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ANDROGEN RECEPTOR TRANSCRIPTIONALLY REGULATES μ-OPIOID RECEPTOR EXPRESSION IN RAT TRIGEMINAL GANGLIA

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Abstract

The involvement of testosterone in pain, inflammation, and analgesia has been reported, but the role of androgen receptor (AR), a steroid receptor for testosterone, is not well understood. We have previously shown that peripheral inflammation upregulates μ-opioid receptor (MOR) in rat trigeminal ganglia (TG) in a testosterone-dependent manner. In this study, we hypothesized that testosterone regulates MOR expression via transcriptional activities of AR in TG. We first examined whether AR is co-expressed with MOR in TG neurons. Our immunohistochemical experiment revealed that AR staining is detected in neurons of all sizes in TG and that a subset of AR is expressed in MOR as well as in TRPV1-positive neurons. We identified the promoter region of the rat MOR gene contains putative AR binding sites. Using chromatin immunoprecipitation assay, we demonstrated that AR directly binds to these sites in TG extracts. We confirmed with luciferase reporter assay that AR activated the MOR promoter in response to androgens in a human neuroblastoma cell line (5H-5YSY). These data demonstrated that AR functions as a transcriptional regulator of the MOR gene activity. Finally, we showed that flutamide, a specific AR antagonist, prevents complete Freund's adjuvant (CFA)-induced upregulation of MOR mRNA in TG, and that flutamide dose-dependently blocks the efficacy of DAMGO, a specific MOR agonist, on CFA-induced mechanical hypersensitivity. Our results expand the knowledge regarding the role of androgens and their receptor in pain and analgesia and have important clinical implications, particularly for inflammatory pain patients with low or compromised plasma testosterone levels.

Keywords

testosterone; inflammation; peripheral; sensory neurons; flutamide

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CONFLICT OF INTEREST

There are no conflicts of interest associated with the present study

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INTRODUCTION

Androgen receptor (AR) plays pivotal roles not only in the regulation of male development, but also in many other physiological processes (Matsumoto et al., 2013). Most biological effects of AR are mediated by the action of androgens, such as testosterone and its 5α-reduced derivative, dihydrotestosterone. AR, which belongs to the nuclear receptor super-family, exerts its effects as a transcription factor that regulates the activity of target genes (Heinlein and Chang, 2002).

The involvement of AR in pain and analgesic processing has been suggested by the distribution of AR in brain regions known for pain modulation (Murphy et al., 1999; Hamson et al., 2004; Loyd and Murphy, 2008). A few studies report the involvement of testosterone in pain, inflammation and analgesia, (Claiborne et al., 2006; Fischer et al., 2007; Nag and Mokha, 2009; Torres-Chavez et al., 2012) and mechanisms underlying testosterone-mediated sexually dimorphic responses under pathological pain conditions have been proposed (Sorge et al., 2011; Niu et al., 2012; Zhang et al., 2014). In most studies that investigated the role of testosterone in pain and analgesia, it is assumed that testosterone effects are mediated by AR. However, the functional role of AR itself and its target genes in the pain circuitry have been rarely explored.

Recently, we have demonstrated that AR mRNA is expressed in trigeminal sensory ganglia (TG), that AR binds to the promoter region of cannabinoid receptor type 1 gene (*Cnr1*), and that AR transcriptionally activates *Cnr1* expression in response to testosterone or dihydrotestosterone treatment (Lee et al., 2013). These results were suggested as the mechanistic basis for sexually dimorphic antihyperalgesic effects of a peripherally administered cannabinoid agonist under inflammatory pain condition (Niu et al., 2012). Along with cannabinoid receptors, μ-opioid receptors (MORs) also show testosterone-dependent modulation of their expression levels in the midbrain periaqueductal gray (Loyd and Murphy, 2008) and in somatic sensory ganglia (Zhang et al., 2014). MOR upregulation induced by inflammatory cytokines is prevented in TG of gonadectomized (GDX) male rats and testosterone replacement in GDX male rats restores the expression and function of MOR in TG, suggesting that AR could also regulate the MOR gene expression (Zhang et al., 2014).

The primary objective of the present study was to elucidate the role of AR in the MOR gene (*Oprm1*) expression in sensory neurons. Specifically, we explored whether AR binds directly to *Oprm1* in TG and regulates its transcription and whether pharmacological blockade of AR prevents MOR upregulation during peripheral inflammation and attenuates the anti-hyperalgesic effects of a peripherally administered MOR agonist. AR as a transcription factor has been rigorously studied in other systems (Chang et al., 2013), but such data are limited in the pain literature. Therefore, our studies exploring AR as a transcription factor of the MOR gene can offer novel perspectives on how male gonadal hormone receptors modulate analgesic responses to endogenous or exogenous opioid treatments.

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EXPERIMENTAL PROCEDURES

Animals

A total of 40 male Sprague–Dawley rats (8 weeks old) were used in the present study. Immunohistochemistry (n=3), RT-PCR (n=11) and behavioral (n=24) experiments were conducted with only male rats. In the chromatin immunoprecipitation (ChIP) assay, two age-matched female rats were used along with two male rats. All animals were housed in a temperature-controlled room under a 12:12-h 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.

