THE ROLE OF ANDROGEN RECEPTOR IN TRANSCRIPTIONAL MODULATION OF CANNABINOID RECEPTOR TYPE 1 GENE IN RAT TRIGEMINAL GANGLIA

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Abstract

We have previously shown that anti-hyperalgesic effects of cannabinoid agonists under inflammatory condition are much greater in male than female, and that inflammatory cytokines upregulate cannabinoid receptor type 1 (CB1) expression in male, but not female, trigeminal ganglia (TG) in a testosterone-dependent manner. In this study, we investigated the mechanisms underlying the testosterone-mediated regulation of peripheral CB1 expression. We hypothesized that testosterone upregulates CB1 through transcriptional modulation by androgen receptor (AR). Interleukin-1 beta (IL-1β), a proinflammatory cytokine, upregulated CB1 mRNA expression in TG of male rats. The cytokine-induced upregulation was prevented by the pre-treatment with flutamide, a specific antagonist for AR, but not by ICI 182,780, a specific antagonist for estrogen receptor, suggesting that the effects of testosterone are not mediated by estradiol, a testosterone metabolite. The expression levels of AR and IL-1β receptors were comparable between male and female TG, suggesting that the male specific IL-1β effects on CB1 upregulation occurs downstream to these receptors. The chromatin immunoprecipitation assay showed AR binding to the CB1 promoter in the rat TG. Furthermore, luciferase reporter assay revealed that AR activated the CB1 gene in response to testosterone or dihydrotestosterone treatment. These experiments provided compelling evidence that testosterone regulates CB1 gene transcription in TG through AR following cytokine stimulation. These results should provide mechanistic bases for understanding cytokine–hormone–neuron interactions in peripheral cannabinoid systems, and have important clinical implications for pain patients in whom testosterone level is naturally low, gradually declining or pharmacologically compromised.

Keywords

testosterone; inflammation; cytokines; sensory neurons; rat

INTRODUCTION

Cannabinoid receptor type 1 (CB1) belongs to a family of G-protein-coupled receptors that are activated by endogenous or synthetic cannabinoids (Breivogel and Sim-Selley, 2009). CB1, though widely distributed throughout the body, is primarily expressed in the CNS (Felder et al., 1996; Ong and Mackie, 1999). Cannabinoids have emerged as attractive

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CONFLICT OF INTEREST

There are no conflicts of interest associated with the present study.

alternatives or adjuncts for the treatment of pain and inflammation (Hazekamp and Grotenhermen, 2010). However, psychotropic effects mediated by cannabinoid receptors in the CNS limit the therapeutic use of cannabinoids as analgesics.

Recently, studies supporting the role of peripheral CB1 in the management of various types of chronic pain conditions, without producing centrally mediated side effects, have been accumulating. In addition to the CNS, CB1 mRNA is also detected in dorsal root ganglia (DRG) as well as trigeminal ganglia (TG) neurons (Price et al., 2003; Amaya et al., 2006). A peripherally acting CB1-selective agonist, SAB-378, inhibits pain-related responses to colorectal distension in a visceral pain model (Brusberg et al., 2009). Systemic administration of another peripherally restricted CB1 agonist, AZ11713908, produces robust analgesia in inflammatory and neuropathic pain (Yu et al., 2010). Nociceptor-specific loss of CB1 in the peripheral nervous system in mice substantially reduces the analgesic effect produced not only by local, but also systemic delivery of cannabinoids (Agarwal et al., 2007), lending further support for the role of peripheral CB1. Therefore, better understanding of detailed mechanisms underlying CB1 expression should prove valuable for effective pain management by targeting peripheral CB1.

