variability within groups or result in the exclusion of values that are exponentially high or undefined. By quantifying the results only after evaluating drug-mediated alterations in pixel-changes via this novel raster plot subtraction method, we were able to eliminate these outcomes and produce more uniform results.

Baseline responses were similar between genotypes and sexes. However, there was a significantly lower peak response in the SR in the G/G animals. This could result from enhanced tonic GABAergic activity, possibly suggesting either a reduction in efficacy of endogenous MOPR modulation of GABA activity or, alternatively, a

reduction in endogenous opioidergic tone in G/G animals. However, this effect was not seen for other measures of responses in the SR or SO, suggesting only a subtle consequence of these potential baseline alterations. Previous work with these mice did not uncover any robust baseline differences, but only reductions in morphine-mediated behaviors. One experiment, however, showed that non-morphine-dependent female G/G mice responded aversively to acute naloxone administration, which might suggest an *increased* endogenous opioidergic tone in G/G female mice (see Chapter 2; Mague et al., 2009). However, further studies are needed to evaluate the endogenous tone of opioid peptides in this mouse model.

Despite similar basal responses, there was a pronounced difference between genotypes following application of DAMGO. Normalization of ROI and raster plot results revealed similar increases in the area of activation and peak responses between genotypes and sexes, but a significant reduction in the duration of the response for G/G animals in the SR. The raster plot subtraction analysis, however, revealed more robust MOPR deficits in the G/G animals in all CA1 regions tested. Though both genotypes show an initial peak disinhibitory effect of DAMGO, this response was more intense and prolonged in A/A animals. This resembles previous studies in which G/G animals responded to the locomotor-activating and antinociceptive properties of acute morphine administration, albeit at levels greatly reduced compared to A/A mice. Since the extent and duration of responses seems to be most affected, these data suggest that there could be alterations in the desensitization or trafficking of the receptor; these effects have not been evaluated in these mice.

In the current experiment, we also found significant reductions in the female responses to MOPR activation compared to males, regardless of genotype. This was not surprising given the frequency of reported sex-differences in response to opioid administration (Craft, 2008). Opioids have been shown to be more efficacious in males compared to females in both rodent (Kepler et al., 1989) and human (Cepeda and Carr, 2003) studies investigating sex-differences in the analgesic properties of opioids. In contrast, female rats respond more robustly to the rewarding properties of opioids (Cicero et al., 2003) and it has been shown that women are more likely to abuse prescription opioid analgesics (Roe et al., 2002). Specifically in the hippocampus, ovarian steroid hormones have been shown to influence levels of opioid peptides (Roman et al., 2006; Williams et al., 2011) and the availability of MOPRs on the surface of PV cells (Torres-Reveron et al., 2009). In contrast to our previous studies, however, we did not demonstrate interactions between genotype and sex. This could suggest that differences in CA1 responses to MOPR activation may not underlie the sex-specific reduction in morphine-conditioned place-preference studies. Alternatively, these behaviors may be linked by shared U-shaped dose-responses in these experiments. Though there was not an interaction between genotype and sex, there were similarities in the patterns of response for both VSDi and place-conditioning studies. In the present studies, there was a reduction in response to DAMGO for both G/G mice and female mice compared to their respective A/A or male counterparts. As a result, the female A/A and the male G/G mice displayed similar MOPR-activated responses, while the male A/A mice responded more robustly than all other groups and the female G/G hardly responded at all. Likewise, female A/A and male G/G mice displayed similarly strong preferences for morphine-

paired environments, whereas male A/A mice showed slightly lower preferences and the G/G female mice did not demonstrate morphine reward at all (see Chapter 2, Figure 4). Other studies have demonstrated inverted U-shaped dose-responses to the acute locomotor activating effects of morphine (Bardo et al., 1997). Since CA1 MOPR-mediated GABAergic modulation has been shown to be necessary for the expression of morphine CPP (Rezayof et al., 2007), it is possible that in our CPP studies the dose tested provided the optimal CA1 responses in the female A/A and male G/G mice, resulting in maximal morphine reward. A complete dose-response experiment might help elucidate this hypothesis.

Since VSDi responses show net activity of entire circuits, we were unable to isolate responses of specific subpopulations of interneurons and, thus, cannot unequivocally ascribe our findings to MOPR modulation of PV basket cells. However, previous studies have shown that MOPRs are found predominantly on these interneurons (Drake and Milner, 1999, 2002) and that stimulation of these cells disinhibits glutamatergic dendrites (Glickfeld et al., 2008). This is supported by the findings provided by the stimulation paradigm utilized in these studies, in which a single 200-μs pulse was administered, as the PV interneurons have been shown to respond with high reliability to initial, but not repeated, afferent input (Pouille and Scanziani, 2004; Spruston, 2008). Also, $GABA_A$ receptors located opposite PV cell terminals produce IPSPs that rise and decay very rapidly (Lavoie et al., 1997; Klausberger et al., 2002). Indeed, other studies evaluating MOPR-mediated elevations of CA1 responses to Shaffer collateral stimulation found that paired current pulses, similar to the single pulses utilized in the present studies, were mediated by $GABA_A$ receptors (McQuiston and Saggau,

