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Morphine-induced antinociception and reward in “humanized” mice expressing the mu opioid receptor A118G polymorphism

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Abstract

The rewarding and antinociceptive effects of opioids are mediated through the mu-opioid receptor. The A118G single nucleotide polymorphism in this receptor has been implicated in drug addiction and differences in pain response. Clinical and preclinical studies have found that the G allele is associated with increased heroin reward and self-administration, elevated post-operative pain, and reduced analgesic responsiveness to opioids. Male and female mice homozygous for the “humanized” 118AA or 118GG alleles were evaluated to test the hypothesis that 118GG mice are less sensitive to the rewarding and antinociceptive effects of morphine. We found that 118AA and 118GG mice of both genders developed conditioned place preference for morphine. All mice developed tolerance to the acute antinociceptive and hypothermic effects of morphine. However, morphine tolerance was not different between AA and GG mice. We also examined sensitivity to the acute antinociceptive and hypothermic effects of cumulative morphine doses. We found that 118GG mice show reduced hypothermic and antinociceptive responses on the hotplate for 10 mg/kg morphine. Finally, we examined basal pain response and morphine-induced antinociception in the formalin test for inflammatory pain. We found no gender or genotype differences in either basal pain response or morphine-induced antinociception in the formalin test. Our data suggests that homozygous expression of the GG allele in mice blunts morphine-induced hypothermia and hotplate antinociception but does not alter morphine CPP, morphine tolerance, or basal inflammatory pain response.

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Keywords

A118G; morphine tolerance; antinociception; morphine reward; formalin test; pain; mu opioid receptor; MOR

1.1 Introduction

Chronic pain is a highly prevalent and expensive public health condition that afflicts over 100 million Americans and costs society in excess of \$635 billion annually (Gaskin and Richard, 2012). Chronic pain generally, although not exclusively, arises from previously experienced unrelieved acute pain, and for both, opiates are frequently prescribed. Concomitant with the rise in physician-prescribed opiates is a dramatic increase in prescription opiate abuse which is now the fastest growing drug problem in the United States (CDC, 2012). Prescription opioid abuse results in over 1.2 million emergency visits and 15,000 fatal overdoses annually, exceeding those of cocaine and heroin combined (CDC, 2011).

The analgesic and rewarding effects of prescription opioids are primarily mediated through the mu-opioid receptor (MOR) (Wise, 1996; Koob, 2006). A number of allelic variants that could modulate MOR function and be involved in the risk for opiate addiction have been identified for the MOR (*Oprm1*) gene. Of these, the A118G variant is the most common single nucleotide polymorphism (SNP), with an allelic frequency of 40-60% among those of Asian, 15-30% among those of Caucasian, and 1-3% among individuals of African and Hispanic descent (Bergen et al., 1997; Bond et al., 1998; Gelernter et al., 1999; Tan et al., 2003). This SNP results in an asparagine to aspartic acid amino acid substitution (N40D) at a putative glycosylation site in the receptor (Bergen et al., 1997; Bond et al., 1998). Originally it was suggested that the A118G SNP caused a gain-of-function due to increased binding of endogenous opioid peptides (Bond et al., 1998). However, more recent work suggests that the A118G SNP may be a loss-of-function mutation due to MOR instability caused by the loss of one of the receptor's five glycosylation sites (Zhang et al., 2005; Mague et al., 2009; Huang et al., 2012; Wang et al., 2012; Weerts et al., 2013).

The objective of this study was to understand how the A118G SNP influences morphine reward, pain sensitivity, and tolerance to the antinociceptive effects of morphine, in order to better understand whether patients expressing this SNP may require more opioid analgesics and might be more vulnerable to opioid use disorder. Although a great deal of work has been done investigating pain and opioid reward in male rodent models, much less has been done on these topics with females. In order to investigate whether the effects of the A118G SNP are gender-specific, male and female mice homozygous for the "humanized" 118A or 118G alleles were evaluated in this study. To this end, we tested the hypotheses that 118GG mice are less sensitive to the rewarding effects of morphine as well as the hypothermic and antinociceptive effects of morphine using the tail-flick, hotplate, and formalin tests.