We have recently shown that inflammatory cytokines such as Interleukin-1 beta (IL-1β) or IL-6 increase CB1 expression in rat TG, which correlates with the increased peripheral CB1 efficacy under inflammatory condition (Niu et al., 2012). Interestingly, the cytokine-induced upregulation of CB1 was dependent on the presence of testosterone such that the same concentration of cytokines did not result in increased CB1 expression in TG of either gonadectomized male or female rats. These observations prompted us to further investigate detailed mechanisms involved in testosterone-dependent regulation of CB1 in sensory neurons under inflammatory conditions. In this study, we sought to determine whether (1) the cytokine effect is androgen receptor (AR)- or estrogen receptor (ER)-dependent, since testosterone is aromatized to estradiol (Santen et al., 2009), and (2) the potential role of AR in CB1 gene regulation in TG.

EXPERIMENTAL PROCEDURES

Animals

Age-matched adult male and female Sprague–Dawley rats (8 weeks old) were used in the present study. In this study, we did not determine the estrous stage in female rats since our previous study showed that circulating estrogens do not affect CB1 expression in TG (Niu et al., 2012). 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.

TG primary culture

TG from each animal were extracted and dissociated by sequential digestion with collagenase D (0.1%) in DMEM-F12 medium (with L-glutamine) at 37 °C for 30 min, followed by collagenase D (0.1%), trypsin (0.25%), DNase (50 μg) and EDTA (0.02%) in DMEM-F12 medium at 37 °C for 15 min. After trituration, cells were plated on laminin pre-coated 12-well plates and kept in a 37 °C incubator at 5% CO_2 for 3 days.

Real-time reverse transcription polymerase chain reaction (RT-PCR) and conventional RT-PCR

Total RNA was extracted from cultured TG using Trizol (Sigma–Aldrich, St Louis, MO, USA) and purified with an RNeasy kit (Qiagen, Gaithersburg, MD, USA) that included a

DNase treatment to remove genomic DNA. Reverse transcription was carried out using the Superscript First Stand synthesis kit (Invitrogen Carlsbad, CA, USA). Superscript II (Invitrogen Carlsbad, CA, USA) was used to generate cDNA from 1 μg of RNA along with 2.5 ng of random primer per reaction. All primer pairs used in this study are described in Table 1. To assess cytokine-induced changes in CB1 mRNA level, and to compare AR and IL-1β receptor (IL-1R) expression between male and female TG, real-time PCR analysis of cDNA equal to 25 ng RNA was performed on the Eppendorf Mastercycler ep realplex 2.0. 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. The amount of a given mRNA was normalized to the GAPDH mRNA in the same sample and relative quantification of the mRNA of interest was calculated by the comparative CT method (ΔΔCT method) between the groups. For conventional RT-PCR, we followed the procedure previously described (Nñnéz et al., 2007). Briefly, PCR products were fractionated on a 3% agarose gel, visualized by ethidium bromide, scanned by Image Station 440CF and analyzed with 1D software (Kodak, Rochester, NY, USA).

Western blotting

Total proteins were extracted from TG of naïve male and female rats. The protein samples were dissolved in radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail. The protein concentration in lysates was determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Fifty micrograms of protein for each sample were separated on 4–12% NuPAGE gel with 3-(N-morpholino)propanesulfonic acid Sodium Dodecyl Sulfate (MOPS SDS) running buffer and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-rad, Hercules, CA). After blocking 1 h in 5% milk Phosphate Buffer Saline-Tween (PBST) at room temperature, membranes were probed with primary antibodies for AR (1:750, Millipore, Billerica, MA, USA) and GAPDH (1:5000, Calbiochem, Billerica, MA, USA), used as an internal control protein, diluted in blocking solution. Membranes were incubated with primary antibodies overnight at 4 °C and washed four times with PBST. HRP-conjugated anti-rabbit secondary antibody (Cell Signaling) for AR and anti-mouse secondary antibody (Millipore, Billerica, MA, USA) for GAPDH were diluted to 1:5000 in PBST and incubated with membranes for 1 h at room temperature. Bands were visualized using Enhanced Chemiluminescence (ECL) (Western Lightning, PerkinElmer Inc.) or ECL plus Western blotting detection reagent (Lumigen PS-3, GE Healthcare, Laurel, MD, USA). Protein level for AR was normalized to that of GAPDH in the same sample.