2003), while DAMGO-induced augmentations of CA1 responses following prolonged stimulation were mediated by $GABA_B$ receptors (McQuiston, 2007). These features of PV-containing, fast-spiking interneurons enable them to induce a reliable and brief, yet intense, somatic shunting of postsynaptic conductance (Vida et al., 2006; Bartos et al., 2007). Thus, reduced PV interneuron inhibition is a plausible explanation for the augmentation of CA1 responses following DAMGO administration in these current experiments. In addition, the reduced disinhibition demonstrated by the G/G animals following DAMGO administration suggests a disruption in MOPR modulation of these PV interneurons resulting from the A112G SNP.

A consequence of the increase in excitatory responses, demonstrated in wild-type mice, could be a reduction in both neuronal synchrony and the formation of high-frequency oscillatory activity. Indeed, PV interneurons have been shown to be important in generating gamma-band oscillations in the hippocampus (Bartos et al., 2007; Fuchs et al., 2007). Given the reduced DAMGO-mediated augmentation of responses in the G/G animals, we would predict that reductions in gamma-activity resulting from MOPR activation (Sun et al., 2006; Zuo et al., 2007) would be inhibited in these animals. Future studies investigating EEG activity in CA1 following morphine administration will address these predictions.

The reduced effect of DAMGO in G/G animals further supports a loss of function of the MOPR as a consequence of this SNP. Previous work with this mouse line has provided evidence for reduced MOPR expression and decreased behavioral responses to acute morphine administration; likewise, clinical findings have demonstrated a reduced response to the analgesic properties of opioids (Chou et al., 2006b; Sia et al., 2008). In

support of these findings, authors often cite *in vitro* studies showing decreases in MOPR expression (Befort et al., 2001; Zhang et al., 2005). However, the data presented here suggest that receptor function may be disrupted irrespective of alterations in expression. The hippocampus was chosen, in part, due to the lack of differences in expression between genotypes and sexes. The reduction in DAMGO-mediated responses suggests that MOPR function is impaired in a region in which expression is similar. One caveat, however, exists in the specificity of the circuit utilized for VSDi studies. While responses to DAMGO application were tested only in CA1 of the hippocampus in slices taken from the middle of the structure (i.e., neither dorsal nor ventral), expression level changes (e.g., mRNA and protein) were evaluated for the region as a whole. It is possible that expression-pattern differences between genotypes of sexes could explain our results, (e.g., differences in CA1 MOPR expression may be obscured when evaluating the entire structure). Future work using quantitative receptor autoradiography will determine more specific patterns of MOPR expression.

In summary, we utilized VSDi in a hippocampal slice preparation in order to evaluate circuit responses in a mouse model of the human A118G *OPRM1* SNP. Our experiments show that MOPR activation by DAMGO increases net excitatory responses in A/A mice, an effect that was significantly reduced in G/G mice. Additionally, these studies demonstrate that these deficits occur despite similar MOPR expression levels. Future work will establish how the changes in circuit function may affect CA1 synchrony and hippocampal function.

FIGURE LEGENDS

Figure 1. VSDi procedure, quantification, and analysis (a) A diagram of hippocampus circuitry illustrates the stimulus paradigm utilized in this study. A stimulating electrode was placed in the Shaffer collateral axons from CA3 pyramidal cells and a recording electrode was placed in the distal end of SR in CA1. The light gray line represents the pyramidal cell layer and the dotted black line delineates the path of the Shaffer collateral axons. The dark gray box depicts the area visualized in *b* and *c*. **(b)** Horizontal slices containing the hippocampus were visualized under a 10x lens. The black triangles show the stimulating electrode placement and the white triangle shows the placement of the recording electrode. The structures and regions are labeled thusly: SO – stratum oriens, SR – stratum radiatum, SLM – stratum lacunosum moleculare, CTX – cortex, DG – dentate gyrus. **(c)** The average normalized pixel-changes for the duration indicated following stimulation demonstrates the peak excitatory (c_i) and inhibitory (c_{ii}) responses for a representative wild-type animal. Changes in membrane voltage are illustrated in red (excitation) or blue (inhibition). The dotted area indicates the ROI from which the *area of activation* is determined and whose 2D trace is quantified in *d*. The black line corresponds to the raster plot shown in *e*. **(d)** A 2D trace of the average pixel-changes over time for all the pixels contained within the SR region outlined in *c* was used to quantify the *peak amplitude* and *tau*. The scale of response amplitudes corresponds to the numerical axis of the color scale drawn to the right. **(e)** A raster plot corresponding to the pixels along the black line drawn in *c* shows the average pixel-changes over time for the SO, SR, and SLM.