Immunohistochemistry

Animals were perfused transcardially with phosphate buffer solution (PBS) followed by 4% paraformaldehyde in PBS (250 ml; pH 7.2). TG from each rat was extracted and post-fixed for 90 min, placed in 30% sucrose solution at 4 °C overnight and sectioned coronally at 12 μm. Every fourth section was collected and mounted on gelatin-coated slides for immunohistochemistry. The sections were incubated overnight with primary antisera for AR (1:50, rabbit polyclonal, Santa Cruz, SC-816). The specificity of this antibody has been previously determined (Nilsson et al., 2003; Atanassova et al., 2006). For immunofluorescence the sections were incubated at 37 °C for 30 min with Cy-3 conjugated goat anti-rabbit antiserum (1:500; Jackson Immunoresearch). The primary antibody for AR was omitted from the processing of selected sections to control for non-specific background staining. AR-positive cells were counted from 12 representative sections per ganglion from three TG. The soma sizes were measured from labeled neurons that showed clear nucleolus. Labeled TG neurons were classified as small (>400 μm^2), medium (400–1000 μm^2), and large cells (<1000 μm^2) (Ichikawa and Sugimoto, 2001).

For double-labeling immunohistochemistry, the sections were incubated overnight with primary antisera for AR, and incubated with Daylight 488 anti-rabbit antiserum (1:250, Jackson Immunoresearch) for 60 min at 37 °C for immunofluorescence. After staining for AR, the same sections were incubated overnight with primary antisera for either TRPV1 (1:1000, anti-goat, Neuromics, GT15129) or MOR (1:50, anti-goat, Santa Cruz, SC-27072) followed by 60 min with Cy-3 anti-goat antiserum (1:500; Jackson Immunoresearch) for immunofluorescence. We chose to examine AR in TRPV1- and MOR-positive cells since MOR is expressed primarily in peptidergic, TRPV1-positive neurons in sensory ganglia (Scherrer et al., 2009) and AR is required in MOR-positive neurons for transcriptional regulation of MOR expression. The primary antibody for TRPV1 or MOR was omitted from the processing of selected sections to control for non-specific background staining. Both TRPV1 and MOR primary antibodies have been characterized in previous studies (Czaja et al., 2008; Wu et al., 2015)

ChIP assay

TG dissected from two male to two female rats were homogenized with gradient needles and fixed in 1.42% formaldehyde to cross-link the chromatin at room temperature with shaking

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for 20 min. The reaction was stopped by the addition of glycine to a final concentration of 125 mM at room temperature with shaking for 5 min. Tissues were disaggregated with Dounce Homogenizer and collected by centrifugation. The pellet was washed with ice-cold PBS with protease inhibitor cocktail (Roche, NJ, USA), re-suspended in nuclei lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS), and incubated on ice for 15 min. The cell lysates were sonicated to shear the chromatin into lengths of less than ~500 bp and were centrifuged at 12,000 rpm for 10 min at 4 °C to remove debris. The sheared chromatin was diluted in radio-immunoprecipitation assay (RIPA) lysis buffer (10 mM Tris, pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) and pre-cleared by incubation with protein A/G agarose with rotation at 4 °C for 2 h and centrifugation. The chromatin supernatant was then incubated with anti-AR antibody (sc-13062, Santa Cruz, TX, USA) overnight at 4 °C. Immunocomplexes were collected by incubation with protein A/G agarose for 2 h at 4 °C with rotation. Beads were washed twice on a rotating platform with 1 ml of low-salt wash buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 2 mM EDTA, 1% Triton X-100, 0.1% SDS) and high-salt wash buffer (500 mM NaCl, 20 mM Tris, pH 8.0, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), followed by two washes with 1 ml of lithium chloride wash buffer (0.25 M LiCl, 10 mM Tris, pH 8.0, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate), and then once with TE buffer. Immunocomplexes were eluted by incubation at 65 °C with 150 μl pre-warmed elution buffer (1% SDS, 50 mM $NaHCO_3$) for 30 min with gentle vortexing. NaCl was added to a final concentration of 0.2 M, and the eluents were incubated at 65 °C overnight to reverse the cross-linking. The eluents were digested with proteinase K and incubated for 2 h at 55 °C. DNA was recovered by phenol: chloroform extraction and ethanol precipitation. Immunoprecipitated DNA and input-sheared DNA were subjected to PCR using rat MOR primer pairs: (1) forward, 5′-CTTTCCACAACCAAGGGAGA-3′; reverse, 5′-CCTCGAGGAACCAGAATGC-3′, which amplify a 193-bp region, (2) forward, 5′-CAGAAGCCTGTTCCCA GAAG-3′; reverse, 5′-CCCCACTCCCTCAATTTCTC-3′, which amplify a 231-bp region spanning the AR binding motif of the MOR promoter. The PCR products were run on 1% agarose gel and stained with ethidium bromide for visualization.

