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Sex differences in mu opioid receptor expression in trigeminal ganglia under a myositis condition in rats

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Abstract

Background—Peripheral opioid receptor expression is up-regulated under inflammatory conditions, which leads to the increased efficacy of peripherally administered opioids. Sex differences in the effects of inflammation, cytokines and gonadal hormones on μ -opioid receptor (MOR) expression in trigeminal ganglia (TG) are not well understood.

Methods—MOR mRNA and protein levels in TG from male and female Sprague Dawley rats following complete Freund's adjuvant (CFA)-induced muscle inflammation were assessed. Cytokine-induced changes in MOR mRNA expression from TG cultures prepared from intact and gonadectomized male and female, and gonadectomized male rats with testosterone replacement were examined. Behavioral experiments were then performed to examine the efficacy of a peripherally administered MOR agonist in male, female and gonadectomized male rats under a myositis condition.

Results—CFA and cytokine treatments induced significant up-regulation of MOR expression in TG from male, but not from female, rats. The cytokine-induced up-regulation of MOR mRNA expression was prevented in TG from orchidectomized (GDX) male rats, which was restored with testosterone replacement. Peripherally administered DAMGO, a specific MOR agonist,

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Author Contributions

Xia Zhang: Acquired RT-PCR data from organ culture of TG, participated in manuscript writing and revision, and approved the final version of this manuscript.

Yueping Zhang: Acquired data from behavioral experiments, participated in manuscript writing and revision, and approved the final version of this manuscript.

Jamil Asgar: Acquired RT-PCR data from normal male and female rats, participated in manuscript revision, and approved the final version of this manuscript.

Katelyn Niu: Acquired RT-PCR data from *in vivo* cytokine experiment, participated in the design of experiments, manuscript revision, and approved the final version of this manuscript.

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significantly attenuated CFA-induced masseter mechanical hypersensitivity only in intact male rats.

Conclusions—Collectively, these data indicate that testosterone plays a key role in the regulation of MOR in TG under inflammatory conditions, and that sex differences in the anti-hyperalgesic effects of peripherally administered opioids are, in part, mediated by peripheral opioid receptor expression levels.

1. INTRODUCTION

Peripheral inflammation modulates μ -opioid receptor (MOR) expression in dorsal root ganglia (DRG) and trigeminal ganglia (TG). There is a significant increase in the MOR mRNA content and MOR protein positive neurons in DRG following hindpaw inflammation induced by complete Freund's adjuvant (CFA) (Mousa, 2003; Puehler et al., 2004). A marked up-regulation of MOR protein occurs in DRG after carrageenan-induced hindpaw inflammation (Ji et al., 1995). Inflammation of the deep tissues, such as visceral or muscle tissue, also results in an increase in MOR expression in sensory ganglia (Pol and Puig, 2004; Nnz et al., 2007). The increase in MOR density has been proposed as one of the major mechanisms underlying pronounced anti-hyperalgesic effects of peripheral opioids under inflammatory conditions (Zllner et al., 2003). However, cellular mechanisms underlying the modulation of MOR expression in sensory ganglia as well as potential mediators that induce such changes under inflammatory conditions have not been systematically investigated. Available data show cytokines such as interleukin (IL)-1 β , IL-4, IL-6, and TNF α induce MOR expression in neuronal as well as in non-neuronal cell lines (Ruzicka et al., 1996; Vidal et al., 1998; Kraus et al., 2001; Brner et al., 2002, 2004). There are no data on whether cytokines also modulate MOR expression in intact sensory ganglia.

Humans and animals show sex differences in analgesia to systemic opioid treatments (Kest et al., 2000; Craft et al., 2004; Fillingim and Gear, 2004). Multiple mechanisms underlying sex differences in opioid analgesia may exist, including cellular signaling mechanisms and hormonal and genetic effects (Kest et al., 1999; Cicero et al., 2002; Mitrovic et al., 2003; Selley et al., 2003). Numerous studies also provide evidence for a sexual dimorphism in the opioid receptor density in various regions of the CNS, rendering opioid receptor expression level as the basis for sex differences in opioid mediated behaviors (Ostrowski et al., 1987; Wilson et al., 2002; Flores et al., 2003; Carretero et al., 2004; Harris et al., 2004). In the periphery, a local morphine administration in the jaw joint of male, but not female, rats significantly reduces nociceptive jaw muscle activity (Cai et al., 2001), and activation of peripheral MOR produces more potent analgesia in male than in female rats in a visceral pain model (Ji et al., 2006). However, the mechanisms underlying sex differences in peripheral MOR mediated analgesia have not been fully explored.