2.1 Materials and Methods

2.2 Subjects

Subjects used in this study were 153 experimentally naïve adult male (N=77) and female (N=76) A118G mice of both genotypes (118GG and 118AA). “Humanized” A118G mice were created by replacing exon 1 of the mouse *Oprm1* gene with the corresponding sequence of the major human 118A allele. Thus, producing a chimeric protein derived from both human (exon 1) and mouse DNA sequence. Site-directed mutagenesis was done to induce the mutant G allele at position 118 (Mahmoud et al., 2011; Ramchandani et al., 2011). The mice used in this study were back-crossed for at least 10 generations onto a C57BL/6 background. Mice were group housed on a 12:12 hour light/dark cycle with *ad libitum* access to food and water. Animal care procedures were conducted in accordance with NIH guidelines for the Care and Use of Laboratory Animals (2011) and with Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee (IACUC) approval.

2.2 Genotyping

Mice were genotyped as previously described (Freet et al., 2015). Specifically, ear snips were obtained and sent to Transnetyx (Cordova, TN) for DNA sequencing.

2.3 Drugs

Morphine sulfate (NIDA Drug Supply, Bethesda, MD) was dissolved in 0.9% sterile saline and administered subcutaneously (SC), at a dose of 10 mg/kg in an injection volume of 10 ml/kg, immediately prior to morphine conditioned place preference (CPP) testing or 60 minutes prior to testing for tail-flick and hotplate antinociception. For the cumulative morphine dose response, 1.0 mg/ml of morphine was prepared and serially diluted to give cumulative doses of 0.1, 0.3, 1.0, 3.0, and 10.0 mg/kg. Each injection (SC) was given 30 minutes prior to testing for antinociception and hypothermia. Morphine was given intraperitoneally (IP) 60 minutes prior to formalin testing. A 2.5% aqueous formalin solution was prepared by combining one part formaldehyde (Fisher Scientific, Pittsburgh, PA) in 39 parts water.

2.4 Conditioned Place Preference (CPP)

CPP for 10 mg/kg of morphine was examined in male and female 118GG and 118AA mice. Mice were tested in standard three chambered place conditioning boxes (Med Associates, St. Albans, VT) individually housed in sound-attenuating chambers. The experiment consisted of four phases: habituation (one session), baseline place preference (one session), conditioning (eight sessions), and place preference testing (one session). Sessions were scheduled over 11 consecutive days. On day 1 (habituation), mice were given 30 minutes to explore all three chambers of the CPP apparatus. On day 2 (baseline place preference), mice were given 30 minutes of access to all three chambers of the CPP apparatus to determine their baseline preferences (pre-conditioning scores). Mice were then randomly assigned to have morphine (CS⁺) paired with either the black or white conditioning chambers (half of which contained mesh flooring while the other half contained metal bar flooring) and saline

(CS⁺) paired with the other set of visual and tactile cues. During the conditioning phase (days 3-10), mice were injected with either saline or morphine 10 mg/kg (SC) on alternating days (days 3, 5, 7, 9 or 4, 6, 8, 10). Immediately following injections, mice were confined to the designated conditioning chamber for 30 minutes. The assignment of subjects to receive saline or morphine in the different conditioning environments was counterbalanced. On day 11 (following 4 morphine and 4 saline sessions), mice were given a 30-minute place preference testing session in the absence of morphine. CPP was determined by assessing the amount of time (in seconds) that mice spent in the drug-paired chamber before (pre) versus after (post) conditioning.

2.5 Morphine Tolerance

Male and female 118GG and 118AA mice were assessed for tolerance to the antinociceptive and hypothermic effects of daily morphine. Mice were injected once daily with 10 mg/kg morphine (SC) for 10 consecutive days. Antinociception was measured using a Columbus Instruments TF-1 tail-flick analgesia meter (Columbus, OH) and a hot plate analgesia meter (Columbus, OH). The heat source was calibrated to elicit a tail-flick latency of 3-4 seconds (intensity setting 5) in untreated wild-type mice and the hotplate was set to 55°C. Cutoff times of 10s and 30s for tail-flick and hotplate testing were used, respectively, to avoid tissue damage. Tail-flick and hotplate latencies were measured prior to morphine treatment and 60 minutes afterwards to calculate the antinociceptive responses as the percent maximal possible effect (%MPE) with the following calculation: $\%MPE = [(post\text{-}morphine\text{ latency}) - (pre\text{-}morphine\text{ latency})] / [10 - (pre\text{-}morphine\text{ latency})] \times 100$. Hypothermia was assessed by taking body temperatures using a mouse rectal thermometer (Physitemp Instruments, Clifton, NJ), before and after (60 and 120 minutes) morphine injections. Values were converted to the percent change in body temperature ($\% \Delta BT$) = $[(pre\text{-}morphine\text{ temperature}) - (post\text{-}morphine\text{ temperature})] / [pre\text{-}morphine\text{ temperature}] \times 100$.