Plasmid construction

The MOR1 gene upstream region (−1251 bp to +88 bp) was amplified by PCR using specific primers (forward, 5′-TTGCTGGAATTGCACCCTCT-3′; reverse, 5′-ATGG ACCAGCCCTAGTGGAA-3′) designed from NCBI reference sequences (rat genomic DNA, NC_005100.4 (genomic), NCBI Reference Sequence: NM_001038597.2 (mRNA). The PCR product was inserted into the pGL3 basic vector expressing firefly luciferase (Promega, WI, USA) using the KpnI and NheI sites of multiple cloning regions. This construct was designated as pGL3-MOR1. To clone cDNA encoding rat AR, we purified mRNAs from rat TG and performed reverse transcription. The full-length nucleotide of rat AR was PCR amplified using specific primers (forward, 5′-GAGG TGCAGTTAGGGCTGGGA-3′; reverse, 5′-TCACTGTGT GTGGAAATAGAT-3′). The PCR product of 2.7 kb was subcloned into an expression vector pFLAG-CMV (Sigma–Aldrich, MO, USA) using BglII and BamHI restriction sites, which tag a FLAG epitope at amino terminus of AR. This construct was designated as pFLAG-AR.

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SH-SY5Y cell culture, transfection, and reporter gene assay

SH-SY5Y cells were grown in DMEM-F12 (Sigma) medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C with 5% CO_2. Cells were plated in 24-well plates at a density of 1×10^5 cells/well and cultured overnight before transfection. Transient transfection for luciferase activity assays was performed by lipofectamine 2000 (Invitrogen, CA, USA). Plasmids used were: pGL3-MOR (480 ng); pRL-CMV encoding renilla luciferase (20 ng); pFLAG-AR or empty pFLAG-CMV vector (200 ng). Twenty-four hours after transfection, the cells were treated with either 100 nM dihydrotestosterone (Steraloids, RI, USA) or testosterone (Sigma–Aldrich) for 24 h. Activity of the firefly and renilla luciferase in extracts of the transfected cells was determined using the Dual-Luciferase Reporter Assay System (Promega, WI, USA). For each sample, the activity of the firefly luciferase was divided by the activity of the renilla luciferase to correct for transfection efficiency. The corrected firefly luciferase activity of experimental constructs was expressed relative to that of the β-actin promoter-driven firefly luciferase. At least three independent experiments in triplicate were performed.

Behavioral studies

The behavioral model used in this study for assessing mechanical sensitivity of the rat masseter muscle has been previously described (Ren, 1999; Shimizu et al., 2009; Niu et al., 2012). Briefly, we applied a series of calibrated von Frey filaments 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 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 calculated and determined as a mechanical threshold. A reduction of EF50 value indicated the presence of mechanical hypersensitivity.

Mechanical sensitivity in the masseter muscle is significantly increased following the induction of masseter inflammation with an inflammatory agent, complete Freund's adjuvant (CFA), with the peak hypersensitivity occurring in the first 3 days (Ambalavanar et al., 2006; Shimizu et al., 2009; Niu et al., 2012). We have previously shown that DAMGO ([D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin acetate salt), a selective agonist for MOR, administered directly in the inflamed muscle during this time period effectively reverses CFA-induced mechanical hypersensitivity. In this study, DAMGO (10 μg in 20 μl) was used to assess MOR efficacy in rats treated either with flutamide, a selective antagonist for AR, or with its vehicle control. Flutamide (1, 5, or 10 mg/kg, Sigma Aldrich) or vehicle was administered (i.p) daily for three consecutive days. Twenty-four hours after the first administration of flutamide or its vehicle, CFA (1:1 isotonic saline, 50 μl) was injected into the masseter muscle as described previously (Niu et al., 2012). Baseline mechanical threshold was assessed 10 minutes before the CFA treatment. Effects of DAMGO on CFA-induced mechanical hypersensitivity were assessed two days after the CFA treatment, the time point coinciding with 3 days after the flutamide or vehicle treatment in the same animals. The post DAMGO effect was measured 30 min after the drug injection. The proposed dose of DAMGO was adapted from our previous studies, which confirmed that DAMGO at this dose specifically targets peripheral MOR in a receptor dependent manner

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without producing central effects (Nunez et al., 2007; Zhang et al., 2014). The dose and injection protocol for flutamide were adapted from published studies (Luo et al., 1997; Singh et al., 2000). In order to maintain consistency in assessing behavioral responses an experimenter who was blinded to treatment conditions conducted the behavioral experiment.