These observations led us to hypothesize that inflammatory cytokines modulate MOR expression in sensory ganglia in a sex dependent manner, which lead to sex differences in peripheral opioid anti-hyperalgesia. In the present study, we used a rat model of orofacial myositis to evaluate (1) whether inflammation differentially modulates MOR expression in TG between the sexes; (2) the role of inflammatory cytokines, specifically IL-1 β , IL-6 and TNF- α , on MOR expression in TG; (3) whether cytokine-induced MOR expression is modulated by sex hormones, and (4) whether sex differences in MOR expression are related to anti-hyperalgesic effects of a peripherally administered MOR agonist under an inflammatory condition.

2. MATERIALS AND METHODS

2.1 Animals

Age matched adult male, female, orchidectomized (GDX) male and ovariectomized (OVX) female Sprague Dawley rats (8 weeks old; 250–300 g for males and 225–260 g for females, Harlan, Indianapolis) were used in the present study. GDX rats were used three weeks, and OVX animals two weeks, after gonadectomy surgery. The estrous cycle phase in intact female rats was not determined in this study. All animals were housed in a temperature-controlled room under a 12:12 light-dark cycle with access to food and water ad libitum. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under a University of Maryland approved Institutional Animal Care and Use Committee protocol. Experimental and control groups for real time RT-PCR and western blot studies consisted of 5–7 rats per group. For behavioral experiments, 6–8 rats were used per group. Both TG from one rat were used for each culture experiment and the experiment was repeated 6–7 times.

2.2 Muscle inflammation

Inflammation was induced by injecting 50 µl of 50 % CFA in isotonic saline (Sigma, St. Louis, MO) into the mid-region of the masseter muscle via a 27 gauge needle. Rats were briefly anesthetized with 3 % isoflurane for the injection procedure. The characteristics of inflammation following CFA injections in the rat masseter have been described previously (Ambalavanar et al., 2006; Imbe et al., 1999).

2.3 TG culture

The rats were euthanized with pentobarbital (100 mg/kg, i.p.) and decapitated. Both TG from each rat were quickly dissected and kept in ice-cold HBSS solution. Sheaths of connective tissue were carefully removed under a microscope. Approximately 2 mm of axon fibers of the TG from both ends were preserved. Each TG was cut into two equal sections in the horizontal plane. Each section was split into three pieces in the rostro-caudal direction, and then transferred to a 12-well culture plate with 1 ml of culture media (Dulbecco's Modified Eagle Medium, DMEM-F12 supplemented with penicillin 100 U/ml, streptomycin 100 µl/ml, and 10 % fetal bovine serum) and incubated in a 37 °C incubator at 5 % CO₂ for 48 hours. TG cultures were then treated with either vehicle or different cytokines. A similar protocol for TG culture has recently been published by another lab (Lei et al., 2012).

2.4 Real-Time RT-PCR

Total RNAs were extracted from TG of naïve male and female rats, and from male and female rats 1, 3, and 7 days following CFA injection in the masseter muscle, as well as 4 hours after vehicle or cytokine treatments from TG organ cultures. All samples were processed using Trizol (Invitrogen, Carlsbad, CA, USA) and purified according to the RNeasy kit (Qiagen, MD, USA) that included a DNase treatment to remove genomic DNA. Reverse transcription was carried out using the SuperScript First strand synthesis kit (Invitrogen). SuperScript II (Invitrogen) was used to generate cDNA from 500 ng of RNA along with 2.5 ng of random primer per reaction. Real-time PCR analysis of cDNA equal to 15 ng of RNA was then performed using Maxima SYBR Green/ROX qPCR Master Mix in an Eppendorf Mastercycler Ep Realplex 2.0 (Fermentas, Forest City, CA, USA). The following primer pairs were used to detect MOR mRNA: forward 5'-GCC CTC TAC TCT ATC GTG TGT GTA -3', reverse 5'-GTT CCC ATC AGG TAG TTG ACA CTC-3', and actin mRNA: forward 5'-GGT CCA CAC CCG CCA CCA G-3', reverse 5'-CAG GTC CAG ACG CAG GAT GG-3'. We obtained the ratios between MOR and actin to calculate the relative abundance of mRNA levels in each sample. Relative quantification of the MOR

mRNA was calculated by the comparative CT method ($2^{-\Delta\Delta CT}$ method) between control and experimental groups.