2.6 Cumulative Morphine Dose-Response

Male and female 118AA and 118GG mice were tested across a range of doses to determine the effects of morphine on antinociception and hypothermia. Mice were cumulatively injected with morphine (SC) resulting in final doses of 0 (saline only), 0.1, 0.3, 1.0, 3.0, and 10.0 mg/kg. Tail-flick, hotplate, and body temperature measurements were taken prior to saline injection and 30 minutes after saline and each dose of morphine. Tail-flick and hotplate antinociception were calculated as %MPE and body temperature was calculated as $\% \Delta BT$.

2.5 Formalin Testing

Male and female 118GG and 118AA mice were used to assess basal differences in inflammatory pain response and sensitivity to the antinociceptive effects of morphine in the formalin test as previously described (Marcus et al., 2015). The formalin test is a well-established model of persistent pain characterized by a transient, biphasic pattern of pain behavior (Coderre et al., 1992; Tjolsen et al., 1992). Prior to testing, mice were acclimated for 20 minutes to a Plexiglass (5"×5"×5") observation chamber placed on a transparent elevated platform. A mirror angled at 45° was placed underneath the platform to allow constant observation of the animal's paws. Following the acclimation period, mice were

administered 10 μ L of 2.5% formalin solution into the plantar surface of a single hind paw using a 0.5 mL syringe with a 28 $\frac{1}{2}$ gauge needle (Becton Dickinson, Franklin Lakes, NJ). Mice were returned to the Plexiglas observation set-up immediately after formalin injection and nociceptive behavior was measured for 60 minutes and quantified for each of the 12 five minute time bins in the hour observation period. During each five-minute time bin, the amount of time that the mouse spent exhibiting three different response behaviors was recorded. The nociceptive behaviors were separated into the following three categories: (0) the injected paw has little weight placed on it; (1) the injected paw is held off the platform surface; (2) the injected paw is licked, shaken, or bitten. The amount of time spent in each category was quantified and weighted with the composite pain score-weighted scores technique (CPS-WST_{0,1,2}), resulting in a composite pain score (CPS) for each five-minute interval between 0 (no pain behaviors) to 2 (maximal pain behavior; (Watson et al., 1997; Guindon et al., 2011). The area under the curve (AUC, CPS $\times$ time(m)) was calculated using the trapezoidal rule for the acute phase (0-15 minutes; phase I) and the inflammatory phase (15-60 minutes; phase II). To assess the antinociceptive effects of morphine, mice were injected (IP) with either saline or 10 mg/kg morphine 60 minutes prior to the formalin injection.

2.6 Data Analysis

Animals were randomly assigned to experimental conditions. Data were examined in terms of group (genotype/sex combination: i.e., Female AA; Male AA; Female GG; Male GG) as the between subjects factor and dose/day/conditioning as the repeated measure. Data for each treatment group were expressed as mean $\pm$ SEM. Data were analyzed using an analysis of variance (ANOVA) for repeated measures or one-way ANOVAs where appropriate. The Greenhouse-Geisser correction was applied to all repeated factors; degrees of freedom reported for significant interactions are the uncorrected values. The source of significant interactions was further evaluated by performing one-way ANOVAs at each individual time point, followed by Bonferroni post hoc tests. The different components of the total variation were settled *a priori* using multiple regression analysis (Draper, 1998). Analyses were performed using SPSS statistical software (version 22.0; SPSS Incorporated, Chicago, IL, USA). $p < 0.05$ was considered significant.

3.1 Results

3.2 Conditioned place preference for morphine in GG and AA Mice

GG mice developed a robust CPP for 10 mg/kg morphine ($F_{1,26}=8.89$, $p=0.006$). There were no gender differences between male and female GG mice ($p=0.568$; Figure 1A) and no interaction (conditioning $\times$ gender) was observed ($p=0.405$; Figure 1A). AA mice also developed a morphine CPP ($F_{1,22}=7.10$, $p=0.014$) (Figure 1B). There were also no gender differences in morphine CPP between male and female AA mice ($p=0.209$ (Figure 1A) and no interaction (conditioning $\times$ gender) was observed ($p=0.521$; Figure 1B).