Realtime RT-PCR assay

Effects of flutamide on CFA-induced changes in MOR expression in TG were assessed by quantifying MOR mRNA levels. The dose and protocol for flutamide and CFA treatments were the same as those used for the behavioral study except DAMGO was not used in this experiment. Total RNA was extracted from TG of naïve rats and from CFA-inflamed rats treated with either flutamide or vehicle using Trizol (Sigma) and purified with an RNeasy kit (Qiagen Sciences, MD, USA) that included a DNase treatment to remove genomic DNA. Reverse transcription was carried out using the Superscript First Stand synthesis kit (Invitrogen, CA, USA). Superscript II (Invitrogen) was used to generate cDNA from 1 μg of RNA along with 2.5 ng of random primer per reaction. Real-time PCR analysis of cDNA equal to 25 ng RNA was performed on the Eppendorf Mastercycler ep realplex 2.0. 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′ (Zhang et al., 2014). The amount of a given MOR mRNA was normalized to the GAPDH mRNA in the same sample. The primer pairs for detecting GAPDH mRNA were: sense primer: 5′-TCACCACCATGGAGAAGGC-3′, antisense primer: 5′-GCTAAGCAGTTGGTGGTGCA-3′. The cycling protocol used was 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 50 °C for 15 s and 72 °C for 20 s. Relative quantification of the MOR mRNA was calculated by the comparative C_T method (C_T method) between control and experimental groups.

Data analysis

Statistical comparisons of two independent groups were made with Student's *t*-test. For multiple group comparisons, one-way analysis of variance (ANOVA; *F* score) or Kruskal–Wallis one-way ANOVA on ranks (*H* score) was performed depending on the outcome of the normality test, followed by Dunnett's post-hoc test. Data are presented as mean±SE and differences were considered significant at $p<0.05$.

RESULTS

AR is co-expressed with MOR in TG neurons

We have recently demonstrated the expression of AR mRNA and its protein product in the rat TG (Lee et al., 2013). In this report, we provided immunohistochemical evidence for the expression and distribution of AR in TG neurons (Fig. 1A, D). AR-positive neurons were seen in all three divisions of TG. AR expression was primarily observed in small to medium size cells. AR staining was detected predominantly in the cytoplasm, the pattern consistent with spinal motor neurons (Sheean et al., 2015). Of 237 AR-positive cells measured in TG, 47.3% were small (<400 μm^2), 44.7% were medium (400–1000 μm^2) and only 8% were large (>1000 μm^2) (Fig. 2). AR can be found in a subset of TRPV1-positive neurons,

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suggesting that AR can modulate small to medium size nociceptors in TG (Fig. 1B, C). More importantly, AR was co-expressed with MOR in TG neurons. AR-positive neurons were reliably detected in MOR-positive small to medium size TG neurons (Fig. 1E, F). MOR and TRPV1 co-localization in sensory ganglia has been amply documented (Endres-Becker et al., 2007; Yamamoto et al., 2008; Scherrer et al., 2009) We confirmed that MOR and TRPV1 are co-expressed in TG neurons (Fig. 1G–I). These data suggested that most MOR-positive TG neurons also contain AR, and that AR can regulate MOR expression.

AR binds to the MOR gene in TG

In order to examine direct interactions between AR and the MOR gene, we used ChIP assays to examine whether AR binds to the MOR gene in native TG. JASPAR database (Mathelier et al., 2014) and PROMO (http://alggen.lsi.upc.es/cgi-bin/promo), a bioinformatic analysis tool, were used to identify putative binding sites in promoter sequences of the rat *Oprm1*. The analysis yielded two potential AR binding sites for the rat *Oprm1* (Fig. 3A). We performed ChIP assays using a specific antibody against AR and PCR primers designed to amplify two regions specific to the predicted AR binding sites as shown in Fig. 3A). Fragments of chromatin immunoprecipitated by anti-AR antibody showed clear PCR products of the *Oprm1* with expected sizes (Fig. 3B). These results suggest that AR binds to the promoter region of the rat MOR gene in genomic DNA of TG and likely regulates the gene expression. Interestingly, AR binding was prominent in male TG but was barely detected in female TG. Given that AR expression levels are comparable between male and female TG (Lee et al., 2013), it is possible that the nuclear portion of AR in female TG is low, possibly due to the low level of circulating testosterone.

AR transcriptionally activate the MOR gene

Since it is well known that testosterone-bound AR functions as a transcription factor (Heinlein and Chang, 2002), we examined whether AR can induce the MOR gene transcription. To test this possibility, we evaluated the activity of AR on the *Oprm1* promoter by a luciferase reporter gene assay in a neuroblastoma cell line (5H-SY5Y) in which multiple exogenous genes can be readily expressed. SH-SY5Y cells transfected with pFLAG-AR and pGL3-MOR were treated with either testosterone (100 nM) or dihydrotestosterone (100 nM). We exogenously applied testosterone and dihydrotestosterone since these cells were not exposed to circulating testosterone. The luciferase activity was determined after 18 h following the treatment. Application of testosterone or dihydrotestosterone in cells transfected with pGL3-MOR alone did not show significant activity compared to control. Co-expression of AR and MOR slightly increased the luciferase activity but did not reach statistical significance. When testosterone or dihydrotestosterone was applied in cells co-expressing AR and MOR, luciferase activity was significantly increased by approximately three fold (Fig. 4). These results suggest that testosterone and AR complex can enhance transcription of the MOR gene.