Figure 3.1

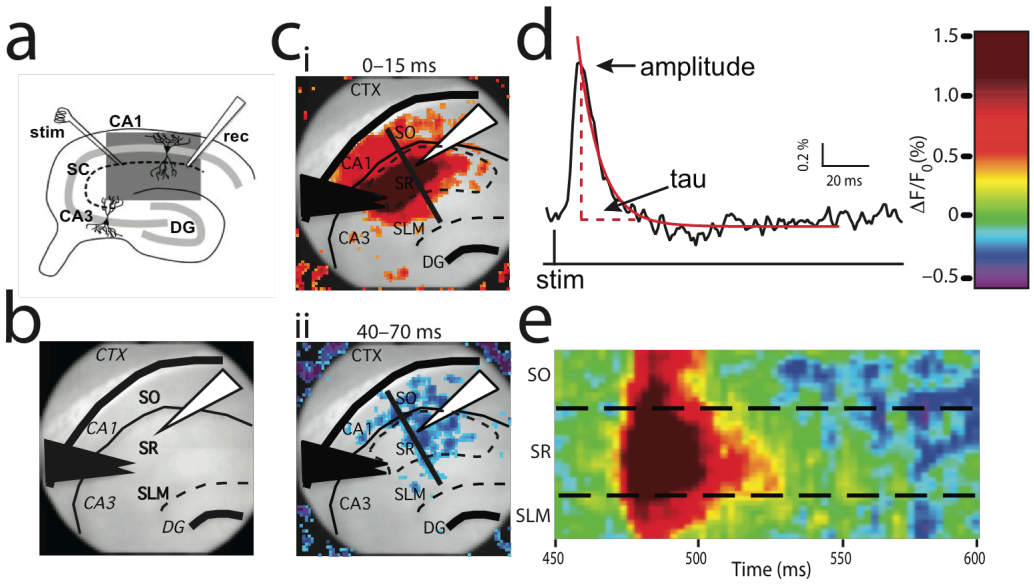

a
stim
CA1
rec
SC
CA3
DG
b
CTX
SO
CA1
SR
CA3
SLM
DG
c i
0–15 ms
ii
40–70 ms
d
amplitude
tau
0.2 %
20 ms
stim
1.5
1.0
0.5
0
−0.5
ΔF/F0 (%)
e
SO
SR
SLM
450
500
550
600
Time (ms)

Figure 2. Raster plot quantification and subtraction analysis (a) Raster plots corresponding to the pixels along the black line drawn in *figure 1c* show the average pixel-changes over time for the SO, SR, and SLM during baseline (a_i) or DAMGO application (a_{ii}). Subtraction of the baseline plots from the DAMGO plots shows the net disinhibition resulting from MOPR activation (a_{iii}). Changes in membrane voltage are illustrated in red (excitation) or blue (inhibition). **(b)** 2D traces of the average pixel-changes over time for all the pixels contained with the SR region of the raster plot were used to quantify the *amplitude* and *tau* ($b_{i,\ ii}$). A 2D trace of the SR region shows the quantification of subtracted raster plots. The *amplitude* was determined by the peak disinhibitory response. The *duration*, shown as the horizontal dashed red line, measured the time (ms) during which disinhibition was elevated above noise. The *area under the curve* (AUC; diagonal red lines) was calculated for a 50-ms window following stimulation. For all 2D plots, the scales of response amplitudes correspond to the numerical axis of the color scales drawn to the left of each trace.