2.5 Western blotting

Total proteins were extracted from TG of naïve male and female rats, and from male and female rats 1 and 3 days following CFA injection in the masseter muscle. The protein samples were dissolved in RIPA buffer containing protease inhibitor cocktail. The protein concentration of lysates was determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Fifty micrograms of protein for each sample were separated on 4–12 % NuPAGE gel with MOPS SDS running buffer and transferred to a PVDF membrane (Bio-rad, Hercules, CA). After blocking 1 h in 5 % milk PBST at room temperature, membranes were probed with primary antibodies for MOR (1:1000, Millipore AB5511, rabbit) and GAPDH (1:5000, Calbiochem), used as an internal control protein, diluted in blocking solution. The MOR antibody was raised against the c-terminus of rat MOR and detects 60–67 kDa proteins which disappear in brain lysates obtained from MOR-null mice (Kasai et al., 2011)

Membranes were incubated with primary antibodies overnight at 4° C and washed four times with PBST. HRP conjugated secondary antibodies [anti-rabbit secondary antibody (Cell Signaling) and anti-mouse secondary antibody (Millipore)] were diluted to 1:5000 in PBST and incubated with membranes for 1 h at room temperature. Bands were visualized using ECL (Western Lightning, PerkinElmer Inc.) or ECL plus Western blotting detection reagent (Lumigen PS-3, GE Healthcare). Protein level for MOR was normalized to that of GAPDH in the same sample. Data from CFA-inflamed rats were normalized to that of naive rats and expressed as mean percent changes $\pm$ standard error of the mean (S.E.M.).

2.6 Validation of MOR antibody in a heterologous system

In order to further validate the MOR antibody in our system, we transfected human embryonic kidney (HEK) 293 cells with an expression vector encoding rat OPRM1 with a Myc-DDK epitope tag, pCMV-rOPRM1 (Origene Technologies, Rockville, MD). HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % FBS at 37 °C, 5 % CO₂ incubator. Transfection procedures were performed according to the Invitrogen protocol for Lipofectamine 2000 transfection. Briefly, one day before transfection, HEK 293 cells were cultured in a 6-well plate. On the day of transfection, culture media of these cells at 90 % confluence was replaced with Opti-MEM I Reduced Serum Medium (Invitrogen). 4 µg/well of OPRM1 cDNA and Lipofectamine 2000 reagent (Invitrogen) were diluted in Opti-MEM I Reduced Serum Media and then combined to form complexes that were added to the cells. The cells were incubated overnight before media replacement with complete growth media. Forty-eight hours after transfection, the cells were lysed and processed for immunoblotting with either the MOR or anti-c-myc tag antibody, clone 9E10 (1:200, Boehringer Mannheim, mouse). For immunoprecipitation, the protein lysates were incubated with the anti-c-myc antibody at 4 °C overnight, and then the substrate/antibody complex was incubated with protein A/G Agarose beads for 2 h at 4 °C. Lithium dodecyl sulfate (LDS) sample buffer including sodium dodecyl sulfate (SDS) was added to elute proteins from the protein A/G beads. Samples were separated by 4ed byNuPage gel electrophoresis and subjected to immunoblotting using antibody against MOR.

2.7 Behavioral studies

In this study, we utilized a behavioral model specifically developed for testing masseter sensitivity in rats (Ren, 1999; Shimizu et al., 2009). In this model, a series of calibrated von Frey filaments were applied to the region over the masseter muscle. An active withdrawal of the head from the filament application was defined as a positive response. Each von Frey

filament was applied five times and the response frequencies [(number of responses/ number of stimuli) $\times$ 100 %] to a range of filament forces were determined. After a non-linear regression analysis, an EF50 value, the filament force (g) necessary to produce a 50 % response frequency, was determined. The EF50 value was used as a measure of mechanical threshold. A reduction of EF50 after inflammation suggested the presence of mechanical hypersensitivity.

Mechanical sensitivity of the masseter muscle was determined before and 1, 2 and 3 days after the CFA injection in the masseter muscle. The effect of a MOR agonist, D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin acetate salt (DAMGO) (Tocris, MS, USA) on mechanical sensitivity was examined on the third day following CFA injection, a time point at which mechanical hypersensitivity is pronounced. On day 3, DAMGO (10 μ g in 20 μ l) or the same volume of vehicle was administered directly in the masseter muscle. The post DAMGO or vehicle effect was measured 30 minutes after the drug injection. The proposed dose of DAMGO was adapted from our previous study, which confirmed that DAMGO at this dose specifically targets peripheral MOR without producing central effects (Núñez et al., 2007). In order to maintain consistency in assessing behavioral responses an experimenter who was blinded to treatment conditions conducted all behavioral experiments.