3.3 Tolerance to morphine in GG and AA mice

Daily pretreatment with 10 mg/kg morphine resulted in tolerance to the antinociceptive and hypothermic effects of morphine (Figure 2). Daily pretreatment with 10 mg/kg morphine

resulted in a loss of morphine-induced tail-flick antinociception in GG ($F_{9,108}=9.52$, $p<0.001$; Figure 2A) and AA ($F_{9,162}=14.72$, $p<0.001$; Figure 2B) mice due to tolerance that develops in a time-dependent manner. No gender differences [$p=0.054$ (GG mice) and $p=0.069$ (AA mice)] or interaction effects [$p=0.361$ (GG mice) and $p=0.357$ (AA mice)] were observed for tolerance to morphine-induced antinociception in the tail-flick test (Figures 2A and 2B). Comparison between GG and AA mice receiving daily morphine (10 mg/kg; SC) revealed time-dependent tolerance development to the antinociceptive effect of morphine ($F_{9,288}=23.24$, $p<0.001$; Figure 2C). No difference in the response to morphine between genotypes (GG relative to AA mice) ($p=0.124$) or an interaction (day $\times$ genotype) ($p=0.133$) was observed.

Daily pretreatment with 10 mg/kg morphine caused time-dependent tolerance to the morphine-induced hot plate antinociception for GG ($F_{9,108}=6.01$, $p<0.001$; Figure 2D) and AA ($F_{9,162}=9.05$, $p<0.001$; Figure 2E) mice. Similar to the tail-flick test, there were no gender differences [$p=0.068$ (GG mice) and $p=0.347$ (AA mice)] or any day $\times$ gender interactions [$p=0.850$ (GG mice) and $p=0.7100$ (AA mice)] observed (Figures 2D and 2E) in tolerance for the morphine-induced hot plate antinociception. Comparison of GG and AA mice receiving morphine (10 mg/kg SC) revealed time-dependent morphine tolerance for both genotypes ($F_{9,288}=12.64$, $p<0.001$; Figure 2F). No genotype difference in morphine tolerance (GG relative to AA mice) ($p=0.598$) or an interaction (day $\times$ genotype) ($p=0.083$) effect was observed.

Daily pretreatment with 10 mg/kg morphine resulted in time-dependent tolerance to the hypothermic effects of morphine in GG ($F_{9,108}=4.00$, $p<0.001$; Figure 2G) and AA ($F_{9,162}=7.54$, $p<0.001$; Figure 2H) mice with no gender differences [$p=0.948$ (GG mice) and $p=0.881$ (AA mice)]. Comparisons between GG and AA mice receiving morphine (10 mg/kg SC) revealed a time-dependent loss of hypothermic effects due to tolerance ($F_{9,288}=10.48$, $p<0.001$; Figure 2I). No genotype difference in morphine response (GG relative to AA mice) ($p=0.086$) or an interaction effect (day $\times$ genotype) ($p=0.221$) was observed.

3.4 Antinociceptive and hypothermic effects of different doses of morphine

Morphine treatment caused dose-dependent antinociception and hypothermia (Figure 3). Morphine treatment (0.1, 0.3, 1, 3 and 10 mg/kg SC) caused a dose-dependent increase in tail flick latencies for both GG ($F_{4,52}=21.12$, $p<0.001$; Figure 3A) and AA ($F_{4,56}=28.19$, $p<0.001$; Figure 3B) mice. However, no gender differences [$p=0.248$ (GG mice) and $p=0.702$ (AA mice)] or interaction effects [$p=0.113$ (GG mice) and $p=0.648$ (AA mice)] were observed (Figures 3A and 3B). Comparison between GG and AA mice showed a dose-dependent effect of morphine on tail-flick latencies ($F_{4,116}=46.44$, $p<0.001$; Figure 3C). No difference in the response to the different doses of morphine was found between the two genotypes (GG relative to AA mice) ($p=0.744$) nor was there an interaction effect (drug $\times$ genotype) ($p=0.342$).