Cytokine and drug preparation

IL-1β (PeproTech–Rocky Hill, NJ, USA) was dissolved in phosphate-buffered saline (PBS) to 200 ng/20 μl and applied to the culture medium to a final concentration of 100 ng/mL as we described in our previous study (Niu et al., 2012). Flutamide (Sigma–Aldrich, St Louis, MO, USA), an AR antagonist, was dissolved in ethanol and prepared to final concentrations of 1, 100, and 1000 nM. Flutamide is a potent and specific AR antagonist (Luo et al., 1997; Singh et al., 2000). ICI 182,780 (Tocris–Minneapolis, MN, USA), an ER antagonist, was dissolved in DMSO and prepared to a final concentration of 1 μM. TG cultures were incubated with either flutamide or ICI 182,780 or their respective vehicle (IL-1 β, 0.002% bovine serum albumin + 2% PBS; flutamide, 0.0001% ethanol; ICI 182,780, 0.06% DMSO) for 2 h followed by IL-1β for an additional 30 min.

Plasmid construction

The CB1 gene upstream region (−1469 to +33 bp) was amplified by PCR using specific primers (forward, 5′-GAAGGGTAAGACCTGGCACAGT-3′; reverse, 5′-GCCTGTTCACAGTAAGTTCTCCTA-3′) from rat genomic DNA (NCBI Reference

Sequence: NC_005104.3 (genomic), NCBI Reference Sequence: NM_012784.4 (mRNA); Fig. 3A). The PCR product was inserted into the pGL3 basic vector expressing firefly luciferase (Promega–Madison, WI, USA) using the KpnI and NheI sites of multiple cloning regions. This construct was designated as pGL3-CB1. 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′-GAGGTGCAGTTAGGGCTGGGA-3′; reverse, 5′-TCACTGTGTGTGGAAATAGAT-3′). The PCR product of 2.7 kb was subcloned into an expression vector pFLAG-CMV (Sigma–Aldrich, MO) using BglII and BamHI restriction sites, which tag a FLAG epitope at the amino terminus of AR. This construct was designated as pFLAG-AR.

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, Carlsbad, CA, USA). Plasmids used were: pGL3-CB1 (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–Newport, 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). 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.

Chromatin immunoprecipitation (ChIP) analysis

TG dissected from male and female rats were homogenized with gradient needles and fixed in 1.42% formaldehyde to cross-link the chromatin at room temperature with shaking 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 a Dounce Homogenizer and then collected by centrifugation. The pellet was washed with ice-cold PBS with protease inhibitor cocktail (Roche–Branchburg, 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 RIPA lysis buffer (10 mM Tris, pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate), and then was 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–Dallas, 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 then 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 CB1 primer pairs as follows: (1) forward, 5′-GCAAATGTTTGCTGTGATGG-3′; reverse, 5′-CCTTTGAGAGATGACCCATGT-3′, which amplify a 102-bp region (−837 to −736). (2) forward, 5′-CCTCCTTGGACATTTAATCCA-3′; reverse, 5′-AGCGACCCCACAGATAATCA-3′, which amplify a 183-bp region (−610 to −427) spanning the AR binding motif of the CB1 promoter. The PCR products were run on 1% agarose gel and stained with ethidium bromide for visualization.