2.8 Cytokines and drug preparation and administration

For TG organ culture studies, IL-1 β , IL-6, TNF- α (PeproTech) and soluble recombinant mouse IL-6 receptor were dissolved in phosphate buffer saline (PBS). IL-1 β , TNF- α and IL-6 along with soluble recombinant mouse IL-6 receptor were applied to culture medium to a final concentration of 100 ng/ml for 4 hrs. Medium containing cytokines was warmed to 37 °C before being applied to cultured TG.

Recombinant human IL-1 receptor antagonist (IL-1RA; PeproTech) and recombinant human gp130 (R&D Systems) were dissolved in PBS. Cytokine antagonists were prepared to a final concentration of 2 μ g/20 μ l and injected into the muscle 30 minutes prior to CFA administration. DAMGO was dissolved in PBS. In order to make sure that all drugs were injected in the same target region of the muscle, the injection site was determined by palpating the masseter between the zygomatic bone and the angle of the mandible. All drug injections were made via a 27-gauge needle for 5–10 seconds.

2.9 Testosterone administration

Testosterone (Sigma-Aldrich, T-1500) was dissolved in safflower oil (16 mg/kg/100 μ l) and administrated subcutaneously to GDX rats for seven days in the morning between 9:00–11:00 AM. The testosterone replacement protocol was adapted from Montico et al., (2011) for Sprague Dawley rats. For RT-PCR experiments, the testosterone replaced GDX rats were subjected to organ culture four hours after the last testosterone injection, followed by cytokine treatments as described above.

2.10 Statistics

A student t-test was used to compare baseline MOR mRNA levels between intact male and female TG samples. All other data obtained from RT-PCR and western blot experiments were analyzed with a One-Way ANOVA on means or Kruskal-Wallis One-Way ANOVA on ranks depending on the outcome of a normality test. The time-dependent changes in mechanical thresholds (EF50) before and after CFA and drug treatments were analyzed with a Two-Way ANOVA with repeated measures. All multiple group comparisons were followed by Dunnett's post hoc test. Data are presented as mean $\pm$ S.E.M. and a $p < 0.05$ was considered significant.

3. RESULTS

3.1 Sex differences in inflammation-induced MOR expression in TG

In order to compare the impact of inflammation on MOR expression between the sexes we measured the changes in the MOR mRNA content in TG following intramuscular injection with CFA. No sex differences in the level of MOR mRNA in TG were observed in naïve rats (Fig 1A; $t=1.03$, $p=0.34$). CFA-induced masseter inflammation resulted in a time-dependent increase in the level of MOR mRNA in TG from male, but not from female rats (Fig 1B,C). In male rats, the level of MOR mRNA was significantly greater in CFA treated groups on days 1 and 3 compared to the naïve group ($H=13.84$, $p=0.003$). The CFA treatment did not alter MOR expression in TG contralateral to the injected side in either male or female rats (data not shown).

To determine whether the changes in mRNA contents were followed by changes in protein levels, we evaluated the changes in MOR protein levels. To detect MOR proteins, we used a commercially available antibody (Millipore AB5511), which has been validated previously (Kasai et al., 2011). To further verify the specificity of MOR antibody in our system, we tested the antibody using recombinant MOR proteins obtained from heterologous transfection of MOR. In order to take advantage of highly specific antibody against c-myc protein, we used cDNA encoding MOR protein tagged with c-myc.

The Western blot detected multiple bands in samples from mock-transfected HEK293 cells that are treated with only transfection reagent or HEK293 cells transfected by myc-MOR or empty vector (pcDNA3.1). A broad protein band around ~64 kDa was detected only in cells transfected with MOR, but not in mock-transfected cells or empty vector transfected cells (Fig 2A). When the same membrane was probed using anti-c-myc antibody, the same sized band was also detected. A broad MOR band over similar size range was previously reported in mouse brain using the same antibody, which disappeared in MOR-null mice (Kasai et al., 2011). Immunoprecipitation with the anti-Myc followed by immunoblotting with the anti-MOR further confirmed the specific detection of proteins of approximately 64 kDa in our preparations (Fig 2B). Therefore, we decided to quantify a protein band around 64 kDa in western blot analyses on TG samples.