Morphine treatment (0.1, 0.3, 1, 3 and 10 mg/kg; SC) dose-dependently increased hot plate antinociception in AA mice ($F_{4,56}=4.54$, $p<0.003$; Figure 3E), but not in GG mice ($p=0.478$; Figure 3D). No gender differences [$p=0.492$ (GG mice) and $p=0.644$ (AA mice)] or an

interaction [$p=0.571$ (GG mice) and $p=1.000$ (AA mice)] were observed (Figures 3D and 3E). Comparison between GG and AA mice showed a dose-dependent effect of morphine in the hot plate ($F_{4,116}=3.04$, $p<0.020$; Figure 3F). No change between genotypes for the different doses of morphine (GG relative to AA mice) ($p=0.202$) was observed. However, an interaction (drug $\times$ genotype) ($F_{4,116}=3.174$, $p=0.016$; Figure 3F) was revealed such that following 10 mg/kg of morphine, AA mice ($F_{1,29}=4.95$, $p=0.034$; Figure 3F) showed a greater degree of morphine-induced antinociception on the hot plate relative to GG mice.

Morphine treatment (0.1, 0.3, 1, 3 and 10 mg/kg; SC) dose-dependently induced hypothermia in AA mice ($F_{4,56}=6.39$, $p<0.001$; Figure 3H), but not in GG mice ($p=0.622$; Figure 3G). No gender differences [$p=0.765$ (GG mice) and $p=0.710$ (AA mice)] or interactions [$p=0.928$ (GG mice) and $p=0.950$ (AA mice)] were observed (Figures 3G and 3H) for morphine-induced hypothermia. Comparison of GG and AA mice revealed a dose-dependent effect of morphine on hypothermia ($F_{4,116}=3.07$, $p<0.019$; Figure 3I). No change in response to the different doses of morphine between genotypes (GG relative to AA mice) ($p=0.243$) was observed. However, an interaction (drug $\times$ genotype) ($F_{4,116}=5.968$, $p<0.001$; Figure 3I) showed that AA mice ($F_{1,29}=10.19$, $p=0.003$; Figure 3F) displayed greater hypothermia than GG mice at 10 mg/kg morphine.

3.5 Peripheral antinociceptive effects of morphine in the formalin test

Analysis of the AUC of pain behavior revealed that in GG mice morphine (10 mg/kg; IP) produced antinociception relative to vehicle in both the acute ($F_{1,6}=8.94$, $p=0.024$) (Figure 4A) and inflammatory phases ($F_{1,6}=16.10$, $p=0.007$) (Figure 4D) of the formalin test. No differences in the responses to morphine between the male and female GG mice [$p=0.290$ (Acute Phase) and $p=0.491$ (Inflammatory Phase)] and no interactions (drug $\times$ gender) [$p=0.240$ (Acute Phase) and $p=0.246$ (Inflammatory Phase)] (Figures 4A and 4D) were observed in either phase of the formalin test.

Analysis of the AUC of pain behavior revealed that in AA mice morphine (10 mg/kg; IP) produced antinociception relative to vehicle in both the acute ($F_{1,6}=19.08$, $p=0.005$) (Figure 4B) and the inflammatory phases ($F_{1,6}=15.09$, $p=0.008$) (Figure 4E) of the formalin test. However, no differences in the response to morphine between the male and female AA mice [$p=0.280$ (Acute Phase) and $p=0.851$ (Inflammatory Phase)] or any interactions (drug $\times$ gender) were observed [$p=0.372$ (Acute Phase) and $p=0.348$ (Inflammatory Phase)] (Figures 4B and 4E) for either phase of the formalin test.

Analysis of the AUC of pain behavior revealed that 10 mg/kg morphine produced antinociception compared to vehicle in both GG and AA mice in both the acute ($F_{1,6}=23.76$, $p<0.001$) (Figure 4C) and inflammatory phases ($F_{1,6}=27.70$, $p<0.001$) (Figure 4F) of the formalin test. Neither a genotype difference in the response to morphine (GG relative to AA mice) [$p=0.718$ (Acute Phase) and $p=0.304$ (Inflammatory Phase)] nor an interaction (drug $\times$ genotype) was observed [$p=0.816$ (Acute Phase) and $p=0.189$ (Inflammatory Phase)] (Figures 4C and 4F) for either phase of the formalin test.