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

The inflammatory cytokine IL-1β upregulates the level of CB1 mRNA in male TG, but not in TG of gonadectomized male, intact and ovariectomized female (Niu et al., 2012). These data suggested testosterone as a key gonadal hormone required in the cytokine-induced CB1 regulation. In this study, we confirmed that IL-1β application reliably and significantly increases CB1 expression in TG primary cultures prepared from intact male rats (Fig 1A). The extent of upregulation was similar to our previous study (Niu et al., 2012). To determine the mechanisms of testosterone effects on CB1 expression, we first examined whether testosterone exert its effects directly via AR or indirectly via ER since testosterone can be aromatized to estradiol (Santen et al., 2009). To test this, we pretreated the TG cultures with flutamide, a specific antagonist for AR, or ICI 182,780, an antagonist of ER, prior to the IL-1β application. IL-1β-induced CB1 mRNA upregulation was significantly inhibited in TG cultures pretreated with flutamide in a dose-dependent manner, but not in ICI 182,780-treated cultures (Fig 1B, C). Flutamide at 100 nmol produced a significant block of IL-1β effects and a higher dose (1 μm) did not produce a greater effect. We did not examine the effects of a higher dose of ICI 182,780 since the dose used in this study (i.e. 1 μM) has been amply demonstrated to be efficacious in blocking both ERα and ERβ (Ivanova et al., 2009; Grassi et al., 2013). Each vehicle used to dissolve IL-1β, flutamide, and ICI 182,780 did not significantly alter the CB1 mRNA level compared to that of naïve untreated TG samples (Fig 1D). Either flutamide or ICI 182,780 alone did not alter the CB1 mRNA level (Fig 1E).

Next, we examined a possibility that the male-specific upregulation of CB1 expression by IL-1β (Niu et al., 2012) can be mediated by the differential expression of AR in TG between the two sexes. Our data showed that the expression levels of AR mRNA as well as AR protein were comparable between TG of male and female rats (Fig 2). Real time RT-PCR analysis on the level of IL-1R mRNA was also similar between male and female TG ($t = 0.727$, $p > 0.05$). We did not examine IL-1R protein expression due to the poor antibody specificity. These data suggested that sex-specific modulation of CB1 expression cannot be explained by the level of AR or cytokine receptor.

Since it is well known that testosterone-bound AR functions as a transcription factor (Heinlein and Chang, 2002), we examined whether AR can induce Cannabinoid receptor 1 (*Cnr1*) transcription. To test this possibility, we identified two potential AR binding sites (AR1 and AR2) in the promoter region of *Cnr1* gene encoding CB1 receptor (Fig. 3A). We then evaluated the activity of AR on the *Cnr1* 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-CB1 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-CB1 alone did not show significant activity compared to control. Co-expression of AR slightly increased the luciferase activity but did not reach statistical significance. When testosterone or dihydrotestosterone was applied in cells co-expressing AR and *rCnr1*, luciferase activity was significantly increased by approximately three fold (Fig 3B). These results suggest that testosterone and AR complex can enhance the transcription of *Cnr1*. It is possible that IL1β application can further increase CB1 activity, which we did not examine in this study.

We then examined whether AR actually binds to the putative promoter region of *Cnr1* in the rat TG. We performed a ChIP assay using a specific antibody against AR and PCR primers designed to amplify two regions specific to the predicted AR binding sites (AR1 and AR2 in Fig. 3A). Fragments of chromatin immunoprecipitated by anti-AR showed clear PCR products with expected sizes (Fig. 4). These results suggest that AR binds to the promoter region of *Cnr1* in genomic DNA of TG and likely regulates gene expression. It is noteworthy that the PCR products in ChIP assay was more prominent in male TG than female TG suggesting AR binding to Cnr1 promoter might be greater in male.

DISCUSSION

The main findings of the present study are that IL-1 β-induced regulation of CB1 in trigeminal sensory neurons is mediated by testosterone via AR, and that AR exerts a transcriptional modulation of *Crn1* in TG. The function of AR as a transcription factor in sensory neurons is a novel finding, which should have multiple implications in the role of gonadal hormones in somatic sensory function. Inflammation or injury has been shown to alter the expression of CB1 in the periphery as well as in the CNS (Izzo et al., 2001; Amaya et al., 2006; Benito et al., 2008). Of the many factors that are released during inflammation, cytokines exert profound regulatory effects on cannabinoid receptor expression (Jean-Gilles et al., 2010). Cytokines such as TGF-β and INF-γ regulate CB2 in various immune cells and microglia in the CNS (González et al., 2000; Riebe et al., 2010). Similarly, IL-4 upregulates CB1 in human T lymphocytes (Börner et al., 2007). We have previously demonstrated that inflammatory cytokines that are released at the local site, such as IL-1β and IL-6, increase CB1 expression in sensory ganglia (Niu et al., 2012). Although inflammation increases the IL-1β level in the muscle tissue (Niu and Ro, 2011), we do not know what concentration is needed to mimic the endogenous levels in the muscle tissue in TG culture. Thus, the high concentration of IL-1β we applied in TG culture may only occur under pathological conditions.