Western blot analysis on TG samples confirmed that masseter inflammation differentially regulates the expression of MOR between male and female TG. There was a significant increase in the MOR protein level from day 1 and day 3 following CFA treatment in the male TG ($H=9.2$, $p<0.05$; Fig 2C). The change in protein level is consistent with the RT-PCR data, which also showed significant up-regulation of the MOR message at those time points. In female TG, the MOR protein level following inflammation was slightly, but significantly decreased at day 1, but remained unaffected on day 3 ($H=6.2$, $p<0.05$; Fig 2D). We did not measure the protein level for day 7. These data confirmed that masseter inflammation induces changes in MOR mRNA levels in TG from male rats (Núñez et al., 2007), and provided new evidence that similar changes do not occur in female rats.

3.2 Effects of inflammatory cytokines on MOR expression

In this experiment, we investigated whether inflammatory cytokines induce differential up-regulation of MOR expression in male and female rats using TG organ cultures. In TG cultures from male rats, the level of MOR mRNA was significantly higher following the application of TNF- α , IL-1 β , or IL-6 along with its soluble α -receptor subunit (IL-6R) compared to the vehicle condition (Fig 3A; $F=8.9$, $p<0.001$). The vehicle treatment itself, in this case PBS, did not significantly alter the basal level of MOR mRNA. The same

concentration of IL-1 β , IL-6R, or TNF- α did not induce significant changes of MOR mRNA in TG cultures prepared from female rats (Fig 3B; $F=0.64$, $p=0.6$).

We then investigated whether these inflammatory cytokines are involved in CFA-induced up-regulation of MOR mRNA in TG *in vivo*. IL-1 receptor antagonist (IL-1RA) or soluble gp130 (an inhibitor of IL-6 signaling complex) was directly administered into the masseter muscle 5 min prior to the induction of inflammation by CFA in male rats. TG from these rats were extracted 3 days later and the level of MOR mRNA was assessed. We did not examine the effects of TNF- α receptor antagonist since CFA does not increase the level of TNF- α in the masseter muscle (Niu and Ro, 2011). Pretreatment of the muscle with PBS failed to block the CFA-induced up-regulation of MOR mRNA (Fig 3C; $H=14.2$, $p=0.03$). However, IL-1RA, at a dose that has been shown to effectively attenuate the IL-1 β effect (2 μ g) (Hook et al., 2011), prevented the up-regulation of MOR mRNA in TG. Similarly, soluble gp130 at a dose that effectively antagonizes the IL-6 effect (2 μ g) (Obreja et al., 2002) prevented the CFA-induced up-regulation of MOR mRNA in intact TG (Fig 3C).

3.3 Testosterone is required for cytokine-induced MOR mRNA up-regulation

Since cytokine-induced up-regulation of MOR mRNA was only observed in TG from male rats, we investigated whether sex hormones play a role in modulating cytokine-induced MOR expression. In order to examine the role of testosterone, we applied the same cytokine treatments to TG cultures prepared from GDX rats. Under this condition, none of the cytokines were effective in up-regulating MOR mRNA (Fig 4A). Instead, IL-1 β application resulted in a slight, but significant down-regulation of MOR mRNA expression ($F=3.1$, $p=0.05$). We then applied the same set of cytokines to TG cultures prepared from GDX rats that received testosterone replacement for 7 days. In TG cultures from these rats, all three cytokines significantly increased the MOR mRNA level (Fig 4B; $H=13.1$, $p=0.004$).

Since it is possible that estrogen could have persistent inhibitory effects on cytokine-induced MOR expression, we applied the same concentrations of the three cytokines to TG cultures prepared from OVX female rats. None of the cytokines exerted a significant effect on MOR mRNA expression (Fig 4C; $F=0.62$, $p=0.6$).

3.4 Sex differences in the effect of peripheral MOR activation on masseter hypersensitivity

Masseteric injection of CFA in the rat induces a time-dependent and significant decrease in mechanical thresholds as early as 30 min that lasts over 12 days (Ambalavanar et al., 2006; Shimizu et al., 2009). We have recently confirmed the development of mechanical hypersensitivity following CFA injection in the masseter with a peak decrease in EF50 on 1–3 days post CFA injection (Niu et al., 2012). In order to examine the effect of MOR activation on CFA-induced masseter mechanical hypersensitivity, we injected the muscle with DAMGO (10 μ g) or vehicle 45 min prior to behavioral testing on the third day following CFA treatment. At this dose, DAMGO effectively blocks masseter nocifensive responses without producing systemic effects by specifically targeting peripheral MOR (Núñez et al., 2007).