Blockade of AR prevents MOR upregulation and peripheral DAMGO effects under inflammatory condition

Since AR is involved in transcriptional regulation of MOR expression, we hypothesized that the increased expression of MOR mRNA in TG during inflammation will be prevented if

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AR activity is blocked. We used flutamide to block AR since it has been shown to block the action of both endogenous and exogenous testosterone and, it is capable of inhibiting nuclear uptake of androgen (Sufrin and Coffey, 1976). Daily treatments with flutamide, but not its vehicle, prevented the inflammation-induced upregulation of MOR mRNA (Fig. 5A). The mRNA level in TG of flutamide-treated rats was significantly lower than that of CFA-inflamed rats with no treatment (t=2.652 p<0.05).

Since the inflammation-induced upregulation of MOR expression in TG is blocked with flutamide treatment, we predicted that the efficacy of MOR agonist during inflammation should also be reduced with flutamide treatment. There was no significant difference in the baseline mechanical thresholds between flutamide and vehicle-treated groups (t=1.246, p>0.05). Consistent with our previous study (Niu et al., 2012; Zhang et al., 2014), CFA treatment produced profound mechanical hyperalgesia in both groups. On day two following CFA treatment, the mean mechanical thresholds were significantly reduced. There was no significant group difference in the mean mechanical thresholds at this time point (t=0.112, p>0.05). DAMGO administered directly into the masseter muscle significantly reversed the CFA-induced mechanical hypersensitivity in vehicle-treated rats. However, flutamide dose-dependently blocked anti-hyperalgesic effects of DAMGO (Fig. 5B). DAMGO effects were significantly reduced in flutamide 5 and 10 mg/kg groups, but not in 1 mg/kg group, compared to that of the vehicle group (F=8.16, p<0.001, Fig. 5B). It is possible that systemic administration of flutamide influenced pain responses by indirectly modulating pain perception or motor behaviors. However, our rats did not show any unusual signs of discomfort or pain hypersensitivity during the flutamide treatment. Behavioral phenotypes other than pain responses were identical to those rats that received control injections. These results provided strong support that AR is required for inflammation-induced upregulation of functional MOR in trigeminal sensory neurons.

DISCUSSION

The main findings of the present study are that AR is co-expressed with TRPV1-positive and MOR-positive trigeminal sensory neurons, and that AR binds directly to the promoter region of the MOR gene in TG and exerts transcriptional modulation of MOR expression. We also demonstrated that the blockade of AR activity pharmacologically prevents the inflammation-induced upregulation of MOR expression and decreases the efficacy of a MOR agonist administered into the inflamed site. The function of AR as a transcription factor in adult sensory neurons is not well known. Our data should have important clinical as well as scientific implications as multiple genes involved in pain and analgesia may be regulated by androgens via AR.

Ontogenetic expression of AR transcripts in somatic ganglia and the brain suggests that androgens have a great influence on the development and maintenance of the nervous system through AR (Young and Chang, 1998). In addition to its ontogenetic role, AR has been suggested to play an important role in modulating visceral and somatic reflexes (Keast and Gleeson, 1998). This study by Keast and Gleeson demonstrated AR immunoreactivity in almost half of the neurons in L6 and S1 dorsal root ganglia (DRG) of adult male rats. AR staining was present in both large and small neurons and over 80% of CGRP neurons

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contained AR in DRG. Recently, we have shown that AR mRNA is reliably detected in TG of adult male and female rats (Lee et al., 2013). In the present study, we showed AR expression in TRPV1-positive neurons, providing further evidence that AR plays functional roles in modulating somatic sensory processing at the level of primary afferent neurons. Available studies that showed the involvement of testosterone in antinociception induced by opioid receptor like-1 receptor in the spinal cord and MOR in TG support our findings (Claiborne et al., 2006; Zhang et al., 2014). In addition, testosterone exerts anti-inflammatory and antinociceptive effects in the temporomandibular joint (Ji et al., 1995; Li et al., 1998; Fischer et al., 2007; Yamamoto et al., 2008; Wang et al., 2010; Torres-Chavez et al., 2012). However, how testosterone mediates these effects is not well understood. MOR is preferentially expressed in small to medium size peptidergic neurons and co-localizes with TRPV1-positive neurons in DRG (Ji et al., 1995; Li et al., 1998; Yamamoto et al., 2008; Wang et al., 2010). Our data demonstrating that MOR is co-expressed with AR in TG neurons provides an anatomical substrate for regulation of MOR by AR. Thus, circulating testosterone can exert pain modulatory effects through the actions of AR on anti-nociceptive receptors, such as MOR, in primary afferent nociceptors.