It appears that both male and female gonadal hormones are important factors that regulate CB1 expression, but the nature and extent of regulation depend on cell types. Estrogens induce CB1 expression in colon cancer cells (Notarnicola et al., 2008). In hippocampus and hypothalamus, ovariectomized females have higher amounts of cannabinoid receptor binding relative to cycling females (Riebe et al., 2010), suggesting a higher level of CB1 in the absence of estrogens. Similarly, estrogenic downregulation of CB1 mRNA transcript is demonstrated in the anterior pituitary gland (González et al., 2000). It is well established that male animals generally express a higher density of CB1 levels than their female counterparts in many brain regions (Rubino and Parolaro, 2011). In the periphery, gonadectomy significantly reduces CB1 mRNA in male rats, which is restored by testosterone replacement (González et al., 2000; Busch et al., 2006). IL-1β-induced upregulation of CB1 in TG is also testosterone dependent (Niu et al., 2012). Neither estrogen depletion nor replacement

affected the cytokine-induced CB1 expression in female rats. However, since testosterone is converted to estrogen in the presence of aromatase (Santen et al., 2009), and TG express ERs (Bereiter et al., 2005), there remained a possibility of estrogenic effect in male rats. In this study, however, we demonstrated that the IL-1β induction of CB1 expression was prevented only in the presence of flutamide, but not ICI 182,780. Neither the vehicle nor ICI 182,780 antagonist blocked the CB1 induction. We have previously reported that neither ovariectomy nor estrogen supplement alters CB1 expression in female TG (Niu et al., 2012). Taken together, these observations provide compelling support that testosterone is a key gonadal hormone mediating the cytokine effects on CB1 in TG neurons.

Since the AR receptor expression in TG is comparable between male and female rats it is reasonable to assume that the circulating testosterone level in female is not sufficient to induce CB1 induction. It is also possible that sex-dependent mechanisms in AR-mediated CB1 induction, regardless of circulating testosterone level, may exist. It is not known how testosterone mediates its effects in cultured TG neurons in which circulating testosterone is no longer present. There are at least three explanations for the IL-1β effects in our culture preparation. First, testosterone can be newly synthesized in TG cells. Steroidogenesis of estradiol, progesterone and testosterone has been demonstrated in various regions of the brain and spinal cord (Sinchak et al., 2003; Evrard and Balthazart, 2004; Dean et al., 2012). *De novo* synthesis of testosterone from steroid precursors, such as cholesterol, has been demonstrated in rat hippocampal slice cultures (Munetsuna et al., 2009). Thus, it is possible that the levels of newly synthesized testosterone may be different between male and female TG. Second, cells that do not normally synthesize testosterone are capable of taking up testosterone from the culture environment such as culture medium and exogenous testosterone added to the preparation (Ceccarelli et al., 2009; Sedelaar and Isaacs, 2009). Finally, circulating plasma testosterone in male rats may induce persistent effects on cytokine-induction of CB1 that are preserved even after culture preparation. Persistent estrogen-induced effects on the excitability of dissociated TG neurons have been reported (Flake et al., 2005). Additional studies are needed to clearly demonstrate how TS effects are exerted in TG culture cells.