4.1 Discussion

Results from basic and clinical research have been mixed regarding the effect of the A118G SNP on morphine reward, addiction, and analgesia. Some clinical studies have reported that at least one copy of the G allele potentiates opiate addiction (Szeto et al., 2001; Bart et al., 2004; Drakenberg et al., 2006) and causes reduced analgesic responsiveness to opioids (Klepstad et al., 2004; Chou et al., 2006a; Chou et al., 2006b; Reyes-Gibby et al., 2007; Campa et al., 2008; Tan et al., 2009; Sia, 2008#138), while others have reported negative or no association between the G allele and these phenotypes (Bond et al., 1998; Tan et al., 2003; Janicki et al., 2006; Ginosar et al., 2013; Xu et al., 2015). A number of reasons may underlie such discrepancies, including the translatability of *in vitro* work to animal models used in clinical studies, and the tremendous, inherent heterogeneity within human populations used in clinical studies. One additional potential factor contributing to mixed results in clinical studies is the relative lack of homozygous carriers of the G variant. One strategy to decipher the role of the A118G SNP in pain and addiction uses transgenic and knock-in animal models that are completely homozygous for the major A and minor G alleles. Two such animal models exist to accomplish this goal. The first is a transgenic mouse that expresses a homologous A112G SNP in the mouse *Oprm1* gene that causes the same N40D amino acid substitution that occurs in humans with the A118G SNP (Mague et al., 2009). The second model, involves humanized mice that express a chimeric version of the MOR with the A118G SNP found in human populations (Mahmoud et al., 2011). Both mouse models have provided considerable insight into the role of this SNP in addiction and pain sensitivity.

Bond and colleagues (1998), using *in vitro* heterologous cell expression systems, found that the A118G SNP caused increased binding of β -endorphin and receptor activation (Bond et al., 1998). However, others have reported decreased binding and receptor activation for opioids such as morphine, methadone, and [D-Ala², N-MePhe⁴, and Glyol]-enkephalin (DAMGO) (Kroslak et al., 2007), or no effect on receptor signaling for the A118G SNP (Befort et al., 2001; Beyer et al., 2004). Interestingly, differences in receptor coupling, but not ligand binding, were detected in secondary somatosensory (SII) cortex (but not thalamus) from human GG homozygotes (Oertel et al., 2009), raising the possibility of region-specific effects of the A118G SNP on receptor signaling.

Brain imaging using fMRI found increased activation of the SII cortex during physical pain processing in heterozygous carriers of the G allele (Bonnenberger et al., 2015). Work done using A112G transgenic mice also found evidence of region- and gender-specific differences in receptor signaling (Wang et al., 2012; Wang et al., 2014). These findings raise the possibility that the decreased sensitivity to morphine in 118GG mice reported here might be due to region-specific differences in mu opioid receptor (MOR) signaling. Another possible explanation for the decreased morphine sensitivity in 118GG mice is that the G allele causes decreased amounts of MOR protein. Previous reports showing that the G allele causes decreased MOR mRNA expression, protein levels, and/or protein stability support this possibility (Zhang et al., 2005; Mague et al., 2009; Huang et al., 2012; Wang et al., 2012; Wang et al., 2014; Robinson et al., 2015).

Our findings indicate that humanized 118AA and 118GG mice of both genders develop a morphine CPP. Other studies using transgenic mice found that both genders of 112AA mice and male 112GG mice developed a morphine CPP while female 112GG mice did not (Mague et al., 2009). The use of different genetic models (humanized vs. mouse transgenic) might be responsible for the slight discrepancies between these two studies. However, Robinson and colleagues recently found that humanized male 118GG mice display less morphine-stimulated dopamine release in the nucleus accumbens and decreased morphine reward [assessed using an intra-cranial self-stimulation (ICSS) model] compared to 118AA controls (Robinson et al., 2015). Despite the reduction in dopamine release and morphine reward, transgenic 112GG mice self-administer more heroin than 112AA mice (Zhang et al., 2015). The decreased sensitivity of male 118GG mice to morphine reward, using the ICSS model, contrasts with the CPP that we observe in male 118GG mice and the morphine CPP observed in male 112GG transgenic mice (Mague et al., 2009). It is unclear whether the discrepancy in results is related to differences in the testing methods and/or mouse models. However, it is apparent that additional work should be undertaken to fully understand the role of the A118G polymorphism in morphine-motivated behavior.

Another major objective of this work was to examine the role of the A118G SNP in pain and sensitivity to the antinociceptive effects of morphine. Previous work has found that morphine modulation of voltage-gated calcium channels is less potent and efficacious in sensory neurons from 118GG mice compared to 118AA mice (Mahmoud et al., 2011). Morphine-induced antinociception, assessed with the hotplate test, was reduced in both transgenic 112GG mice (Mague et al., 2009) and humanized 118GG knock-in mice (Mahmoud et al., 2011). For 118GG humanized mice, we observed a blunted antinociceptive response to morphine in the hot plate test as well as blunted morphine-induced hypothermia. Surprisingly, we observed no effect of the A118G SNP on morphine-stimulated antinociception using the tail-flick assay. It is possible that different neural circuitries, spinal (hotplate or tail-flick) versus supra-spinal (hotplate or tail-flick), are involved in mediating these responses.