The EF50 of baseline mechanical thresholds were comparable between male and female rats (59.9 ± 2 g and 55.1 ± 3.98 g, respectively). Intramuscular CFA injection resulted in a significant mechanical hypersensitivity for the three days we monitored in both male and female rats. In those rats, the vehicle injection into the masseter muscle on day 3 did not alter the mechanical thresholds in either male or female rats (Fig 5A; Time: $F=76.5$, $p<0.001$; Sex: $F=2.4$, $p=0.162$). In contrast, DAMGO treatment in the masseter muscle significantly reversed the CFA-induced mechanical hypersensitivity in male, but not in female, rats (Fig 5B; Time: $F=95.3$, $p<0.001$; Sex: $F=20.3$, $p<0.001$). Finally, neither

DAMGO nor vehicle given 3 days post CFA treatment significantly altered the mechanical hypersensitivity in GDX male rats (Fig 5C; Time: 75.2, $p < 0.001$; Drug: $F = 1.2$, $p = 0.29$).

4. DISCUSSION

Sex differences in MOR expression have been reported in many regions of the brain. In the rat anterior pituitary gland, MOR is more abundantly expressed in males compared to females (Carretero et al., 2004). Similarly, the MOR protein levels in the midbrain and the spinal cord are higher in male rats than in female rats, and CFA-induced arthritis treatment significantly increases MOR expression in the midbrain only in male, but not in female rats (Kren et al., 2008). In the rat periaqueductal gray (PAG), males exhibit significantly higher expression of MOR compared with cycling females (Lloyd et al., 2008). Although changes in MOR expression levels following CFA-induced inflammation were not assessed in their study, microinjection of morphine into the PAG more effectively relieves CFA-induced thermal hyperalgesia in males than in females, suggesting a higher morphine analgesia that correlates with a higher level of MOR expression in males.

In the periphery, opioid receptor efficacy is augmented under inflammatory or injury conditions due to the increase in opioid receptor density in primary afferent neurons (Hassan et al., 1993; Zöllner et al., 2003; Pol and Puig, 2004; Mousa et al., 2010). We have previously shown that masseter inflammation up-regulates MOR mRNA in TG (Núñez et al., 2007), which we confirmed in this study. Here we report that similar changes do not occur in female rats. To the best of our knowledge, we do not know whether such profound sex differences in MOR expression in sensory neurons have been demonstrated in other inflammatory models.

In a visceral pain model, the peripherally restricted MOR agonist loperamide produces greater potency in males compared with females, and the peripherally restricted MOR antagonist naloxone methiodide dose-dependently attenuates the systemic morphine effects (Ji et al., 2006), which could be mediated by a higher level of peripheral MOR expression in male rats. Loperamide also exerts greater anti-hyperalgesic effects in CFA treated arthritic male than female rats (Cook and Nickerson, 2005), and direct morphine injection into the TMJ significantly suppresses glutamate-evoked jaw muscle activity only in male rats (Cai et al., 2001). These studies clearly implicate sex differences in peripheral MOR-mediated analgesia, but MOR expression levels were not directly assessed. Our data provided important clues that sex differences in MOR function are, in part, mediated by the MOR expression levels in sensory neurons. While full dose-response functions of DAMGO for male and female rats are required to assess the extent and nature of sex differences, our data revealed novel information on the impact of inflammation on peripheral MOR expression that could explain greater anti-hyperalgesic effects in males by peripheral opioids.

Cytokines are the most widely studied factor that modulates opioid receptor expression in various cell types (Kraus, 2009). The promoter regions of the MOR gene contain binding sites for many transcription factors that regulate gene transcription. IL-1 α and IL-1 β treatment induces MOR expression in neural microvascular endothelial cells, possibly by activating the cytokine response element, NF-IL6 in the opioid receptor gene (Vidal et al., 1998). However, NF-IL6 in the opioid receptor gene is not functional in immune cells (Im et al., 1999). Using transcription factor decoy oligonucleotides, Börner et al. (2004) showed that STAT1 and STAT3, but not other transcriptional factors such as NF-IL6 and AP1, are involved in MOR transcription in the human neuroblastoma cell line. In the same cell line, AP1 is required for MOR gene expression following the activation of protein kinase C signaling pathways (Börner et al., 2002). TNF α also induces MOR gene expression in immune cells in a nuclear factor-kappa B, but not AP1, dependent manner (Kraus et al.,

2003). Thus, cytokine-mediated MOR gene expression seems to be determined by transcriptional factors and cell types. Of direct relevance to this study, IL-1 β up-regulates kappa opioid receptors (KOR) in DRG (Puehler et al., 2006). Our data provide the first evidence that inflammatory cytokines such as TNF α , IL-1 β and IL-6 induce MOR expression in TG. These data should prove important for further studying transcriptional mechanisms involved in opioid receptor systems in somatic sensory ganglia.