While the role of estrogens in pain and analgesia has received much attention (Bodnar and Kest, 2010), very little is known about the contribution of testosterone. Available studies that showed the involvement of testosterone in 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) supports our findings. In addition, TS exerts anti-inflammatory and anti-nociceptive effects in the temporomandibular joint (Fischer et al., 2007; Torres-Chávez et al., 2012). How testosterone mediates these effects remains to be elucidated. It is well established that androgens mediate a wide range of physiological responses through transcriptional activities of AR (Heinlein and Chang, 2002). The data on AR expression in TG are scarce (Young and Chang, 1998), let alone the sex comparisons of AR expression in TG. Here, we provided novel information on steroid receptors in sensory neurons that could mediate sex differences in somatic sensory function. Our ChiP data suggested that AR in TG is capable of transcriptional regulation of the CB1 gene in both male and female rats, and the luciferase reporter assay showed that AR functions as an activator rather than repressor. While cytokines can induce direct modulation of the CB1 gene by the transcription factors GATA3 and STAT6, which bind to a *cis*-active, regulatory element in T cells (Börner et al., 2008), whether such activity could also lead to sex differences in CB1 expression is not known. Based on these observations, we can hypothesize that testosterone induces AR translocation to the nucleus to activate CB1 gene expression in TG, which is facilitated by inflammatory cytokines.

In this paper, we provided evidence that AR transcriptionally modulates CB1 gene expression in rat TG. Understanding cellular and molecular mechanisms underlying AR-mediated CB1 regulation under inflammatory conditions in sensory neurons bears important clinical implications for the development of effective treatment strategies targeting peripheral CB1 for men, women, and testosterone compromised patients.

Acknowledgments

Abbreviations

ANOVA	analysis of variance
AR	androgen receptor
CB1	cannabinoid receptor type 1
ChIP	chromatin immunoprecipitation
Cnr1	cannabinoid receptor 1
ER	estrogen receptor
EDTA	ethylenediaminetetraacetic acid
IL-1β	interleukin-1 beta
IL-1R	interleukin-1 beta receptor
PBS	phosphate-buffered saline
RIPA	radio-immunoprecipitation assay
RT-PCR	reverse transcription polymerase chain reaction
TG	trigeminal ganglia