Our finding of decreased sensitivity to morphine is consistent with clinical studies suggesting that higher doses of morphine are required for pain management in patients possessing at least one copy of the variant G allele. Recent meta-analyses have found an association between the variant G allele and decreased opioid-induced analgesia and nausea (Walter and Lotsch, 2009; Hwang et al., 2014). Higher morphine doses for pain control in cancer patients who carry either one (Campa et al., 2008) or two copies (Klepstad et al., 2004; Reyes-Gibby et al., 2007) of the variant G allele have also been reported. In addition, the amount of patient-controlled morphine for analgesia after total abdominal hysterectomy (Chou et al., 2006a), total knee arthroplasty (Chou et al., 2006b), and cesarean delivery (Sia et al., 2008; Tan et al., 2009) is higher for patients possessing the G allele.

One of the other principal objectives of this study was to further understand the clinical implications of the A118G SNP in pain sensitivity. Healthy individuals possessing at least one copy of the variant G allele have been reported to exhibit lower pressure pain sensitivity (Fillingim et al., 2005) and women heterozygous or homozygous for the variant G allele reported experiencing less pain after sexual assault (Ballina et al., 2013). The allelic

frequency of the A118G SNP was lower in fibromyalgia patients, suggesting a possible protective role for this chronic pain condition (Solak et al., 2014). However, a meta-analysis of eight clinical studies failed to find a consistent association between the A118G SNP and pain response (Walter and Lotsch, 2009). Consistent with that meta-analysis, we find no effect of the G allele on baseline antinociception for any of the pain tests that we applied.

In conclusion, we found that homozygous expression of the G allele does not alter basal pain response in the formalin test for inflammatory pain, the hot plate test, or the tail-flick test. However, we do observe decreased sensitivity to the antinociceptive and hypothermic effects of morphine in 118GG mice. Our findings are consistent with previous basic research and the clinical literature suggesting that the variant G allele confers decreased morphine sensitivity. Therefore, our data is consistent with the idea that the genotype or this SNP should be considered when designing a personalized pain treatment plan in the clinic.

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Abbreviations

ANOVA	analysis of variance
AUC	area under the curve
BT	bodytemperature
CPP	conditioned place preference
DAMGO	[D-Ala ² , N-MePhe ⁴ , and Gly-ol]-enkephalin
ICSS	intra-cranial self-stimulation
IP	intraperitoneal
MPE	maximal possible effect
MOR	mu-opioid receptor
PCA	patient-controlledanalgesia
SII	secondary somatosensory
SNP	single nucleotide polymorphism
SC	subcutaneous

Figure 1. Conditioned place preference in GG and AA mice

GG and AA mice developed a conditioned place preference for morphine. GG (A) and AA (B) mice receiving morphine developed conditioned place preference for 10 mg/kg morphine. Comparisons between GG and AA mice revealed that there were no genotype or gender differences in morphine CPP. Data are expressed as mean $\pm$ SEM (n=10-16 per group). * $p < 0.01$ for post-conditioning versus pre-conditioning

Figure 2. Tolerance to the antinociceptive and hypothermic effects of morphine in GG and AA mice

Daily pretreatment with 10 mg/kg morphine resulted in the rapid development of tolerance to its antinociceptive and hypothermic effects. Daily pretreatment with morphine (10 mg/kg) caused a time-dependent loss in morphine-induced tail-flick antinociception, hot plate antinociception, and hypothermia in GG (A, D, G respectively) and AA mice (B, E, H respectively) with no gender differences or interaction observed. Comparisons between GG and AA mice receiving morphine (10 mg/kg; SC) revealed a time-dependent decrease in tail-flick latency (Figure 2C), hot plate latency (Figure 2F) and hypothermic response to morphine (Figure 2I) with no genotype differences or interaction effects. Data are expressed as mean $\pm$ SEM (N=5-20 per group).