Since masseter inflammation led to the up-regulation of MOR mRNA in TG in a sex dependent manner and inflammatory cytokines are potent modulators of opioid receptors, it is reasonable to propose that inflammation produces different cytokine profiles in local tissue that might be correlated with MOR expression between the sexes. However, CFA-induced inflammation produces a similar rise in IL-1 β and IL-6 in both male and female rat muscle tissue (Niu and Ro, 2011). Muscle inflammation does not significantly increase local TNF α levels (Loram et al., 2007; Niu and Ro, 2011). Despite the similarities in local cytokine levels, direct application of cytokines resulted in MOR mRNA increase only in TG of male rats. These observations indicate that MOR up-regulation in TG cannot be explained solely by local cytokine effects. While it is possible that functional transcriptional factors or cytokine receptors are differentially expressed in TG of the two sexes, our data from GDX rats and GDX rats with TS replacement clearly implicate the involvement of testosterone in the regulation of MOR expression. These results are consistent with previous studies that showed testosterone is required for the antinociception induced by opioid receptor like-1 receptor in the spinal cord and alpha(2)-adrenoceptor in the trigeminal sensory nuclear complex (Claiborne et al., 2006; Nag and Mokha, 2009). It is possible that TS effects we observed in GDX animals are due to a supra-physiological level of TS (Fischer et al., 2007). However, TS replaced at 5 mg/kg for 30 days in older Sprague Dawley rats restores the physiological level of plasma TS (Montico et al., 2008). Regardless of how various TS replacement protocols affect plasma TS levels, it is unknown as to how much circulating TS or what type of androgen (TS or DHT) is required to exert changes in receptor expression in peripheral sensory neurons under inflammatory conditions. A significant role of estrogen in expression is not supported from our data from OVX females. We could not, however, determine the estrous stage of female rats since CFA-induced inflammation disrupts estrus cycling (Wang et al., 2006). Therefore, the impact of fluctuations in estrogen levels in normally cycling female rats on MOR is yet to be determined. Further studies are required to examine detailed mechanisms that underlie immune-hormone-MOR interactions in sensory ganglia.

Cytokine-induced up-regulation of peripheral cannabinoid type 1 receptors (CB1R) is also dependent on the presence of testosterone (Niu et al., 2012). The up-regulation of CB1R is prevented in TG cultures from GDX male rats, only to be restored by testosterone replacement. The cytokines do not alter the CB1R mRNA level in TG from intact as well as OVX female rats, and neither estradiol supplement nor estrogen receptor blockade produces any effects on CB1R expression. Since the promoter regions of both CB1R and MOR genes contain putative androgen receptor binding sites (unpublished observation) we speculate that cytokines may promote a testosterone-androgen receptor complex, which directly modulates peripheral MOR and CB1R in sensory ganglia. Together, these data provide an intriguing possibility that testosterone may play a key role in modulating multiple peripheral anti-nociceptive systems under inflammatory conditions.

The analgesic efficacy of peripheral opioids in a clinical setting has been most extensively studied after knee arthroscopy. While the majority of studies on intra-articular application of opioids support the role for peripheral opioid receptors, some do not (Rosseland, 2005). Similarly, intra-articular morphine in the temporomandibular joint (TMJ) effectively relieves pain in temporomandibular disorder patients (Ziegler et al., 2010), but render

ineffective in patients following TMJ arthroscopy (Bryant et al., 1999). While the discrepant findings seem to undermine the clinical importance of targeting peripheral opioid receptors methodological factors such as study sensitivity, tissue inflammation, or superimposition of general or local anesthetics, which can mask the local opioid effects need to be carefully considered (Stein, 2006; Stein and Küchler, 2012).