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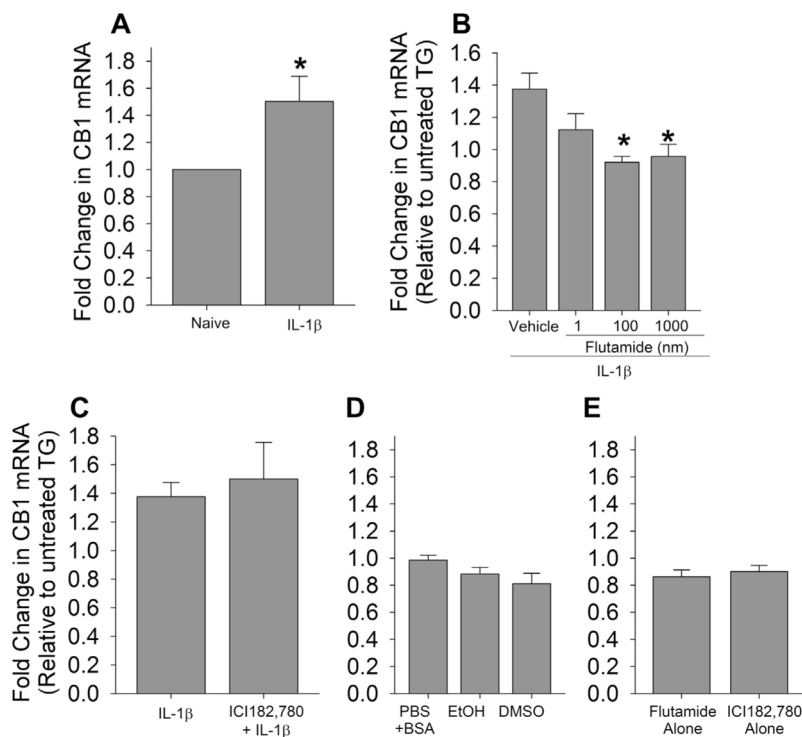
Fig. 1.
CB1 mRNA levels in TG primary cultures prepared from male rats. CB1 mRNA level is significantly higher in IL-1β-treated TG compared to naïve untreated TG (A). Effects of three doses of flutamide (1, 100, 1000 nM) (B) and ICI 182,780 (1 µM) (C) on IL-1β-induced CB1 upregulation. CB1 mRNA levels following the application of vehicles for IL-1β, flutamide and ICI 182,780 (D), and flutamide or ICI 182,780 alone (E). Data are normalized to TG samples from naïve untreated rats and presented as fold change. *Denotes significant effect at p < 0.05. All experiments were repeated six times.
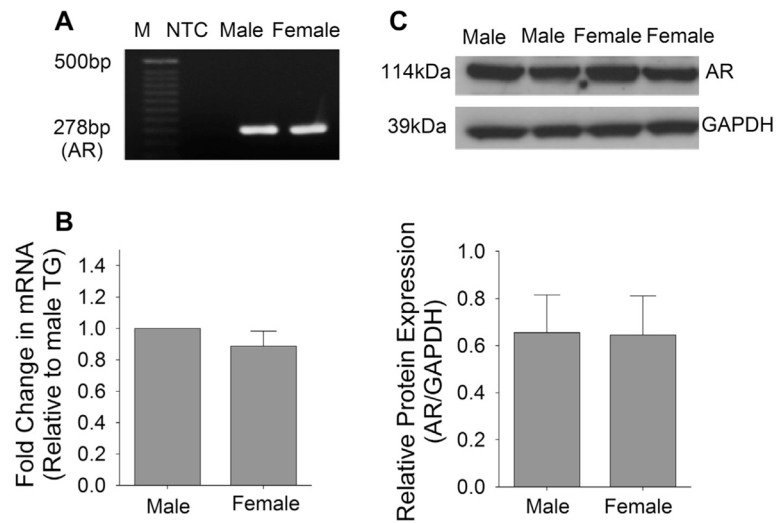
Fig. 2.
mRNA and protein expression for AR in TG of male and female rats. (A) PCR of TG cDNA produced a single band of the expected size for AR (278 bp, A) in male and female TG. M, marker, NTS, no template control. (B) Bar graphs showing quantitative comparisons between male and female rats. (C) Top: Western blot experiments confirmed that AR protein with molecular weight 114 kDa is expressed in TG. Two examples of immunoblots for AR along with GAPDH from TG of males and females are shown. Bottom: The group data showed that AR protein levels in male and female TG were comparable. Each group consisted of four rats.