Figure 3.2

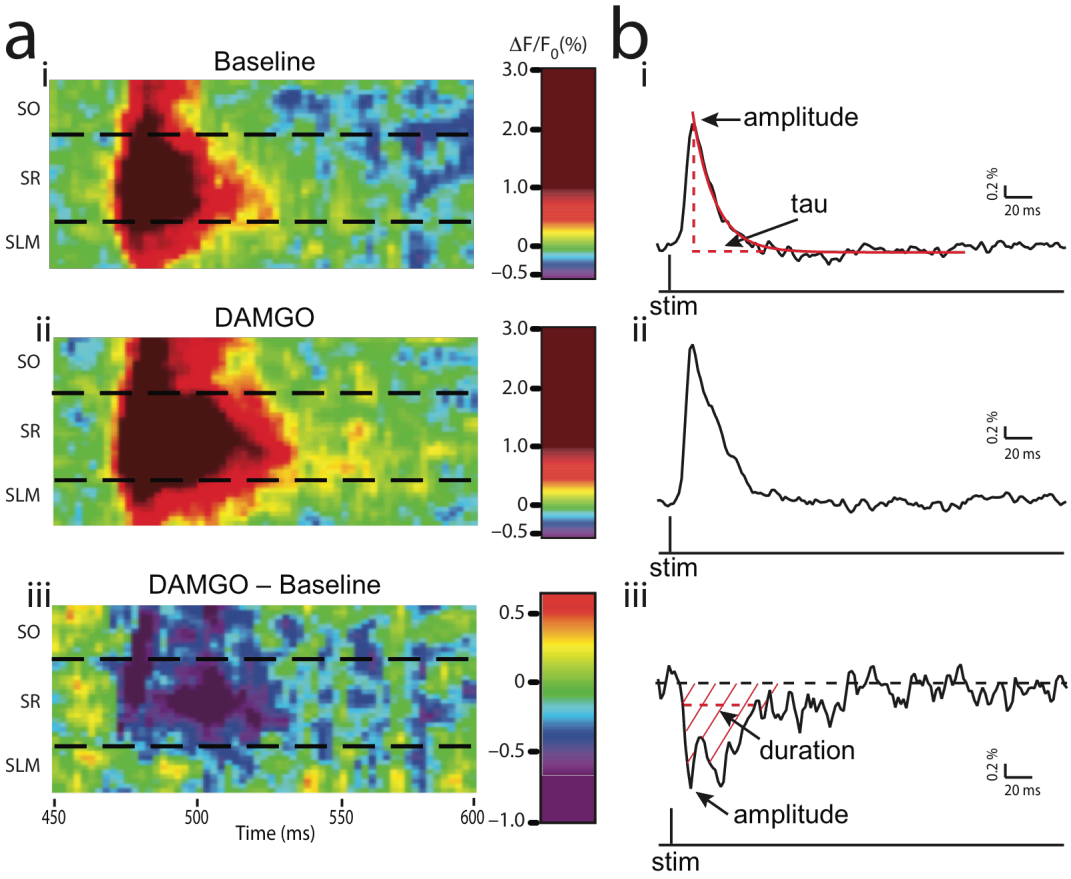

a
i
Baseline
SO
SR
SLM
ΔF/F0(%)
3.0
2.0
1.0
0
−0.5
ii
DAMGO
iii
DAMGO – Baseline
0.5
−1.0
450
500
550
600
Time (ms)
b
amplitude
tau
0.2 %
20 ms
stim
duration

Figure 3. Baseline responses in SR There were no differences between genotypes or sexes for ROI-quantified basal responses in area of activation **(a)**, amplitude **(b)**, or tau **(c)**. For raster plot quantifications, there was a significant reduction in the peak excitation for G/G mice **(d)** but not for the tau **(e)**. All data are presented as mean ± SEM, n = 5; * $p < 0.05$ compared to A/A.

Figure 3.3

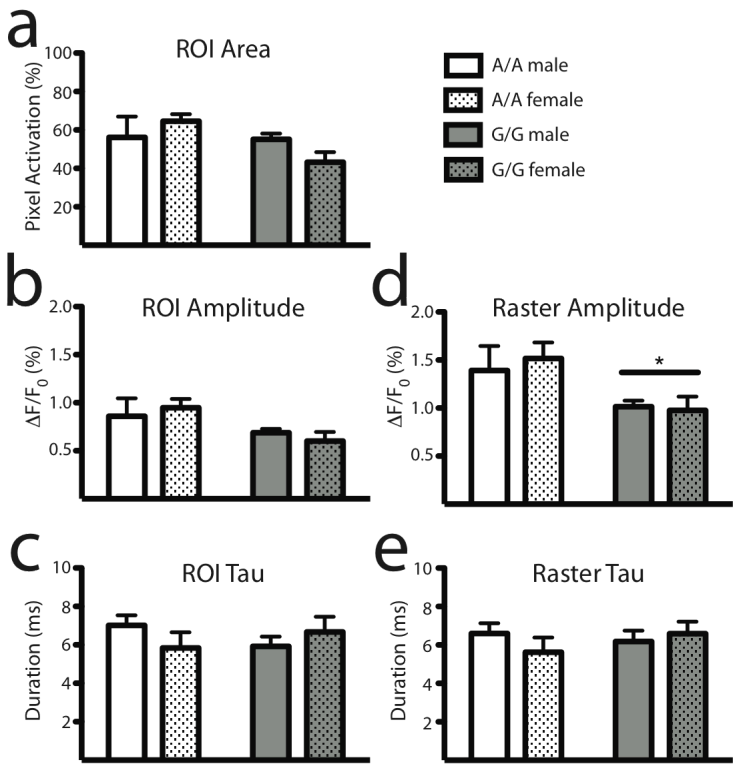

a
ROI Area
Pixel Activation (%)
100
80
60
40
20
A/A male
A/A female
G/G male
G/G female
b
ROI Amplitude
ΔF/F0 (%)
2.0
1.5
1.0
0.5
d
Raster Amplitude
*
c
ROI Tau
Duration (ms)
10
8
6
4
2
e
Raster Tau

Figure 4. Baseline responses in SO There were no differences between genotypes or sexes for ROI-quantified basal responses in area of activation **(a),** amplitude **(b)**, or tau **(c)** nor were there differences for raster plot quantifications of amplitude **(d)** or tau **(e)**. All data are presented as mean ± SEM, n = 4–5.