It is well established that androgens mediate a wide range of physiological responses through transcriptional activities of AR (Heinlein and Chang, 2002). Our ChiP data suggested that AR in TG is capable of regulating MOR expression transcriptionally by binding to the promoter region of the MOR gene, and the luciferase reporter assay showed that AR functions as a transcriptional activator rather than repressor. These data, along with our immunohistochemical demonstration of AR expression in MOR-positive TG neurons, provide mechanisms by which testosterone exerts antihyperalgesic effects under inflammatory pain conditions, i.e., by activating transcriptional machinery of the MOR gene in primary afferent nociceptors. AR mediates its transcriptional effects by binding to genomic androgen response elements (AREs) of AR target genes (Bolton et al., 2007). Numerous AR target genes that contain ARE have been characterized and the number is still growing (Jariwala et al., 2007; Perets et al., 2012; Rana et al., 2014). Interestingly, many of the genes that contain AREs are also expressed in sensory neurons and have been implicated in pain processing (Chang et al., 1996). Inflammatory cytokines, such as IL-4 and γ-interferon, that mediate pathological pain responses (Tsuda et al., 2009; Kiguchi et al., 2015) are transcriptionally regulated by AR in murine T cells (Araneo et al., 1991). We have also demonstrated that AR regulates the expression levels of antinociceptive receptors, such as cannabinoid receptors type 1 (CB1), in TG neurons during inflammation (Lee et al., 2013). Therefore, future studies identifying additional target genes of AR as well as those elucidating molecular machineries responsible for AR-mediated gene expression in nociceptors would greatly increase our understanding on how gonadal hormones modulate nociceptor activities and ultimately shape nociceptive responses under pathological pain conditions.

MOR expression in sensory ganglia is increased during peripheral inflammation (Ji et al., 1995; Puehler et al., 2004; Nunez et al., 2007). Rapid upregulation of MOR mRNA in DRG in response to peripheral inflammation depends on neuronal conduction (Puehler et al., 2004). However, molecular mechanisms leading to a prolonged increase in MOR expression following inflammation are not well understood. Our data showed AR-mediated

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transcriptional regulation of the MOR gene as an underlying mechanism leading to a greater MOR expression under inflammatory conditions in sensory neurons. In addition, we confirmed that the AR blockade leads to the attenuation of functional MOR activities that correlate with reduced MOR expression in TG. It is possible that systemic flutamide could lead to the attenuation of hyperalgesia by blocking AR activities at other brain regions involved in pain modulation. It is also possible that flutamide could block non-genomic actions of AR (Benten et al., 1997; Revelli et al., 1998) thereby directly modulating nociceptor excitability, although such possibility has never been demonstrated. Nevertheless, our data showed that even systemic flutamide effectively prevented MOR mRNA upregulation within TG, which correlated with the reduced efficacy of peripherally administered DAMGO in flutamide-treated animals. These data support the notion that AR plays an important role in functional expression of MOR in sensory ganglia.

These findings bear important implications for the development of effective treatment strategies targeting peripheral MOR. Since the level of AR receptor expression in TG is comparable between male and female rats (Lee et al., 2013), and that AR is hereby shown to bind to the *Oprm1* promoter region in female TG, it is reasonable to assume that the circulating testosterone level in female is not sufficient to elicit MOR induction under inflammatory conditions. Such testosterone dependent regulation of MOR within TG has been demonstrated in a myositis model (Zhang et al., 2014) as well as in a tendon ligation model (Bai et al., 2015). In both models, inflammation- or injury-induced upregulation of MOR in TG is impaired in female and GDX rats and that testosterone replacement in GDX male rats restores the expression of MOR and the efficacy of peripherally administered MOR agonists. These studies further suggest that peripheral MOR efficacy could be impaired under conditions in which plasma testosterone level is compromised. Epidemiological data show that testosterone levels decline with normal aging (Harman et al., 2001; Feldman et al., 2002), which could lead to reduced opioid efficacy. Recent clinical studies provide compelling evidence that testosterone therapy enhances pain management in elderly men and hypogonadic chronic pain patients (Aloisi et al., 2011; Dedov et al., 2011; Tan et al., 2013). Clinically, low testosterone levels have been linked to various disease states such as diabetes and ischemic heart disease (Barrett-Connor and Khaw, 1988; Barrett-Connor, 1992). Testosterone level is significantly compromised in a substantial population of men with spinal cord injury and traumatic brain injury from all causes (Durga et al., 2011; Wilkinson et al., 2012; Rosario et al., 2013). Based on our data, systemic or local treatment with opioid drugs in these patient populations may not provide effective pain relief. However, there is little clinical data that compare opioid efficacy between testosterone-compromised patients and those with normal testosterone level. Thus, future studies exploring cellular and molecular mechanisms underlying androgenic regulation of MOR in sensory neurons as well as in the brain should prove valuable for the development of effective treatment strategies targeting MOR for men, women, and testosterone-compromised patients.

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Abbreviations

5H-5YSY	human neuroblastoma cell line
ANOVA	analysis of variance
AR	androgen receptor
AREs	androgen response elements
CFA	complete Freund's adjuvant
ChIP	chromatin immunoprecipitation
DRG	dorsal root ganglia
GDX	gonadectomized
MOR	μ-opioid receptor
Oprm1	mu opioid receptor
PBS	phosphate buffer solution
TG	trigeminal ganglia