Figure 3. Sensitivity to the antinociceptive and hypothermic effects for different doses of morphine

Morphine treatment (0.1, 0.3, 1, 3 and 10 mg/kg; SC) caused a dose-dependent increase in tail flick latency for GG (A) and AA (B) mice. Dose-dependent effects on morphine-induced hot plate antinociception were observed in AA mice but not in GG mice (E and D, respectively) and also for morphine-induced hypothermia (H and G, respectively). Dose-dependent effects were observed between GG and AA mice for the tail flick, hot plate and hypothermia assays. Following 10 mg/kg morphine AA mice showed a greater degree of hot plate antinociception and hypothermia than GG mice. Data are expressed as mean $\pm$ SEM (N=7-16 per group). * $p < 0.05$.

Figure 4. Morphine-induced antinociception in the formalin test for inflammatory pain
Morphine suppressed formalin-induced pain behavior in GG (A, D), AA (B, E) and AA relative to GG (C, F) mice for both the acute (A, B, C) and inflammatory (D, E, F) phases of the formalin test. In the acute phase, morphine at 10 mg/kg decreased the area under the curve (AUC) in GG (A), AA (B) and GG relative to AA mice (C). In the inflammatory phase, morphine at 10 mg/kg decreased the area under the curve (AUC) in GG (D), AA (E) and GG relative to AA mice (F). Data are expressed as mean $\pm$ SEM (N=4-6 per group).
* $p < 0.05$ for morphine vs. vehicle group

Table 1
Basic research implications of the A118G SNP for reward, addiction, and analgesia

Model	Opioid Addiction Potential	Analgesic Response	Binding for opioids	Receptor activation or coupling	mRNA and/or protein expression	Reference(s)
A118G Transfected cells	-	-	Increase for β -endorphin	Increase for β -endorphin	-	Bond et al., 1998
A118G Transfected cells	-	-	Decrease for DAMGO	Decrease for DAMGO, morphine, and methadone	-	Krosiak et al., 2007
A118G Transfected cells	-	-	No effect	No effect	-	BeFort et al., 2001
A11GG Transfected cells	-	-	No effect	No effect	Decrease	Beyer et al., 2004
A118G Humanized mice	Decreased morphine reward using intra-cranial ICSS	-	Decrease	-	-	Robinson et al., 2015
A118G Humanized mice	-	Decreased morphine response	-	Decrease, in sensory neurons	-	Mahmoud et al., 2011
A118G Transfected cells	-	-	-	-	Decrease	Zhang et al., 2005
A112G Transgenic mice	Gender-specific decrease in morphine reward	Decreased morphine response	-	-	Decrease	Mague et al., 2009
A118G Transfected cells	-	-	-	Signaling	Decrease	Huang et al., 2012
A112G Transgenic mice	-	-	Region and gender-specific decrease for DAMGO	-	-	Wang et al., 2012
A118G	-	-	-	Gender specific decrease for DAMGO	-	Wang et al., 2014
A112G Transgenic mice	Increase heroin self-administration	-	-	-	-	Zhang et al., 2015

Table 2
Clinical implications of the A118G SNP for reward, addiction, and analgesia

Model	Opioid Addiction Potential	Reduction in Analgesic Response (to):	Basic or Clinical Research	Reference(s)
A118G	Yes	-	Clinical	Szeto et al., 2001
A118G	Yes	-	Clinical	Bart et al., 2004
A118G	Yes	-	Clinical	Drakenberg et al., 2006
A118G	-	Yes	Clinical	Klepstad et al., 2004
A118G	-	Yes	Clinical	Chou et al., 2006a, b
A118G	-	Yes	Clinical	Reyes-Gibby et al., 2007
A118G	-	Yes	Clinical	Campa et al., 2008
A118G	-	Yes	Clinical	Tan et al., 2009
A118G	-	Yes	Clinical	Sia et al., 2008
A118G	-	Yes, cancer pain	Clinical	Campa et al., 2008
A118G	-	Yes, cancer pain	Clinical	Klepstad et al., 2004
A118G	-	Yes, cancer pain	Clinical	Reyes-Gibby et al., 2007
A118G	-	Yes, hysterectomy	Clinical	Chou et al., 2006a
A118G	-	Yes, knee arthroplasty	Clinical	Chou et al., 2006b
A118G	-	Yes, cesarean delivery	Clinical	Sia et al., 2008
A118G	-	Yes, cesarean delivery	Clinical	Tan et al., 2009
A118G	-	Reduced threshold for pressure pain	Clinical	Fillingim et al., 2005
A118G	-	Less pain after sexual assault	Clinical	Ballina et al., 2013