Figure 3.4

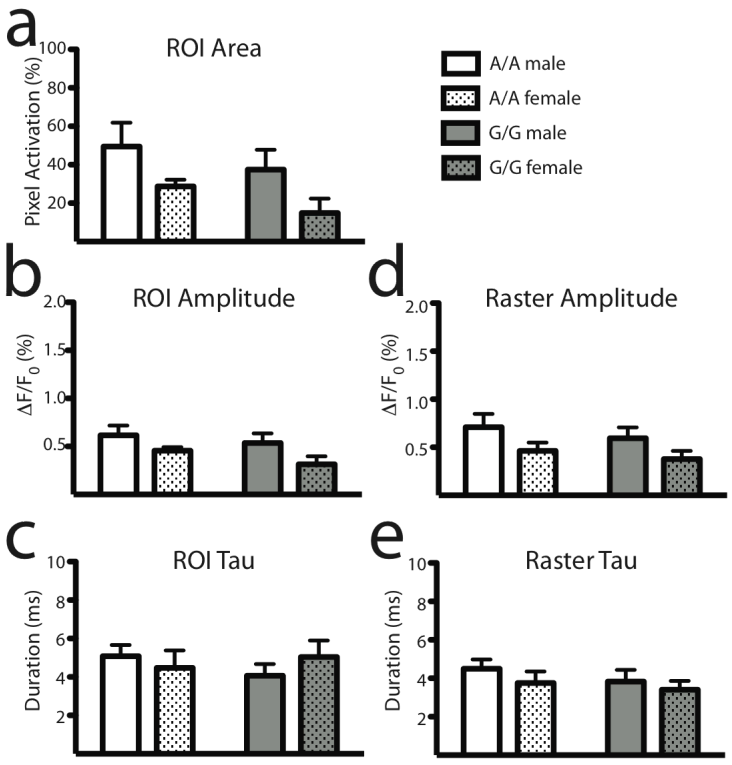

a
ROI Area
Pixel Activation (%)
100
80
60
40
20
A/A male
A/A female
G/G male
G/G female
b
ROI Amplitude
ΔF/F0 (%)
2.0
1.5
1.0
0.5
d
Raster Amplitude
ΔF/F0 (%)
2.0
1.5
1.0
0.5
c
ROI Tau
Duration (ms)
10
8
6
4
2
e
Raster Tau
Duration (ms)
10
8
6
4
2

Figure 5. Normalized DAMGO-mediated responses in SR For ROI-quantified results, there were no gentoype or sex differences in the ability of DAMGO to affect the area of activation **(a)** or amplitude **(b)**. DAMGO-mediated elongations of response duration were reduced in G/G mice **(c)**. For raster plot quantifications, there was not an alteration in amplitude responses to DAMGO application **(d)**. DAMGO-mediated elongations of response duration were reduced in G/G mice **(e)**. All data are presented as mean ± SEM, n = 5; ** $p < 0.01$, *** $p < 0.001$ compared to A/A.

Figure 3.5

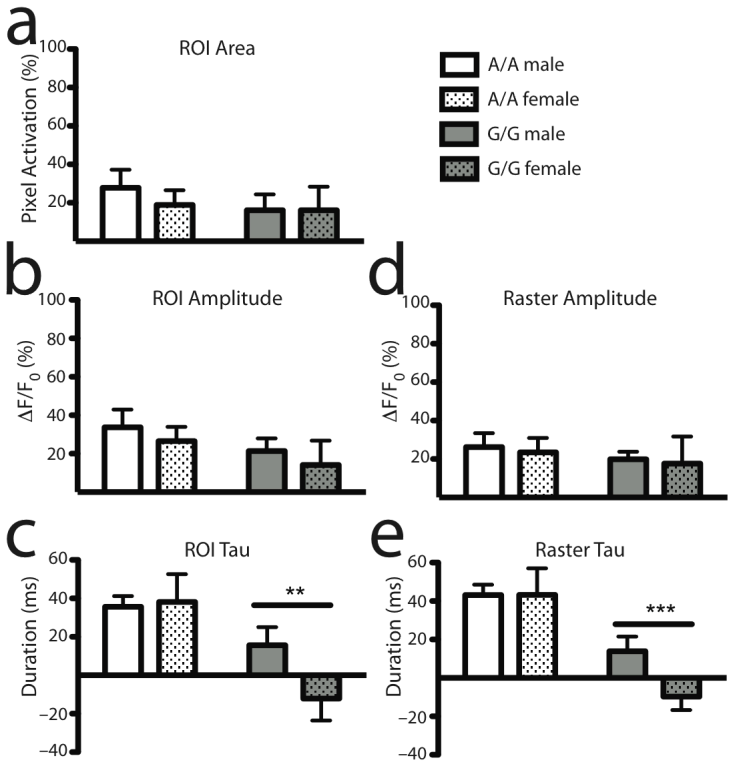

a
ROI Area
Pixel Activation (%)
100
80
60
40
20
A/A male
A/A female
G/G male
G/G female
b
ROI Amplitude
ΔF/F0 (%)
d
Raster Amplitude
c
ROI Tau
Duration (ms)
60
40
20
−20
−40
**
e
Raster Tau
***

Figure 6. Normalized DAMGO-mediated responses in SO For ROI-quantified results, there were no gentoype or sex differences in the ability of DAMGO to affect the area of activation **(a)** or amplitude **(b)** or tau **(c)**. For raster plot quantifications, there were no alterations the ability of DAMGO to affect the amplitude **(d)** or tau **(e)**. All data are presented as mean ± SEM, n = 3–5.