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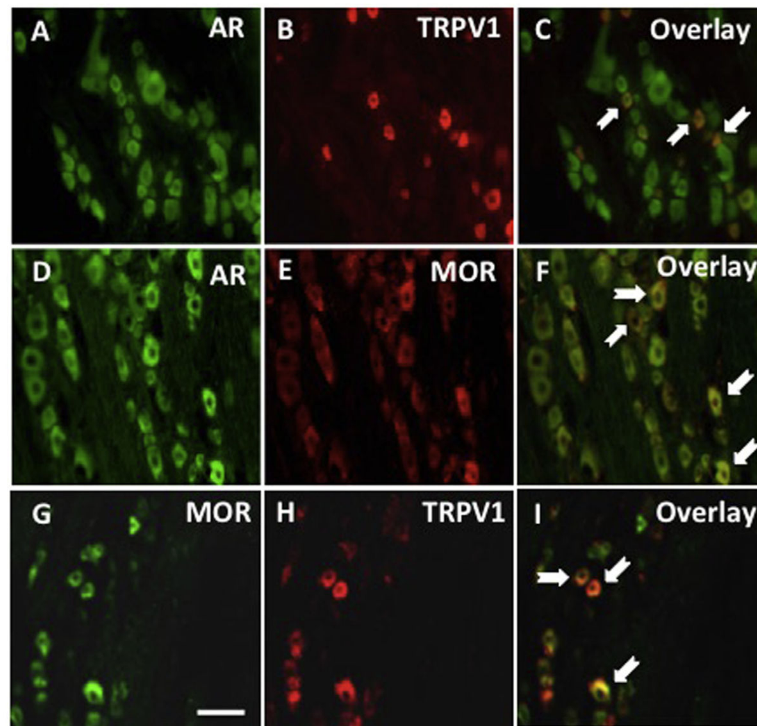

Fig. 1.

AR is co-expressed with MOR in TG. Photomicrographs show immunohistochemical staining of AR in TRPV1 (A–C) and MOR (D–F)- positive neurons in TG. MOR in TRPV1-positive neurons in TG is also shown (G–I). Arrows indicate examples of co-expression. Scale bar=30 μm.

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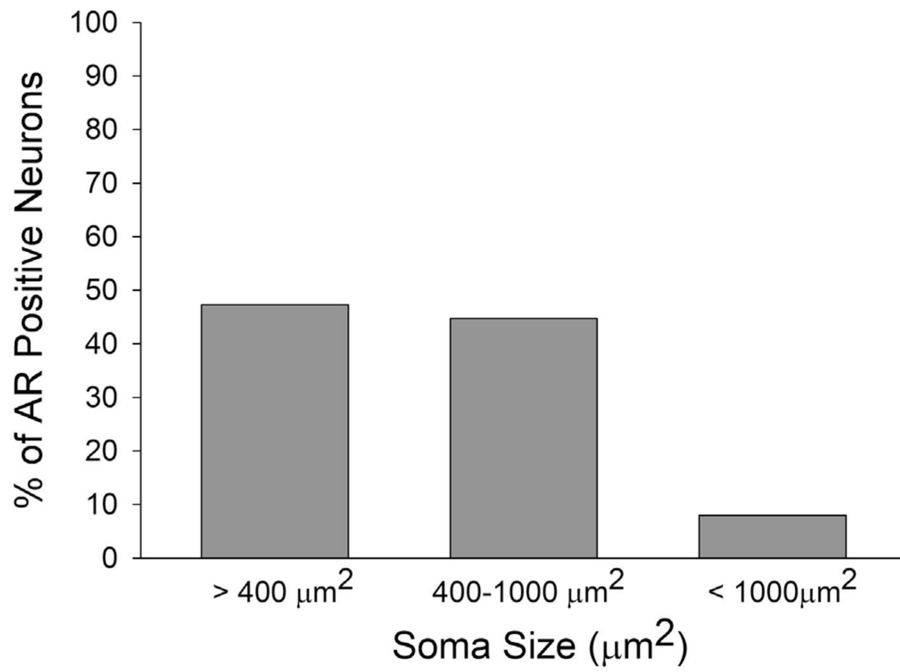

Fig. 2.
Soma size distribution of AR-positive neurons in TG based on cell body area (μm^2). The histograms were constructed from area measurements of 237 AR-positive neurons from two TG.

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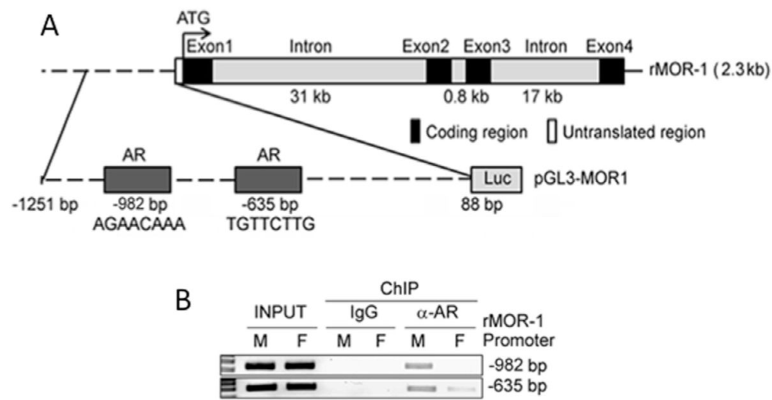

Fig. 3.