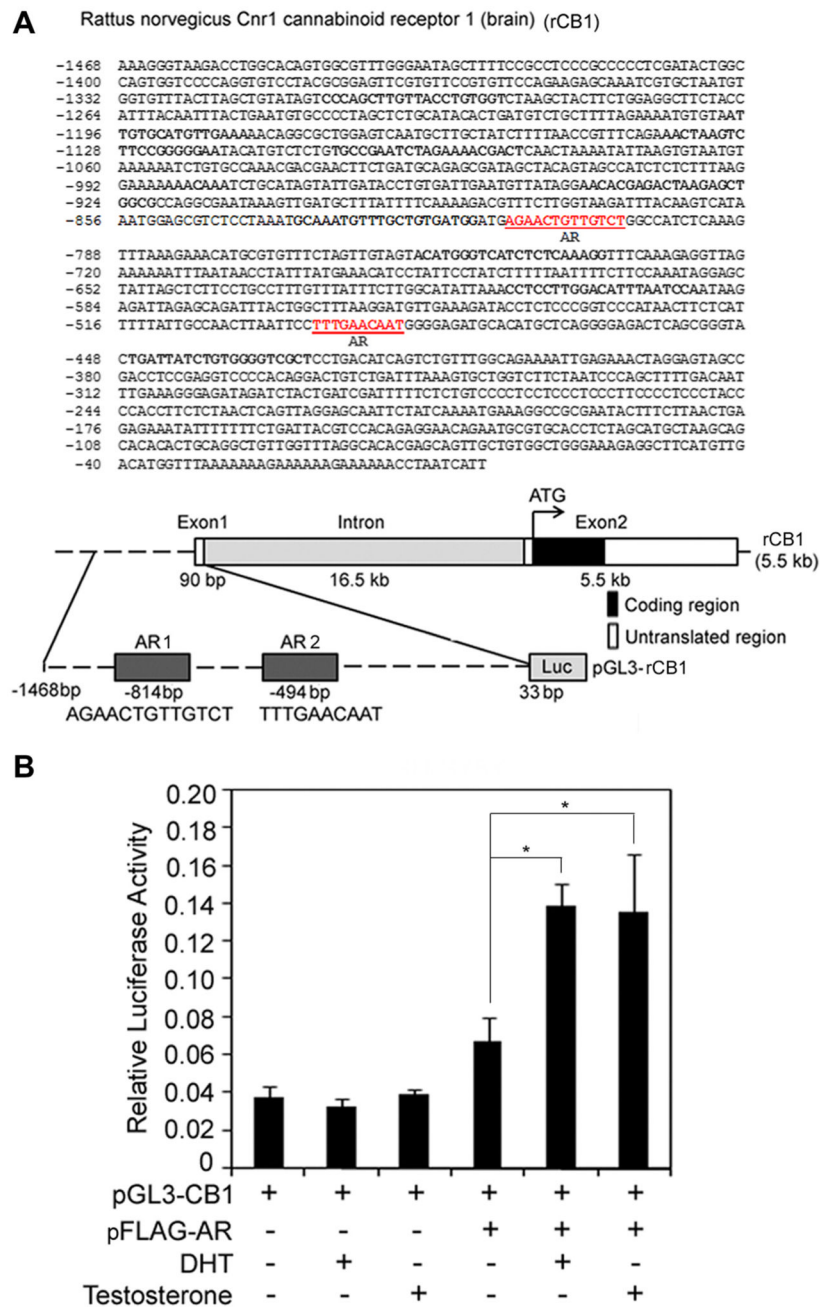
Fig. 3.
AR transcriptionally regulates CB1 expression. (A) A schematic diagram of the cloned 1.5-kb rat CB1 promoter showing two putative AR-binding sites located at −837 to −736 bp and −610 to −427 bp. AR, putative AR-binding site; Luc, firefly luciferase (B) The SH-SY5Y cells were co-transfected with pFLAG-AR and 1.5-kb CB1 promoter-luciferase construct (pGL3-CB1) 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. $n = 3$. *$p < 0.05$ in unpaired Student's t-test.
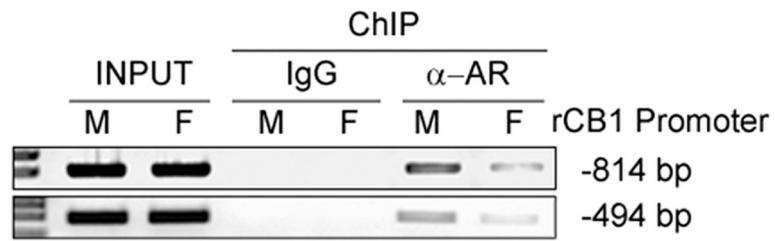
Fig. 4.
AR directly binds to the CB1 promoter in the rat TG. The rat TG were subjected to ChIP assay using anti-AR antibody and PCR primers specific for CB1 promoter region as shown in Fig. 3. IgG was used as a negative control for IP. The PCR products were analyzed on 1% agarose gel and stained with ethidium bromide for visualization. The input represents PCR product amplified from 10% ChIP assay input material.

Table 1

A list of primer sequences used for RT-PCR experiments

Target	Sense primer	Anti-sense primer
AR	5′-GGGGCAATTCGACCATATCTG-3′	5′-CCCTTTGGCGTAACCTCCCTT-3′
IL-1R	5′-TCCTCCATCCTGGAGAATATGA-3′	5′-CTCATTTGGATACTCCGTGCAT-3′
CB1	5′-CTACTGGTGCTGTGTGTCATC-3′	5′-GCTGTCTTTACGGTGGAATAC-3′
GAPDH	5′-TCACCACCATGGAGAAGGC-3′	5′-GCTAAGCAGTTGGTGGTGCA-3′