Figure 3.6

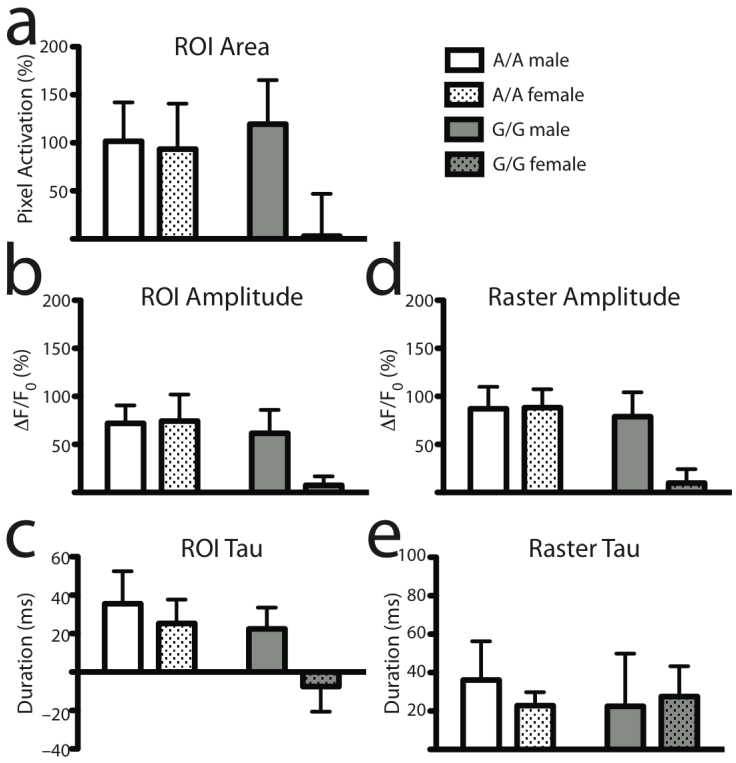
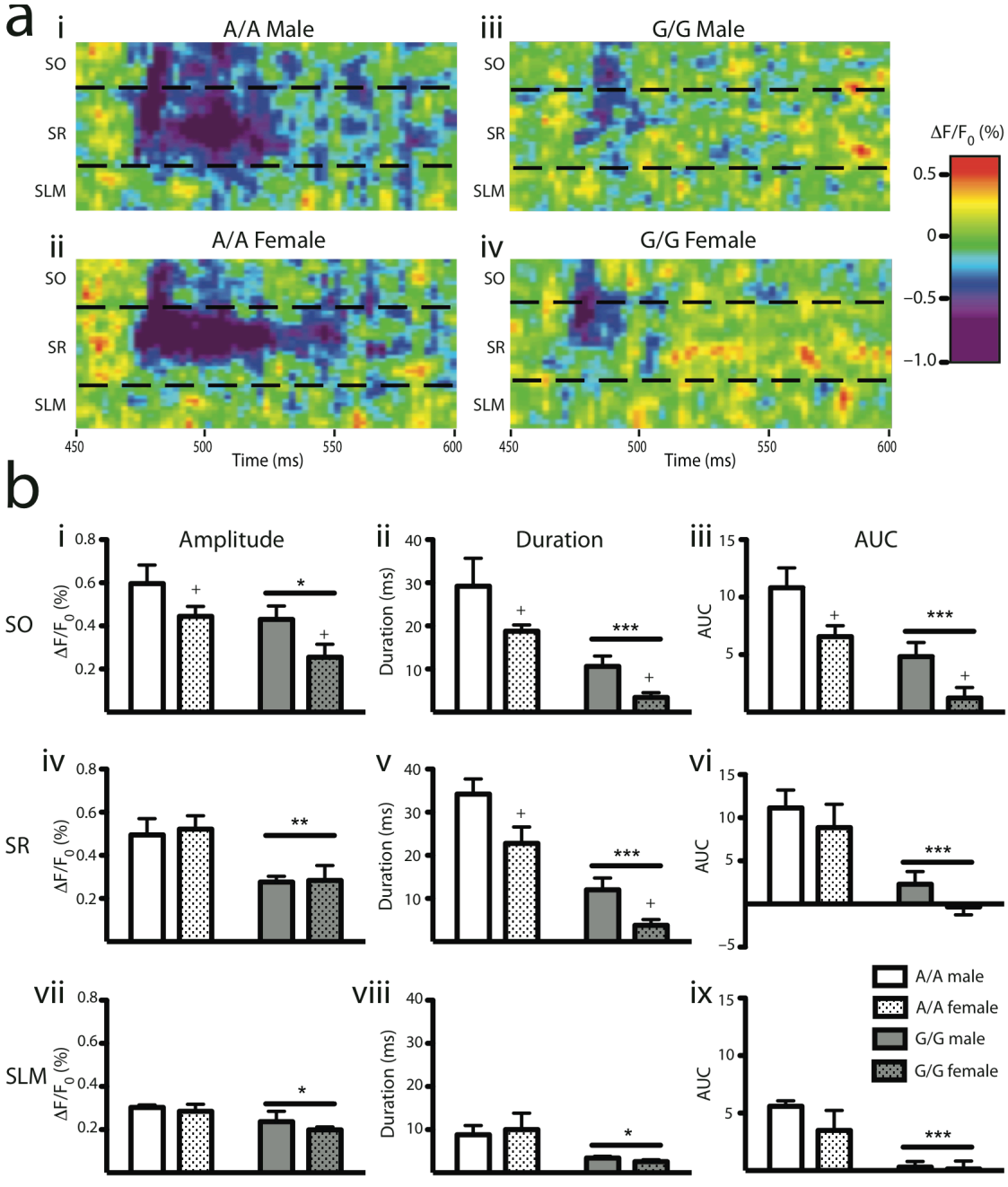