AR directly binds to the MOR promoter in rat TG. (A) A schematic diagram of the cloned 2.3-kb rat MOR promoter showing two putative AR-binding sites located at −982 bp to −789 bp and −635 bp to −404 bp. Luc, firefly luciferase (B) The rat TG were subjected to ChIP assay using anti-AR antibody and PCR primers specific for the two putative AR binding sites in the MOR promoter region. IgG was used as a negative control for IP. The PCR products were analyzed on 1% agarose gel and stained with ethidium bromide for visualization. The input represents PCR product amplified from 10% ChIP assay input material. M, male TG. F, female TG.

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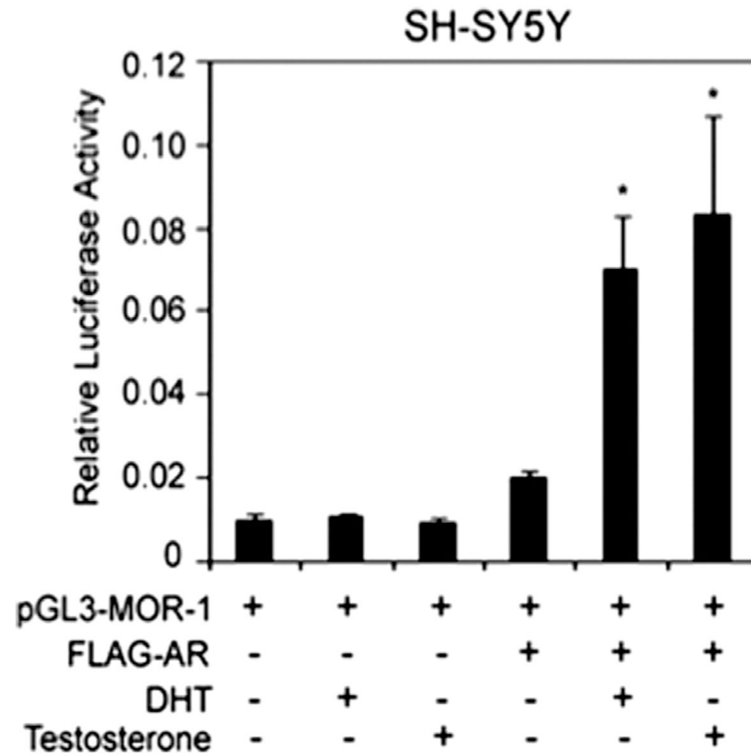

Fig. 4.

AR transcriptionally activates MOR expression. The SH-SY5Y cells were co-transfected with pFLAG-AR and 2.3-kb MOR promoter-luciferase construct (pGL3-MOR-1) as shown in Fig. 2A and then treated with 100 nM dihydrotestosterone (DHT) or testosterone (TS) for 24 h. Cell lysates were then analyzed for luciferase activity. The means±SEM are presented. *$p<0.05$ in Student's *t*-test.

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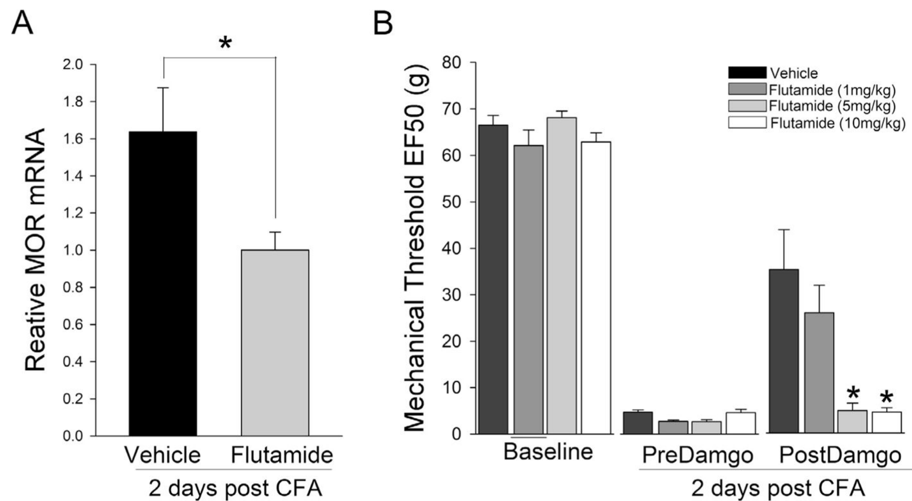

Fig. 5.

Flutamide blocks CFA-induced MOR expression and function. (A). MOR mRNA levels in TG were compared between vehicle (n=5) and flutamide (n=6)-treated rats under CFA-induced inflammatory condition. The relative RNA levels in both groups were normalized to the mean RNA contents of the flutamide-treated group. (B) Effects of peripheral DAMGO (10 μg) on mechanical hyperalgesia before and after CFA injection in vehicle (n=6) and flutamide (1, 5, and 10 mg/kg, i.p.; n=6 per group)-treated rats. For both RT-PCR and behavioral experiments, flutamide was administered daily for 3 days. The data are presented as means±SEM. $^*p<0.05$.