In other inflammatory or injury related clinical conditions, such as chronic rheumatoid, osteoarthritis, chronic tooth inflammation and postoperative bladder surgery, application of opioids at the local site effectively relieves pain and hyperalgesia (Duckett et al., 1997; Likar et al., 2001; Rorarius et al., 1999; Dionne et al., 2001). It is also important to note that pre-clinical studies demonstrating the effectiveness of peripheral opioid receptors are continuously being accumulated, even including those under neuropathic pain conditions (Shinoda et al., 2007; Guan et al., 2008; Sánchez et al., 2010; Saloman et al., 2011; Auh and Ro, 2012; Martins et al., 2012; Ni et al., 2013). Therefore, peripheral opioid receptors still remain as a viable target for pain management, especially under inflammatory conditions, and additional human experimental as well as clinical studies are definitely warranted. Our data suggest that testosterone may be involved in maintaining the peripheral MOR system in chronic muscle pain conditions and that effective treatment strategies targeting peripheral MOR should consider the hormonal status of patients. Our data also offer important clues to further investigate cellular mechanisms that link cytokines and sex hormones in various inflammatory conditions for the development of mechanism-based sex-specific treatment alternatives that can be directed at the peripheral anti-nociceptive system.

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What's already known about this topic?

- Peripheral opioid efficacy is augmented under inflammatory conditions due to increased opioid receptor expression in sensory ganglia

What does this study add?

- Inflammation and cytokines modulate mu opioid receptor expression in trigeminal ganglia in a sex dependent manner
- Testosterone is required for cytokine-induced up-regulation of mu opioid receptors in trigeminal ganglia

Figure 1.

(A) Real time RT-PCR data showing MOR mRNA levels in TG of age-matched naive male and female rats. The MOR mRNA level in female was normalized to that of male rats. (B,C) Real time RT-PCR data showing CFA-induced changes in MOR mRNA levels in TG of male and female rats, respectively. Data from CFA inflamed rats were normalized to naïve rats and presented as a fold change. * denotes significant effects with respect to naïve condition at $p < 0.05$.

Figure 2.

(A) Protein samples from HEK293 cells were immunoblotted (IB) using antibody against MOR-1 (left) followed by c-myc (right). HEK293 cells were treated with the transfection reagent only (Mock), or transfected by cDNA encoding myc-MOR-1 or empty vector (pcDNA3.1). (B) Protein lysates HEK293 cells were immunoprecipitated (IP) with anti-c-myc and immunoblotted (IB) with anti-MOR. (C–D) Western blot analysis of TG obtained from male (C) or female (D) in naïve group, or 1 or 3 days following the masseteric injection of CFA. (top) Examples of immunoblots for MOR along with GAPDH from TG of naïve and inflamed rats. (bottom) Bar graphs show comparisons of relative MOR protein levels between naïve and inflamed rats. $n=6$ for male and 5 for female groups and * denotes significant effects with respect to naïve condition at $p < 0.05$.

Figure 3.

(A,B) Effects of cytokines on MOR mRNA expression in TG organ cultures. Real time RT-PCR data following individual cytokine applications were compared to those of PBS treatment in cultures prepared from male and female rats. (C) Role of inflammatory cytokines in CFA-induced up-regulation of MOR mRNA in intact TG of male rats. CFA-induced changes in MOR mRNA in intact TG in the presence of IL-1 β or IL-6 receptor antagonist or PBS were compared to that of untreated rats. Data are shown as mean $\pm$ S.E.M. and * denotes significant differences compared to control group at $p < 0.05$.

Figure 4.

Role of gonadal hormones in MOR mRNA expression in TG. (A, B) Real time RT-PCR data following individual cytokine applications were compared to that of PBS treatment in GDX rats and in GDX rats that received testosterone (TS). (C) MOR mRNA levels were assessed from TG of OVX rats treated with the same cytokines. All data were normalized to vehicle treated samples and presented as fold changes in MOR mRNA transcripts. Data are shown as mean $\pm$ S.E.M. and * denotes significant differences compared to PBS group at $p < 0.05$.

Figure 5.

(A,B) Effects of intramuscular DAMGO or vehicle on mechanical sensitivity 3 days after CFA treatment in intact male and female rats. Bar graphs show mean EF50 values in vehicle and DAMGO treated rats. (C) Effects of DAMGO on mechanical sensitivity 3 days after CFA treatment of GDX rats. + denotes significant sex differences, and * and # significant time dependent changes for each group at $p < 0.05$ compared to the pretreatment condition. All data are shown as mean $\pm$ S.E.M. and each group consisted of 6–8 animals.