a
ROI Area
Pixel Activation (%)
200
150
100
50
A/A male
A/A female
G/G male
G/G female
b
ROI Amplitude
ΔF/F0 (%)
200
150
100
50
d
Raster Amplitude
ΔF/F0 (%)
200
150
100
50
c
ROI Tau
Duration (ms)
60
40
20
–20
–40
e
Raster Tau
Duration (ms)
100
80
60
40
20

Figure 7. Raster plot subtraction analyses for SO, SR and SLM (a) Representative subtracted raster plots for A/A male (a_i), A/A female (a_{ii}), G/G male (a_{iii}), and G/G female (a_{iv}) show the loss of inhibition resulting from DAMGO administration. **(b)** Analysis of 2D traces from each strata of CA1 reveals alterations in genotype or sex responses to DAMGO administration. In the SO, both the G/G animals and females, each compared to their respective counterparts, showed decreases in the amplitude (b_i), duration (b_{ii}) and AUC (b_{iii}). In the SR, G/G animals showed reductions in amplitude (b_{iv}), duration (b_v), and AUC (b_{vi}); additionally, there was a significant reduction in females compared to males for duration only (b_v). In the SLM, levels of disinhibition were lower compared to the other CA1 regions; however, G/G animals still showed a decreased response to DAMGO administration measured by the amplitude (b_{vii}), duration (b_{viii}), and AUC (b_{ix}). All data are presented as mean ± SEM, n = 5; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, † $p < 0.0001$ compared to A/A; + $p < 0.05$ compared to males.

Figure 3.7


a
i
A/A Male
iii
G/G Male
ii
A/A Female
iv
G/G Female
SO
SR
SLM
450
500
550
600
Time (ms)
ΔF/F0 (%)
0.5
0
−0.5
−1.0
b
i
Amplitude
ii
Duration
iii
AUC
iv
v
vi
vii
viii
ix
ΔF/F0 (%)
Duration (ms)
AUC
A/A male
A/A female
G/G male
G/G female

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The previous chapters describe my work characterizing a novel mouse model of a common human variation in the gene encoding the μ-opioid receptor. Through detailing alterations in receptor expression and function using this model, I have helped extend our understanding of the functional consequences of this SNP. A myriad of clinical studies have found associations of the A118G SNP with several drug and behavioral responses; however, limitations in our abilities to directly study receptor function or determine causative outcomes in human populations precludes uncovering the mechanisms mediating these changes. Conversely, *in vitro* studies, well-equipped to establish molecular adaptations resulting from this SNP, are often performed in heterologous cellular systems, rendering functional implications difficult to interpret. As such, we generated a mouse containing an equivalent SNP in order to evaluate both the changes in receptor expression/function and the behavioral consequences that result from these alterations.

Our initial work, detailed in Chapter 2, determined that there is a reduction in MOPR expression and a deficit in several morphine-evoked responses in mice possessing the G118 allele compared to their wild-type counterparts. Interestingly, we demonstrated a sex-specific reduction in the rewarding properties of morphine administration and the

dysphoria associated with morphine withdrawal in female G112 allele-carriers; all other differences between genotypes were present in both males and females. I continued this research, described in Chapter 3, by examining how changes in receptor function might integrate and alter circuit activity by analyzing hippocampal responses using voltage-sensitive dye imaging techniques. Similar to my original findings, I demonstrated deficits in receptor function in G/G animals. DAMGO application disinhibited cells in wild-type animals, and effect that was significantly reduced in male and female G112 mice. These results, together with clinical and *in vitro* findings from other labs, validate this mouse model of the A118G SNP and justify continued work to determine the mechanisms underlying the changes seen in human populations.

The use of mouse models allows for an important, bidirectional evaluation of genetic contributions to human illness and drug response: observations of human traits can direct investigation in mice and, inversely, new discoveries in the mouse can target new traits for study in humans. Firstly, using the mouse model to study known human phenotypes can help us understand the mechanisms underlying these changes, which can be utilized to either circumvent problems in the human carriers or mimic the putative advantages. For instance, individuals with at least one copy of the G118 allele respond poorly to the analgesic properties of opioids and, consequently, endure more discomfort following surgical procedures (Chou et al., 2006a; Sia et al., 2008). Thorough evaluation of opioidergic- and non-opioidergic-mediated reductions in pain responses in the animal model might identify compounds that are more therapeutically efficacious for those with the G118 allele. Additionally, it has been shown that G118 allele-carriers respond more effectively to treatments for alcohol (Crowley et al., 2003) and nicotine (Lerman et al.,

2004) cessation. Investigation into the mechanisms mediating these outcomes could generate improved treatment options for those carriers of the A118 allele who do not benefit from the putative advantages conferred by the SNP. Indeed, another group has generated a mouse model of the same A118G SNP and identified responses to alcohol that may help explain differences conferred by the SNP (Ramchandani et al., 2010).

Secondly, analysis of the mouse model may identify new phenotypes that have not been recognized in clinical studies. For example, our results have shown multiple occurrences of sex-specific reductions in function due to the SNP. As many clinical studies are underpowered for the identification of sex-differences, significant effects may be obscured. Our results highlight the importance of evaluating sex-differences when investigating this SNP. Additionally, I have shown several other features of this SNP that do not directly correspond to any known clinical effects. For instance, G112 mice showed a reduction in the locomotor-activating effects of morphine and did not sensitize to repeated administration. Sensitization has been associated with drug "wanting" and is considered a cause for addicted behavior (Robinson and Berridge, 1993). Consequently, the G118 should be associated with a decreased risk for developing opioid addiction. Though this has been reported (Schinka et al., 2002), there are other studies that suggest that the G118 allele is actually a risk for the disease (Zhang et al., 2006a), while others found no influence of this SNP on this behavior (Gelernter et al., 1999; Franke et al., 2001). As drug addiction is a complex disorder involving many genes, transmitter systems, brain regions, and behaviors, it is difficult to assign vulnerability based on this one SNP. However, it is perhaps more feasible to identify aspects of the disorder that are affected by allele-differences. For instance, it is possible that populations of addicted

patients could be distinguished by certain reasons for or responses to drug taking. In our studies, though male G118 mice failed to sensitize to repeated morphine, they did develop preferences for environments previously paired with morphine, showing they still respond to the rewarding properties of the drug. Future clinical studies may find parallel responses in humans.

Since this SNP has been associated with a variety of clinical responses, the purpose for generating this mouse model was to address these many effects. As this mouse has a specific mutation in the MOPR, I wanted to first report μ-opioid-specific alterations, related to receptor changes and morphine responses, before encompassing other drugs or transmitter systems. A large portion of the clinical literature is concerned with direct, exogenous application of opioid ligands as they appertain to the maintenance of pain and the development of drug dependence. However, a prevailing hypothesis explaining the rewarding properties for alcohol and nicotine is that they stimulate the release of endogenous opioids (Gianoulakis and Barcomb, 1987; Davenport et al., 1990), which might explain why alterations in receptor function resulting from the SNP might affect responses to these drugs. As such, it is important to next evaluate potential differences in endogenous opioidergic modulation. Though a few of my results suggest that there could be altered tonic MOPR activity, we have yet to specifically evaluate basal or drug-evoked levels of β-endorphin in these mice. This would also provide insight into how the stress system is affected by the SNP, as β-endorphin is released in response to stress and provides negative feedback to stress circuitry. Studies have reported a differential response to psychological versus pharmacological stressors: G118 allele-carriers had lower CORT responses while giving a public speech, but an increased CORT

response to naloxone (Chong et al., 2006), suggesting a dysregulation of tonic β-endorphin transmission. Future studies with these mice could evaluate the response to physical, pharmacological, or behavioral stress.

Initially, I described reductions in responses to morphine-evoked behaviors, which seemed to coincide with decreases in receptor expression. However, further investigation uncovered functional differences in MOPR responses in the hippocampus, a region that did not demonstrate expression-level changes, suggesting that these alterations are mediated by something other than reductions in receptor number. Though there were not differences in MOPR binding affinity, we have yet to investigate alterations in downstream signaling or receptor trafficking, as these would greatly affect receptor function and availability. Future work would identify how these processes may contribute to changes conferred by this SNP.

It has been contested whether the A118G SNP confers a gain or loss of function. Clinical studies that report elevations in biochemical or behavioral traits typically describe elevations in β-endorphin binding (Bond et al., 1998) as the putative mechanism. Alternatively, studies reporting deficits in behavior typically refer to decreases in MOPR expression (Zhang et al., 2005) to explain the effects. While the current experiments did not confirm increases in binding affinity or signal transduction, they did corroborate decreases in MOPR expression. However, I have also shown that the functional and behavioral consequences of the SNP do not seem to follow patterns predicted by these changes in receptor number. This suggests that a more complex interaction between receptor number, availability, and function must be occurring. Future work with this model should help us to better understand the ways in which this SNP

affects receptor function and, consequently, the alterations in behavior seen in the human population.

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